Role of Cytochrome P450 Polymorphism in Activation and Metabolism of Tamoxifen in ER Positive Breast Cancer Patients

Submitted

By

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In the name of Allah,
the Most Beneficent,
the Most Merciful

DEDICATION

This thesis is dedicated to

My family and my worthy supervisor

Dr Aneesa Sultan

There is no doubt in my mind that without her continued support and counsel I could not have completed this process.

Certificate of Approval

This is to certify that the research work presented in this thesis, entitled: **"Role of Cytochrome P450 Polymorphism in Activation and Metabolism of Tamoxifen in ER Positive Breast Cancer Patients"** was conducted by **Mr. Muhammad Usman Tareen** under the supervision of Prof. Dr. Aneesa Sultan.

No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan in partial fulfillment of the requirements for the **Degree of Doctor of Philosophy** in the field of Biochemistry from Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan.

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LIST OF ABBREVIATIONS

µl	Microliter
µM	Micro molar
2C19*2	CYP2C19*2
4-OH-Tam	4-Hydroxy Tamoxifen
ACD	Acid citrate dextrose
ACE	Angiotensin converting enzyme
ADP	Adenosine diphosphate receptor
ADRs	Adverse drug reactions
BC	Breast Cancer
BRU	Brahui
BSK	Burusho
℃	Degree Centigrade
CAR	Constitutive Androstane Receptor
CO	Carbon monoxide
CYP	Cytochrome P450
DBP	Diastolic blood pressure
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
Endo	Endoxifen
EMs	Extensive metabolizers
ER	Estrogen receptor
ERE	Estrogen response element
EtBr	Ethidium bromide
FDA	U.S. Food and Drug Administration
HTN	Hypertension
HZR	Hazara
IARC	International Agency for Research on Cancer
IC	Internal Control
IMs	Intermediate metabolizers
kDa	Kilo Dalton
$MgCl_2$	Magnesium Chloride
mM	Milimolar
NDMTam	NDesmethyl-Tamoxifen
OD	Optical density
PAT	Pathans
PCR	Polymerase chain reaction
P450	Pigment 450
PMs	Poor metabolizers
PPI	Proton Pump Inhibitors
PRS	Parsi
PUN	Punjabi
RAAS	Renin-angiotensin-aldosterone system
SBP	Systolic blood pressure
SDH	Sindhi
SERM	Selective estrogen receptor modulator

SDS	Sodium dodecyl sulphate
SNP	Single Nucleotide Polymorphism
SNS	Sympathetic Nervous System
STE	Saline Tris EDTA
SULT1A1	Sulfotransferases 1A1
Tam	Tamoxifen
TCAs	Tricyclic Antidepressants
TE buffer	Tris-EDTA buffer
Ums	Ultra rapid metabolizers
UV	Ultra violet
WHO	World Health Organization
XERM	Xenobiotic-Responsive Enhancer Module

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M. Usman Tareen

Abstract

Breast cancer remains the most prevalent cancer among women worldwide. Tamoxifen or aromatase inhibitors are recommended in endocrine therapy because most newly diagnosed breast cancer cases are estrogen-receptor positive. After two or three years of endocrine therapy, tamoxifen has been recommended as either a monotherapy or in combination with an aromatase inhibitor. When administered adjunctively, tamoxifen decreases breast cancer mortality and relapse rates; however, when combined with other metastatic breast cancer treatments, it has been shown to increase survival durations. It is unfortunate that tamoxifen's effectiveness varies significantly. The study objective was to investigate the CYP2C9(*2,*3), CYP2C19(*2,*3 &*17) CYP3A4(*22) and CYP3A5(*3,*6) frequency between Pakistani estrogen positive-breast cancer patients and unrelated healthy controls, and also effect of gene variants on the pharmacogenetics and pharmacokinetics of tamoxifen in patients with estrogen-positive breast cancer.

ER-positive breast cancer patients receiving 20 mg/day of tamoxifen (n = 430) and healthy, unrelated individuals (n = 410; control group) comprised the study population. High-Performance Liquid Chromatography was utilized to determine the steady-state plasma concentrations of tamoxifen and its three metabolites in the patients. DNA was extracted and analyzed by ARMS PCR and AS-PCR followed by RFLP. Five phenotypes observed are as follows, extensive metabolizer (EM), poor metabolizer (PM), intermediate metabolizer (IM), Rapid Meabolizer (RM) and ultra-rapid metabolizer (UM). Further statistical analysis were performed on median plasma concentration of tamoxifen and derivatives. Plasma metabolic ratio and total metabolic ratio were determined and correlated with each genotype.

In our population, *CYP2C9*2* heterozygous (OR: 0.4; 95% CI: 0.53–0.56; $p = 0.0001$) and homozygous mutant (OR: 3,12; 95% CI: 1.80–5.43; P = 0.0001) condition were identified as breast cancer risk factors. The *CYP2C9*2* gene variant had no significant effect on the metabolic ratio between tamoxifen and its three metabolites. However, an insignificant decrease was recorded in the median plasma concentration of 4-OH-Tam in the subjects having heterozygous (**1/*2*) ($P = 0.747$) and mutant (**2/*2*) ($P = 0.223$) genotypes.

More than 65% of healthy individuals were extensive metabolizers (*1/*1) for CYP2C19, whereas more than 70% of ER-positive BC patients were rapid and ultra-rapid metabolizers (*1/17*, *17/17*). The polymorphism CYP2C19*17 is significantly

associated with higher 4-OH-Tamoxifen. Patients with the *17/*17 genotype exhibited 1 to 1.5-fold higher 4-OH-Tamoxifen, which was also high in patients with the *1/*2 and *2/*2 genotypes. Which suggests that CYP219*17 has a significant effect on the higher production of 4-OH-Tamoxifen.

The allele frequencies of the *CYP3A4*22* variant in ER positive breast cancer patients and healthy controls, demonstrated no noteworthy difference between the allele frequencies of *CYP3A4*22*. Plasma metabolic ratios of tamoxifen and its metabolites indicates that CYP3A4*22 does not have any significant effect on the metabolism of tamoxifen and metbolities.

CYP3A5*3 and *6 genotyping results for unrelated healthy individuals and ER positive breast cancer patients in Pakistani population indicates that most of the individuals in both the groups belongs to extensive metabolizer. The frequencies of the CYP3A5 alleles did not differ noticeably from one another. Our study demosttrates that *CYP3A5* do not have any significant impact on the metabolism of tamoxifen and its metabolites.

Tamoxifen and its metabolites were tested for their biological effects with different CYP2C19*17 genotypes (EM, RM and UM) on cell proliferation and estrogen-responsive gene regulation in the MCF-7 breast cancer cell line. Real clinical levels of tamoxifen metabolites in breast cancer patients, as well as actual amounts of estrogens reported in premenopausal individuals receiving tamoxifen, were employed in vitro. Interestingly, tamoxifen anaits primary metabolites were not able to fully inhibit the estrogen-stimulated expression of estrogen-responsive genes in MCF-7 cells (***P*** < .05 for all genes), but the addition of endoxifen was able to produce additional antiestrogenic effect on these genes. Our results suggest that the circulating levels of tamoxifen and its metabolities are not sufficient enough to completely block the estrogen stimulated growth in cell line.

Abstract

Breast cancer remains the most prevalent cancer among women worldwide. Tamoxifen or aromatase inhibitors are recommended in endocrine therapy because most newly diagnosed breast cancer cases are estrogen-receptor positive. After two or three years of endocrine therapy, tamoxifen has been recommended as either a monotherapy or in combination with an aromatase inhibitor. When administered adjunctively, tamoxifen decreases breast cancer mortality and relapse rates; however, when combined with other metastatic breast cancer treatments, it has been shown to increase survival durations. It is unfortunate that tamoxifen's effectiveness varies significantly. The study objective was to investigate the CYP2C9(*2,*3), CYP2C19(*2,*3 &*17) CYP3A4(*22) and CYP3A5(*3,*6) frequency between Pakistani estrogen positive-breast cancer patients and unrelated healthy controls, and also effect of gene variants on the pharmacogenetics and pharmacokinetics of tamoxifen in patients with estrogen-positive breast cancer.

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Chapter 1

1. Introduction

A succession of molecular processes that profoundly disrupt the typical cell features causes cancer. These modified cells replicate and increase in the existence of signals that ordinarily limit cell growth; hence, they no longer need unique signals to trigger cell division and growth. As these cells multiply, they acquire new properties, such as alterations in cell shape, reduced cell adhesion, and the synthesis of novel enzymes. These heritable alterations let the cell and its descendants divide and develop even in the existence of normal cells, which normally limit the proliferation of neighboring cells. Such alterations let the cancer cells spread and infect surrounding tissues. Figure. 1.1, illustrates the characteristics of cancer.

Only a small percentage of the roughly 35,000 human genes have been linked to cancer. Alterations in the same gene are frequently related to many cancer types. These defective genes can be roughly split into three classifications. The first class, proto-oncogenes, generates protein products that promote cell proliferation or prevent natural apoptosis. These genes' mutated variants are known as oncogenes. The second class, tumor suppressors, produces proteins that ordinarily inhibit cell proliferation or induce cell death. The third group comprises DNA repair genes that prevent cancer-causing mutations.

1.1. Breast Cancer

Diverse genetic and chromosomal differences and clinical outcomes make breast cancer more difficult to treat (Vargo-Gogola *et al.*, 2007). Most breast cancers are invasive or infiltrating. Most commonly, breast cancer originates in breast lobules (milk production site) and the ducts connecting these lobules to the nipple. At the same time, some breast cancers are also associated with fatty, lymphatic, and connective tissue (Alteri *et al.*, 2015).

Several speculated factors are identified to accelerate the possibility of developing breast cancer, including null parity, estrogen (hormone) replacement therapy, post-menopausal obesity, use of oral contraceptives, and intake of high-energy products (Dossus *et al.*, 2014). Nevertheless, recognized breast cancer risk factors comprise gender, age, race/ethnicity, menarche (and post-menopausal status), breast cancer

history, benign breast illness, genetic factors, late age at first pregnancy (Helmrich *et al.*, 1983), and exposure to ionizing radiation (Brenner *et al.*, 2003) in early life.

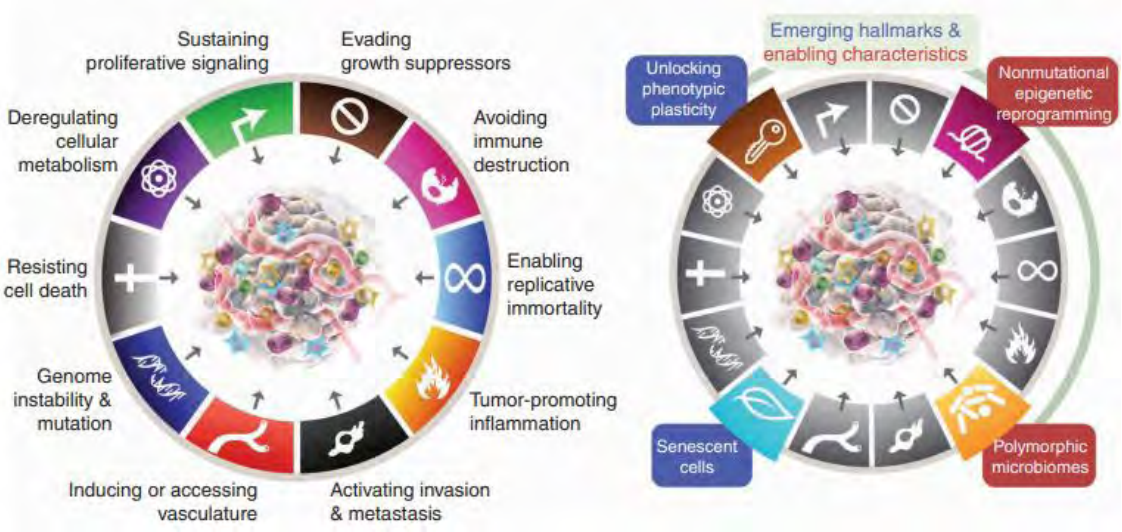

Figure 1.1: Hallmarks of cancer (Hanahan, 2022)

1.1.1. Breast Cancer Global Prevalence and Mortality Rate

Breast cancer is the highly frequent cancer among women. In 2008, there were reported 1,4 million cancer sufferers (Bray *et al.*, 2012)1.6 million in 2010 (Forouzanfar *et al.*, 2011), 1.67 million were diagnosed in 2012 (25% of all cancers). It is expected that by 2030 this number will rise to 2.1 million (Bray *et al.*, 2012). Developed countries have high prevalence rates, where one out of nine women develops breast cancer. Lifestyles such as smoking, less physical activity, poor diet, and high rate of birth control are causing an increase in breast cancer incidence by up to 5% every year in the western population. Its incidence rate varies 10-fold globally (Jemal *et al.*, 2010). Among 1.67 million breast cancer patients in 2012, 522,000 died (Ferlay *et al.*, 2013), and massive casualties arose in underdeveloped areas (324,000 deaths, 14.3% of the total). Breast cancer is a leading death cause triggered by cancer (after lung cancer), causing 198,000 deaths. Mortality rates, however, may differ among different ethnic groups. Korean women have the lowest mortality rate, while the black women in United States have the maximum mortality rate. According to the International Agency for Research on Cancer (IARC), in 2012, around 690,000 new cases were diagnosed annually in developed and less developed nations. The death rates in different regions of the world

are not as high as the improved breast cancer survival rate in urbanized regions.

1.1.2. Breast Cancer Prevalence in Pakistan

Pakistan has the greatest breast cancer incidence rate among Asian countries. One in every nine women in the Pakistani population has breast cancer. The occurrence of breast cancer malignancies in Pakistan is almost 25 times greater than in India and Iran (Garwood *et al.*, 2010). Each year at minimum, 90,000 women (in Pakistan) have breast cancer. From 1998-2002, the rate of breast cancer reported cases in Karachi was 69 per 100,000. Among the Asian nations, specifically in the Pakistani populace, there has been a disconcerting increase in breast cancer cases (I. Malik, 2002). Even though in Pakistan, there is rarely national breast disease occurrence, mortality, or hazard figure information available yet it was reported as the highly common malignancy, representing 34.6 % of female diseases (Shamsi *et al.*, 2013).

1.1.3. Treatment approaches for breast cancer

Several advances for the breast cancer treatment involve surgery, radiotherapy and chemotherapy, targeted therapy, and the hormone therapy. Radiations halt the growth of the cancerous cells. Radiations have a significant effect on the rapidly dividing cancer cells, as they cannot reverse the damage easily. While radiotherapy is only confined to a specific area; chemotherapy can work throughout the body. Chemotherapy treats cancer using drugs that are given orally as well as intravenously. Chemotherapy is accompanied by many side effects and therefore is not suitable for some breast cancer patients. Adjuvant therapy is the therapy that is given post-primary treatment to facilitate the initial treatment and lessen the chances of recurrence of cancer. It may include chemotherapy, radiotherapy and hormonal therapy. Nastrozole and lentrozole are FDA-approved aromatase inhibitor adjuvants (Network, 2003).

1.2. Tamoxifen

Women with estrogen receptor (ER)-positive malignant breast cancer are cured with Tamoxifen, a Selective Estrogen Receptor Modulator (SERM-TAM). U.S. Food and Drug Administration (FDA) approved this drug as adjuvant therapy for breast cancer patients in 1977 (Osborne, 1998). Tamoxifen (TAM), a synthetic anti-estrogen, is a well-known therapeutic drug for treating ER-positive BC patients at any stage of this disease (Group, 1998). It is commonly employed as a chemopreventive drug in women

at greater hazard for developing breast cancer (Fisher *et al.*, 1998b). In "early" breast cancer, the tumor is localized in the breast tissue or may present in the nearby lymph nodes in females. Therefore surgery can be done for the removal of breast tumors. However, once the disease progresses to an advanced stage, micrometastatic deposits of cancer, which initially are not detected, may develop into a clinically detectable form over time and ultimately result in the patient's death (Group, 1998).

1.2.1 Tamoxifen Function and Role in Adjuvant Therapy

Tamoxifen is a non-steroidal anti-estrogen pro drug, having antagonist activity against estrogen receptors. Tamoxifen is a pioneering therapy for curing and inhibiting ER positive breast cancer. Tamoxifen has been claimed to reduce disease relapse and mortality rates by up to 50 and 30%, respectively (Fisher *et al.*, 1998a).

Tamoxifen action is conditional on spreading estrogen quantities, which are lower in post-menopausal women and higher in pre-menopausal women. Tamoxifen was originally thought an antagonist and is presently categorized as a SERM which is a mixture demonstrating tissue-specific Estrogen Receptor (ER) antagonist activity. It competitively binds to the ERs, thus preventing E_2-dependent tumor growth, gene transcription, and cell proliferation (Maximov, McDaniel, *et al.*, 2014b; Osborne, 1998).

Tamoxifen binds to ER having lower affinity than estrogen and detaches the HSP90 protein from the receptor. This tamoxifen-estrogen receptor combination homo or hetero dimerizes, then translocate into the nucleus, activating the AF1 domain and inhibiting the activation factor 2 (AF2) domain of ER. This tamoxifen-ER dimer then binds to DNA at specific Estrogen Response Element (ERE) sequences in the promoter region of E_2 genes. Transcription of the E_2 gene is reduced because the AF2 (ligand-dependent domain) is dormant, and ER co-activator attaching ability is diminished by the AF1 domain, which persists active in the tamoxifen-ER complex, contributes to partial agonist action (Howell *et al.*, 2000).

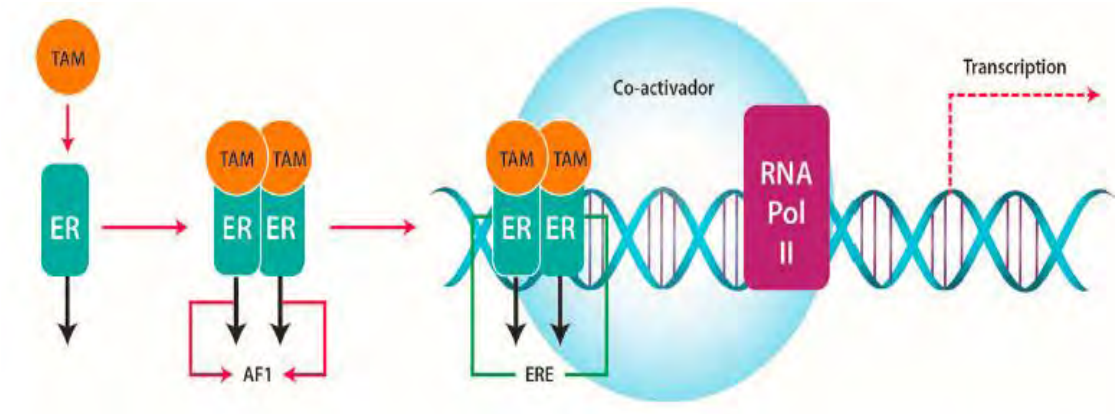

Figure 1.2: The action mechanism of Tamoxifen in the estrogen genes' promotor region inactivation of the AF2 domain results in diminished gene activity. (Johnson *et al.*, 2004a).

Tamoxifen-estrogen receptor complex triggers the AF1 domain and prevents the AF2 domain stimulation. This tamoxifen-estrogen receptor complex then attaches to DNA at 13 bp specific palindromic sequence (ERE sequence) in the estrogen genes' promotor region inactivation of the AF2 domain results in diminished gene activity (Figure 1.2).

Tamoxifen and its primary metabolites combine to the estrogen receptors with various binding affinities. They prevent the estrogen binding to estrogen receptors, inhibiting conformational alterations required to recruit co-activators. The binding of tamoxifen metabolites also leads to the co-repressors (including NCOR1) recruitment. The reduction in estrogen receptors transcriptional action, affects tumor growth, as estrogen-regulated genes are involved in proliferation and angiogenesis (Johnson *et al.*, 2004a).

Endoxifen, a key metabolite, is responsible for the *in vivo* action of tamoxifen. Endoxifen acts differently on the two estrogen receptors. It steadies ERβ and promotes receptor heterodimerization. In addition, its inhibitory effects on the target genes expression are increased. In breast cancer cells, however, endoxifen targets ERα for proteasomal destruction.

It has been reported in several studies that tamoxifen is responsible for halting the cell cycle and can induce apoptosis by modifying some growth factors, e.g., by down-regulating expression of TGFα, stromal TGFβ1 expression induction and reduction in

the production IGF-1 which is a powerful mitogen (Notas *et al.*, 2015).

Several studies demonstrate that tamoxifen can also induce cell cycle detention in the G0/G1 phase, hence limiting cellular growth. Tamoxifen can also accelerate cellular proliferation by acting on numerous signalling pathways(consist of *c-MYC* and MAPKs) (Ariazi *et al.*, 2011; Miller *et al.*, 1999; Mizutani *et al.*, 2004).

In premenopausal and postmenopausal women having estrogen receptor-positive breast cancer, adjunct tamoxifen treatment has been proven to cut yearly recurrence rates and decrease death by one-third (Group, 2005). Tamoxifen is also qualified to avoid breast cancer in women at elevated risk (Fisher *et al.*, 2005).

1.2.2 Tamoxifen Metabolism by CYP Family

Tamoxifen is mostly processed by the liver, with minimal local breast metabolism. The Cytochrome P450 (CYP) enzyme system in the liver metabolizes tamoxifen into multiple main and secondary metabolites (Figure 1.3). Some of these metabolites have greater anti-estrogenic activities than tamoxifen in breast cancer cells (Desta *et al.*, 2004; Mürdter *et al.*, 2011). Polymorphisms in many CYP enzymes implicated in tamoxifen metabolism influence metabolites' relative amount and availability and, subsequently, their effects on E2-dependent cell proliferation of breast cancer.

The metabolic pathway directly transforms tamoxifen to N-desmethyl tamoxifen by *CYP3A4/5*, followed by change to endoxifen. This N-desmethyl tamoxifen conversion to endoxifen is catalyzed by *CYP2D6*. Moreover, some tamoxifen is initially metabolized to the 4-hydroxytamoxifen (4OH-TAM) by *CYP2D6*. This 4-hydroxy tamoxifen can be degraded or converted to endoxifen by *CYP3A4/5*.

Endoxifen is a very efficient metabolite of the tamoxifen metabolizing routes (4-hydroxylation and N-demethylation) that are widely observed. The initial focus was on the 4-hydroxylation pathway, mediated by many CYPs, including *CYP2D6*, because the immediate resultant metabolite, 4-hydroxy-tamoxifen, was 30 to 100 times more effective as an antiestrogen than tamoxifen itself (Desta *et al.*, 2004). However, this pathway only provides nearly 7% of the total tamoxifen metabolism.

Tamoxifen's N-demethylation to N-desmethyl tamoxifen is mainly catalyzed by *CYP3A4/5* with small influences by *CYP2D6*, *CYP1A2*, *CYP2C9*, and *CYP2C19* and contributes roughly 92% of tamoxifen metabolism (Desta *et al.*, 2004; Kiyotani *et al.*, 2012; Stearns *et al.*, 2003). N-desmethyl tamoxifen is oxidized to various tamoxifen-

active metabolites, the most remarkable of which is endoxifen (Kiyotani *et al.*, 2012; Stearns *et al.*, 2003).

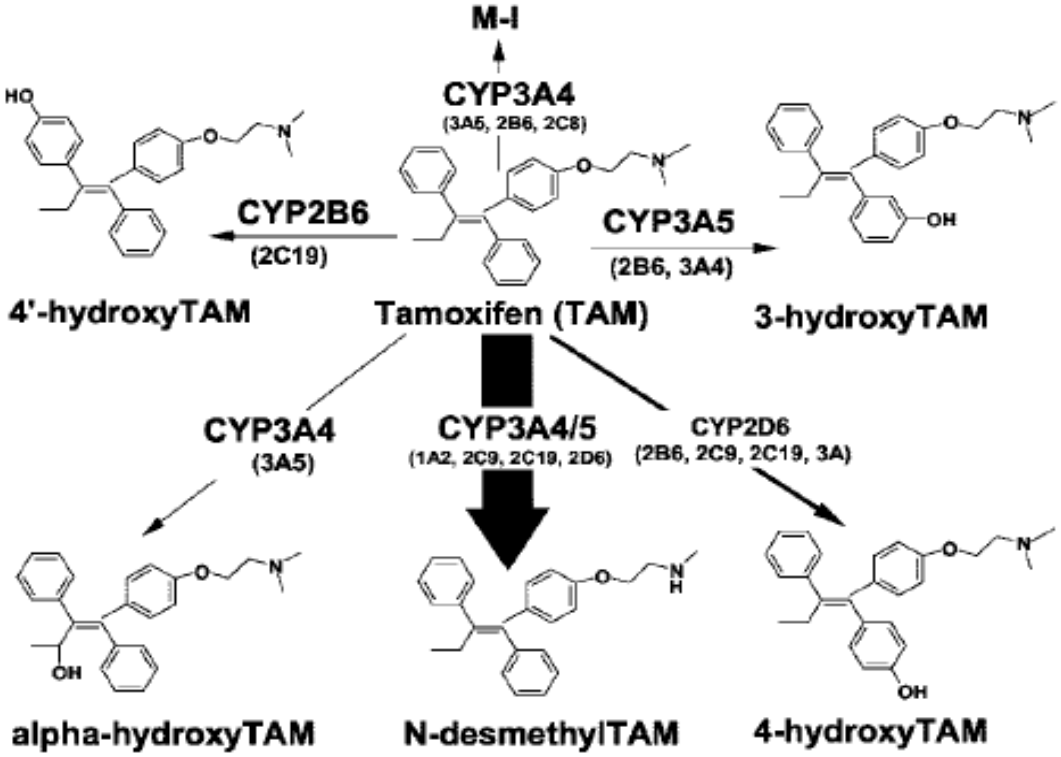

Figure 1.3: Role of several cytochromes in tamoxifen metabolism. The Cytochrome P450 (CYP) enzyme system in the liver metabolizes tamoxifen into multiple main and secondary metabolites (Desta *et al*., 2004)

1.2.3. Xenobiotics metabolism and Cytochrome P450 function

In most breast cancer cases, chemotherapy as adjuvant or neo-adjuvant treatment is most effective when combining numerous drugs. Most anticancer drugs used in this cocktail of drugs are xenobiotic. Some anthracycline (doxorubicin), taxanes, e.g., paclitaxel and docetaxel, 5-fluorouracil, cyclophosphamide, and carboplatin common drugs used as a combination of two or three drugs in neo and adjuvant chemotherapy. Tamoxifen, also known as nolvadex, is an important xenobiotic drug employed as hormonal adjuvant therapy to prevent the reoccurrence of breast cancer. Xenobiotics (including anti-cancer medications) are digested by a series of chemical processes following absorption in the organism. Metabolism of a xenobiotic is ensured through two discrete successive phases known as phase I and II, though this is not a complete

order. Phase I does not always follow Phase II, and Phase II does not constantly follow Phase I (Iyanagi, 2007).

Cytochrome P450 is a distinct group of hepatic catalytic enzymes engaged in the drugs biotransformation. This enzyme allows the xenobiotic to be transformed into forms that are easily excreted and are less toxic. Cytochrome P450 is a phase I biotransformation enzyme, which helps in hydrolysis, reduction, and oxidation reactions (Monk *et al.*, 2014). About 1/4th of *CYP*s are involved in mammalian drug metabolism which include *CYP*3A4/5, *CYP*1A2, *CYP*2B6, *CYP*2D6 and *CYP*2C. They convert lipophilic endogenous substances and drugs into easily excretable hydrophilic metabolites (Wolf *et al.*, 1999). The *CYP*2C subfamily plays an active role in 24% of mammalian drug metabolic reactions, followed by *CYP*2D6, which accounts for 20% of metabolic drug reactions (Ye *et al.*, 2014).

1.3 Cytochrome P450

Cytochrome P450 (CYP) is an enzymes superfamily that performs a specialized function in fat-soluble vitamin metabolism, eicosanoid production, and xenobiotic metabolism (Hasler *et al.*, 1999). Cytochrome P450's involvement in the drug metabolism system has appeared as an important determining factor in various drug-drug interactions resulting in drug toxicities, diminished pharmacological effects, and Adverse Drug Reactions (ADRs). About 57 different CYP proteins, called isozymes, are encoded by the human genome that plays crucial roles by catalyzing different reactions (Guengerich, 2005; N. Lewis *et al.*, 2004). Out of these, six CYP isozymes play a major character in drug metabolism, including *CYP3A4/5, CYP1A2, CYP2E1, CYP2D6, CYP2C9* and *CYP2C19* (Ogu *et al.*, 2000). CYP enzymes account for over 75% of the total drug biotransformation via oxidation (Guengerich, 2005). They convert the lipid-soluble drug into a hydrophilic metabolite. In addition to detoxifying harmful xenobiotic, CYPs activate some medicines into reactive species via biotransformation (Ortiz de Montellano, 2015; Parkinson *et al.*, 2001). Clinical data of 248 drugs shows that *CYP3A4/5* is involved in 30% of drug metabolism, accounting for 29% of CYP 450 concentration in the liver (Zanger *et al.*, 2013). CYP2C sub-gene family is vital in 24% of metabolic drug reactions and accounts for 18% CYPs concentration in the liver, followed by *CYP1A2*, which is involved in 9% of drug

metabolic reactions and accounts for about 13% of total CYPs concentration in the liver as shown in Figure 1.4 (Chang, 2014).

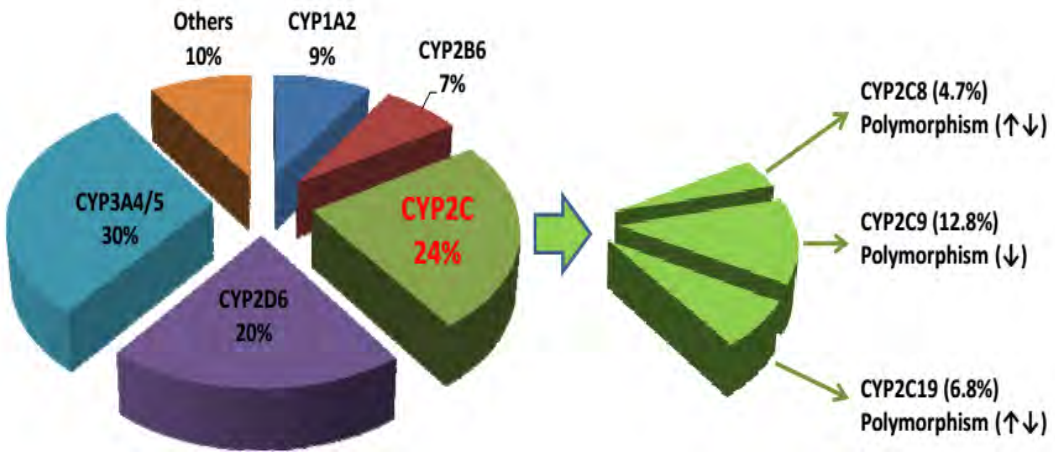

Figure 1.4: Role of *CYP3A4/5, CYP2B6, CYP1A2, CYP2D6, CYP2C* with further breakdown into *CYP2C19, CYP2C9* AND *CYP2C8* in xenobiotic metabolism. CYP2C sub-gene family is vital in 24% of metabolic drug reactions and accounts for 18% CYPs concentration in the liver. (Chang, 2014).

The CYPs are a heme group containing membrane proteins located in the gut and liver and bound on the smooth endoplasmic reticulum and inner membrane of mitochondria in various tissues in the body (Ogu *et al.*, 2000). Biotransformation and drug metabolism are mainly catalyzed in the hepatocytes by CYPs and other drug-metabolizing enzymes. During drug metabolism, the oily soluble compounds are converted into less toxic water-soluble compounds that are easily expelled out of the body by urine. Once the drug enters the liver cells, it moves through the smooth endoplasmic reticulum wall, where it gets involved in the CYP metabolic pathway (Coleman, 2010).

1.3.1. Sites of Cytochrome P450

Most Cytochrome P450 enzymes are in the liver and are hydrophobic. CYP450 enzymes are also in the gut, kidney, and small intestine. However, most of them are in hepatocytes bounded by the endoplasmic reticulum and inner mitochondrial membrane, as they are membrane-associated proteins (Guengerich, 2005). When entering hepatocytes, a drug or any other xenobiotic molecule passes through the wall of the smooth endoplasmic reticulum and enters the CYP metabolic pathway, as described in Figure 1.5 (Coleman, 2010).

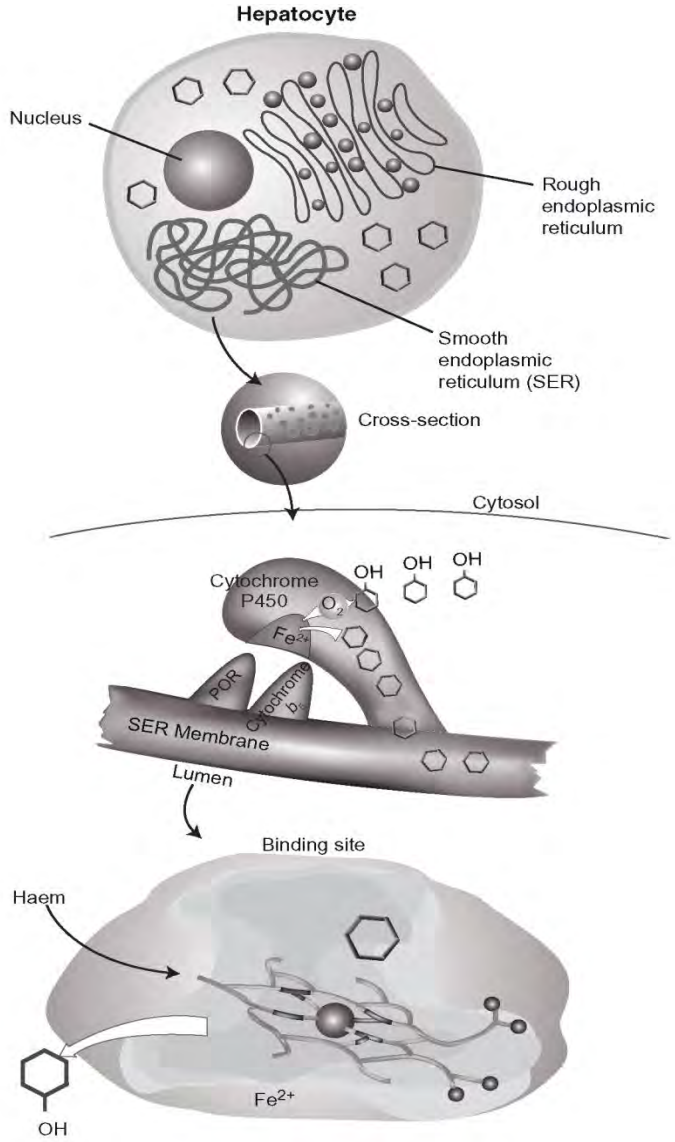

Figure 1.5: CYP Location and their redox partners in hepatocytes**.** Drug or any other xenobiotic molecule passes through the wall of the smooth endoplasmic reticulum and enters the CYP metabolic pathway (Coleman, 2010)**.**

1.3.2. CYP450 Structure

Structurally, CYP450 enzymes are classified into two types. Type 1 includes mitochondrial and bacterial enzymes. The mitochondrial enzyme system has 3 components; FAD, NADH containing flavoprotein, FeS protein comprising Fe, S, and phytochrome 450. Type II comprises microsomal enzymes, which have a eukaryotic nature and comprises of two components, NADH and phytochrome 450 (Smith *et al*., 1994). CYP450 proteins have a 3-dimensional globular structure with a beta (β) sheet at the N terminal and helix-rich C terminal. The C terminal of these enzymes is reported to be highly conserved.

The signature motif "FXXGXXXCXG" is present in all, where X indicates non-specific amino acid. The heme-binding region, consisting of 50 amino acids, is located at the protein's C-terminal (Smith *et al*., 1994). The structural components of bacterial and mitochondrial CYP450 differ from that of fungi, plants, and animals. It has been reported that prokaryotic CYP450 has three components: a Heme domain, ferredoxin with clusters of Iron (Fe) and Sulfur (S), and NADH-dependent reductase. While microsomal eukaryotic enzymes only contain the heme domain and reductase unit (Smith *et al*., 1994).

1.3.3. Nomenclature of Cytochrome P450

In 1987, a nomenclature scheme for CYP isoforms was first proposed. As a result of the extent of similarities among primary amino acid sequences determined via gene sequencing, the CYPs are categorized into families and subfamilies (Nebert *et al*., 1987). The term cytochrome P450 is originated as the human loci for Cytochrome P450 are designated as CYP. At the same time, P450 represents "pigment," which exhibits a spectrophotometric peak at 450 nm when complexed with carbon monoxide and a reduced state. Usually, amino acid sequences with 40% or more resemblance are classified in the identical family, labeled by a digit (e.g., CYP1), but those having match more than 55% are placed in the similar subfamily, labeled by an alphabet (e.g., *CYP1A*), and the ones with 97% homology, specify the gene by again labeling with a number after the letter (e.g., *CYP1A1*). Distinct alleles are labeled by adding an asterisk or a numeral and, in particular cases, an alphabet letter (e.g., CYP1A1*1A), with *1 or *1A representing the wild-type allele (Danielson, 2002) as shown in Figure 1.6. Therefore, this nomenclature system for CYP450 is not dependent on the enzyme's function and reaction that they catalyze.

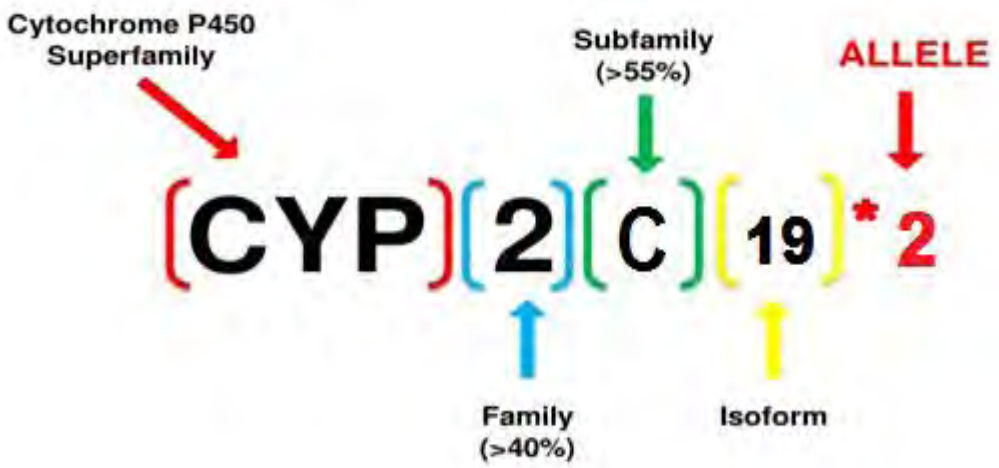

Figure 1.6: Nomenclature of CYP450 superfamily. Cytochrome P450 is labeled with the abbreviation "CYP," resulting in a number representing the gene's family, an alphabet representing the subfamily, and a further number representing an individual gene (isoform). The allele variant is represented by a number before a star symbol (www.cypalleles.ki.se).

1.3.4. Factors Affecting Enzymatic Activity of CYP Family Members

In general, the phenotypic expression of all genes is a manifestation of both genetic and physiological parameters. The existence or deficiency of exogenous compounds, certain hormones, and cytokines determine the expression of respective CYP enzymes. The catalytic activity is deeply influenced by variation in copy number and variant alleles, either present in considerable proportion or rare in a population. Almost all drug-metabolizing cytochrome P450s are polymorphic. Alleles variation from normal to mutant are due to substitution or change in amino acids, base deletions, frameshift mutation, missense mutation, defective splicing, and whole gene deletion (Ingelman-Sundberg, 2004). The genetic variability gives rise to four major phenotypes when both alleles are defective or non-functional, producing a truncated or null protein incapable of catalyzing a reaction and stated as having no enzyme activity. The term used for such a condition is **poor metabolizer (PM)**. PMs have an increased risk of ADRs due to the slow rate of drug metabolism and lack of therapeutic efficacy of prodrugs to produce their active compounds, e.g., tamoxifen.

Therefore, alternative medication is advised to lessen the adverse effects of PMs (Zanger and Schwab 2013). The term **intermediate metabolizer (IM)** is stated for heterozygous for a defective allele or when one of the alleles is functional and translating normal protein. In contrast, the second allele is defective, decreasing enzyme activity.

IM needs a lower drug dose than normal for the best treatment response (Sundberg *et al*., 2007). **Extensive metabolizers (EM)** possess 2 normal alleles resulting in a normal phenotype. Therefore, a normal drug quantity is required to achieve the best results (Zanger and Schwab, 2013). **Ultra-rapid metabolizer (UM)** phenotype is defined when due to certain defects increase in enzyme production or enzyme activity is observed or carrying more than two active gene copies (Scott *et al*., 2011). Ultra-rapid metabolism is due to gene duplications, which cause proportionally increased enzyme expression and regulatory elements which control the transcription. Ultra-rapid metabolizers require higher doses of medicine than normal to achieve the required response.

1.4. CYP2C Sub gene family

The CYP2C subfamily metabolizes 20 to 25% of clinically used medicines and several endogenous compounds, including arachidonic acid. CYP2C sub-gene family comprises four gene clusters in a sequence of *CYP2C8, CYP2C9, CYP2C19, and CYP2C18* from telomere to the centromere (Zanger *et al.*, 2013). These four gene clusters are 80% identical at the amino acid level, accounting for 20% of the liver's encoded proteins of all cytochrome P450 content. All CYP2C sub-gene families express genetic polymorphism. *CYP2C19*, *CYP2C9*, and *CYP2C18* have more clinical value among these enzymes. Generally, CYP2C enzymes have overlapping and broad-spectrum substrate specificities. Some enzymes exhibited significant activity against steroids such as testosterone (F. J. Gonzalez *et al.*, 1990). *CYP2C9* have high expression level than any other CYP2Cs member (Naraharisetti *et al.*, 2010; Ohtsuki, 2012), followed by *CYP2C19* and *CYP2C8*. The *CYP2C9* expression is 10-fold greater than *CYP2C19,* but *CYP2C19* shows broad-spectrum activity in the metabolism of widely prescribed drugs as a result of its polymorphic expression (Danielson, 2002).

1.4.1 *CYP2C9*

The *CYP2C9*, a key isozyme of the CYP2C family, represents about 20% of the total liver CYP P-450 content. The cDNAs of *CYP2C9* code for a protein of 490 amino acids, all of which have a molecular mass of 55.6 kDa. The *CYP2C9* gene' transcribed protein is identified as mephenytoin 4-hydroxylase.

The substrate or drug catalyzed by *CYP2C9* enzymes is called a *CYP2C9* substrate. *CYP2C9* metabolizes around 15% of therapeutically effective medications, including anti-diabetic, angiotensin receptor blockers, and anticonvulsant treatments (Table 1.1) (H.-G. Xie *et al.*, 2002). More than 60 drugs are identified to be metabolized by the CYP2C9 enzyme. Most known substrates for *CYP2C9* are either lipophilic or weakly acidic, with pKa values that range from 3.8 to 8.1. The electropositive group on the enzyme and the electronegative group on the substrate give rise to electrostatic interaction, which appears to be of key significance in determining the affinity of these medications. (Miners *et al.*, 1998).

It has been determined that CYP2C9 is accountable for approving up to 15% of all medicines through Phase I metabolism. **"**Probe Drug" or "Marker Drug" is a term that was introduced in the early 1970s. A probe drug is formulated to provide information about important enzyme activity issues. Warfarin, an anti-coagulant, is extensively oxidized in humans by the P450 enzyme. Several studies indicate the predominant affinity of warfarin towards *CYP2C9*. Therefore Warfarin is suggested as a promising probe drug in order to determine *CYP2C9* activity *in vivo* (Pelkonen *et al.*, 2008)

Diclofenac, Phenytoin, and Tolbutamide are other important drugs used as probe drugs for *CYP2C9.* Several important drugs are the substrate of *CYP2C9,* which include hydroxylation of tolbutamide and hexobarbital, as well as phenytoin and warfarin.

Table 1.1: Therapeutic class frequency of CYP2C9 substrates (Miners *et al.*, 1998)

Class	**Anti Inflammatory agents**	**Anticancer agents**	**Endogenous compounds**	**Anticonvulsants**	**Oral hypoglycaemics**
Substrates	flurbiprofen	Cyclophosphamide (Tamoxifen)	Arachidonic acid	Phenytoin (Phenobarbital) (Thimethadione)	Tolbutamide
	Diclofenac		5-hydroxy tryptamine		Glyburide
	Naproxen		Linoleic acid		Glipizide

1.4.1.1. *CYP2C9* Gene Transcriptional regulation

The *CYP2C9* gene is in the 10q24.33 region of the chromosome and comprises of nine exons. *CYP2C9* gene codes a protein constituting 490 amino acid residues. The expressed gene product of the *CYP2C9* gene is 92% homologous to the gene product of its neighboring gene *CYPC19*, varying only by 43 amino acids. However, these two enzymes have entirely distinct substrate specificities. Primarily, gene expression is regulated at the transcription level. Within a 2.2- kb region of the CYP2C9 gene, there are many consensus sequences for glucocorticoid response element (GREs) and identified binding sequences for transcription factors including TATA box, CAAT box, HNF-1, AP-1, and C/EBP. This area is extremely polymorphic in humans, with at least seven single-nucleotide polymorphisms, a few of which coexist to provide six sequence patterns (Demorais *et al.,* 1993). Some of these polymorphisms are correlated with decreased enzyme activity *in vitro* relative to the wild type.

A luciferase reporter gene study revealed that pattern 2 had roughly 40% of the activity of pattern 1 (called wild-type), and those carrying pattern 2 demonstrated a 38% drop in the obvious intrinsic phenytoin clearance relative to persons carrying pattern 1 (Shintani *et al.*, 2001).

1.4.1.2. Genetic Polymorphism of *CYP2C9*

Polymorphism is the term used to explain a trait that exists in a population as two or more phenotypes. Vogel and Motulsky (1986) stated: A polymorphism is a Mendelian or monogenic characteristic that occurs in a population in no less than two phenotypes and likely at least two genotypes, none of which are uncommon and none of which occur with a frequency of less than 1-2%

CYP2C9, like other members of the 2C subfamily, is vastly polymorphic. *CYP2C9* polymorphism was first reported, in the early 1970s, in the metabolism of phenytoin and tolbutamide (J. Scott *et al.,* 1978). Tolbutamide polymorphism was reported due to a rare allele that carries an Ile359Leu mutation, also known as *CYP2C9*3* (Sullivan-Klose *et al.*, 1996). Two homozygous poor metabolizers (PMs) of losartan reported the exact problem. One of these individuals was phenotyped for tolbutamide and proven to be homozygous for the *CYP2C9*3* allele; thus, he or she was determined to be a tolbutamide-sensitive individual (Kidd *et al.,* 1999). Another patient with the

CYP2C9*3 genotype was shown to have a worsened response to warfarin and a lower ability to remove S-warfarin (Steward *et al.*, 1997).

1.4.1.3 *CYP2C9* VARIANTS

In recent years, several *CYP2C9* single nucleotide polymorphisms have been documented (Bhasker *et al.*, 1997). Due to single nucleotide polymorphism (SNPs), allelic variants have been produced, resulting in a difference in amino acid (Figure1.7). They differ in a few positions in the gene' coding region (Goldstein *et al.*, 1994). Among 24 *CYP2C9* variants, ***CYP2C9*1*** is also called a wild-type allele. Both alleles are functional and produce a normal protein (Bhasker *et al.*, 1997). In *CYP2C9*2*, substituting T (C430T) on exon 3 occurs, resulting in Arg144Cys conversion and reduced activity enzyme (Crespi *et al.*, 1997). ***CYP2C9*3*** variant allele results on exon 7 by A1075C substitution, leading to altered protein with the substitution of Leucine residue (Ile359Leu). Such protein has reduced enzyme activity compared to *CYP2C9** (Surendiran *et al.*, 2011).

CYP2C9*4 variant result due to (1076T>C) conversion on exon 7, resulting in the isoleucine replacement by threonine residue at 359 position (Imai *et al.*, 2000). Recently, the ***CYP2C9*5*** variant allele has been identified. It is characterized by the transversion of C1080G on exon 7, resulting in Asp360Glu conversion (Dickmann *et al.*, 2001). **CYP2C9*6**, a new null polymorphism, has been identified in which deletion of adenine nucleotide at 818 positions on exon 5 occur, resulting in protein inactivation (Kidd *et al.*, 2001). **CYP2C9*11** is due to the substitution of 1003C>T in exon 7, which results in the substitution of Arg335Trp (Higashi *et al.*, 2002).

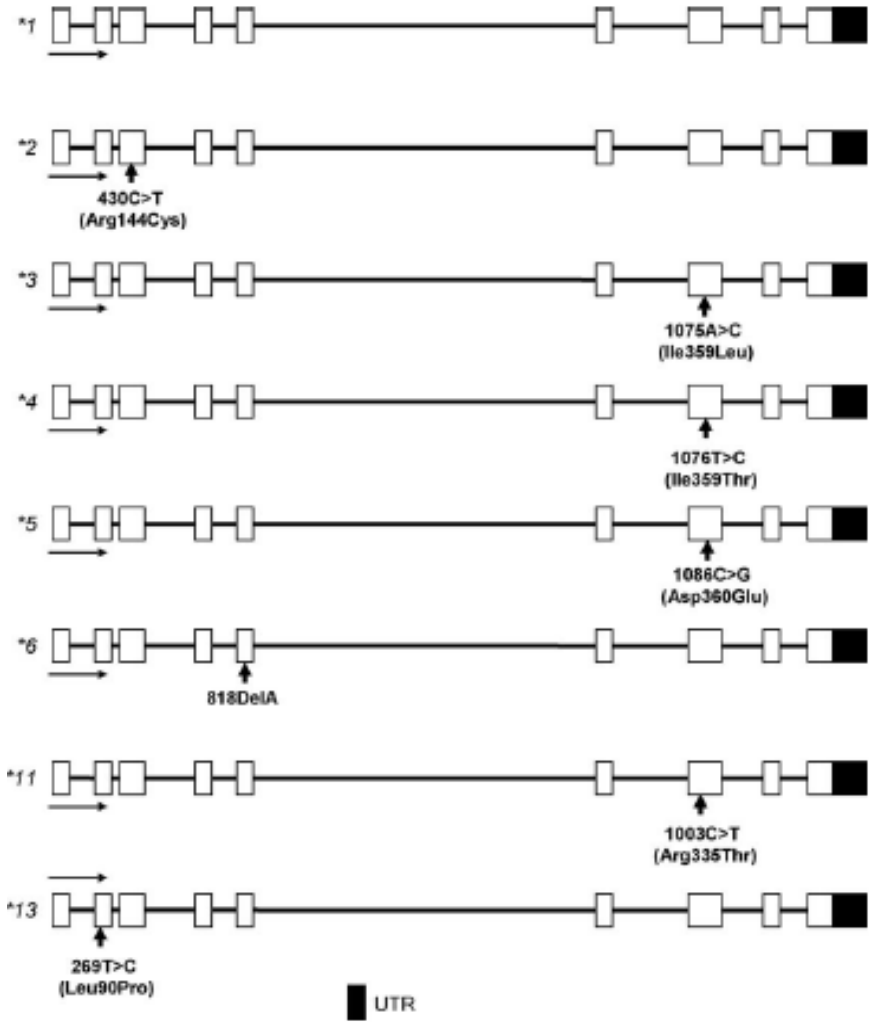

Figure 1.7: Functionally important allele of the human *CYP2C9* gene. To date, more than 15 variant alleles (*1B to *16) and a series of subvariants of the CYP2C gene have been identified (S.-F. Zhou *et al.*, 2010)(S.-F. Zhou *et al.*, 2010)

1.4.1.4 Phenotypes of *CYP2C9*

The *CYP2C9* gene, which codes for the *CYP2C9* enzyme, is extremely polymorphic and comprises functional variants of key pharmacogenetic significance (Table 1.2). Genetic variation in *CYP2C9* leads to changes in metabolic activity, which is critical in pathogenesis due to ADRs. An inclusive literature review has identified two common SNP variants inside the *CYP2C9* gene, Arg144Cys and Thr359>Leu, which leads to poor metabolism (PM) phenotype. Individuals identified with **2* and **3* genotype exhibit the most noticeable pharmacokinetic effects for several drugs, particularly in the heterozygous carrier of both variants. It is reported that patients who are identified as a carrier of *2 and *3 variants, which are associated with poor metabolism, need

lower dosages of warfarin to obtain the same anticoagulant effect as those with the wild-type (*CYP2C9*1*) genotype (Aithal *et al.*, 1999; Lindh *et al.*, 2009).

Table 1.2: Mutation and resulting phenotypes of *CYP2C9* variant Allele.

Allele	Mutation	Effect on Enzyme activity	Phenotype	Reference
CYP2C9*1	None	None	Extensive Metabolizer (UM)	(Leishman, 1991)
CYP2C9*2	430C>T	Decrease in activity	Poor metabolizer (PM)	(García-Martín *et al.*, 2001)
CYP2C9*3	1075A>C	Decrease in activity	Poor metabolizer (PM)	(García-Martín *et al.*, 2001)
CYP2C9*5	1090C>G	Decrease in activity	Poor metabolizer (PM)	(Dickmann *et al.*, 2001)
CYP2C9*6	818delA	Decrease in activity	Poor metabolizer (PM)	(Kidd *et al.*, 2001)
CYP2C9*13	269T>C	Decrease in activity	Poor metabolizer (PM)	(Si *et al.*, 2004)

1.4.1.5. Frequencies of CYP2C9 Allelic Variants in the Population

*CYP2C9*2* is lacking in the East Asian populations, including Chinese, Korean, and Japanese, according to reports. However, the American, African, and Ethiopian populations have reported a general allele frequency of 3.2% for *CYP2C9*2.* Substantial *CYP2C9**2 allele frequency heterogeneity occurs among several Caucasian

populations, ranging from 8 to 19%. In the case of Hispanics, *CYP*2*C*9*2 has an allele frequency of 12.0%, relative to Caucasians but greater than blacks.

In the case of *CYP*2*C*9*3, an allele frequency of 1.1% in Koreans, 2.2% in Japanese, and 3.3% in Chinese is reported (Inoue *et al.*, 1997) (Leung *et al.*, 1999; Odani *et al.*, 1997). However, blacks have an allele frequency of 1.3% for *CYP2C9*3;* in the case of Caucasians, for *CYP*2*C*9*3 allele frequency, significant heterogeneity is revealed. Hispanics have exhibited an allele frequency of 3.4%, comparable to Chinese 3.3%.

*CYP*2*C*9 *5, first identified in African-Americans, has an allelic frequency of 0.2–1.7% (Dickmann *et al.*, 2001; Strom *et al.*, 2000; H.-G. Xie *et al.*, 2002) For Hispanic-Americans, the frequency for *CYP*2*C*9 *5 ranges from 0–0.5% (Strom *et al.*, 2000; H.-G. Xie *et al.*, 2002) and was found absent or extremely scarce in Caucasians, Chinese, and Japanese population (H.-G. Xie *et al.*, 2002). *CYP*2*C*9 *6, a rare and uncommon allele, has an allele frequency of 0.6 to 1.5% in African-American (Kidd *et al.*, 2001).

1.4.1.6. Interethnic Variability

Substantial ethnic differences are found in the *CYP2C9* allelic variants frequency. Several studies have been designed and implemented to report the phenotypic character and frequency of poor metabolizers in different populations. The *CYP2C9*2* variant is most prevalent in the Caucasian population, where 1% are homozygous (carriers) and about 22% are found to be heterozygous (Sullivan-Klose *et al.*, 1996). *CYP2C9*2* and *3 are either rare or absent in the Asian population (Kirchheiner *et al.*, 2005). Patients with *2 and *3 genotypes have been reported to have shown reduced intrinsic clearance of oral anticoagulants and were more susceptible to bleeding events. Patients who were a carrier for *2 or *3 genotype have shown a higher frequency of bleeding complications during warfarin therapy initiation (Aithal *et al.*, 1999; Sullivan-Klose *et al.*, 1996) (Takahashi *et al.*, 2001)

Furthermore, it has been reported that the East Asian population has a higher sensitivity to warfarin due to the absence of the *CYP2C9*2/*3* variant compared to Caucasian, indicating poor metabolizer status.

*CYP2C9*5* is assessed to be inherited in 3% of the African-American population as a C1080>G mutation (Allabi *et al.*, 2005; Allabi *et al.*, 2004). Additionally, African Americans have fundamentally lower *CYP2C9*2* and *3 inheritance rates than the Caucasian population, with an allele frequency of 2.5% and 1.25%, respectively. In the

case of the African American-population, the presence of *5 and *6 alleles may be vital in ADRs caused by *CYP2C9* substrate drugs.

Table 1.3: Interethnic variability of *CYP2C9* variant in different populations.

Ethnic group	No of subjects	Allele frequency %			References
		*1	*2	*3	
Russian	290	83.9	9.1	7.0	(Gaikovitch *et al.*, 2003)
Turkish	499	79.4	10.6	10.0	(Aynacioglu *et al.*, 1999)
Ethiopian	150	94.0	4.0	2.0	(Scordo *et al.*, 2005)
Chinese	376	96.7	0	3.3	(Hong *et al.*, 2005)
Japanese	218	97.9	0	2.1	(Nasu *et al.*, 1997)
Korean	358	93.4	0	6.0	(Bae *et al.*, 2005)

1.4.1.7. Role of *CYP2C9* in Breast cancer

Estrogen is an important growth factor that can stimulate cancer cell growth by interacting with ER and triggering the cellular proliferation pathway. In addition to having a crucial role in the metabolism of anticancer medicines and carcinogens, cytochrome p450 are powerful enzymes that enable estrogen metabolism (Husbeck *et al.*, 2002). Expression of various CYPs has been reported in breast cancer (Forrester *et al.*, 1990). Although several CYP2C have been reported in breast cancer studies, the frequency of *CYP2C9* protein expression as measured by western blotting ranged from 0% (Albin *et al.*, 1993) to 100% (Forrester *et al.*, 1990). However, a recent study reported weak *CYP2C9* protein expression in all tested tumor samples (Schmidt *et al.*, 2004). *CYP2C9* metabolizes estrone sulfate to 16-αhydroxy sulfate metabolite, often linked with increased breast cancer risk. 16α-hydroxy estradiol and 16α-hydroxy estrone are both metabolites that have a strong affinity for estrogen receptors which can lead to the activation of ER. Evidence suggests that there is significant involvement of 16-OH product, a product of *CYP2C9* metabolism, in the progression of breast carcinoma (Zhu *et al.*, 1998).

1.4.1.8. Phenotype of *CYP2C9* for Tamoxifen Metabolism

Among >30 variations of *CYP2C9,* *2 and *3 have been reported to be associated with a large yet substrate-dependent drop in intrinsic clearance (C. R. Lee *et al.*, 2002). The *3 allele has a greater effect than *2, with a decrease in enzyme activity of up to 90% for some specific drugs (King *et al.*, 2004).

Numerous studies fail to find any link between *CYP2C9* polymorphisms and tamoxifen pharmacokinetics (Jin *et al.*, 2005). A recent study suggested that *CYP2C9* is vital in the endoxifen formation by the metabolic transformation of tamoxifen into 4-hydroxytamoxifen, which is the ancestor of endoxifen (Mürdter *et al.*, 2011).

In contrast to *CYP2C9*1* (Wild type), expressed *CYP2C9*2* and *CYP2C9*3* variant proteins showed a reduction in the tamoxifen's intrinsic clearances to 4-hydroxytamoxifen. The study also indicated that this reduction would be greater in patients having *CYP2C9*3/*3* genotype than those with *CYP2C9*2/*2* or *CYP2C9*2/*3* genotypes (Coller *et al.*, 2002).

1.4.2. CYP2C19

CYP2C19 is the most clinically important form of the CYP2C subfamily. It is a 490 amino acid enzyme with a heme moiety at the active site. Neutral substrate molecules, or slightly alkaline molecules, bind. The substrates or drug molecules catalyzed by *CYP2C19* enzymes are termed *CYP2C19* substrates. Depending upon the reaction, the *CYP2C19* substrate can either act as an inhibitor or an inducer (D. F. Lewis, 1999). A vast category of medicines can be metabolized by *CYP2C19* enzymes, such as antidepressants like Citalopram, certain proton pump inhibitors (PPIs) like Omeprazole, antiepileptic drugs like Mephenytoin and Diazepam, antiplatelet drugs like clopidogrel, antimalarial drugs, e.g., proguanil and B-adrenoceptor blocker, e.g., propranolol (Sim *et al.*, 2006).

Chromosomal location for *CYP2C19* gene cluster at 10q24, spanning a region of 90kb with 9 exons (Riddell *et al.*, 1987). According to an FDA report in 2012, certain inhibitors are listed, but none is specific to the *CYP2C19* enzyme. *CYP2C19* enzyme is inhibited by several inhibitors that reduce the rate of *CYP2C19* substrate turnover. Two inhibition mechanisms are classified as reversible and irreversible (Bjornsson *et al.*, 2003; Yueh *et al.*, 2005). The probability of inhibition was restricted, as *in vitro*

experimental conditions lack true physiological cell state. Therefore, clinical evaluation of inhibition is a powerful approach when studies are held with context to clinical aspects. For *CYP2C19* inhibition in humans, Omeprazole might be regarded as a diagnostic probe. However, it is listed as a moderate inhibitor by FDA.

The *CYP2C19* gene encodes the *CYP2C19* enzyme with a vital role in estrogen catabolism, as it is responsible for 16 α-hydroxylation and 17 β-hydroxy dehydrogenation catalysis of estradiol (Cheng *et al.,* 2001). *CYP2C19* is vital in the tamoxifen metabolism during BC therapy, as it is involved in the 4-hydroxylation of tamoxifen (Crewe *et al.,* 2002) . Reduced *CYP2C19* activity via haploinsufficiency might be linked to the rise in BC risk, possibly through life-long greater estrogen (Tervasmäki *et al.,* 2014). The nature of *CYP2C19* is inductive. The increased intrinsic metabolic clearance is projected if the enzyme concentration or activity is increased in hepatocytes.

1.4.2.1. History of *CYP2C19* Polymorphism

In the late 1940's, S-Mephenytoin (racemic drug) was marketed as an anticonvulsant drug. Adrian Kupfer, a Ph.D. student at the university of Brene in the 1970s, started research on the enantiomer project and reported stereo-specific mephenytoin metabolism in dogs (Küpfer *et al.,* 1984). Kupfer and his co-worker discovered stereo specific metabolism of S-mephenytoin in humans. One of the healthy participants in his study suffered from severe sedation after intake a low dosage of Mephenytoin, whereas a normal response was shown by the other individuals. To find the reason, radiolabeled Mephenytoin was analyzed through urine. Results revealed a marked reduction in 4-hydroxy mephenytoin concentration in the patient's urine sample. These findings suspected polymorphism of mephenytoin hydroxylation (Küpfer *et al.,* 1984). A familial study involving 28 families unveiled that *CYP2C19* is strongly involved in this poor metabolism of (Inaba *et al.*, 1986). Later on, the immunoblot assay performed by Wrighton and his co-workers provided strong evidence supporting the poor metabolism of mephenytoin by *CYP2C19* enzymes (Wrighton *et al.*, 1993).

Due to *CYP2C19*'s role in converting S-mephenytoin to 4-OH mephenytoin, *CYP2C19* polymorphism is also called "S-mephenytoin polymorphism." Expression analysis of cDNA showed that all *CYP2C9* variants produced negative results toward S-mephenytoin. Goldstein and Wrighton's group separately identified *CYP2C19* as a key

enzyme accountable for the production of 4-OH Mephenytoin using the cDNA expression system of yeast and immunoblot analysis (De Morais *et al.*, 1994)

1.4.2.2. Clinical Important Allele Variants of *CYP2C19*

At present, 34 genetic *CYP2C19* variants have been catalogued (http://www.CYPalleles.ki.se). Most mutations are unique and have no clinical significance.

*CYP2C19*1*, a wild-type allele, has normal enzyme function and no SNP. *CYP2C19*2* genetic variant arises due to a single base alteration 681G>A, rs4244285 located in exon five. This mutation is responsible for the premature stop codon, which is twenty amino acids downstream of the amino acid at position 215. As a result, truncated or non-functional protein fails to conduct any function (De Morais *et al.*, 1994)

Exon 4 mutation 636G>A, rs4986893, produced premature stop codon and shortened inactive protein product, forming the *CYP2C19*3* allele variant (Morais *et al.*, 1994a).

*CYP2C19*4* is characterized by C>T, rs28399504 situated at the first base of exon 1, producing initiation codon, and hence no protein product is formed (Ferguson *et al.*, 1998)

*CYP2C19*5* shows a mutation 1297C>T, rs56337013 in exon 9. This mutation is responsible for $Arg^{433}Trp$ amino acid substitution. Hence, enzyme activity is reduced (Ibeanu *et al.*, 1999).

Exon 3 of *CYP2C19*6* has the mutation 395G>A. This mutation causes amino acid substitution $Arg^{132}Gln$. As a result of this mutation, protein structure and stability are affected (Ibeanu *et al.*, 1998)

*CYP2C19*7* mutation is caused by T>A inversion at the 5´ splice site, producing a splicing flaw in intron 5. Therefore protein synthesis is affected (Ibeanu *et al.*, 1998).

*CYP2C19*8* is associated with 358T>C mutation in exon 3. This mutation is responsible for amino acid $Try^{120}Arg$ substitution, causing enzyme activity to decrease (Ibeanu *et al.*, 1999).

*CYP2C19*17* variant has 2 linked mutations in total non-disjunction equilibrium at -806C>T and -3402C>T in the 5´ regulatory region. This mutation increases enzyme activity and expression (Sim *et al.*, 2006).

1.4.2.3. *CYP2C19* Genetic Polymorphism

CYP2C19 is a gene with high polymorphism. *CYP2C19* genetic variation and its molecular mechanism have been studied through various methodologies. An initial study on liver biopsy reported a reduced concentration of CYP450 isozyme in extensive and poor metabolizers (Meyer *et al.*, 1986). The first mutation in CYP2C19 was identified using liver samples, which were designated as *CYP2C19m1* (*CYP2C19*2*) using reverse transcription and mRNA amplification techniques. Then the second mutation was identified in the Japanese poor metabolizer (PM) subject, and it was designated as *CYP2C19*m2 (*CYP2C19*3*) (De Morais *et al.*, 1994).

In 2006, Sim and his co-worker found a novel *CYP2C19*17* allele as an ultrarapid metabolizer (UM) phenotype, which has tremendous *CYP2C19* activity by increasing *CYP2C19* gene expression.

1.4.2.4. Transcription Regulatory Mechanism of *CYP2C19*

CYP2C19 regulatory mechanism involves nuclear receptors ERα (NR3A1), which are activated after the binding of a ligand ((Mwinyi, 2010)), constitutive androstane receptor (CAR), Estrogen response element (ERE) (Wortham *et al.*, 2007) and pregnane X receptor-(PXR) (Tirona *et al.*, 2005). The functional activity of the constitutive androstane receptor response element (CAR-RE) in the CYP2C19 promoter region is shown. Binding of the ligand to this site is hindered (Chen *et al.*, 2003). In humans, rifampicin, a classic ligand that binds to PXR, is a strong inducer of *CYP2C19,* starts the transcription process, and synthesizes functional enzymes. As the level of induced enzyme activity is less than the induced mRNA level, the induced enzyme activity is concentration-dependent (Dixit *et al.*, 2007; Xing *et al.*, 2012). When compared with CYP3A4, the induction activity of CYP2C19 is moderate.

1.4.2.5. Phenotypes of *CYP2C19*

CYP2C19 is one of the main xenobiotics metabolizing enzymes in humans and is polymorphically expressed. Individuals are categorized into four types when polymorphism is implicated in drug metabolism.: normal or extensive, poor, intermediate, and ultra-rapid metabolizer for a specific drug; this classification is based on Phenotyping results. The phenotype of *CYP2C19* is measured by comparing the drug metabolites via urine or plasma samples. *CYP2C19* phenotypic evaluation is done by giving mephenytoin drug 150mg (Küpfer *et al.*, 1984) and omeprazole drug 20 mg.

FDA has recommended omeprazole for phenotypic evaluation (H. M. Gonzalez *et al.*, 2003).

Extensive Metabolizer (EM)

*CYP2C19*1/*1* allele is referred to as a normal or extensive metabolizer when both alleles are functional and produce 490 amino acid proteins. Phenotypic analysis by probe mephenytoin and omeprazole drug for *CYP2C19*1/*1* has shown the estimated concentration of metabolites in urine when the enzyme is fully functional (Chang *et al.*, 1995).

Intermediate Metabolizer (IM)

It is a phenotype in which one allele is not functional. In comparison, just one single allele produces a functional protein (Ingelman-Sundberg *et al.*, 2020), and the phenotypic expression of the protein is less than the EM. The possible allele combination for IM is *1/*2-*8; here, one allele is non-function (S. Scott *et al.*, 2011).

Poor Metabolizer (PM)

When both alleles are non-functional and produce truncated or null protein, such a phenotype is termed PM. Allele combinations, e.g., *2-*8/*2-*8, that show no or very reduced enzyme activity are included in this class. *CYP2C19*2* and *CYP2C19*3* are mainly regarded as poor metabolizers of drugs (H. M. Gonzalez *et al.*, 2003)

Ultra-rapid Metabolizer (UM)

It is a phenotype with more than one gene, or the enzyme function is enhanced due to a certain defect. *CYP2C19*17* is the only allele that is responsible for rapid xenobiotic metabolism. The allele combination *1/*17 plays a part as UM for some drugs (S. Scott *et al.*, 2011)

1.4.2.6. Inter-individual Variability

Inter-individual variations in the different drug metabolisms within the human body result from genetic polymorphism. Clinical data suggests that because of these widespread inter-individual differences, the TAM and its metabolites' amount in the plasma differ broadly among patients, affecting the therapeutic outcome. It can be due to CYP450 variable activity that may modify the pattern of TAM metabolism within the body causing variations in TAM and its active metabolites' systemic concentration (Desta *et al.*, 2004).

1.4.2.7. Interethnic Variability

Different ethnic groups show differences in the metabolism of *CYP2C19*. Mutations in a gene encoding a drug-metabolizing enzyme can produce enzyme variants with no activity, moderate activity, or even high activity. Frequency of UM i-e. *CYP2C19*17* variant was identified in different ethnic groups and is found to be 18% in the Ethiopian and Swedish populations. In comparison, 1.3% of its allele frequency is found in the Japanese and Chinese populations (Sim *et al.*, 2006). *CYP2C19*2* poor metabolizer allele frequency is relatively higher in Chinese, Korean, and Japanese populations (Roh *et al.*, 1996), but it is quite low in Arabs, African, and Caucasian ethnic groups (Persson *et al.*, 1996; H. Xie *et al.*, 1999) whereas, in Cuna Indians of Panama, frequency of poor metabolizer was so low that it could not be detected (Inaba *et al*., 1988). However, 79% of the inhabitants of the Pacific Ocean islands of Vanuatu are poor metabolizers (Kaneko *et al.*, 1997).

1.5. *CYP3A* Subgene Family

The CYP3A enzyme family consists of enzymes that have a part in metabolizing 50% of therapeutically used drugs and several xenobiotics. These also metabolize important endogenous compounds like retinoic acid, bile acids, and steroid hormones. The *CYP3A* family consists of four genes, i.e., *CYP3A43*, *CYP3A7*, *CYP3A5*, and *CYP3A4*. In addition, three pseudogenes are also present in the family, i.e., *CYP3AP1*, *CYP3AP2*, and *CYP3AP3*. All these genes are on human chromosome 7 at position q21-q22.1(Ingelman-Sundberg *et al.*, 2020). The CYP enzymes expression varies with the tissue type. In the fetal stage, the maximum expression of hepatic CYP3A7 and CYP3A4 is not present, while in adults, the case is vice versa, but some adults might have CYP3A7 expression (Saiz-Rodríguez *et al.*, 2020). CYP3A5 expression is the same at all stages of development. The three enzymes, CYP3A4, CYP3A5, and CYP3A7, share a comparable affinity for their respective substrates (X. Liu *et al.*, 2019). There is more than 70% homology present in amino acids sequences of the members of the CYP3A family, (Plant, 2007).

1.5.1. *CYP3A4*

The *CYP3A4* gene is present on human chromosome no. 7, having 27,592 base pairs and comprising 13 exons. CYP3A4 is known to involve the metabolism of therapeutic drugs and various xenobiotics; it also participates in the oxidation of various

endogenous substrates like fatty acids, steroids, and sex hormones. CYP3A4 involves in the conversion of testosterone into 15β-hydroxy testosterone, 2β-hydroxy testosterone, and 6β- hydroxy testosterone by oxidation reactions (Saiz-Rodríguez *et al.*, 2020)

CYP3A4 is the highly present hepatic protein of the CYP superfamily, constituting about 60% of total CYP enzymes in the liver. CYP3A4 is engaged in the metabolism of >120 different therapeutic drugs and is known to metabolize approximately 60% of currently used drugs (Danielson, 2002). Many compounds have molecular weight variations, i.e., cyclosporine (MW = 1203 Dal) to metyrapone (MW = 226 Dal), metabolized by CYP3A4. The enzyme can oxidize massive substrates because of a large binding cavity for a substrate such as statins, cyclosporine, macrolide antibiotics, and taxanes. CYP3A4 catalyzed reactions include about 7% O-dealkylation, 27% N-dealkylation, and 43% aromatic or aliphatic hydroxylation reactions (X.-Y. Zhou *et al.*, 2019). CYP3A4 also metabolizes various drugs with anticancer activity, like paclitaxel, docetaxel, tamoxifen, cyclophosphamide, vinblastine, and irinotecan (Bozina *et al.*, 2009). In addition to drugs, CYP3A4 is also known to metabolize numerous environmental and dietary chemicals like sterigmatocystin, pesticides, aflatoxin B1, G1, mycotoxins, PAH-diols, flavonoids, and a variety of food additives (Bozina *et al.*, 2009)

1.5.1.1. *CYP3A4* Substrates, Inducers, and Inhibitors

CYP3A4 protein comprises 502 amino acids having 57 kDa molecular weight (S.-J. Lee *et al.*, 2005). Many therapeutic medications, including calcium channel blockers, immunosuppressants, cancer chemotherapeutic agents, sedatives, antihistamines, and synthetic estrogens, are metabolized by the CYP3A4 enzyme. Certain endogenous steroids like cortisol, estradiol, and testosterone are metabolized by the CYP3A4 protein (X.-Y. Zhou *et al.*, 2019). There are a large number of CYP3A4 enzyme substrates, some of them are as follows (Li *et al.*, 1995)

Various chemicals (dietary and drugs) present as an inducer for CYP3A4 protein, such as rifampicin, glucocorticoids, and anticonvulsants like carbamazepine. The components of Hypericum perforatum, especially hyperforin, are known as potent *CYP3A4* enzyme inducers. In the inhibition of CYP3A4 protein, different chemicals and drugs are involved, such as the azole antifungal agents including ketoconazole,

inhibitors of HIV protease like saquinavir, macrolide antibiotics like troleandomycin and antidepressants like as fluoxetine. The probes that are used as a substrate for the study of CYP3A4 protein include erythromycin, midazolam, testosterone, and cortisol (Bozina *et al.*, 2009)

1.5.1.2. *CYP3A4* Transcriptional Regulation

The *CYP3A4* gene is placed on chromosome 7; its promoter region consists of basal transcription elements (from -35 to -50). AP-3 binding site, Estrogen Response Element (ERE), glucocorticoid response element, p53 binding motif (DNA sequence that acts as a binding site for p53 protein). HNF-4 (hepatocyte nuclear factor-4 element) and two HNF-5 are appeared in the 5' UTR (untranslated region). CYP3A4 protein formed is also known as P-450-PCNI, nifedipine oxidase, and NF-25 (Albertsen, 2005).

In *CYP3A4* gene regulation, various signaling pathways are involved. In constitutive transcription regulation, both regulators, i.e., negative, and positive, are involved, especially CCAAT/Enhancer Binding Protein (C/EBPα and C/EBPβ), USF, HNF1α, HNF3γ, and HNF4α. A range of xenobiotics and cis-acting modules accountable for inducible-transcriptional control of genes are the proximal PXR (Pregnane X Receptor) Responsive Element prPXRE, the Constitutive Liver Enhancer Module 4 (CLEM4) (-11.4 to -10.5 kb) and Xenobiotic-Responsive Enhancer Module (XREM) (-7.2 to -7.8 kb). The drug ligands bind, the xenosensors CAR (Constitutive Androstane Receptor) and PXR translocate to the nucleus, and then heterodimerization of Retinoid X Receptor (RXR) occurs and thus transcription enhanced to several folds. The ligand-dependent regulation of gene transcription is driven by the bile acid receptor FXR, glucocorticoid receptor, oxysterol receptor LXR, and the vitamin D receptor. PPARα is also involved in gene's constitutive and inducible transcriptional regulation (Zanger *et al.*, 2013)

1.5.1.3. *CYP3A4* Genetic Polymorphism

CYP3A4 enzyme exhibit variation in its activity in different individuals, leading to its unpredictable role in xenobiotic (especially drug) metabolism within a population. This variation arises from gene expression regulation, genetic polymorphism, and xenobiotics interactions with the enzyme. It was reported that genetic polymorphism is accountable for 90% of the variation in CYP3A4 activity (Sukprasong *et al.*, 2021). The variation in nucleotide sequence is termed polymorphism only if its frequency is ≥

0.01 in a population; otherwise (frequency is < 0.01), it is known as mutation (Albertsen, 2005). Genetic polymorphism is a persistent alteration in the nucleotide sequence at a particular gene locus. SNPs (single-nucleotide polymorphisms) are the most prevalent genetic variants in human cytochrome P450 genes. The nonsynonymous SNPs are present in the gene's coding region and are very important as these are involved in amino acid sequence change of the relative CYP protein. On average, 14.6 nonsynonymous SNPs exist for each cytochrome P450 gene (Sukprasong *et al.*, 2021). *CYP3A4* polymorphism is not only because of variation in the exonic region of the gene but also involves variation in the non-coding, i.e., intronic region. According to the Human Cytochrome P450 (CYP) Allele Nomenclature Database (http://www.cypalleles.ki.se), 19 subtypes of wild type *CYP3A4*, i.e., *CYP3A4*1* having 18 different SNPs but these do not affect the expression of mRNA and the complementary sequence of DNA. The SNPs in exonic regions are reported to be 24 (Werk *et al.*, 2014).

1.5.1.4. Allelic Variants of *CYP3A4*

The null allele for *CYP3A4* has not been reported yet, unlike other enzymes of the CYP family. Variations in the functions and level of protein due to genetic change in the intronic, exonic, or flanking region are possible. However, full-length mRNA has been found in all adult individuals studied until now (Lin *et al.*, 2019). Some of the important alleles of *CYP3A4* are mentioned in the Table 1.4 given below (Werk *et al.*, 2014) http://www.cypalleles.ki.se/cyp3a4.htm)

1.5.1.5. *CYP3A4* Allele Frequency

CYP3A4 shows a vast inter-individual variation in its activity, having a difference of about 40-fold. One of the most common and frequent variants is *CYP3A4*1B*. It was found in 1998 that the *CYP3A4*1B* variant is coupled with prostate cancer in relevance to its higher grade and stage. In the *CYP3A4*1B* variant, the point mutation, i.e., SNP (A392G), exists in a 5' flanking region, i.e., 392 base pairs upstream from the translational initiation site (Werk *et al.*, 2014). The *CYP3A4*1B* variant frequency was estimated as 53% in the African American population, 9% in the Caucasian population, and 0% in the Taiwanese population. The polymorphism is known to alter NFSE (Nifedipine Oxidase Specific Element), an important regulatory element in the transcription process and essential for *CYP3A4* normal expression. (Hsieh *et al.*, 2001; Seredina *et al.*, 2012; Wojnowski *et al.*, 2006; Zanger *et al.*, 2013).

*CYP3A4*2* allele encodes the protein that has kinetics depending on the substrate. It was found that *CYP3A4*2* frequency is 2.7% in Caucasians, but Chinese and blacks lack the variant (Hu *et al.*, 2017). The *CYP3A4** , *CYP3A4*5,* and *CYP3A4*6* frequency was determined to be 1.5, 0.98, and 0.5%, respectively, in the Chinese population (Hu *et al.*, 2017). In the Asian population, *CYP3A4*18* polymorphism is the most frequent. The frequency reported in the Chinese, Japanese, Korean, and Malaysian populations to be 0.008∼0.01, 0.013, 0.012∼0.017, and 0.021, respectively (J. S. Lee *et al.*, 2013). *CYP3A4*22* is known to have decreased enzyme activity and has been reported in the Caucasian population. The allelic frequencies of important *CYP3A4* variants in Caucasians, African and Asian populations are as follows (Vichi *et al.*, 2021):

Table 1.4: *CYP3A4* allelic frequencies variants in different populations.

CYP3A4 Allele	**Allelic frequency**		
Caucasians (%)	**Africans (%)**	**Asians (%)**	**Caucasian (%)**
*CYP3A4*1B*	5.5	76.2	0.0
*CYP3A4*2*	0.3	0.0	0.0
*CYP3A4*3*	1.6	0.0	0.0
*CYP3A4*4*	0.0	0.0	0.2
*CYP3A4*5*	0.0	0.0	0.3
*CYP3A4*7*	0.5	0.0	0.0
*CYP3A4*11*	0.1	0.2	0.0
*CYP3A4*12*	0.3	1.0	0.0
*CYP3A4*15*	0.0	1.6	0.0
*CYP3A4*16*	0.0	0.0	0.5
*CYP3A4*18*	0.0	0.0	1.7
*CYP3A4*19*	0.0	0.0	2.3
*CYP3A4*22*	5.3	0.0	0

1.5.1.6. *CYP3A4* and Breast Cancer

Estrogen hormones interact with the estrogen receptor (ER) and trigger cell proliferation, functioning as a cancerous cell growth development element. Cytochrome P450 members are critical in the metabolism of estrogen, anti-cancerous drugs, and carcinogens (Husbeck *et al.*, 2002). CYP3A4 contributes to the 4- and 16α-hydroxylation process of estrogen, especially estrone, most commonly present in post-menopausal women. CYP3A4 and CYP1A2 (catalyze 2-hydroxy estrone formation) play roles in determining 2-hydroxy estrone: 16α- hydroxy estrone ratios related to breast carcinoma (Keshava *et al.*, 2004).

The correlation between **CYP3A4** overexpression and breast cancer is also described in certain studies. In a study, the expression of mRNA of CYP family members was determined in normal and tumor tissues of breast cancer, and CYP3A4 mRNA expression was reported in 70% normal and 18% tumor tissues (Huang *et al.*, 2004). In China (Shanghai), a population-based study in a women subgroup determined the association of urinary cortisol ratios with breast carcinoma risk. It was found that urinary cortisol: 6β-hydroxy cortisol ratio was correlated with breast cancer risk, and this ratio is also the measure of enzymatic activity of CYP3A4. It has also been shown that breast cancer risk increases dose-dependently (Zheng *et al.*, 2001).

1.5.1.7. *CYP3A4* and Tamoxifen Metabolism

Tamoxifen, a pro-drug, is metabolized by CYP family proteins into more potent metabolites. These metabolites have different binding affinities for ER. *CYP3A4/5* and *CYP2D6* are the major enzymes implicated in these metabolic reactions. N-desmethyl tamoxifen is a primary and major metabolite formed by *CYP3A4/5* catalyzed reaction, and *CYP2D6* mediates the development of 4-OH-tamoxifen. In secondary metabolic reactions, these metabolites, i.e., N-desmethyl tamoxifen and 4-OH-Tamoxifen, are converted into 4-OH-N-desmethyl-tamoxifen (likewise called Endoxifen) by *CYP2D6* and *CYP3A4/5* respectively. Norendoxifen is also produced during metabolic reactions of tamoxifen by *CYP3A4/5* activity, inhibiting *CYP19A1*. The most important and active metabolites of tamoxifen are 4-OH-tamoxifen and endoxifen. They have similar binding affinity for ER, i.e., 33 times more than tamoxifen, but plasma endoxifen concentration is 5-10 times more than 4-OH-tamoxifen.

The other enzymes of the CYP family involved in tamoxifen metabolism are *CYP1A1*, 1B1, 2B6, 2C9, and 2C19 (Fig 5). The enzymes Sulfotransferases 1A1 (SULT1A1) and UDP glucuronosyltransferases (UGT) are responsible for the inactivation of tamoxifen and its metabolites. Certain xenobiotics induce or inhibit various CYP proteins (Singh *et al.*, 2011)**.**

1.5.2. *CYP3A5*

CYP3A5 is the CYP3A predominant extra-hepatic isoform expressed in the prostate, kidneys, breast, lungs, and small intestine besides the liver (J. K. Lamba *et al.*, 2002). CYP3A5 causes the cortisol to 6β-hydroxy cortisol metabolism in the kidneys, which is the Na^+ transport regulator in kidney epithelium and causes its retention. Thus any variation leading to increased CYP3A5 expression can lead to salt-sensitive hypertension (A. R. Khan *et al.*, 2020). CYP3A5 is also vital in the estrogen and 16α-hydroxy estrogen metabolism to 2 and 4- hydroxy estrogens in prostate and breast tissues. These metabolites, specifically 4- hydroxy estrogen, are carcinogenic and thus responsible for disease risk in these organs (J. K. Lamba *et al.*, 2002). A study by Kuehl et al. (2001) indicates that 50% hepatic content of CYP3A is contributed by CYP3A5 in individuals possessing both functional alleles of the gene (Kuehl *et al.*, 2001). CYP3A5 shows highly overlapping substrate specificity with CYP3A4, so it also metabolizes many CYP3A4 substrates in the liver. The metabolic activity of CYP3A5 is lower than CYP3A4 (Lolodi *et al.*, 2017). The *CYP3A5* gene is located at 7q22.1 on the negative strand of the long arm of chromosome 7. The gene is ~33 kb in length, has thirteen exons and twelve introns, and encodes a protein with 502 amino acid residues and a molecular mass of 57 kDa (Rodriguez-Antona *et al.*, 2022).

1.5.2.1. Transcriptional regulation of *CYP3A5*

All *CYP3A* genes are clustered on the chromosome, but their regulation is quite different. In order to analyze the transcriptional regulation for *CYP3A5* polymorphism, (Lolodi *et al.*, 2017) *CYP3A5* promoter was analyzed by (Jounaıdi *et al.*, 1996), and two mutations were found to be responsible for *CYP3A5* variations. However, later it was revealed through sequencing that these SNPs were present in *CYP3AP1*(pseudogene) promoter, which shows great homology with the *CYP3A5* gene, thus, not responsible for *CYP3A5* polymorphism (Kuehl *et al.*, 2001).

The transcription regulation of *CYP3A5* is different from *CYP3A4* in that no nuclear hormonal inducers or distant enhancers are involved in its regulation (Lolodi *et al.*, 2017). In *CYP3A5* transcriptional regulation, the CCAAT box and Basal Transcription Element (BTE) cis-elements are involved, located at the -90 to -40 region of the *CYP3A5* gene. Moreover, transcription factors implicated in transcriptional regulation are Nuclear Factor Y proteins (NF-Y) and Specificity-proteins (SP family), including SP1 and SP3 nuclear factors (Iwano *et al.*, 2001). Both these classes are constitutively active transcription factors and are the reason for the vast expression of CYP3A5 in different body tissues (Kolell *et al.*, 2002).

1.5.2.2. CYP3A5-mediated drug metabolism

More than half of clinically treated drugs are metabolized by the CYP3A subfamily, where CYP3A4 and CYP3A5 are predominant. These enzymes have a huge metabolic diversity because of their large active site, which can bind to several substrates, often more than one (Guo *et al.*, 2020). *CYP3A5* cDNA shows 90% sequence similarity with *CYP3A4*. Thus both share many substrates, inducers, and inhibitors (Y.-T. Liu *et al.*, 2007). Thus, it must be noted that it is uncertain whether the alteration in metabolism is because of *CYP3A5* alone or because both enzymes have equal efficiencies for *CYP3A* substrates metabolism and work competently together. However, some careful studies indicated a substantial function of *CYP3A5* in the metabolism of certain drugs; saquinavir for HIV-I infections treatment (Fröhlich *et al.*, 2004; Mouly *et al.*, 2005), quinine for malarial infections (Mirghani *et al.*, 2006) haloperidol and chlorpromazine for depression and mental illness, Tacroliums which is used after an organ transplant (Y.-T. Liu *et al.*, 2007).

1.5.2.3. CYP3A5 inter-individual and inter-ethnic variability

CYP3A5 was first discovered by Aoyama and Schuetz in 1989, who noted that CYP3A5 protein levels were not the same in every individual. Instead, some individuals express extremely high levels, while others show reduced or negligible levels (Huang *et al.*, 2004). Such observations suggest variable expression of CYP3A5. Inter-individual differences might be due to previous exposure to drugs, CYP inhibition by other drugs, first-pass effect after oral intake, dietary factors, or due to genetic mutations (J. S. Lee *et al.*, 2013; H.-G. Xie *et al.*, 2004). It was suggested that about 70-95% of inter-individual variability of CYP3A is due to genetic variations (Oleson *et al.*, 2010).

Further studies indicated that in addition to inter-individual variability, inter-ethnic differences in CYP3A5 expression are also present, which can be termed as 'Polymorphism.' Interethnic variability ranges from 6 to 99% in ethnic groups (Roy *et al.*, 2005). According to research, almost 10-25% of Europeans, 55- 95% of African Americans, and 30-50% of Asians and South Americans showed considerable levels of CYP3A5 protein in the liver (J. K. Lamba *et al.*, 2002). The CYP3A5 variant frequency, which causes normal metabolism, is 5% in Europeans, 27% in Chinese, 30% in Koreans, 29% in Japanese, and 73% in African-Americans (Hustert *et al.*, 2001). These variations are because of underlying genetic reasons on the one hand, while environmental factors and drug-drug interactions can also be the source of polymorphisms on the other hand (Roy *et al.*, 2005). Genetically, SNPs are responsible for formation of different alleles with variable phenotypes (Xie *et al*., 2004). About 25 allele variants of CYP3A5 have been recognized. Four alleles, *CYP3A5*1*, *CYP3A5*3*, *CYP3A5*6*, and *CYP3A5*7*, occur most commonly and are majorly accountable for CYP3A5 variable expression (Lamba *et al*., 2012). Environmental factors like selection pressure on alleles might also cause such variations, e.g., CYP3A5 genetic distribution in industrialized countries differs from that of developing countries (Roy *et al*., 2005).

1.5.2.4. *CYP3A5* Genetic Polymorphism

Genetic polymorphism is the manifestation of two or more alleles of the same gene with a minimum 1% frequency in a population. CYP3A5 shows a highly polymorphic expression than any other CYP3A isoform.

Consequently, it is accountable for inter-individual and inter-ethnic variation in CYP3A-mediated drug metabolism (Lee *et al*., 2003). There are about 25 allelic variants of CYP3A5 narrated by different researchers. Seven alleles are present in the exonic region of the gene: *CYP3A5*2*, *CYP3A5*7*, *CYP3A5*4*, *CYP3A5*6*, *CYP3A5*8*, *CYP3A5*9* and *CYP3A5*10*. In addition, some variants are also present in intronic regions, including *CYP3A5*3* and *CYP3A5*5* (Xie *et al*., 2004)

Positions of these alleles in the *CYP3A5* gene locus are shown in (**Figure 1.8)** *CYP3A5*1*, *CYP3A5*3*, *CYP3A5*6*, and *CYP3A5*7* occur most commonly and are chiefly responsible for all this inter-individual and inter-ethnic CYP3A5 polymorphism (Lamba *et al*., 2012).

*CYP3A5*1* is a fully operational wild-type allele, while the remaining three exhibit SNPs in their sequences and result in nonfunctional alleles.

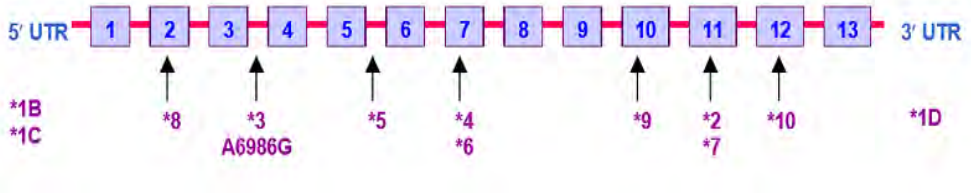

Figure 1.8. Localization of genetic polymorphisms in the human *CYP3A5* gene and Positions of these alleles in the *CYP3A5* gene locus (J. Lamba *et al.*, 2012)..

1.5.2.5. Clinically important variants of *CYP3A5*

To date, 25 CYP3A5 allelic variants have been stated (http://www.cypalleles.ki.se/cyp3a5.htm). Only *CY3A5*1* is functional, and the remaining are non-operational forms.

CYP3A5*1 is the only allele that forms a full-length functional CYP3A5 protein. It is a wild type of allele with no SNP.

CYP3A5*2 was identified by Jounaidi *et al.* in 1996 in Caucasian liver samples. *CYP3A5*2* shows a substitution mutation at 27289C>A, rs28365083 in exon 11, thus forming a non-functional protein.

CYP3A5*3 is the most prevalent non-functional allele with an SNP at 6986A>G, rs776746. The change from A to G occurred at this position, creating a cryptic splice site in intron three, ultimately leading to modified mRNA splicing and truncated protein formation. According to a study by (Kuehl *et al.*, 2001), individuals expressing *CYP3A5*3* allele showed a reduced level of CYP3A5 protein in the liver than those possessing wild type allele. *CYP3A5*3* allele is present in all ethnic groups with varying frequencies, and in many populations, it is more widespread than wild type allele (J. K. Lamba *et al.*, 2002). The *CYP3A5*3* frequency is 71-75% in East Asians, 59.8% in South Asians, 29-35% in Black Americans, 60-66% in Hispanics, and 92-94% in Caucasians (H.-G. Xie *et al.*, 2004).

CYP3A5*4 is an SNP in exon seven at 14665A>G, rs56411402 position. *CYP3A5*4* protein shows a non-expresser phenotype (Lim *et al.*, 2011) ***CYP3A5*5*** transitions from T→A at the 5'-end of exon number 5, causing frameshift and formation of non-functional protein (Chou *et al.*, 2001). *CYP3A5*6*, also a non-functional allele, shows SNP at 14690G>A, rs10264272 in exon 7, skipping this exon and changing the reading frame and truncated non-functional protein forms (J. Lamba *et al.*, 2012). *CYP3A5*6* allele is comparatively common in black individuals (7-17%) but rare in Asian and Caucasian populations. ***CYP3A5*7*** reflects an insertion of T nucleotide at exon 11 (rs41303343; 27125_27126insT). This insertion causes the formation of premature termination codons and immature, non-functional proteins (J. Lamba *et al.*, 2012). Like *CYP3A5*6*, *CYP3A5*7* is also comparatively frequent in Black Population (5-12%) and comparatively rare in Asian and American populations (H.-G. Xie *et al.*, 2004). ***CYP3A5*8*** is a substitution at 3699C>T, rs55817950 in exon 2, triggering an amino acid shift at codon 28 and non-functional protein formation (S.-J. Lee *et al.*, 2003) ***CYP3A5*9*** results from SNP at 19386G>A, rs28383479 in exon 10 triggering amino acid shift and non-functional protein (H.-G. Xie *et al.*, 2004) ***CYP3A5*10*** shows a point mutation in exon 12 at 29753T>C, rs41279854 resulting in an amino acid change (Oleson *et al.*, 2010)

1.5.2.6. Phenotypes of *CYP3A5*

CYP3A5 is important in drug metabolism and has about 25 allelic variants. When genetic polymorphism is implicated in drug metabolism, individuals are categorized as normal or extensive poor, intermediate, and ultra-rapid metabolizers according to the phenotype of their expression polymorphism. The phenotype of *CYP3A5* is measured by comparing the polymorphism with drug metabolite concentration in blood serum and urine. The phenotypic evaluation was done for *CYP3A5* polymorphism in different studies.

Extensive Metabolizer (EM)

*CYP3A5*1/*1* variant is an extensive or normal metabolizer, where both alleles are functional and yield full-length CYP3A5 protein. Individuals with the CYP3A5*1/*1 genotype were observed to have higher Tacrolimus drug clearance than any other genotype (Passey *et al.*, 2011).

Intermediate Metabolizer (IM)

Intermediate metabolizer phenotype refers to the heterozygous *CYP3A5* genotype, where one allele is functional, i.e., *CYP3A5*1,* and the other allele is non-functional, e.g., *CYP3A5*3, *6, *7* (Ingelman-Sundberg *et al.*, 2020).

Poor Metabolizer (PM)

When both alleles are non-functional and create truncated or null protein, such phenotype is called Poor Metabolizer (PM) phenotype. Allelic combinations for PM are *CYP3A5*2/CYP3A5*2-*10*, which all show very reduced or no enzymatic activity (Floyd *et al.*, 2003). Among them, *CYP3A5*3* non-functional allele is very common.

1.5.2.7. Role of CYP3A5 in Tamoxifen Metabolism

Among all the CYP enzymes, CYP3A4/5 and CYP2D6 play prominent roles in Tamoxifen metabolism. CYP3A5 is significant in tamoxifen conversion to its primary metabolite, N-desmethyl tamoxifen, and in the metabolism of 4-OH TAM to a secondary metabolite, endoxifen (Charoenchokthavee *et al.*, 2016). Polymorphism in the *CYP3A5* genotype accounts for variability in concentrations of Tamoxifen metabolites individually and inter-ethnically, thus affecting drug response (Charoenchokthavee *et al.*, 2017). Only *CYP3A5*1* is a functional allele, and the individual possessing both alleles is a normal or extensive metabolizer of tamoxifen. All other *CYP3A5* allele variants, like *CYP3A5*3* and **6*, are non-functional and refer to poor metabolizer phenotype (J. Lamba *et al.*, 2012).

1.6. *CYP2D Subfamily*

The subfamily of cytochrome P450 called *CYP2D* comprises three genes: an active or functional gene, CYP2D6 , and two pseudogenes, *CYP2D7P* and *CYP2D8P* (Yasukochi *et al.*, 2011)

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1.6.1. *CYP2D6*

It is present on the long arm of chromosome 22 in band 13 and sub-band 1 (González *et al.*, 2008)). Among all other liver enzymes, the percentage of CYP2D6 is 1-4%. This enzyme hydrolyzes about 25% of drugs, making it the most well-known (Owen *et al.*, 2009). The gene comprises 9 exons, traversing almost 4400 nucleotides (Yang *et al.*, 2017). *CYP2D6* is an enzyme consisting of 497 amino acids or is a CYP2D family member of the superfamily cytochrome P450 for which the highly polymorphic gene called CYP2D6 is positioned on the q arm of chromosome 22 at position 13.1 in a region of 45kb near to the two pseudogenes (*CYP2D7P* and *CYP2D8P*) having similar nucleotide sequences to that of *CYP2D6*. This gene consists of 12 exons in the liver, central nervous system, and small intestine (Kimura *et al.*, 1989; Zanger *et al.*, 2004)

It is among the widely studied CYPs due to its clinical importance. *CYP2D6* has elaborated in the metabolism and removal of about 25% drugs presently available in clinics and phase 1 reactions of xenobiotics. *CYP2D6* is also known as xenobiotic or microsomal monooxygenase and debrisoquine 4-hydroxylase. Moreover, it can also be represented as *CYP2D, CYD6, CYP2DL1, CYP2D7P2, CYP2D7BP, CYP2D7AP, P450-DB1, P450C2D, CYP2D8P2* and *CYP11D6* (Gopisankar, 2017).

1.6.1.1. Polymorphism and Phenotypes of *CYP2D6*

CYPs have 18 families, 43 subfamilies, 57 active, and 58 pseudogenes in humans. The 18 families are represented as *CYP1, CYP2*, 3, 4, 5, 7, 8, 11, 17, 19, 20, 21, 24, 26, 27, 39, 46, and 51, out of which 2, 3 and 4 have more genes than remaining. The 57 active genes are classified on a functional basis into two types denoted by D, and B-types called Detoxification type (35 out of 57 genes belong to this type) and Biosynthesis type (22 genes lie in this category), having more conserved sequences than D-type. Among 18 families, only four, 1, 2, 3, and 4 belong to D and the remaining to B-type (Katsumura *et al.*, 2014; Song *et al.*, 2015). The vastly polymorphic genes 2D6, 2C9, and 2C19 together perform 40% of the oxidative metabolism of the drug (Sadee, 2011). Until now, highly polymorphic 2D6 genes possess >100 variants comprising duplications, SNPs, frameshift mutations, or deletions/insertions of the gene (S.-F. Zhou, 2009). These variants have different frequencies and influence drug response in different groups. Alleles are classified into 4 types on the rate of metabolism of 2D6 substrates basis termed as extensive metabolizer or EM alleles, poor metabolizer (PM)

or null alleles, intermediate metabolizer or IM alleles, and ultrarapid metabolizer or UM alleles (Bradford, 2002; Gaedigk *et al.*, 2007; Ingelman-Sundberg, 2005). 2D6 is the only CYP with copy number variants (CNVs) represented by CYP2D6*(variant) XN reported for, while XN is for no copies of the gene (Lam, 2019).

As a result of 2D6 polymorphism, there exist four phenotypes differing from each other based on the number of active alleles as individuals with PM phenotype have no active 2D6 alleles but rather possess both inactive alleles and have either no production of 2D6 enzyme or kind of 2D6 enzyme produced with no activity. In contrast, those having either one wild type and other inactive allele or two active alleles with decreased function or an allele with decreased activity along with inactive allele are IMs, and EMs have two wild type alleles. UMs contain multiple copies of the 2D6 gene due to duplication or multiplication of the gene (Gaedigk *et al.*, 1991). Table 1.5 shows the 2D6 classification ((Gopisankar, 2017; Lam, 2019).

Table 1.5: Classification of *CYP2D6* Alleles

Types	***CYP2D6* alleles Names**
Null/PM alleles	*3 *4 *5 *6 *7 *8 *11 *12 *13 *14 *15 *16 *18 *19 *20 *21 *38 *40 *42 *44 *48 *56 *62 *68 *92 *10 *101
Partial function/IM alleles	*10 *14 *17 *18 *36 *41 *47 *49 *50 *51 *52 *54 *55 *57 *59 *69 *72
Normal function/EM alleles	*2A, *17 x 2, *27, *35, *39, *41 x 2, *48
Increased function/UM alleles	*****1 x N > 2, *2 x N > 2

1.6.1.2. Prevalence of *CYP2D6* Phenotypes

The frequency of the 2D6 phenotypes gene vastly differs concerning different racial groups. At the same time, on a broad spectrum, a larger proportion of the population (nearly 72-80%) consists of EM phenotype, and the remaining is composed of nearly 1-20% PM phenotype along with UM or increased function phenotype by only 1-10% proportion. EM phenotype is more common than PM and UM phenotypes in the general population, both of which differ in various ethnic groups by their frequency (Dean, 2012).

According to a study by Alva´n et al., 1990, 5-10% of Caucasians have PM phenotype, 3-5% have UM phenotype, while a larger proportion of Caucasians with EM phenotype and 10-17% are IMs. On the other hand, PM and UM phenotypes were also observed in the populations like PM up to 19% in African Americans, about 1% in Chinese and 0.6% South Indians, and the UM phenotype in black Ethiopians up to 16% while up to 2% in Swedish Caucasians (Naveen *et al.*, 2006). In short, UM phenotype is commonly observed in Oceania and North America, the PM phenotype mostly in Europe, and the IM phenotype in Asia (Gopisankar, 2017).

Table 1.6: Different variants of CYP2D6 with their characteristics

***CYP2D6* Alleles**	**Allele Designation**	**Enzyme Activity**
*1, *2, *33, *35	Normal (C, C)	Normal
*3-*8, *11-*16, *18-*21, *36, *38, *40, *42, *44, *56, *62.	Null (T, T)	Negligible
*9, *10, *17, *29, *41, *59	Reduced activity (C, T)	Decreased
Duplicated Alleles	**Allele multiplication**	**Enzyme activity**
*1xN, *2xN, *35xN	Normal (C, C)	Increased
*10xN, *17xN, *29xN, 41xN	Reduced function (C, T)	Decreased
*4xN, *6xN, *36xN	Null alleles (T, T)	Negligible

A major biological factor in the *CYP2D6* polymorphism is ethnicity. Previous studies reported that the *CYP2D6*10* allelic variant is common in almost 50% of the Asian population, while in the Caucasian population, it is less prevalent (3%)(Chin *et al.*, 2016; Yin *et al.*, 2012). In *CYP2D6*10*, the enzyme's function is greatly reduced, as is the concentration of active metabolites of Tamoxifen in the blood ((Areepium *et al.*, 2013; Lan *et al.*, 2018).

1.6.1.3. *CYP2D6*6* and Breast Cancer

*CYP2D6*6* is one of the PM or defective alleles of a gene (2D6) that codes for the 2D6*6 enzyme with no function and so has poorer or no contribution in tamoxifen metabolism in case of breast cancer. It is mostly found in European Caucasians and two other PM alleles, such as 2D6*3 and 2D6*4. 2D6*6 is identified by deleting the single nucleotide T in the third exon at position 1707 (1707delT) near the deletion site, such as CAG.TGG.GTG, a stop codon represented by red (CAG.GGG.TGA), is formed due to the deletion of T located at the 1707 nucleotide position (Bradford, 2002) . Subtypes of 2D6*6 are given in the following Table 1.7) (Rasmussen *et al.*, 2011).

Table 1.7: Subtypes of *CYP2D6*6* Variant

Subtype	Similarity	Difference (Change in Nucleotide)
2D6*6A	1707delT	
2D6*6B	1707delT	Having G>A at position 1976
2D6*6C	1707delT	G>A at 1976 and G>C at 4180
2D6*6D	1707delT	G>A at position 3288
Novel Variant	1707delT	A>G at 1749, CT>AG at 1754-1755 and G>A at 1976

Table 1.8: Distribution of different CYP2D6 allelic variants in different populations

Population	Most recurrent alleles
Africans and African Americans	*CYP2D6*5/*17*[a]
Alaska Natives	*CYP2D6*1*[b]
American Indians	*CYP2D6*1*[b]
Caucasians	*CYP2D6*4*[c]
Chinese	*CYP2D6*10*[a,d]
Malaysian Malay	*CYP2D6*10*[a]
Malaysian Indians	*CYP2D6*4/*10*[a]

(**a: (Chin *et al.*, 2016) b: (B. A. Khan *et al.*, 2018) c: (Moyer *et al.*, 2011) d: (Lan *et al.*, 2018).**

1.6.1.4. Role of CYP2D6 Genetic Polymorphism in Tamoxifen Metabolism and Breast Cancer

Various studies have been done to find the correlation between *CYP2D6* polymorphism and the metabolism of TAM. *CYP2D6*4* and *CYP2D6*10* are the most common *CYP2D6* allelic variants, and their prevalence was found to be associated with the prevalence of BC (Thota *et al.*, 2018). In other studies, it was shown that *CYP2D6* polymorphism (most commonly *5 and *10) has an impact on plasma endoxifen level (active metabolite) and TAM/endo metabolic ratio (B. A. Khan *et al.*, 2018; Lim *et al.*, 2011).

Other studies demonstrated that *CYP2D6*10/*10* (homozygous) and other *CYP2D6* heterozygous null alleles are associated with a low endoxifen level, corresponding to lower TAM hydroxylation. Thus, ethnic groups carrying PM have an improved risk of BC relapse (Motamedi *et al.*, 2012; Teh *et al.*, 2012). Zembutsu *et al.*, in 2017, carried out a study on this topic by identifying Ki-67 expression as a clinical response to TAM therapy. He found that patients having a wild-type genotype (or a minimum of one wild-type allele) underwent higher Ki-67 expression than homozygous mutant alleles (Zembutsu *et al.*, 2017).

Table 1.9: CYP2D6 polymorphism role in breast cancer and therapeutic recommendations accordingly

CYP2D6 Metabolizer Status	Activity Score	Plasma Endoxifen Concentration/Implications	Therapeutic Recommendations
Ultrarapid **Normal**	>2.0 1.5-2.0	Normal therapeutic Endoxifen level	Strong recommendation to start treatment with 20mg TAM/day (Standard dose) and avoid any CYP2D inhibitor.[a]
Intermediate	0.5-1.0	Lower Endo level/ increased Risk of BC relapse	Post-menopausal women are moderately recommended to take estrogen inhibitors (AI), while premenopausal women should carry ovarian function suppression treatment.[b] Another option is to use a high dose of 40mg TAM/day (FDA approved)[c] if AI use is contraindicated.
Poor	0	Very low to none Endo Conc/ higher chances of BC recurrence.	AI therapy and ovarian function suppression therapy are strongly recommended for premenopausal women, but AI alone is needed in the case of postmenopausal women.[b] If AI use is contraindicated, TAM (40mg/day)[c] can be used, but it is noted that this concentration increases but cannot normalize endo concentration.[c]

a: (Horn *et al.*, 2017) **b:**(Group, 2015); (Pagani *et al.*, 2014) **c:** (Hertz *et al.*, 2016)

Chapter 2

2. Materials and Methods

2.1. Study Population

The sample size was assessed with the formula provided {$n = Z_{\alpha/2}^2 pq / (MOE)^2$} using the breast cancer frequency from literature as 12–15%. Therefore, applying an estimated population size of 15% with a 5% error margin, the sample size was 195 (S. S. Malik *et al.*, 2020), where Zα/2 is a statistical constant, p is prevalence, and MOE is the error margin relative precision. After taking patients' and controls' written informed consent, sampling was carried out. This study enrolled 430 subjects (425 female and five male patients). Clinically diagnosed ER-positive breast cancer patients at any disease stage and taking tamoxifen as adjuvant therapy with a day-to-day dosage of 20 mg for at least three months prior to sampling were included in this study.

Demographic characteristics, including age, race, weight, marital status, family history, and smoking, were compiled using a specially designed questionnaire and are shown in Table 2.1. In addition, we recruited patients' reports and hospital records or clinical information such as surgery, chemotherapy, radiotherapy, tumor size, and cancer stage. Patients with liver, kidney, heart, or neurological disorders or those diagnosed with diabetes mellitus or on any other medication except tamoxifen for the last seven days were excluded from the study. To compare the genotyping results with the control population, 410 age and gender-matched healthy individuals from the same cities and socioeconomic backgrounds were also enrolled and are shown in Table 2.2.

Study I Investigated the allelic frequency of CYP*2C9*2* and *CYP2C9*3* variants and their association with tamoxifen and metabolites' metabolism among ER-positive breast cancer patients.

Study II Genotypic allelic frequencies of *CYP2C19*2, CYP2C19*3,* and *CYP2C19*17* were determined, and their impact on the metabolism of tamoxifen and its metabolites among Pakistani ER Breast Cancer Patients.

Study III Impact of *CYP3A4* polymorphism on tamoxifen metabolism in the ER positive breast cancer patients.

Study IV Role of CYP3A5 variants was determined in tamoxifen metabolism in ER-Positive breast cancer patients.

Study V *In vitro* simulation of tamoxifen therapy in breast cancer patients with distinct CYP2C19 genotypes.

Table 2.1. Percentage and frequency of demographic characters of ER Positive BC patients.

Demographic Characters		Percentage (%)
Age	**20-35**	15.11
	36-50	40.69
	51-65	32.55
	66-80	11. 62
Ethnic Groups	**Punjabi**	57.2
	Saraiki	2.55
	Pushtoon	16.04
	Sindhi	10.46
	Kashmiri	8.6
	Hazarvi	1.16
	Hindkoh	3.95
Marital	**Married**	91.16
Status	**Un-married**	4.41
	Divorced	2.79
	Widow	1.62
Weight	**Under-weight**	24.18
	Normal weight	52.32
	Obese	23.48
Educational Status	**Illiterate**	82.79
	Primary	1.62
	Middle	3.02
	Matric	6.04
	Inter-mediate	2.55
	Graduate	3.95
Life Style	**House Wives**	92.7
	Professional	7.2
Family History	**Sporadic**	80
	Familial	20
Smoking	**non-smokers**	92.5
	smokers	7.44
Stage Of Breast	**Stage 1**	5.34
Cancer	**Stage 2**	50.93
	Stage 3	31.86
	Stage 4	11.86
Menopausal	**Pre-menopause**	49.3
Status	**Peri-menopause**	10.01
	Post-menopause	40.69

Table 2.2. Percentage and frequency of demographic characters of Healthy individuals.

Demographic Characters		Percentage (%)
Age	**20-35**	17.07
	36-50	52.19
	51-65	26.09
	66-80	4.63
Ethnic Groups	**Brahui**	19.51%
	Kashmiri	12.20%
	Pashtoon	19.02%
	Punjabi	17.07%
	Saraiki	15.12%
	Sindhi	17.07%
Marital Status	**Married**	64.39
	Un-married	30.24
	Divorced	4.63
	Widow	0.73
Weight	**Under-weight**	10.48
	Normal weight	78.78
	Obese	10.73
Educational Status	**Illiterate**	36.09
	Primary	11.21
	Middle	20.97
	Matric	17.07
	Inter-mediate	6.09
	Graduate	8.533
Life Style	**House Wives**	65.83
	Professional	34.14

2.2. Ethical Approval

The Bioethical Committee (BEC) of Quaid-i-Azam University Islamabad accepted the current study, as shown by protocol number BEC-FBS-QAU-40. Samples were collected from Sir Ganga Ram Hospital (SGRH), Lahore, Pakistan; Institute of Radiotherapy and Nuclear Medicine, Peshawar; Centre for Nuclear Medicine and Radiotherapy (CENAR), Quetta; and Nuclear Oncology Research Institute (NORI), Islamabad, Pakistan.

2.2. Sample Collection

Blood and Plasma samples of ER-positive breast cancer patients taking tamoxifen monotherapy were collected in vacutainers Syringe (BD 0.6mm × 25mm 23G × 1, Becton Dickinson, Pakistan). The sampling criteria were that the patient had undergone surgery, chemotherapy, and radiotherapy before treatment with adjuvant tamoxifen taken as monotherapy for at least 3 months at 20 mg daily. Samples were taken 48 h after the last dosage taken by the patients and stored at 4 ºC till DNA and Plasma extraction. An equal number of healthy individuals were enrolled in this study as a control, and 3 mL of blood was collected in a vacationers Syringe (BD 0.6mm × 25mm 23G × 1, Becton Dickinson, Pakistan). The participants filled out the consent form and were informed about the study.

2.2.1. DNA Extraction from Whole Blood

DNA was extracted by the procedure described by Sambrook *et al.*, 1989. The blood sample (5 mL) was vortexed (VWR Scientific; vortex Genie 2TM) and transferred to the labeled 5 mL falcon tube (50 mL polypropylene conical tube 30 × 115, BD Franklin Lakes, NJ. USA). Lysis buffer (3 times the blood volume) was added to the blood sample into a falcon tube and placed on ice for 30 min to assist the red blood cells lysis. After incubation, the mixture was centrifuged (MSe: Mistrex 3000i, centrifuge) at 1200 rpm for 10 min at 4 ºC. The supernatant was discarded, and the pellet was resuspended by tapping the tube. Again 10 mL of lysis buffer was put into the sample and centrifuged at 1200 rpm (MSe: Mistrex 3000i, centrifuge) at 4 ºC for 10 min. The supernatant was eliminated, and the pellet was resuspended correctly. Briefly,4.75 mL of STE buffer was put into the pellet, adding 10% SDS while vortexed (VWR Scientific; vortex Genie 2TM). Briefly,10 uL of Proteinase K (20 mg/mL, broad-spectrum serine proteinase) was added to the sample and incubated in a water bath at 55 ºC overnight. On the following day, to get rid of proteins, DNA was extracted with equal volume (5 mL) of equilibrated phenol (8.0 pH) and shaken for 10 min by inverting 50 mL falcon tube (30 × 115, BD Franklin Lakes, NJ. USA) and put on ice for 10 min. After centrifugation at 3200 rpm, the aqueous layer was separated by cut tips to another labeled 15 mL falcon tube (8400 × G Maximum RCF, Biologix. USA).

To this aqueous solution, 5 mL of chilled Chloroform-Isoamyl alcohol (24:1) was added, mixed for 10 min, and placed on ice for 10 min. The sample was centrifuged (MSe: Mistrex 3000i, centrifuge) at 3200 rpm for 30 min at 4 ºC, and the aqueous layer was separated by cut tips into labeled 15 mL falcon tube (8400 × G Maximum RCF, Biologix. USA).

To this aqueous layer, 10 uL of RNase (10 mg/ml) was added and incubated in a shaker water bath (US Patent No. DES 288; 600) at 37 ºC for 2 h; optimum for enzyme action. After incubation, 250 uL of 10% SDS and 10 uL of proteinase-K (20 mg/mL) were added and incubated in a shaker water bath at (US Patent No. DES 288; 600) 55ºC for 1 hour. After this, 5 mL of equilibrated Phenol (8.0 pH) was added to the sample, shaken for 10 min, placed on the ice for 10 min, and later centrifuged at 3200 rpm for 30 min at 4 ºC. After centrifugation, the upper aqueous layer was separated into another labeled 15 mL falcon tube. To this aqueous layer, 5 mL of chilled chloroform-isoamyl alcohol (24:1) was added, shaken for 10 min, placed on ice for 10 min, and afterwards centrifuged (MSe: Mistrex 3000i) at 3200 rpm for 30 min at 4 ºC. Then upper aqueous layer was separated by cut tips into separate labeled 15 mL falcon tube (8400 × G Maximum RCF, Biologix. USA). Briefly, 500 uL of Ammonium acetate and 3 mL of chilled isopropanol were added and mixed to this aqueous layer until DNA was precipitated into visible white threads by inverting the falcon tube many times and then placed overnight at -20 ºC.

The sample was centrifuged at 3200 rpm for 60 min at 4 ºC to deposit the precipitated DNA at the base of a 15 mL falcon tube (8400 × G Maximum RCF, Biologix. USA). The pellet was resuspended, and the supernatant was removed. For washing, 5 mL of 70% ethanol was added and centrifuged at 3200 rpm for 40 min at 4 ºC. After centrifugation supernatant was discarded. The pellet was dried by inverting a falcon tube on tissue paper in a laminar flow hood for 3 h. When the pellet was completely dried, 300 uL of Tris-HCl (pH 8.0) was added and incubated at 55 ºC in a water bath shaker. Tapping and incubation were repeated periodically in a day so that DNA dissolved efficiently. The optical density of the DNA sample was taken by Nanodrop (2000c; Thermo Scientific, USA) at 260 and 280 nm. Now DNA sample was shifted to a labeled Eppendorf and stored at 4 ºC. This method will take 2 days if step (xiii) is modified by keeping the sample for 15 min at 70 ºC.

2.2.2. Quantitative Analysis of Extracted DNA

Using Nanodrop technology (2000c; Thermo Scientific, USA), the DNA concentration was determined. The extracted DNA's absorbance was measured at both 260 and 280 nm. The O.D. of genomic DNA less than 2 and around 1.8 are considered good quality. The DNA dilutions of 100 ng/µl were made for a polymerase chain reaction. The PCR result was tested on a 2% agarose gel after DNA amplification to confirm the patients' genotype. Theoretically, the phenotype was deduced from the genotype.

2.2.3. Amplified product Analysis by Gel Electrophoresis

The amplified product was checked by resolving on 2% agarose gel. By suspending 7 g of agarose into 350 mL of 0.5 TAE buffer, 2% agarose gel was prepared. Then the solution was heated in a microwave oven until (approx. 7-10 min) a clear solution was obtained, then 5 µL of Ethidium Bromide was added to this solution. The solution was kept for cooling down in a water bath at 55 ºC for 15 min. The solution was then poured into a gel casting tray, three combs of 40 wells were adjusted, and the gel was left to solidify. After polymerizing the gel, the combs were removed delicately without disturbing the wells. The gel was cut down according to the required wells and transferred to a gel tank (Maxi cell: EC 360M, USA) containing 0.5 TAE buffer.

After amplification, 5 µL of the Orange G dye containing 0.125% Orange G, 20% Ficol, and 100 Mm EDTA was put into each sample. The samples were then loaded on the 2% agarose gel. The 100 bp ladder (Fermentas cat# SM0403) was also stuffed into a well to locate the fragment size of the PCR product. The electrophoresis was conducted at 120 volts for thirty 35 min. The gel was then placed on the Gel Doc system (UVITEC, Cambridge UK) to visualize gel bands of PCR product, and an image was taken.

2.2.4. Reagent Preparation for DNA Isolation and Gel Electrophoresis

a. Cell-lysis buffer (1L)

Briefly, 1 g potassium bicarbonate and 8.29 g ammonium chloride were dissolved in deionized H_2O. A 200 µL of 0.5M EDTA was added, and pH was adjusted by combining 1M HCL and 1M NaOH. The solution was centrifuged at 2500 rpm for 10 min, autoclaved, and stored at room temperature.

b. 1M Tris (1L, pH 8.0)

Approximately 121.1g of Tris Base was added to 800 mL of water, and the pH was corrected to 8.0 by adding concentrated HCl. The volume was brought to 1L by adding deionized water. The solution was filtered through 0.4 µM filter paper and stored at room temperature.

c. 0.5M EDTA (1L, pH 8.0)

A total of 186.15g of EDTA was mixed in 700 mL of deionized water, and pH 8.0 was maintained by adding 4M NaOH. The final volume of 1L was made by adding more deionized water autoclaved and saved at room temperature.

d. TE (Tris EDTA) (1L, pH 8.0)

Briefly, 33.3 mL (100 mM) of 3M NaCl was mixed with 50 mL (50 mM) of 1M Tris (pH 8.0) and 2 mL (1 mL) of 0.5 M EDTA (pH 8.0). The ultimate volume of 1L was achieved by adding deionized water and storing it at room temperature.

e. 10% SDS Solution (250 mL)

A total of 25 g of SDS was carefully weighed at a weighing balance, and 150 mL of deionized water was added. The solution was allowed to stir at a low speed to avoid the formation of foam. The volume was up to 250 mL, filtered through filtration assembly, and stored at room temperature.

f. Chloroform-Isoamyl alcohol (24:1, 500 mL)

A total of 480 mL of Chloroform and 20 mL of Isoamyl-alcohol were mixed at the ratio of 24:1 and stored at 40 ºC.

g. Iso Propanol

Isopropanol was transferred from stock in another bottle, wrapped with aluminum foil, and placed in the refrigerator for future use.

h. Equilibrated Phenol

Usually, distilled phenol is kept at -20°C. When needed, it is melted in a 55 °C water bath (The phenol's melting temperature is 42 °C). To 1 kg melted phenol, 1g of 8-hydroxyquinoline (0.1%) and an equal volume of 1 M Tris were added and mixed by shaking in a fume hood for 2-3 min. The bottle was left in the hood for shaking on a shaker for 30 min. The bottle was then kept stationary in the hood for a few minutes to separate the Tris and the phenol layer; the upper Tris layer was removed. 0.1M Tris was added, and the bottle was stirred for 2-3 min, left still for a few minutes, and removed from the upper Tris layer. The procedure was repeated until the pH was stable at 8.0. Finally, 100 mL of 0.1M Tris-HCl containing 0.2% β- mercaptoethanol (2 mL) was included and stored at 4 °C.

i. 70% Ethanol (chilled, 500 mL)

500 ml of 70% ethanol was prepared by mixing 350ml of absolute ethanol with
150 mL deionized water and stored at -20 °C.

j. Ammonium Acetate (1L)

By suspending 770 g of ammonium acetate in 800 mL of deionized water, a 1 L solution with a concentration of 10 M was produced. The total volume of 1L was made up by adding dH2O and sterilized by filtration through a 0.22 µm filter. The solution was in a sealed bottle in a freezer at 4°C.

k. 0.5X TAE buffer

A total of 5 L 0f 10X TAE buffer was made by adding 242 g Tris base, 57.1 glacial acetic acid, and 100 mL of EDTA, and a final volume of up to 5 L was made up by adding dH2O. This 10XTAE buffer was diluted to 0.5XTAE buffer, which was used for the gel electrophoresis.

l. Loading dye

A total of 25 mg bromophenol blue, 25 mg xylene cyanol, and 4 g sucrose were into deionized water, making a total volume of 10 mL, and used as tracking dye. Bromophenol blue always moves forward to xylene cyanol as its size is smaller.

m. Ethidium Bromide

Dissolve 400 mg into 20 mL of deionized water, and 5 µL was used to locate DNA bands on the gel; EtBr molecules intercalate with DNA and glow under UV rays.

2.3. Primer Design

Reference SNP-4244285, SNP-12248560, SNP-1799853, and SNP-1057910 sequences were taken from the National Centre of Biotechnology Information (NCBI). Primers were created using the primer designing software Primer3 (Rozen and Skaletsky, 2000). The sequence of primers used is mentioned in **Table 2.3**.

Table 2.3: Primers for CYP2C9(*2,*3), CYP2C19(*2,*3 &*17), CYP2D6(*6,*10),CYP3A4(*22), CYP3A5(*3,*6) Genotyping.

No	SNP ID	mer ID	Sequence (5′-3′)	Length	Tm	Size (bp)	Type of PCR Product
1	rs1799853	2C9*2-F	AATAGTAACTTCGTTTGCTGTTATCTC	27	62.2	493	C-allele specific
		2C9*2R-C	GGGCTTCCTCTTGAACACG	19	59.9		
		2C9*2F-T	GGAAGAGGAGCATTGAGGACT	21	61.3	127	T-allele specific
		2C9*2-R	CAGTAGAGAAGATAGTAGTCCAGTAAGGT	29	67.6		
2	rs1057910	2C9*3-F	GCCATTTTTCTCCTTTTCCAT	21	55.5	177	C-allele specific
		2C9*3 R-C	TGGTGGGGAGAAGGTCAAG	19	59.9		
		2C9*3F-A	GCACGAGGTCCAGAGATACA	20	60.5	295	A-allele specific
		2C9*3-R	GGAGAACACACACTGCCAGA	20	60.5		
	rs4244285	9*2-F	CAGAGCTTGGCAATATTGTATC	22	57.1°C	291	Control
		9*2-R	ATACGCAAGCAGTCACATAAC	21	57.4°C		
		9*2-A	GTAATTTGTTATGGGTTCCT	20	52.3°C	169	A-allele specific
		9*2-F	CAGAGCTTGGCAATATTGTATC	22	57.1°C		

		9*2-G	ACTATCATTGATTATTTCCCG	21	55.6°C	202	G-allele specific
		9*2-R	ATACGCAAGCAGTCACATAAC	21	57.4°C		
	rs4986893	*C19*3F2	TATTATTATCTGTTAACAAATATGA	25	52.7ºC	253	Control
		*2C19*3R	AACTTGGCCTTACCTGGATC	20	58.4 ºC		
		*2C19*3F1	TATTATTATCTGTTAACAAATATG	24	51.6ºC	253	T-Allele fragment
		*2C19*3R(T)	**AACTTGGCCTTACCTGGATT**	***20***	***56.4ºC***		
5	**rs12248560**	2C19*17-F	AAGAAGCCTTAGTTTCTCAAG	21	55.5	507	Control
		2C19*17-R	AAACACCTTTACCATTTAACCC	22	56.6		
		2C19*17-T	TGTCTTCTGTTCTCAAAGTA	20	52.3	218	T-allele specific
		2C19*17-R	AAACACCTTTACCATTTAACCC	22	56.6		
		2C19*17-F	AAGAAGCCTTAGTTTCTCAAG	21	55.5	330	C-allele specific
		2C19*17-C	ATTATCTCTTACATCAGAGATG	22	54.7		
6	**rs10264272**	*CYP3A5*6* NF(G)	CTTTGTGGAGAGCACTAAG	19	55.2°C	150	G-Allele Specific
		*CYP3A5*6* R1	AGTGGATGAATTATACGATATGT	23	55.7°C		
		*CYP3A5*6* MF(A)	CTTTGTGGAGAGCACTAAA	19	53.0°C	150	A-Allele Specific
		*CYP3A5*6* NF(G)	CTTTGTGGAGAGCACTAAG	19	55.2°C		
7	**rs776746**	*CYP3A5*3* F1	CAGATGACACAGCTCTAGATGTCC	24	65.3 °C	285	T-Allele Specific
		*CYP3A5*3* NR(T)	ATATGTGGTCCAAACAGGGAAGAGATAT	28	65.7 °C		
		*CYP3A5*3* F2	CAGATGACACAGCTCTAGATGTCC	24	65.3 °C	285	C-Allele Specific
		*CYP3A5*3* F1	CAGATGACACAGCTCTAGATGTCC	24	65.3 °C		
8	**rs35599367**	*3A4*22* F	TTGTCTGATAGTGGGTCTCTGTCTT	25	64.2	232	Control
		*3A4*22* R	CTTCCTCTATGCATGCAACAGG	22	62.1		
		*3A4*22* F	TTGTCTGATAGTGGGTCTCTGTCTT	25	64.2	107	C Allele Fragment
		*3A4*22* C	ATGCAGCTGGCCCTACG	17	57.2		
		*3A4*22* T	TGAATCTCCATCACACCCAGT	21	59.4	162	T Allele Fragment
		*3A4*22* R	CTTCCTCTATGCATGCAACAGG	22	62.1		
9	**rs5030655**	*Cyp2D6*6F*	TCCCAGCTGGAATCCGGTGTCG	22	59.9		
		*Cyp2D6*6R*	GGAGCTCGCCCTGCAGAGACTCC	24	61.1		
		*Cyp2D6*6F*	TCCTCGGTCACCCA	14	56.3		
		*Cyp2D6*6F Tmut*	GTCGCTGGAGCAGG	14	54.8		
10	**rs1065852**	*2D6*10F*	TCAACACAGCAGGTTCA	17	50ºC	433	Control (followed by RFLP)
		*2D6*10R*	CTGTGGTTTCACCCACC	17	54.8ºC		

2.4. Genotyping

2.4.1. Genotyping for CYP2D6 variants.

Amplification Refractory Mutation System (ARMS) Polymerase Chain Reaction (PCR) was utilized to genotype *CYP2D6*6 and CYP2D6*10* in breast cancer patients and unrelated healthy individuals via thermal cycler Bio-rad T100^{TM}. For performing each PCR, 25 µL (final volume) of the reaction mixture for each sample was prepared by using 1X PCR buffer (2.5 µL) (Fermentas), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM each of forward and reverse primer, 0.2 units of Taq DNA polymerase (Fermentas) and DNA sample of 100 ng was used. Optimized conditions for amplification for *CYP2D6*6* and *CYP2D6*10* allele amplification are shown in Tables 2.4 and 2.5.

Table 2.4: PCR Conditions CYP2D6*6 amplification

Steps	**Sub-Cycles**	**Conditions**		**PCR Cycles**
		Temperature	**Time**	
Initial Denaturation		94 ºC	10 min	
PCR Cycles	Denaturation	94 ºC	30 s	42 Cycles
	Primer annealing	63.0 ºC	30 s	
	Primer extension	72 ºC	1 min	
Final Extension		72 ºC	7 min	
Hold		4 ºC	∞	

Table 2.5: PCR conditions for CYP2D6*10 amplification

Steps	**Sub-Cycles**	**Conditions**		**PCR Cycles**
		Temperature	**Time**	
Initial Denaturation		95 ºC	5 min	
PCR Cycles	Denaturation	95 ºC	1 min	35 Cycles
	Primer annealing	52.8 ºC	1 min	
	Primer extension	72 ºC	2 min	
Final Extension		72 ºC	7 min	
Hold		4 ºC	∞	

The amplified products were detected on 2% agarose gels for genotyping.

2.4.2. Genotyping for CYP2C9 variants

Sequence-specific polymerase chain reaction (PCR) and refragment length polymorphism (RFLP) was utilized to genotype *CYP2C9*2* and *CYP2C9*3* in breast cancer patients and unrelated healthy individual via thermal cycler Bio-Rad T100^{TM}. For performing PCR, 25 µL (final volume) of the reaction mixture for each sample was prepared by using 1X PCR buffer (2.5 µL) (Fermentas), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM each of forward and reverse primer, 0.2 units of Taq DNA polymerase (Fermentas) and 100 ng of DNA sample was used. The optimized conditions for amplification for *CYP2D6*6* and *CYP2D6*10* allele amplification are shown in Tables **2.6 and 2.7.**

Table 2.6: Optimized conditions for *CYP2C9*2* amplification

Steps	**Sub-Cycles**	**Conditions**		**PCR Cycles**
		Temperature	**Time**	
Initial Denaturation		95 ºC	5 min	35 Cycles
PCR Cycles	Denaturation	95 ºC	1 min	
	Primer annealing	60 ºC	40 s	
	Primer extension	72 ºC	45 s	
Final Extension		72 ºC	10 min	
Hold		4 ºC	∞	

The amplified products were detected on 2% agarose gels for genotyping.

2.4.2.1 Restriction Fragment Length Polymorphism (RFLP)

Selection of Enzyme

The nucleotide position Arg144Cys was selected using the Ensemble tool based on reference site rs1799853. The mutation site was confirmed via Mutation Taster and dbSNP tool. Restriction enzyme against Arg144Cys was selected from a single base pair cutter using NEB cutter V2.0. The restriction enzyme AvaII having restriction site **GGACC,** was selected.

a. Digestion of the CYP2C9*2 by Ava II

The CYP2C9*2 PCR 396 bp product was digested with 800 U Ava II. 32µl RFLP reaction mixture for each sample was prepared, which consisted of 1 µl restriction enzyme Ava II (10 U/µL), 2 µL of 10X Buffer R (10 mM Tris-HCl (pH 8.5), 100 mM KCl,10mM MgCI2 and BSA (0.l mg/mL)), 10 µL of PCR product and 18 µL of nucleases free water. After adding the reaction mixture to the samples, they were centrifuged at 3000 rpm for 10 s and incubated at 37 °C for 16 h. The enzyme was inactivated by incubating the reaction mixture at 65 °C for 20 mins. Following incubation, restriction enzyme digests were separated on a 2% agarose gel at 90-120 volts for 45 min. Patients 'genotype were analyzed based on the number and size of the band.

Table 2.7: Optimized conditions for *CYP2C9*3* amplification

Steps	**Sub-Cycles**	**Conditions**		**PCR Cycles**
		Temperature	**Time**	
Initial Denaturation		95 °C	10 min	35 Cycles
PCR Cycles	Denaturation	95 °C	1 min	
	Primer annealing	53 °C	1 min	
	Primer extension	72 °C	45 s	
Final Extension		72 °C	10 min	
Hold		12 °C	∞	

2.4.2.2. Restriction Fragment Length Polymorphism (RFLP)

a. Selection of Enzyme KpnI

The nucleotide sequence A1075C was selected using an ensemble tool via Genome assembly based on reference site rs1057910. The restriction enzyme KpnI having restriction site was 5 "GGTACC3" selected.

b. Kpn I, restriction enzyme digestion, and PAGE of the *CYP2C9*3* digests

The 12 µL *CYP2C9*3* PCR product was digested with 10 U/µl Kpn1. The restriction enzyme cocktail comprised of 1 µL restriction enzyme Kpn1 (l0 U/ µl), 3.0), 2 µL of 10X Buffer, 15 µL DEPC treated water for each PCR product sample. The reaction mixture was incubated at 37 °C for 16 h.

The digested products and size marker were separated by PAGE at 140 V for 3 h on the polyacrylamide (l0% T, 2.9% C) gel and visualized. The patient's genotype was analyzed based on the band's size. RFLP product against rs1057910 for 1075A>C generate bands of various size. In case of two bands of size 85 and 20 bp, the subject was designated as a homozygous

mutant (+/+), in the case of 3 bands of size 105 bp, 85 bp, and 20 bp subject was considered as heterozygous mutant(+/-) and band of 105 bp subject was regarded as normal (-/-).

c. Polyacrylamide Gel Electrophoresis (PAGE) of restriction enzyme digests. The 105 bp *CYP2C9*3* PCR digests were evaluated by polyacrylamide gel electrophoresis (PAGE). A loading buffer of 10 µL (70% sucrose comprising 0.01 % BPB) was add up to each sample. Each was blended, centrifuged, and loaded onto an 8% PAGE gel and divided at room temperature on a GIBCOBRL Vertical Gel Electrophoresis Apparatus in 1 X TBE buffer at 140 V for 3 h. A 30% acrylamide stock solution: methylene bisacrylamide, was prepared. 8% PAGE gels were formulated by adding 13.4 mL of 30% polyacrylamide and 5 mL 10 x TBE buffer, and the volume was raised to 50 mL by adding distilled H_20 in a tiny beaker. A 400 µL aliquot of a 10% ammonium persulphate (APS) solution and 25 µL TEMED was included, and the solution was blended well. Combs were adjusted to create wells. At room temperature, the gels were let to polymerize for 20 min. The combs were separated, and the wells washed with lX TBE gels were transferred from the casting device to the GIBCOBRL Vertical Gel Electrophoresis Apparatus. After loading the samples onto the gels, they were operated at 140 V, 42 mA, and 6W. A 20 bp ladder was included in each electrophoretic run. The gels were taken from between the glass plates and immersed for 4 min in a 0.0001% EtBr staining solution produced from a 10 mg/EtBr stock solution. The bands were seen with ultraviolet (UV) light and photographed using an ultraviolet (UV) transilluminator (Biometra, Gottingen, Germany) Gel Documentation System.

2.4.3. Genotyping for CYP2C19 Variants

Amplification Refractory Mutation System (ARMS) Polymerase Chain Reaction (PCR) was utilized to genotype *CYP2C19*2, CYP2C19*3* and *CYP2C19*17* in breast cancer patients and unrelated healthy individuals via thermal cycler Bio-Rad, T100^{TM}.

For PCR, 25 µL (final volume) of the reaction mixture was prepared for each sample using 1X PCR buffer (2.5 µL) (Fermentas), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each forward and reverse primer, 0.2 units of Taq DNA polymerase (Fermentas) and 100 ng of a DNA sample. Optimized Conditions for amplification for CYP2D6*6 and CYP2D6*10 allele amplification are shown in Tables. 2.8, 2.9, and 2.10.

Table 2.8: Optimized conditions for *CYP2C19*2* amplification

Steps	**Sub-Cycles**	**Conditions**		**PCR Cycles**
		Temperature	**Time**	
Initial Denaturation		95 ºC	3 min	35 Cycles
PCR Cycles	Denaturation	95 ºC	1 min	
	Primer annealing	53 ºC	40 s	
	Primer extension	72 ºC	45 s	
Final Extension		72 ºC	10 min	
Hold		4 ºC	∞	

Table 2.9: Optimized conditions for *CYP2C19*3* amplification

Steps	**Sub-Cycles**	**Conditions**		**PCR Cycles**
		Temperature	**Time**	
Initial Denaturation		95 ºC	5 min	35 Cycles
PCR Cycles	Denaturation	95 ºC	1 min	
	Primer annealing	54 ºC	40 s	
	Primer extension	72 ºC	1 min	
Final Extension		72 ºC	10 min	
Hold		4 ºC	∞	

Table 2.10: Optimized conditions for *CYP2C19*17* amplification

Steps	Sub-Cycles	Conditions		PCR Cycles
		Temperature	**Time**	
Initial Denaturation		95 ºC	10 min	35 Cycles
PCR Cycles	Denaturation	95 ºC	1 min	
	Primer annealing	53 ºC	1 min	
	Primer extension	72 ºC	45 s	
Final Extension		72 ºC	10 min	
Hold		12 ºC	∞	

The amplified products were detected on 2% agarose gels for genotyping.

2.4.4. Genotyping for CYP3A4 Variants

Amplification Refractory Mutation System (ARMS) Polymerase Chain Reaction (PCR) was utilized to genotype CYP3A4*22 in breast cancer patients and unrelated healthy individuals via thermal cycler Bio-Rad T100^{TM}. For PCR, 25 µL (final volume) of the reaction mixture was prepared for each sample using 1X PCR buffer (2.5 µL) (Fermentas), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each forward and reverse primer, 0.2 units of Taq DNA polymerase (Fermentas) and 100 ng of a DNA sample. The optimized conditions for amplification for CYP2D6*6 and CYP2D6*10 allele amplification are shown in Table 2.11.

Table 2.11: Optimized conditions for *CYP3A4*22* amplification

Steps	Sub-Cycles	Conditions		PCR Cycles
		Temperature	**Time**	
Initial Denaturation		94 ºC	10 min	34 Cycles
PCR Cycles	Denaturation	94 ºC	1 min	
	Primer annealing	63 ºC	1 min	
	Primer extension	72 ºC	1 min	
Final Extension		72 ºC	5 min	
Hold		4 ºC	∞	

The amplified products were detected on 2% agarose gels for genotyping.

2.4.5. Genotyping for CYP3A5 Variants

Allele-specific PCR (AS-PCR) was performed for genotyping and was utilized for genotyping *CYP3A5*3* and *CYP3A5*6* in breast cancer patients and unrelated healthy individuals by thermal cycler Bio-Rad T100^{TM}. For PCR, 25 µL (final volume) of the reaction mixture was prepared for each sample using 1X PCR buffer (2.5 µL) (Fermentas), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each forward and reverse primer, 0.2 units of Taq DNA polymerase (Fermentas) and 100 ng of a DNA sample. The optimized conditions for amplification for *CYP2D6*6* and *CYP2D6*10* allele amplification are shown in Tables 2.12 and 2.13.

Table 2.12 Optimized conditions for *CYP3A5*3* amplification

Steps	**Sub-Cycles**	**Conditions**		**PCR Cycles**
		Temperature	**Time**	
Initial Denaturation		94 °C	5 min	34 Cycles
PCR Cycles	Denaturation	94 °C	1 min	
	Primer annealing N(A)	63.5 °C	45 s	
	Primer annealing M(C)	62.2 °C	45 s	
	Primer extension	72 °C	1 min	
Final Extension		72 °C	5 min	
Hold		4 °C	∞	

To determine the genotype of *CYP3A5*3*, two sets of PCR were performed. In the the first set of PCR, forward (F1) primer and wild-type allele "A" specific reverse primer (R) were utilized to amplify the wild-type allele fragment of 285 bps. The presence of wild-type allele genotype "AA" was confirmed by 2% agarose gel electrophoresis. In the second PCR, the same sample was used to check the presence of the mutant allele. In allele-specific PCR reaction, the control forward (F2) primer and "G" allele-specific primer R (G) were used to amplify the mutant allele fragment of 285 bps and confirmed by 2% agarose gel electrophoresis.

Table 2.13: Optimized conditions for *CYP3A4*22* amplification:

Steps	**Sub-Cycles**	**Conditions**		**PCR Cycles**
		Temperature	**Time**	
Initial Denaturation		94 ºC	5 min	34 Cycles
PCR Cycles	Denaturation	94 ºC	1 min	
	Primer annealing N(G)	53 ºC	45 s	
	Primer annealing M(A)	54 ºC	45 s	
	Primer extension	72 ºC	1 min	
Final Extension		72 ºC	5 min	
Hold		4 ºC	∞	

second PCR, the same sample was used to check the presence of the mutant allele. In allele-specific PCR reaction, the control forward (F2) primer and the "G" allele-specific primer R (G) were used to amplify the mutant allele fragment of 285 bps and confirmed by 2% agarose gel electrophoresis.

2.5. Tamoxifen and its metabolites quantification in plasma

2.5.1. High-Performance Liquid Chromatography (HPLC)

For determining the tamoxifen and its metabolites in the biosamples, High-Performance Liquid Chromatography (HPLC) is the easily available and cost-effective approach (Antunes *et al*., 2012).

Chemicals for HPLC

The list of chemical reagents required for HPLC is given in Table 2.14.

Table 2.14 List of chemicals for HPLC

No.	Chemicals
1.	Tamoxifen (TAM) (Sigma)
2.	Endoxifen (Sigma)
3.	N-desmethyl tamoxifen (Sigma)
4.	4-hydroxytamoxifen
5.	Tris ammonium methane (Sigma)
	Verapamil (Sigma)
6.	Methanol
7.	Phosphoric acid (85%)
8.	Triethyl-ammonium phosphate buffer (pH=3.0)
9.	Acetonitrile
10.	N-propanol, n-hexane (60%)
11.	HPLC water

2.5.2. Chromatographic conditions.

To determine the association of CYPs in the metabolism of tamoxifen and its metabolites, we employed HPLC Agilent 1100 UV system for the plasma analysis of 430 ER-positive breast cancer patients at a 280nm wavelength. The separation was conducted on a CNW Athena C18 columnn(150 mm × 4.69 mm, particle diameter 5.0 um). The flow rate was adjusted at 1.0 mL min-1 until the end of the analysis. The column temperature was set at 30 ◦C. The overall run time was 16 min. The retention time was 2.05 min for verapamil (IS), 4.14 min for Endoxifen, 4.5 min for 4-OH-Tamoxifen, 11.13 min for N-DesM-Tam, and 13.7 min for tamoxifen Figure 2.1, and chromatogram of sample is shown in figure 2.2.

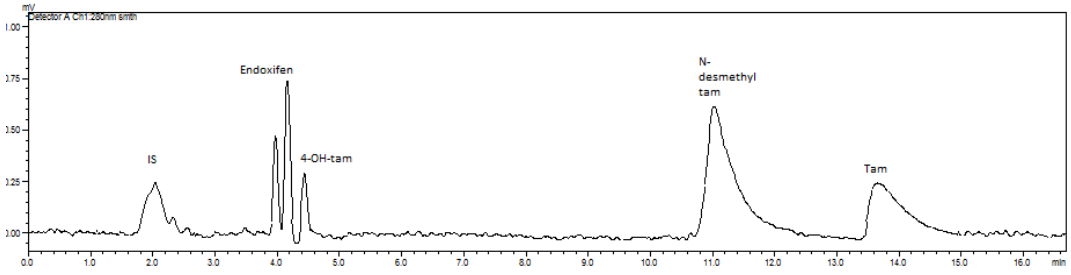


Figure 2.1: Chromatogram of standards: wavelength= 280 nm, the retention time of Verapamil (IS)= 2.05, endoxifen = 4.2, the retention time of 4-hydroxy tamoxifen= 4.5 min, the retention time of N-desmethyl tamoxifen = 11.0 and retention time of tamoxifen= 13.7 min.

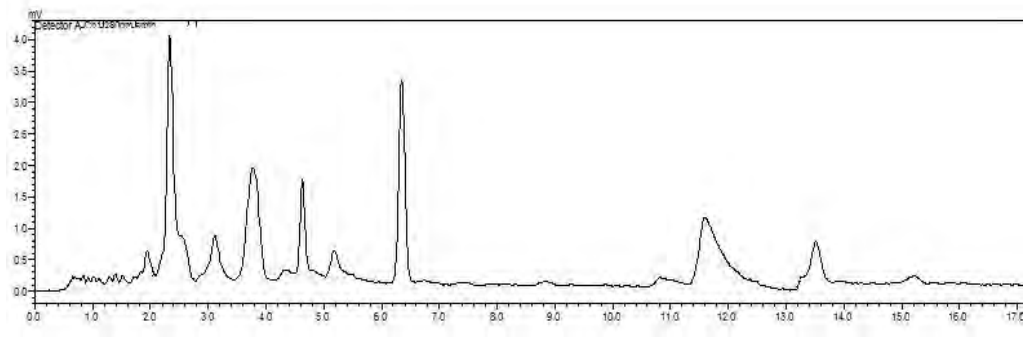


Figure 2.2: Chromatogram of sample: Wavelength = 280 nm, retention time of endoxifen = 4.3, retention time of 4-hydroxy tamoxifen= 4.6 min, retention time of N-desmethyl tamoxifen = 11.1 and retention time of tamoxifen= 13.3 min

2.5.3. Mobile Phase Buffer Preparation for HPLC

500µl of triethyl-ammonium phosphate buffer was diluted with 1000 mL of ultrapure water to formulate mobile phase buffer for HPLC. It was then filtered through a 0.2 µm cellulose acetate membrane, and its pH was adjusted to 3.3 by using triethyl-ammonium phosphate as a base while phosphoric acid as an acid. Before using the mobile phase buffer, it was sonicated for about 15 minutes. To prepare the mobile phase, 5 mM of triethyl-ammonium phosphate buffer (pH = 3.3) and acetonitrile were mixed in a ratio of 45:55, respectively. A total of 95 mL hexane and 5 mL n-propanol were combined to create an extraction solvent (Antunes *et al*., 2013).

2.5.4. Solutions and Standards Preparation for HPLC

Separate solutions for stock of Tam (tamoxifen), 4-OH tamoxifen (4-OH-Tam), N-desmethyl tamoxifen hydrochloride (N-Des-Tam), and N-desmethyl-4 hydroxytamoxifen (Endoxifen/Endo) were composed, at the concentration of 1mg/ml for Tam, the concentration of 1 mg/mL for 4-OH-Tam, 1 mg/mL for N-Des-Tam and 1mg /ml for Endo, by dissolving them in methanol. Verapamil (1 mg/mL) was employed as an internal standard (IS). Working solutions of Tam, 4-OH-Tam, N-Des-Tam, and Endo were prepared by combining standard solution and proper volume of methanol to gain the following concentration of Tam, 4-OH-Tam, N-Des-Tam, and Endo; 40 ng/mL, 20 ng/mL, 10 ng/mL, and 5 ng/mL respectively. Mobile phase buffer was composed by diluting 500 uL triethylammonium phosphate buffer into ultrapure water (1000 mL). The mobile phase buffer was filtered afterward by using a Millex Syringe-driven unit. 2.43 g of Tris-hydroxymethyl aminomethane was dissolved in ultrapure water (100 mL) to prepare Tris buffer. Using 0.1M NaOH, the pH was properly adjusted to 10.0. A solvent for extraction was set up by combining 95 mL hexane with n-propanol (5 mL) (Antunes *et al.*, 2013).

2.5.5. Plasma Sample Preparation

A volume of 2 mL of the patient's plasma sample was mixed in Tris buffer (0.7 mL) with pH 10.0 and 5.2 mL extraction solvent in a 15 mL falcon tube. The samples were gently mixed for 10 min and centrifuged at 2000 g for 10 min. The organic layer was shifted into another falcon tube, and 200 μL of phosphoric acid 0.1% (v/v) was added. After homogenization, it was again centrifuged at 4000 rpm for 15 min. A fresh tube was used to collect the liquid layer, and 20 μL of the aqueous layer was injected into Agilent HPLC 1100 system.

The following formula was used for tamoxifen and metabolites quantification in the plasma sample:

Concentration of Sample = Concentration of Standard/Area of Standard × Purity of Standard/100 × Area of Sample

2.6. Cell Culture Studies

2.6.1. Effects of Estrogens, Tamoxifen and Metabolites on Cell Proliferation

We tried to investigate the effect of CYP2C19 EM (extensive metabolizers), Rapid Metabolizers and UM (Ultra Rapid Metabolizers) metabolites concentration on estrogens on cell proliferation. ER-positive human breast cancer cell line MCF7 and T47D was employed in this study. All cells were attained from the American Type Culture Collection ATCC, Manassas, VA, USA) and were kept in phenol red RPMI 1640 medium, comprising 10% FBS (HyClone Laboratories , Logan, UT, USA), 2 mM glutamine, penicillin at 100 units·mL-1, streptomycin at 100 μg·mL-1, 1× non-essential amino acids (all from Life Technologies, Grand Island, NY, USA), and bovine insulin at 6 ng·mL-1 (Sigma-Aldrich, St. Louis, MO, USA). All cells were cultured in T185 culture flasks (Thermo Scientific, Pittsburgh, PA, USA) and passaged twice a week in 1:3 ratio. All cultures were grown in 5% CO_2 at 37°C.

Cells were cultured in oestrogen-free medium [phenol red-free RPMI 1640 media supplemented with 10% charcoal stripped FBS (SFS)] for three days before the beginning of the proliferation assay. On day 0 of the experiment, cells were seeded in oestrogen-free RPMI media comprising 10% SFS at a density of 10000 cells per well, respectively, in a 24-well cell culture plates (Corning, Tewksbury, MA, USA). After 24 h, cells were treated with combinations of oestrogens, tamoxifen, and its metabolites in different CYP2C19 EM, RM and UM concentrations (Tables 2.15) set in estrogen-free RPMI. All treatments were accomplished in triplicate. The medium comprising the test compounds was replaced on days 4 and 7, and the experiment was halted on day 8. Cells were splashed with cold PBS (Life Technologies) at least twice and analyzed with a fluorescent DNA quantification kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions, and the samples were read on a Mithras LB540 fluorometer/luminometer (Berthold Technologies, Oak Ridge, TN, USA) in black wall 96-well plates (Thermo Scientific).

2.6.2. Real Time PCR

MCF-7 cells were cultured in oestrogen-free medium for three days before seeding and treatment. Cells were seeded after oestrogen deprivation in 6-well cell culture plates (Corning) at a density of 300 000 cells per well. Cells were handled with test

compounds for 48 h. Total RNA was isolated using TRIzol reagent (Life Technologies) and an RNeasy RNA purification kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. Real-time PCR was performed by first synthesizing cDNA by the reverse transcribing 1 μg of total RNA by a high-capacity cDNA reverse transcription kit (Life Technologies) as per the manufacturer's guidelines and consequently diluted to 500 μL with nuclease free water. The real-time PCR was accomplished in a 20 μL reaction, which involved 1× SYBR green PCR master mix (Life Technologies), 125 nM each of forward and reverse primers and 5 μL of diluted cDNA using an ABI Prism 7900 HT Sequence Detection System (Life Technologies). Primers sequences that were used for human *pS2* cDNA amplification are: 5'-CAT CGACGTCCCTCCAGAAGA-3' sense, and 5'-CTCTGGGACTAATCACCGTGCTG-3' anti-sense; human *GREB1* gene: 5'-CAAAGAATAACCTGTTGGCCCTGC-3'sense, 5'-GACATGCCTGCGCTCTCATACTTA-3' anti-sense; human progesterone receptor the reference gene 36B4: 5' GTGTTCGACAATGGCAGCAT-3' sense, 5'-GACAC CCTCCAGGAAGCGA-3' anti-sense. All primers were acquired from Integrated DNA Technologies Inc. (Coralville,IA). Experiments, each containing of three replicates, were executed at least thrice.

2.6.3. Immunoblotting

MCF-7 Cells were kept in oestrogen-free media (oestrogen-starved) for 3 days before seeding. Cells were seeded on 10 cm Petri dishes (Corning) at a density of 3 million cells per plate and were incubated overnight. The cells were treated for 24 h with the actual tamoxifen metabolites' clinical levels in breast cancer patients (Table 2.15) along with actual levels of estrogens examined in postmenopausal patients taking tamoxifen. After media aspiration, cells were rinsed with cold PBS (pH 7.4), trypsinized and pelleted in 15 ml falcon tubes. The cells were lysed with freezing lysis buffer with fresh protease inhibitors (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA) incubated over ice for 20 min. The cells were centrifuged at 14000 *g* for 30 min at 4 °C. From supernatant 30 μg of protein was resolved on 4-12% polyacrylamide gel. After shifting to a nitrocellulose membrane probed with primary antibodies anti-Erα (clone G-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA),8and with anti-β-actin (clone AC-15; Sigma-

Aldrich) diluted in blocking buffer at ratios recommended by the suppliers at 4°C overnight. The membranes were washed thrice (10 min each) the next day with buffer and subsequently incubated with appropriate HRP-linked secondary antibodies (anti-mouse or anti-rabbit from Cell Signaling Technology, Danvers, MA, USA) diluted in blocking buffer for 1 h at room temperature and detected by chemiluminescence autoradiography. Intensity of protein band was measured from scanned images with Image J software. The intensity of control band was normalized to actin and considered as unity (1.0) whereas the intensity of treated band was determined relative to control and data was presented as mean ± SD on three replicates.

Chapter 3

3. Results and Discussion

Blood samples of four hundred and thirty patients were collected. Every Participant filled and signed the consent form (Annexure I), and these forms were kept as a record. The participants were interviewed with the help of a designed questionnaire (annexure II) and information regarding various parameters and risk factors such as age, ethnic group, marital status, stage of breast cancer, family history, treatment undertaken by breast cancer patients, and presence of any other disease, were obtained (Table 2.1). To compare the genotyping results with the control population, 410 age and gender-matched healthy individuals from the same cities and socioeconomic backgrounds were also enrolled (Table 2.2). DNA was extracted by using the phenol-chloroform extraction method. The results were visualized by using 2% agarose gel electrophoresis.

3.1 Study I

A total of 430 ER Positive breast Cancer patients and 400 unrelated healthy individuals were included in the study, which aimed to verify the CYP2C9*2 and *3 gene polymorphism frequency and their impact on the metabolism of tamoxifen and its metabolites.

3.1.1 Genotyping of CYP2C9 Variants

3.1.1.1 CYP2C9*2 SNP at 430C > T in Exon 3

A polymerase chain reaction was conducted to amplify the *CYP2C9*2*. The product size was 396 bp, as shown in Figure 3.1a. The amplified fragment was further digested using the Avall enzyme and generated different fragments. Restricted fragments show wild-type homozygous C allele with two fragments size; 223 and 173 bp. The heterozygous C allele and T allele exhibit three bands of size; 396, 223, and 173 bp, while the mutant T allele did not undergo restriction and showed a band of 396 bp, as shown in Figure 3.1b. *CYP2C9*2* heterozygous (OR: 0.4; 95% CI: 0.53–0.56; P = 0.0001) and homozygous mutant (OR: 3,12; 95% CI: 1.80–5.43; P = 0.0001) condition was at breast cancer risk factor in our population (Table 3.1).

3.1.1.2 CYP2C9*3 SNP at 1075A > C in Exon 7

The *CYP2C9*3* gene was amplified using a polymerase chain reaction. As depicted in Figure 3.1c, a 105-bp-long product was generated. This product was further digested by the Kpnl enzyme, which generates fragments of varying sizes. Homozygous A allele did not undergo restriction, whereas the mutant homozygous CC allele genotype displays two bands of 85 and 20 bp sizes. As shown in Figure 3.1d, heterozygous A and C alleles exhibit three sizes 105, 85, and 20 bp bands. Statistical analysis revealed that *CYP2C9*3* plays no significant role in the breast cancer development in the current study population (Table 3.1).

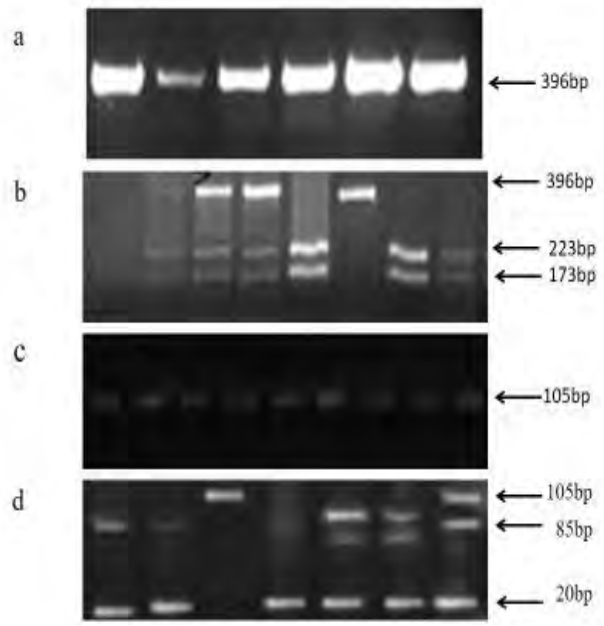


Figure 3.1: 3.1a: Electropherogram of PCR for *CYP2C9*2* allele. Band size: 396bp and 100bp ladder is used for comparison. **3.1b:** Electropherogram of RFLP for *CYP2C9*2* allele. Homozygous mutant type (TT)= 396bp, heterozygous mutant (CT)= 396bp, 223bp & 173bp and homozygous wild type (CC)= 223bp & 173bp. **3.1c:** Electropherogram of PCR for *CYP2C9*3* gene of 410 individuals. Band size: 105bp and 100bp ladder is used for comparison. **3.1d:** Electropherogram of RFLP for *CYP2C9*3* gene of 410 individuals. Homozygous wild type (AA)= 105bp, heterozygous mutant (AC)= 85bp, 20bp & 105bp and homozygous mutant type (CC)= 85bp & 20bp.

Table 3.1 Association of CYP2C9 genotypes with ER Positive breast cancer

Genotype			Controls (410)		Cases (430)			
			n	%	n	%	OR (Upper Limit-Lower Limit)	p-value
CYP2C9*2								
CC	NM [a]	*1/*1	229	55.85	273	63.48	1.37 (1.04-1.81)	0.02
CT	IM [a]	*1/*2	163	39.75	103	23.95	0.47 (0.35-0.64)	<0.0001
TT	PM [a]	*2/*2	18	4.39	54	12..55	3.12 (1.80-5.43)	0.0001
CYP2C9*3								
AA	NM [a]	*1/*1	140	34.1	128	29.76	0.81 (0.61-1.09)	0.17
AC	IM [a]	*1/*3	111	27.2	126	29.30	1.11 (0.82-1.50)	0.47
CC	PM [a]	*3/*3	159	38.7	176	40.93	1.09 (0.82-1.44)	0.52

[a] **NM = Extensive Metabolizer, IM = Intermediate Metabolizer, PM = Poor Metabolizer**

3.1.2 Tamoxifen and its Metabolites Analysis

Tamoxifen is converted to 4-OH-Tamoxifen by enzymes including *CYP2D6, CYP2C19, CYP2C9, CYP2B6,* and *CYP3A. CYP2C9* is the utmost abundant and polymorphic *CYP2C* enzyme. *CYP2C9* plays a minor role in the tamoxifen metabolic pathway (Antunes *et al.*, 2013). Mutants *CYP2C9*2* and *CYP2C9*3* have low enzyme activity. To assess *CYP2C9*2* and *CYP2C*3* role in forming hydroxytamoxifen from tamoxifen and N-desmethyl tamoxifen from Tamoxifen, plasma analysis was performed on 430 ER-positive breast cancer patients using the Agilent 1100 UV HPLC system.

***CYP2C9* polymorphisms impact the tamoxifen and its metabolites' plasma concentrations.**

The median plasma concentrations of tamoxifen and three metabolites measured in patients in each genotypic group (Table 3.4, Figure 3.2). The associations of tamoxifen and its derivatives with the *CYP2C*1/*1* (wild type), *CYP2C9*1/*2* (heterozygous), and *CYP2C9*2/*2* (mutant) genotypes are depicted in Figure 3.2. No substantial difference was detected for the median plasma concentration of tamoxifen, 4-OH-Tam, NDM, and endoxifen concentrations among the three different genotypes of patients. (Table 3.2; Figure 3.2 a,b,c,d). However, an insignificant decrease was observed in the median plasma concentration of 4-OH-Tam in the subjects having heterozygous (**1/*2*) (P = 0.747) and mutant (**2/*2*) (P = 0.223) genotypes in

comparison with the subjects having wild-type genotype (**1/*1*) (Table 3.2; Figure 3.2b).

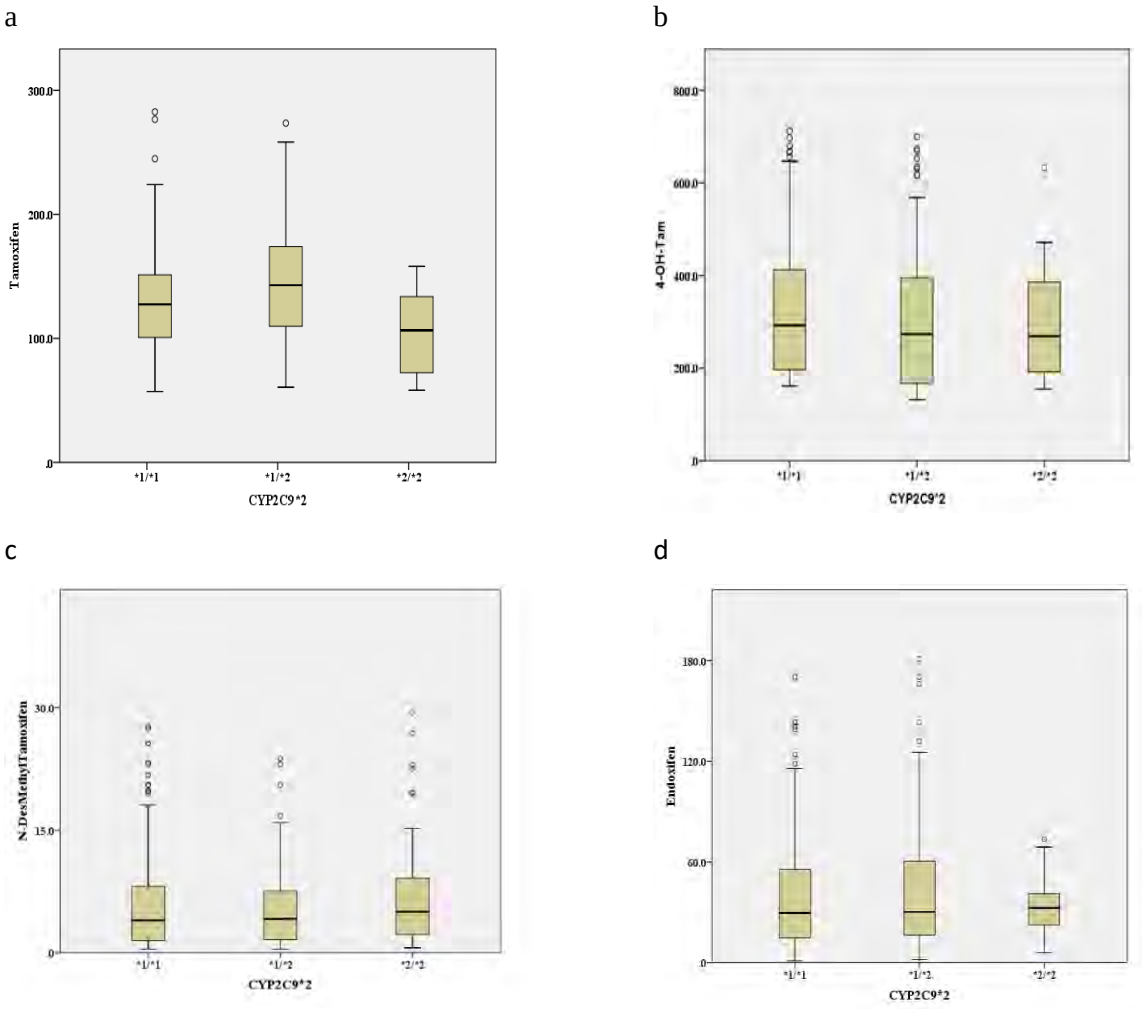


Figure 3.2. Association between CYP2C9*2 genotypes and steady-state plasma concentrations of (A) Tamoxifen, (B) 4-OH-Tam, (C) N-desmethyl tamoxifen (D) Endoxifen.

Table 3.2. Effects of *CYP2C9* polymorphisms on (median) plasma concentrations (ng/ml) of tamoxifen and its analytes.

Parameters ***CYP2C9*2*** **(430 C>T)**	**Genotype *1/*1 (NM)[a1]**	**Genotype *1/*2 (IM)[a1]**	**Genotype *2/*2 (PM)[a1]**
Tamoxifen	145.43	142.50	140.32
4-OHT	252.57	228.79.65	234.40
NDM	3.96	4.13	3.95
Endoxifen	29.49	30.20	32.66
Parameters ***CYP2C9*3*** **(1075 A>C)**	**Genotype *1/*1 (NM)[a2]**	**Genotype *1/*3 (IM)[a2]**	**Genotype *3/*3 (PM)[a2]**
Tamoxifen	141.93	144.13	140.45
4-OHT	255.34	237.50	238.95
NDM	3.92	4.29	3.91
Endoxifen	27.47	28.46	27.51

[a] NM= Normal Metabolizer , , IM = Intermediate Metabolizer, PM = Poor Metabolizer,

[1] for NM n = 273, IM n = 103, and PM n = 54

[2] for NM n = 128, IM n = 126, and PM n = 176

The associations of tamoxifen and its derivatives with the CYP2C9*1/*1 (wild type), CYP2C9*1/*3 (heterozygous), and CYP2C9*3/*3 (mutant) genotypes are shown in Table 3.2 and Figure 3.3. No substantial association was examined for tamoxifen, 4-OH-Tam, or NDM among the wild-type, heterozygous and mutant genotypes for the *CYP2C9*3 locus.

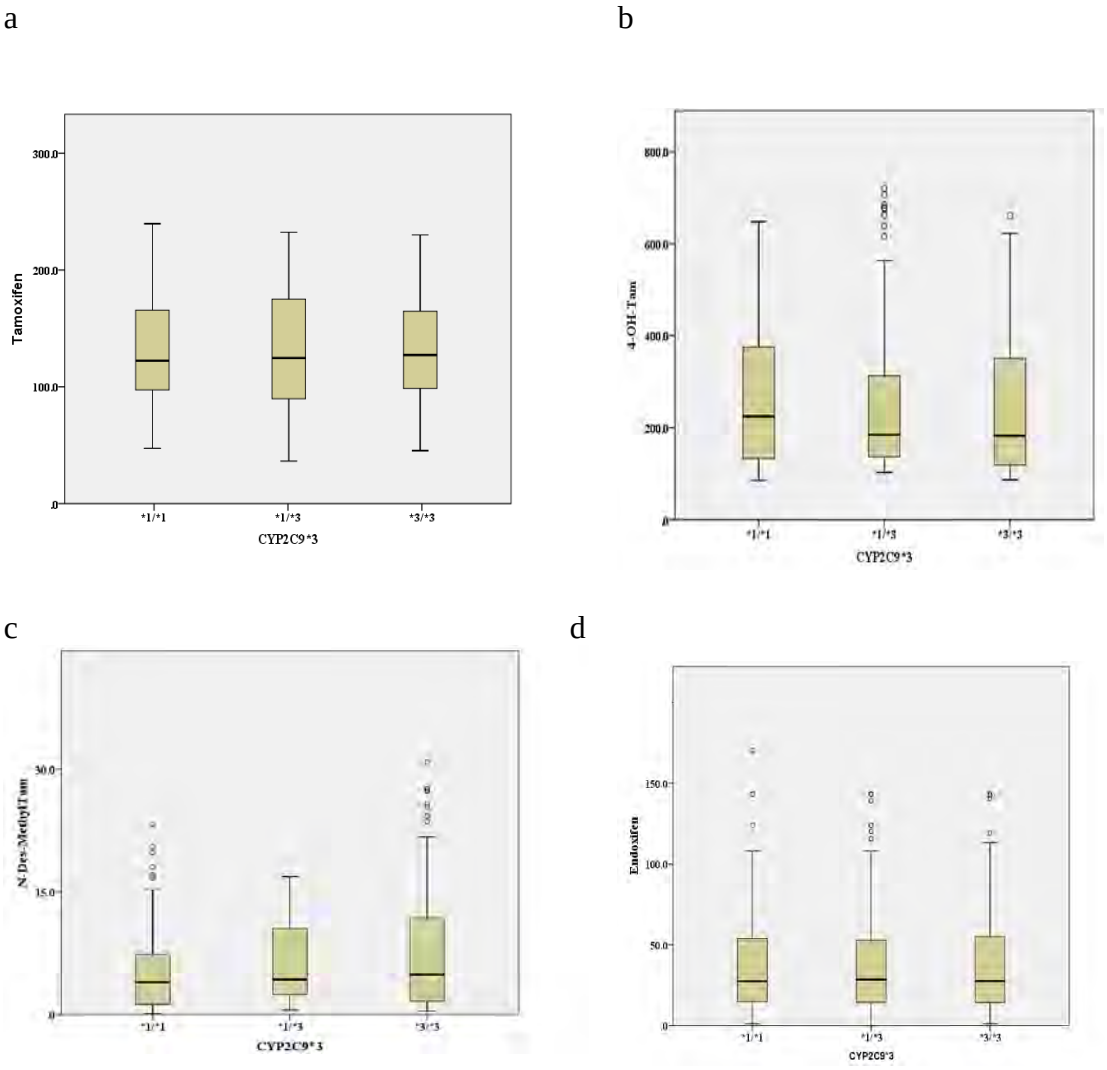


Figure 3.3. Association between CYP2C9*3 genotypes and steady-state plasma concentrations of (A) Tamoxifen, (B) 4-OH-Tam, (C) N-desmethyl tamoxifen (D) Endoxifen

3.1.3. *CYP2C9* polymorphisms impact on Total Metabolic Ratio of tamoxifen and its metabolites

For the *CYP2C9*2* locus, the median Plasma Metabolic Ratio of MR ***NDM-TAM*** was marginally higher in the heterozygous (1.78) (P = 0.85) and mutant (1.80) (P = 0.19) genotypes compared to the wild-type (1.68) genotypes (Table 3.5, Figure 3.4a). No significant difference was detected in Plasma metabolic ratio MR End-4-OHT between the three genotypes (Table 3.3, Figure 3.4b). An insignificant decrease was observed in the total metabolic ratio of 4-OH-Tam in the subjects having heterozygous (**1/*2*) (*P*

= 0.36) and mutant (**2/*2*) (*P* = 0.51) genotypes in comparison with the subjects having wild-type genotype (**1/*1*) (Table 3.3, Figure 3.4c). No substantial difference was observed in the median TMR NDM ratio among the three genotypes (Table 3.3, Figure 3.4d). *CYP2C9*3* locus had no considerable effect on the median plasma metabolic ratio of MR NDM-TAM and MR END-4-OHT (Table 3.3, Figure 3.5a, 3.5b) and the median total metabolic ratio of TMR NDM and TMR 4-OHT (Table 3.3, Figure 3.5c, 3.5d).

Table 3.3. Outcomes of *CYP2C9* polymorphisms on median metabolic ratios of Tamoxifen and its analytes

Parameters *CYP2C9*2* (Exon 3 at 430C>T))	**Genotype *1/*1 (NM)[a1]**	**Genotype *1/*2 (IM)[a1]**	**Genotype *2/*2 (PM)[a1]**
Plasma metabolic ratios (MRs)			
$MR_{NDM-TAM}$	2.01	2.88	2.80
$MR_{END-4-OHT}$	14.54	14.34	14.42
Total metabolic ratios (TMRs)			
TMR_{NDM}	2.52	2.43	2.63
TMR_{4-OHT}	13.96	11.21	12.84
Parameters *CYP2C9*3* *CYP2C9*3*(Exon 7 at 1075A>C)	**Genotype *1/*1 (NM)[a2]**	**Genotype *1/*3 (IM)[a2]**	**Genotype *3/*3 (PM)[a2]**
Plasma metabolic ratios (MRs)			
$MR_{NDM-TAM}$	2.32	2.35	2.31
$MR_{END-4-OHT}$	15.20	15.21	15.20
Total metabolic ratios (TMRs)			
TMR_{NDM}	2.82	2.56	2.11
TMR_{4-OHT}	14.63	14.14	14.75

[a] NM= Normal Metabolizer , , IM = Intermediate Metabolizer, PM = Poor Metabolizer,

[1] for NM n = 273, IM n = 103, and PM n = 54

[2] for NM n = 128, IM n = 126, and PM n = 176

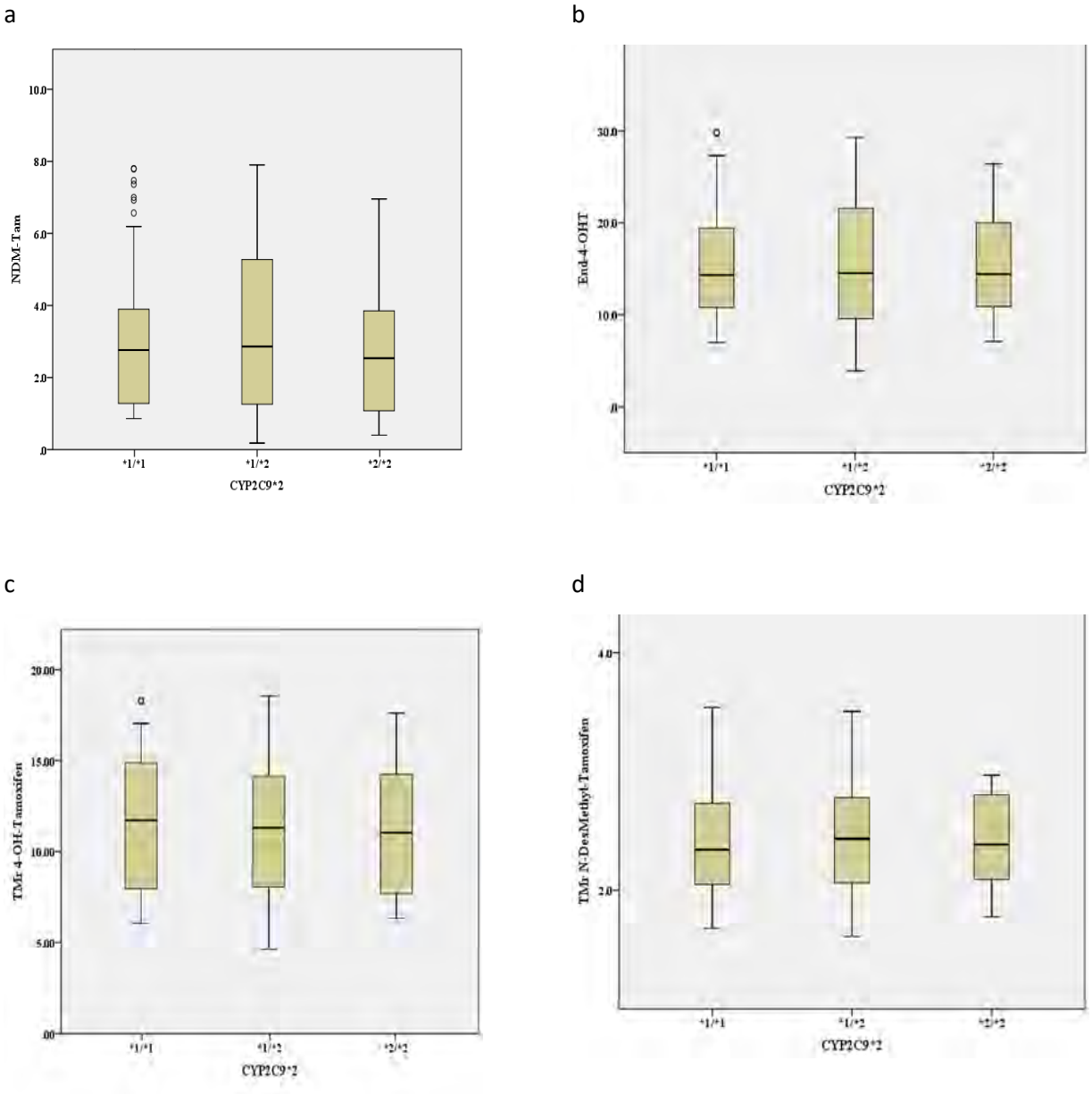


Figure 3.4. CYP2C9*2 genotype association with plasma metabolic ratio of (a) NDM-Tam (b) End-4OHT and total metabolic ratios of (c) 4-HydroxyTamoxifen and (d) N-desmethyl tamoxifen

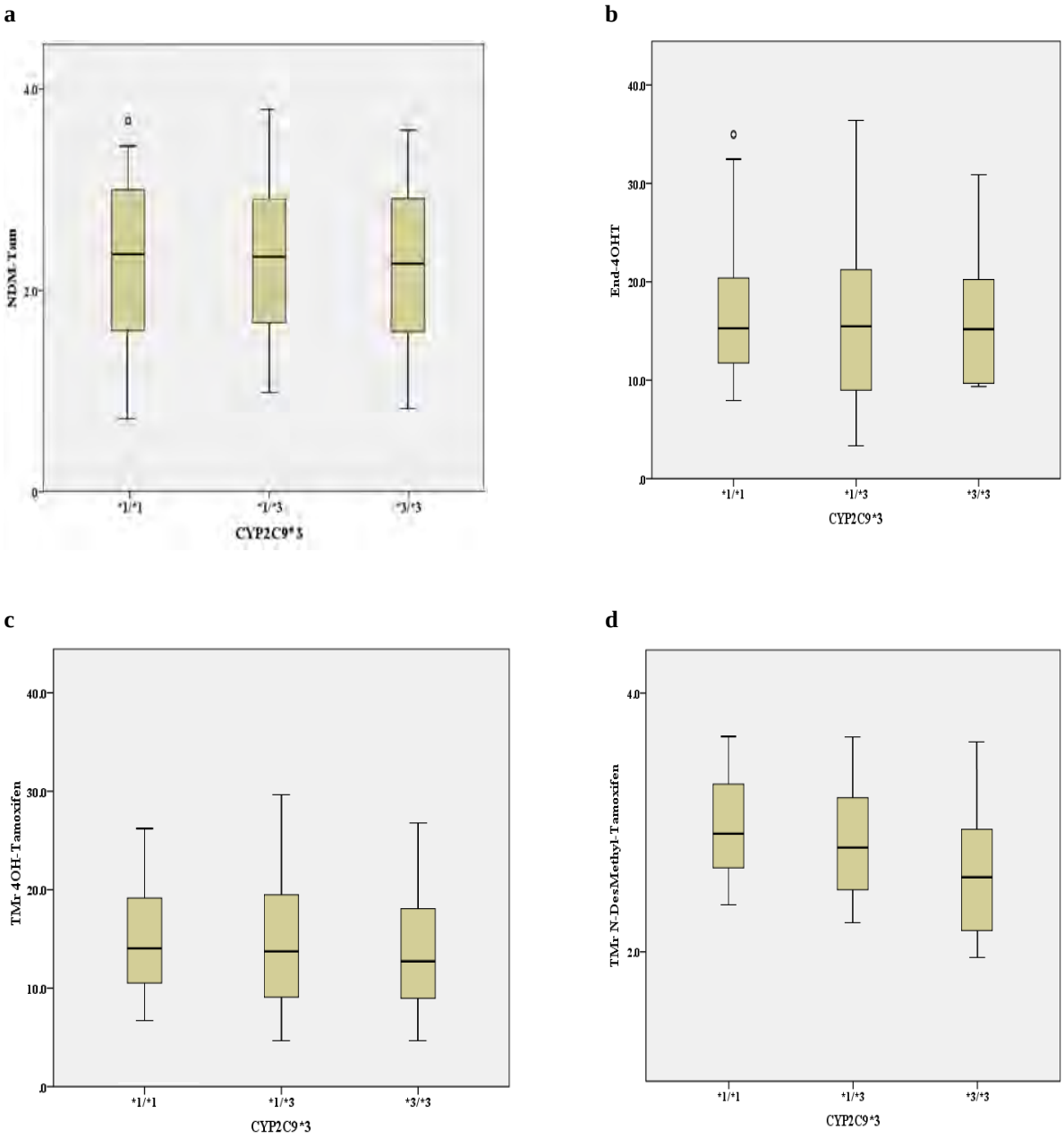


Figure 3.5. CYP2C9*3 genotype association with plasma metabolic ratio of (a) NDM-Tam (b) End-4OHT and total metabolic ratios of (c) 4-HydroxyTamoxifen and (d) N-desmethyl tamoxifen.

Tamoxifen was the first and among most effective targeted cancer therapies. Tamoxifen is widely used for breast cancer risk reduction in high-risk populations and adjuvant and metastatic treatment of estrogen receptor-positive breast cancer

(Davies *et al.*, 2013). A minor pathway for the tamoxifen conversion to endoxifen is believed to occur by Tamoxifen hydroxylation by *CYP*2D6, *CYP*2B6, *CYP*2C9, *CYP*2C19, or *CYP*3A4/55,6; this is followed by conversion of 4-hydroxytamoxifen to endoxifen (Brauch *et al.*, 2009). Multiple cytochrome P450 enzymes metabolize tamoxifen, and polymorphisms in the genes encoding these enzymes may affect tamoxifen and its metabolites' plasma concentrations. Tamoxifen's efficacy is dependent on CYP enzymes' conversion to active metabolites. Tamoxifen metabolites can also be transported out of cells, and transporter proteins should be considered in pharmacogenetics studies (Cronin-Fenton *et al.*, 2014). Tamoxifen metabolites can also be transported.

It was reported that tamoxifen and its primary metabolites in concentrations found in three CYP2D6 genotypes (EM, IM, and PM) are sufficient to constrain estrogen-induced replication in the postmenopausal setting. They recognized the importance of 4OHT as the active primary metabolite in the antiestrogenic action of tamoxifen in the postmenopausal setting. The estrogenic steroids would be anticipated to accrue in the cell by binding to the ER (Maximov, McDaniel, Fernandes, Korostyshevskiy, *et al.*, 2014).

Estrogens activate the ER and induce its transcriptional activity by interaction with the estrogen-responsive gene promoters (Maximov, McDaniel, *et al.*, 2014a). Estrogen receptor protein turnover is essential (Lonard *et al.*, 2000) to provide the continuous mRNA transcription. This turnover is accomplished by proteosomal degradation of the ER protein. The binding of antiestrogens, particularly 4OHT, blocks the ER and promotes stabilization. Estrogens activate the ER and induce its transcriptional activity by interaction with the estrogen-responsive gene promoters (Maximov, McDaniel, *et al.*, 2014a).

In this exploratory study, we examined the effects of formerly reported polymorphisms in the CYP2C9 gene, which is involved in tamoxifen and its metabolites' metabolism, in ER-positive breast cancer patients in Pakistan. Additionally, the genotype frequencies for CYP2C9*2 and CYP2C9*3 were assessed between the two groups, and the allele frequencies of the CYP2C9 variants in healthy controls and ER-positive breast are shown in Table 3.1.

Our findings demonstrated the genotyping results for unrelated healthy individuals and estrogen-positive breast cancer patients from Pakistan. No noteworthy difference was detected between the allele frequencies of *CYP2C9*2,* but conditional logistic regression shows that *CYP2C9*2* heterozygous (OR: 0.4; 95% CI: 0.35–0.64; $P < 0.0001$) and homozygous mutant (OR: 3.1; 95% CI: 1.80–5.43; $P = 0.0001$) condition was at risk of developing breast cancer. The cytochrome P450 variants *CYP2C9*2* and *CYP2C9*3* encode proteins with lowered enzymatic activity. Individuals carrying these variants metabolize drugs more slowly than individuals with wild-type *CYP2C9*1*, potentially altering their response to drugs and their disease risk (Sausville *et al.*, 2018). No significant difference was detected between the allele frequencies of *CYP2C9*3* in unrelated healthy and estrogen-positive breast cancer patients.

*CYP*2C9 contributes to forming the principal tamoxifen metabolites 4-hydroxy-tamoxifen and N-desmethyl-tamoxifen (Antunes *et al.*, 2013), though to a smaller extent than the isoforms (*CYP*2D6 and *CYP*3A5) (Coller *et al.*, 2002). This study explored the effect of previously reported polymorphisms in genes encoding the enzymes responsible for tamoxifen and three metabolites' metabolism in Pakistani breast cancer patients. There was no variation in the tamoxifen or its metabolites median plasma concentrations between patients with two wild-type alleles and those with heterozygous or homozygous *CYP2C9*2* and *CYP2C9*3* variant alleles. However, a minor or insignificant decrease was observed in the median plasma concentrations of 4-OH-tamoxifen patients with *1/*2 and *2/*2 genotypes. Our findings were consistent with those of Teft et al. (no p-values stated) and Jin et al. (p-values 0.05) in that there was no significant difference in tamoxifen or its metabolites plasma concentrations between patients involving two wild-type alleles or carriers of heterozygous or homozygous *CYP2C9*2* and *CYP2C9*3* variant alleles. Lim et al. also found comparable results concerning *CYP2C9*3* and its impact on the concentrations of tamoxifen and metabolites (Lim *et al.*, 2011). However, homozygous wild-type carriers and carriers of the CYP2C9*2 and *3 alleles demonstrated significantly different rates of 4-hydroxy-tamoxifen production from tamoxifen ($p = 0.007$) and significantly lower plasma levels of 4-hydroxy-tamoxifen (Saladores *et al.*, 2015).

3.2 Study II

3.2.1. Genotyping of CYP2C19

3.2.1.1. Genotyping frequency for CYP2C19*2 SNP in Exon 5 at 681 G>A:

An amplification refractory mutation system (ARMS) was used for DNA amplification at this locus, producing three band sizes. 291 bp (positive control for the locus), 202 bp (wild type), and 169 bp (mutant). The subject was considered heterozygous (*CYP2C19*1/*2*) in case three bands of size 291 bp, 202 bp, and 169 bp were detected; homozygous wild type (*CYP2C19*1/*1*) if two bands of size 291 bp and 202 bp were detected; and homozygous mutant (*CYP2C19*2/*2*) if two bands of size 291 bp and 169 bp were observed. *CYP2C19*2* heterozygous condition was a breast cancer risk factor (OR: 0.6; 95% CI: 0.43–0.84; P = 0.003) in our population, whereas no association was found for the homozygous mutant condition. (Fig 3.6 A, Table 3.4)

3.2.1.2. Genotyping frequency for CYP2C19*3 SNP in Exon 4 at 636 G>A

Allele-specific PCR amplified *CYP2C19*3* gene. Normal "C" allele band of 253bp and mutant "T" allele band of 253bp werewere amplified too determine genotype. The allele-specific band marked the presence of respective alleles in the *CYP2C19* gene. Homozygous with major allele 'C' and homozygous with minor allele 'T' have shown one band, whereas heterozygous has shown bands with both primer. Statistical analysis unveiled a substantial provision of the *CYP2C19*3* heterozygous variant (OR: 0.34; 95% CI: 0.24–0.48; *P*<0.001) towards breast cancer development in the current study population. (Fig 3.6 B, Table 3.4)

3.2.1.3. Genotyping frequency for CYP2C19*17 (-806C>T; rs12248560)

PCR technique ARMS was employed for implication of *CYP2C19*17*. Three bands produced in this case were 507 bp (control), 330 bp (wild type), and 218 bp (mutant). If all three bands detected, the subject was considered as heterozygous *(CYP2C19*1/*17)*. The subject was designated as homozygous wild type *(CYP2C19*1/*1)* if 22 bands of 507 bp and 330 bp were detected and homozygous mutant *(CYP2C19*17/*17)* if bands of the sizes 507 bp and 218 bp were observed. Heterozygous individuals (OR: 7.177; 95% CI: 5.088–10.10; *P*<0.001) and homozygous mutant individual (OR: 5.366; 95% CI: 3.180–9.011; *P*<0.001) were at significant risk of breast cancer progression. (Fig 3.6 C, Table 3.4)

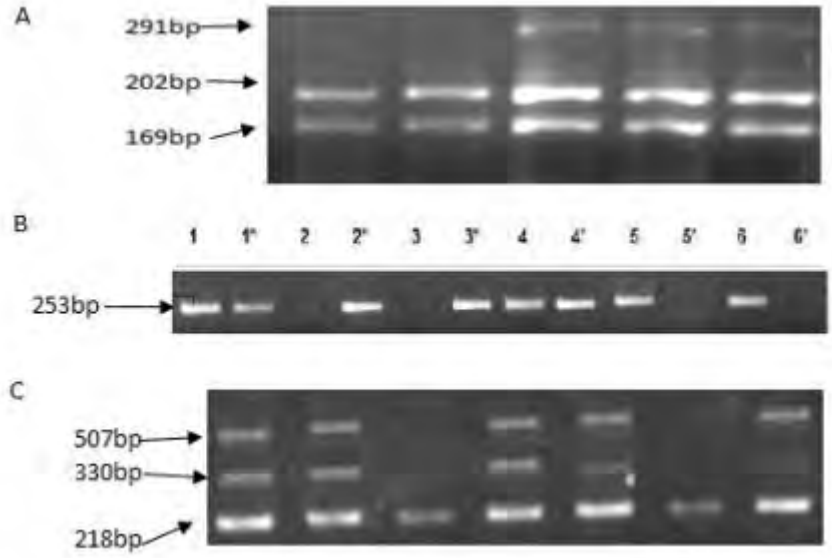


Figure 3.6. Genotyping of CYP2C19 SNPs. a) Electropherogram of ARMS PCR for CYP2C19*2 allele. The control band is 291 bp, the wild-type G-allele band is 202 bp, and the mutated A-allele band is 169bp. b) Electropherogram of PCR for CYP2C19*3 allele. Asterisk (*) represents mutant allele. Wild type C allele band= 253bp, mutant type T allele= 253bp. 1 &1* lane showing heterozygous major C and minor T allele, 2* and 3* showing homozygous minor T allele, while 5 and 6 show homozygous major C allele. c) Electropherogram of ARMS PCR for CYP2C19*17. Control band of 507 bp, wild-type C-allele band of 330 bp, and mutated T-allele band of 218 bp. 100 bp ladder is used for comparison.

Table 3.4. Association of CYP2C19 genotypes with ER Positive breast cancer

Genotype			Controls (410)		Cases (430)			
			n	%	n	%	OR (95% C.I)	P-value
CYP2C19*2								
GG	NM [a]	*1/*1	210	51.2	214	49.8	1.0 (Ref.)	
GA	IM [a]	*1/*2	180	43.9	190	44.2	0.6 (0.43 to 0.84)	0.003
AA	PM [a]	*2/*2	20	4.9	26	6	0.81 (0.39 to 1.71)	0.594
CYP2C19*3								
GG	NM [a]	*1/*1	152	37.1	159	37.0	1.0 (Ref.)	
AG	IM [a]	*1/*3	214	52.2	205	47.7	0.34 (0.24 to 0.48)	>0.001
AA	PM [a]	*3/*3	44	10.7	66	15.3	0.68 (0.39 to 1.16)	0.165
CYP2C19*17								
CC	NM [a]	*1/*1	265	64.6	80	18.6	1.0 (Ref.)	
CT	RM [a]	*1/*17	107	26.1	281	65.3	7.17 (5.08 to 10.1)	>0.001
TT	UM [a]	*17/*17	38	9.3	69	16.1	5.36 (3.18 to 9.01)	>0.001

[a] EM = Extensive Metabolizer, IM = Intermediate Metabolizer, PM = Poor Metabolizer, RM=Rapid Metabolizer and UM = Ultra Rapid Metabolizer

3.2.2. Analysis of Tamoxifen and its metabolites

Tamoxifen is metabolized by enzymes comprising *CYP2D6, CYP2C19, CYP2C9, CYP2B6,* and *CYP3A* into 4-OH-Tamoxifen. *CYP2C19* is a polymorphic gene. *CYP2C19*2* and *CYP2C19*3* mutants have low enzyme action, whereas *CYP2C19*17* heterozygotes and mutants have enhanced enzyme activity.

CYP2C19 polymorphisms impact the plasma concentrations and Total Metabolic Ratio of tamoxifen and its metabolites

Median plasma concentrations of the tamoxifen and the three metabolites measured in patients of each genotypic group are shown in Table 3.5. Figure 3.7 displays the associations off tamoxifen and itss derivatives with *CYP2C19*1/*1* (wild-type), *CYP2C19*1/*17* (heterozygous) *CYP2C19*17/*177* (mutant) genotypes. No significant difference was observed for the the median plasma tamoxifen and endoxifen concentrations among the three different genotypes off patients (Table 3.5; Fig 3.7A & 3.7D). However, median palsaama showed a strong association with this locus .

concentrations off 4-OH-tamoxifens, which was recorded to be 288.59 ng ml/L in the wild-type genotypes, 354.533 ng ml/L (P<0.001) in the heterozygous genotypes (62.0%) increased than the wild-type patients), and 378.255 ng ml/L (P<0.001) for the mutant

genotypes (75.0 % increase compare to the wild-type patients) (Table 3.7; Fig 3.7B). A slight decline (P= 0.3620) for median concentrations of N-DesM-Tam was observed in heterozygous & mutant patient (Table 3.5; Fig. 3.7C).

Figure 3.8 shows the associations of tamoxifen along with derivatives *CYP2C19*1/*11* (wild-type), *CYP2C19*1/*3* (heterozygous), & *CYP2C19*3/*3* (mutant) genotype. No significantt association was observed for tamoxifen & N-DesM-Tam amongst the wild-type, heterozygous as well as mutant genotypes for the *CYP2C19**3 locus. However, an insignificant decreases was observed in the median plasmas concentrations off 4-OH-Tam for heterozygous (240.634 ng ml/L) (P=0.420) or mutants (276.293 ng ml/L) (P=0.879) genotype, compared too the wild-type (288.437 ng ml/L) genotypes for this locus. (Table 3.5, Fig 3.8B). Conversely, an increment for the median plasmas concentrations were noted for endoxifen in the heterozygous (24.737 ng ml/L) and mutants (35.408 ng ml/L) genotype, compares to the wild-type (19.733 ng ml/L) genotype (Table 3.5, Fig 3.8D).

Figure 3.9 depicts the associations of tamoxifen and also the derivatives with *CYP2C19*1/*1* (wild-type), *CYP2C19*1/*2* (heterozygous), and *CYP2C19*2/*2* (mutant) genotype. Weak association for three types of genotypes were noted for the median plasma's concentration of tamoxifen & N-DesM-Tam (Fig. 3.9A & 3.9C). However, an increased for median plasmas concentration of 4-OH-Tam was noted in the heterozygous (272.657 ng ml/L) (P<0.021) and mutants (305.250 ng ml/L) (P<0.004) genotype, in comparison with the concentrations of the wild-type (255. 75 ng ml/L) genotypes (Table 3.5, Fig 3.9B). Similarly, there was stepwise increase noted for median plasma concentration of endoxifen: 19.433 ng ml/L in the wild-type, 27.173 ng ml/L for heterozygous , and 32.501 ng ml/L in the mutant genotype (Table 3.5 , Fig 3.9D).

Table 3.5. Effects of *CYP2C19* polymorphisms on (median) plasma concentrations (ng/ml) of tamoxifen and its analytes.

Parameters CYP2C19*2 *(681G>A; rs4244285)*	*Genotype* ****1/*1*** ***(NM)***[a1]	*Genotype* ****1/*2*** **(IM)**[a1]	*Genotype* ****2/*2*** ***(PM)***[a1]
Tamoxifen	116.96	119.68	118.49
4-OH- Tamoxifen	255.75	272.65	305.25
N-DesM-Tam	3.96	4.10	4.91
Endoxifen	19.34	27.15	32.50
Parameters CYP2C19*3 *(636G>A; rs4986893)*	*Genotype* ****1/*1*** ***(NM)***[a2]	*Genotype* ****1/*3*** **(IM)**[a2]	*Genotype* ****3/*3*** ***(PM)***[a2]*2*
Tamoxifen	116.87	116.99	117.26
4-OH- Tamoxifen	288.43	240.63	276.29
N-DesM-Tam	3.02	3.13	1.68
Endoxifen	19.34	24.73	35.40
Parameters ***CYP2C19*17 (-806C>T; rs12248560)***	*Genotypes* ****1/*1*** ***(NM)***[a3]	*Genotype* ****1/*17*** **(RM)**[a3]	*Genotype* ****17/*17*** ***(UM)***[a3]
Tamoxifen	118.97	120.15	121.88
4-OH- Tamoxifen	288.59	354.53	378.25
N-DesM-Tam	4.78	3.47	3.20
Endoxifen	29.49	27.04	31.40

[a] EM = Extensive Metabolizer, IM = Intermediate Metabolizer, PM = Poor Metabolizer, RM= Rapid Metabloizer and UM = Ultra Rapid Metabolizer

[1] for RM n = 214, IM n = 190, and PM n = 26

[2] for RM n = 159, IM n = 205, and PM n = 66

[3] *for RM* n = 69, UM n= 299, and UM n= 62

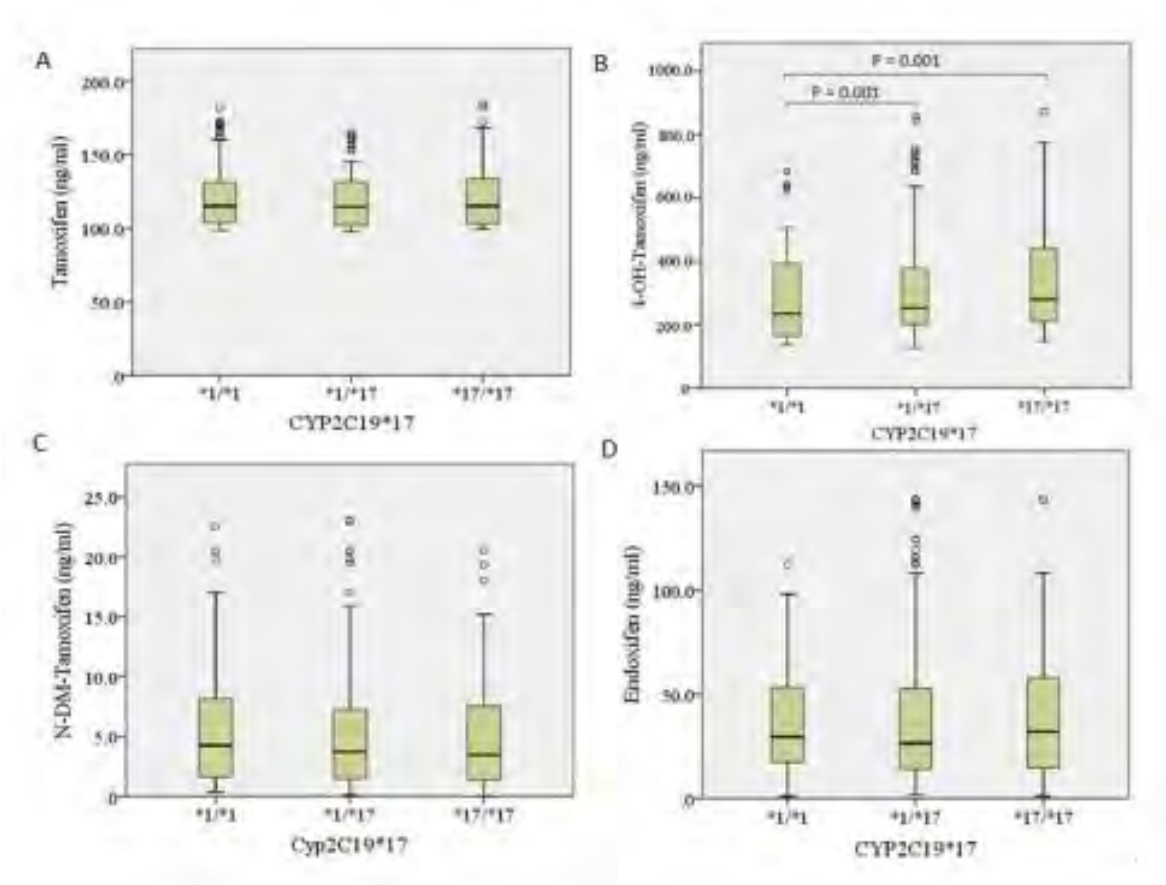


Figure 3.7. Association between CYP2C19*17 (*1/*1, *1/*17 & *17/*17) genotype and steady-state median plasma concentrations of (A) Tamoxifen, (B) 4-OH-Tam, (C) N-desM-Tamoxifen & (D) Endoxifen. No significant difference for median plasma concentration of Tamoxifen and Endoxifen between the three genotypes. A extensively raised amount of 4-OH-Tam concentrations in *17 genotype compared with wild type (P= 0.001). Non-significant reduction in median plasma concentration of N-desM-Tamoxifen (P= 0.3621) in *17 genotype compared with wild type.

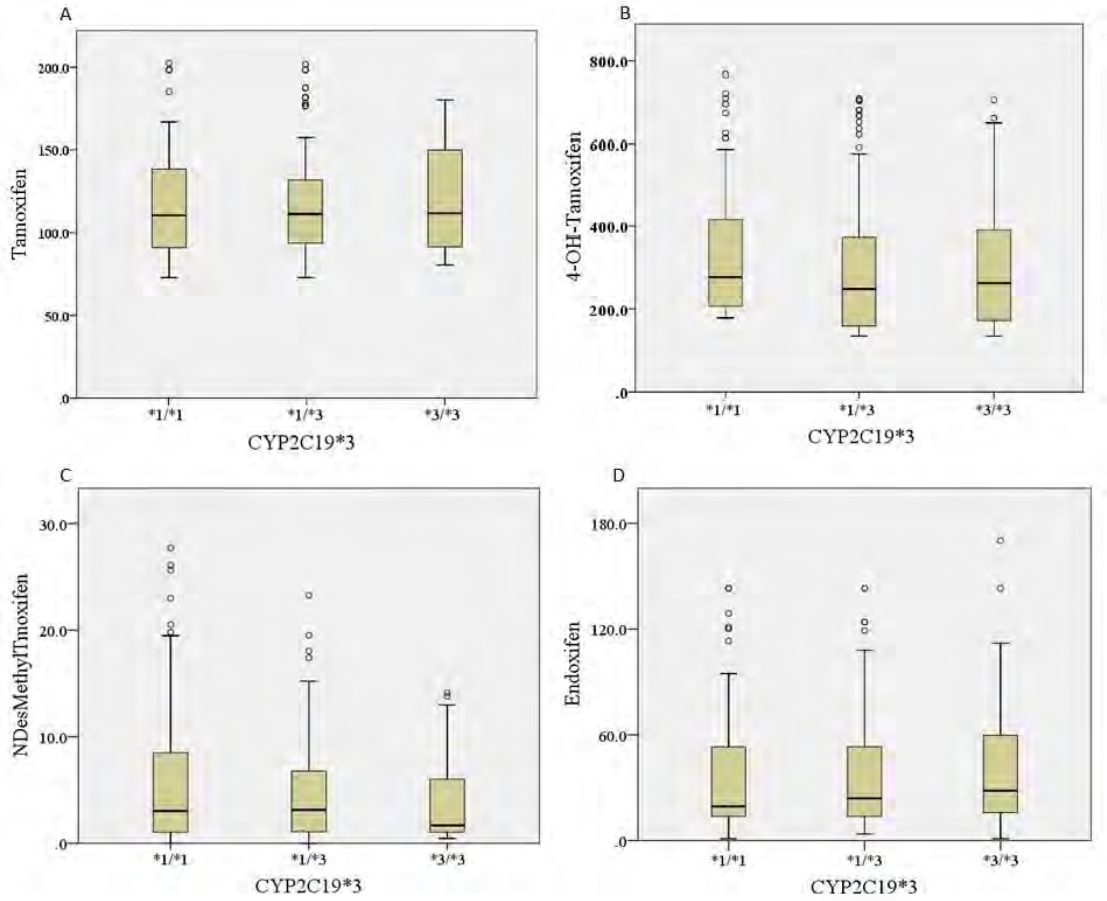


Figure 3.8. Association b/w CYP2C19*3 (*1/*1, *1/*3 and *3/*3) genotype and steady-state median plasma concentration of (A) Tamoxifen, (B) 4-OH-Tam, (C) N-desMTamoxifen & (D) Endoxifen. No measurable change was noted for median plasma concentration of Tamoxifen and N-desM-Tamoxifen between the genotype. Non-significant reduce in concentration 4-OH-Tam in *3 genotype to wild type (P=0.4210), whereas there was a little boost in the median plasma concentrations of Endoxifen in *3 genotypes to *1/*1.

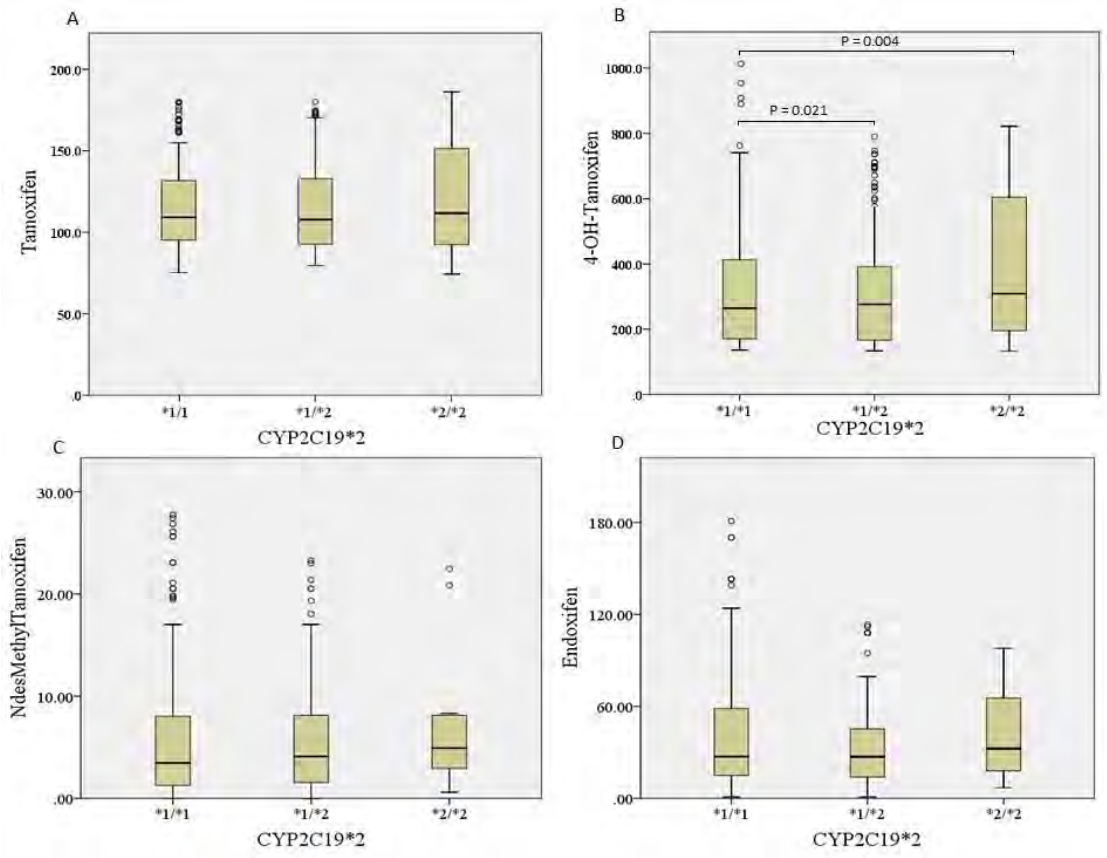


Figure 3.9. Correlation between CYP2C19*2 genotypes and steady-state median plasma concentrations of (A) Tamoxifen, (B) 4-OH-Tam, (C) N-desM-Tamoxifen, & (D) Endoxifen. No recordable difference in Tamoxifen and N-desM-Tamoxifen concentration for the three genotypes, a substantial increment in the median plasma concentration of 4- OH-Tam (P= 0.0212) for *1/*2, and (P= 0.004) for *2/*2 compared to wild type. Endoxifen concentration depicts an non-significant increase in *1/*2 and *2/*2 compared to *1/*1.

For the *CYP2C19*17* locus, the median Total Metabolic Ratio of 4-OH-Tam (TMR-4-OH-Tam) was suggestively higher for heterozygous (187.91) (P<0.0011) and mutant (210.900) (P<0.001) genotype, compared to the wild-type (122.464) genotype (Table 3.6, Fig 6A). Correspondingly, TMRR-N-desM-Tamoxifen median amounts also depict an increasing trend: 7.668 in the wild-type, 8.920 in heterozygous, and 9.213 in the mutant genotype (Table 3.6, Fig 6B).

Although no considerable correlation examined between the *CYP2C19*22* polymorphisms and the TMRR-4-OH-Tam (Table 3.6, Fig 6C), patients sheltering the **1/*22* heterozygous (P<0.001) and **2/*2* homozygous mutants genotype (P=0.2272) displayed increases median TMRR-4-OH-Tam. In contrast, TMRE-N-desM-Tamoxifen were parallel for the patients **1/*1, *1/*2* and **2/*2* (Table 3.6, Fig 6D). Non-significant relationship was noted for the median TMRr-4-OH-Tam and TMRE-N-

desM-Tamoxifen in the patients bearing **1/*1, *1/*3,* and **3/*3* genotypes (Table 3.6, Fig 6 E, F).

Table 3.6. Effects of *CYP2C19* polymorphisms on median metabolic ratios of Tamoxifen and its analytes

Parameters	Genotype	Genotype	Genotype
*CYP2C19*2* (681G>A; rs4244285)	***1/*1 (NM)[a1]**	***1/*2 *(IM)*[a1]**	***2/*2 (PM)[a1]**
Plasma metabolic ratios (MRs)			
MR NDM-TAM	**0.21**	**0.26**	**0.37**
MR END-4-OHT	**7.87**	**7.46**	**12.31**
Total metabolic ratios (TMRs)			
TMR NDM	6.64	7.30	8.76
TMR 4-OHT	143.78	183.79	213.7
Parameters CYP2C19*3 (636G>A; rs4986893)	Genotype ***1/*1 (NM)[a2]**	Genotype ***1/*3 *(IM)*[a2]**	Genotype ***3/*3 (PM)[a2]**
Plasma metabolic ratios (MRs)			
MR NDM-TAM	**0.32**	**0.32**	**0.27**
MR END-4-OHT	**13.72**	**12.67**	**12.01**
Total metabolic ratios (TMRs)			
TMR NDM	7.88	7.81	8.34
TMR4-OHT	93.81	82.04	87.27
Parameters **CYP2C19*17** (**-806C>T; rs12248560**)	Genotypes ***1/*1 (NM)[a3]**3	Genotype ***1/*17 (RM) [a3]**	Genotype ***17/*17 (UM) [a3]**
Plasma metabolic ratios (MRs)			
MR NDM-TAM	0.28	0.28	0.26
MR END-4-OHT	0.26	0.16	0.19
Total metabolic ratios (TMRs)			
TMR NDM	6.68	8.98	9.23
TMR 4-OHT	122.48	187.91	210.90

[a] EM = Extensive Metabolizer, IM = Intermediate Metabolizer, PM = Poor Metabolizer, and UM = Ultra Rapid Metabolizer

[1] for EM n = 214, IM n = 190, and PM n = 26

[2] for EM n = 159, IM n = 205, and PM n = 66

[3] for EM n= *69*, RM n= 299, and UM n= *62*

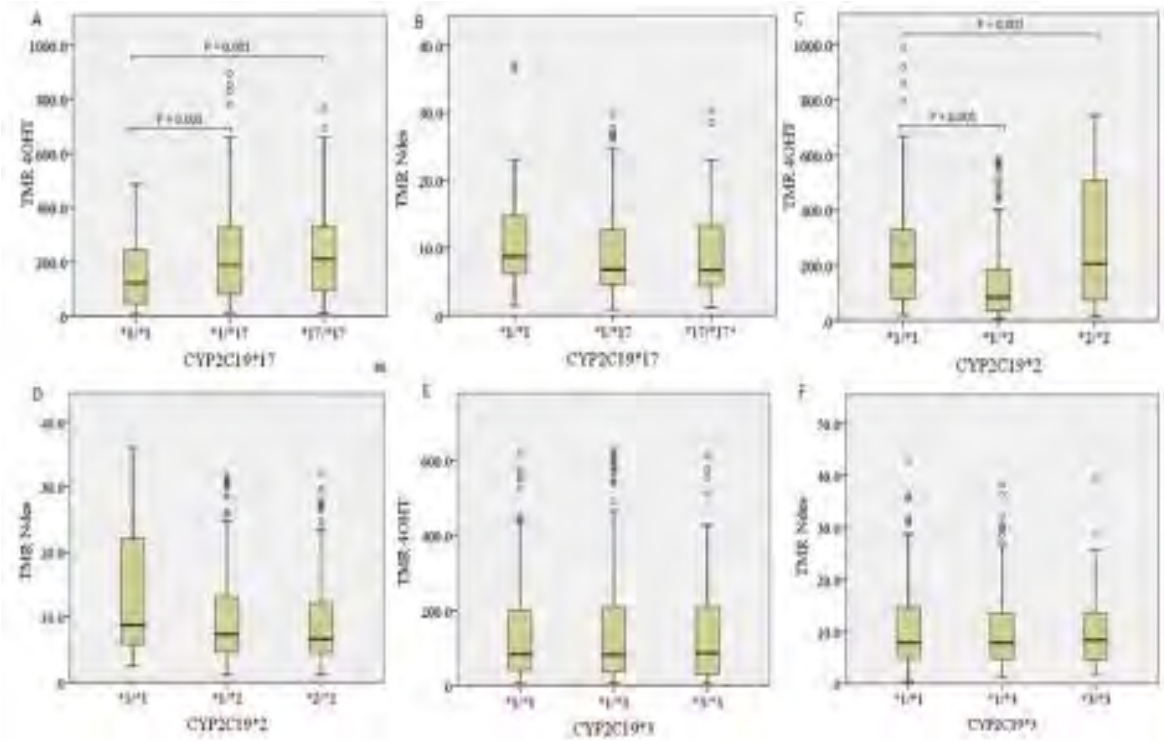


Figure 3.10. CYP2C19 genotypes and metabolic ratios of N-desM-Tamoxifen and 4-HydroxyTamoxifen.

(A) The median TMR of 4-OH-Tam (TMR - 4-OHT) was significantly higher in *1/*17 (P<0.001) and *17/*17 (P<0.001) genotypes than *1/*1 genotype.

(B) An insignificant increase in the median TMR N-desM-Tamoxifen (TMR-N-des) median values with genotype *1/*17 and *17/*17 compared to *1/*1.

(C) Significantly higher median TMR of 4-OH-Tam (TMR- 4-OH-Tam) between the CYP2C19*2 genotypes *1/*2 (P<0.001) and *2/*2 genotype (P=0.2327).

(D) The median TMR N-desM-Tamoxifen (TMR-N-desM-Tam) was similar among *1/*1, *1/*2, and *2/*2 genotypes.

(E) No substantial association was detected in median TMR of 4-OH-Tam (TMR- 4-OH-Tam) between *1/*1, *1/*3 and *3/*3 genotypes.

(F) No significant association was detected for the median TMR N-desM-Tamoxifen (TMR-N-desM-Tam) between *1/*1, *1/*3, and *3/*3 genotypes.

The *CYP2C19* gene plays crucial role in majority Proton Pump Inhibitors (PPI) and antiepileptic drug metabolism. The metabolic pathways have not been completely understood so far. Cytochrome P450 enzymes, major participants in the metabolism of xenobiotics. Breast cancer has been extensively diagnosed cancer in women worldwide (Jemal *et al.,* 2010).

All women, apart from their ethnic or racial origin or familial history, are prone to breast cancer, whereas males are at a decreased risk of developing breast cancer (Naeem *et al.*, 2008). Major influencers in breast carcinoma progression are hormones (endogenous and exogenous) in females, genetic factors, environmental factors.

We earlier reported the frequency of *CYP2C19*2* & *CYP2C19*17* alleles in different racial groups of Pakistan. Moreover, we reportedd that the overall ratio of the expected poor metabolizer (PM) (*2/*2) allele was 29.10% in contrast to the ultra-rapid metabolizer (UM) allele (*17/*17) 23.70% (Riaz *et al.*, 2019). A German study could not find any relation between *CYP2C19*2* polymorphism & breast cancer risk (Justenhoven *et al.*, 2009). Iraqi population suggests that *CYP2C19*2* polymorphisms with the proliferation of breast cancer (Jabir *et al.,* 2018). *CYP2C19*2* heterozygous mutants breast cancer patients taking tamoxifen have greater opportunities of survival (Ruiter *et al.*, 2010) (Bai *et al.*, 2014). Furthermore, the *CYP2C19*3* allele was related to expanded breast cancer. Chinese studies revealed, arachidonic acid's metabolisms can be regulated by *CYP2C19*'s anti-apoptotic effect, subsequently causing breast cancer (Gan *et al.,* 2011)**.** Another study by A. B. Sanchez-Spitman concludes that *CYP2C19* has no notable effect on tamoxifen metabolism or breast cancer relapse (Sanchez-Spitman *et al.,* 2021).

*CYP2C19*17* allele has been linked to the sublime in breast cancer in a German cohort, indicating that *CYP2C19*17* causes increase in estrogen catabolism, declining the risk for breast cancer (Justenhoven *et al.*, 2009).

The study's total sample was 410 unrelated healthy individuals and 430 ER-positive breast cancer patient. The genotypes frequency for *CYP2C19*2* , *CYP2C19*3* and *CYP2C19*17* were calculated for the two groups, and allele frequencies of the *CYP2C19* variants in control and ER Positive breast cancer patient was present, as shown in Table 2.0.

Our result findings (Table 3.4) demonstrates the genotyp result for controlled individuals & estrogen-positive breast cancer patient in the Pakistan. No noticeable change was found among the allele frequencies of *CYP2C19*2;* conditional logistic regressions show that *CYP2C19*2* heterozygous conditions were a risk reason for breast cancer (OR: 0.56; 95% CI: 0.413– 0.84; *P*=0.003). The current findings strongly encourages the statistical contribution of the *CYP2C19*33* heterozygous variant in the formation of breast cancer (OR: 90.34; 95% CI: 0.224–0.418; *P*<0.001). Mostly samples were from to the extensive metabolizer (EM) group in normal individual and ER-positive breast cancer patients. Table 3.4 demonstratess the allelic frequency of *CYP2C19* variants, but a notable difference was recorded for unrelated healthy individual and ER-positive breast cancer patients for *CYP2C19*17* allele frequencies. Over 65.0% of unrelated healthy individuals were extensive metabolizers (**1/*1*) in *CYP2C19*. On contrary, results were very different for ER-positive breast cancer patients, representing more than 70.0% of them were ultra-rapid metabolizers (**1/17**, **17/17*) (OR: 7.717; 95% CI: 5.108–10.11).

Multiple cytochrome P450 enzymes and polymorphisms in the genes producing these enzymes facilitates the multichannel metabolism of tamoxifen, exerting a influential impact on tamoxifen and its various metabolites plasma concentration. In the present research, we emphasized to elucidate the influence of earlier identified polymorphisms in genes coding for the enzymes catalyzing the tamoxifen's metabolism and its metabolites in Pakistani patients with breast cancer.

In the light of the current findings, the *CYP2C19*17* (-806C>T; rs12248560) allele was corelated with higher plasma metabolic ratio of 4-OH-tamoxifen for plasma, whereas the linkage with N-DesM-Tam was non-significant. Thus indicates that, there is accumulation of 4-OH-tamoxifen & tamoxifen in plasma when the metabolic conversion of 4-OH-tamoxifen to endoxifen is not sufficient. Impaired conversation of 4-OH-tamoxifen to endoxifen was also suggested by total metabolic ratios. There was substantial increment was noted for 4-OH-Tam plasma concentrations in patients carrying CYP2C19 **1/*2* & **2/*2* genotype.

N-DesM-Tam plasma concentrations were similar for the patient with **1/*2, *1/*17,* and **2/*2, *17/*17* genotypes. Minor fluctuation for endoxifen plasma concentrations in patient having genotype *CYP2C19*1/*11,*1/2 *2/*2,* and **1/*17,*17/*17* . Plasma concentrations of 4-OH-tamoxifen in patient with **1/*3* and **3/*3* genotype was found to be lower than those have **1/*1* genotype.

An expanded gene expression of the *CYP2C19*17* allele resulting in a putative ultra-rapid (UMm) phenotype (Sim *et al.*, 2006). *CYP2C19* carries the tamoxifen metabolism to anti-estrogenic metabolite 4-OH-tamoxifen, conceiving *in vitro* activities similar to *CYP2D6* (Desta *et al.*, 2004) **(Coller *et al.*, 2002)**. Our data suggest that *CYP2C19*17* has a crucial and significant part in the formation of plasma concentrations of 4-OH-tamoxifen. An active type of *CYP2C19*17* can produce significant results toward the control of breast cancer recurrence, previously published by Schroth and his co-workers (Schroth *et al.*, 2007). But, our study oppses the findings of Joanne S. L. Lim et al., suggested no relationship between *CYP2C19* polymorphisms and tamoxifen's pharmacokinetics (Lim *et al.*, 2011)

3.3 Study III

A total of 430 ER-positive breast cancer patients and 400 unrelated healthy individuals were included in the study, which aimed to regulate the frequency of CYP2C9*2 and *3 gene polymorphism and their impact on the metabolism of tamoxifen and its metabolites

3.3.1. Genotyping

Genotyping of four hundred breast cancer patients for *CYP3A4*1/*1*, *CYP3A4*22/*22*, and *CYP3A4*1/*22* was done with the help of Amplification refractory mutation system (ARMS) by thermocycler Biorad T100TM. The results were visualized on 2% agarose gel.

3.3.1.1 *CYP3A4*22* SNP in Intron 6 at 15389C>T

By ARMS PCR, the *CYP3A4*22* gene was amplified. The control band of 232 bp was amplified, as shown in figure 19. Three bands produced in this case were 232 bp (control), the `C` allele (wild type) 107 bp, and the `T` allele (mutant) 162 bp. In the case of detecting all three bands, the subject was considered heterozygous *(CYP3A4*22/*22)*. The subject was designated as homozygous wild type *(CYP3A4*1/*1)* if two bands of 232 and 107 bp were detected and homozygous mutant *(CYP2C19*17/*17)* if two bands of the sizes 232 bp and 162 bp were observed. Heterozygous individuals (OR: 2.79; 95% CI: 1.45 –5.34; *P*=0.43) and homozygous mutant individuals (OR: 0.42; 95% CI: 0.18–1.23; *P*=0.11). Statistical analysis revealed that *CYP3A4*22* plays no significant role in the breast cancer development in the current study population. (Figure 3.11, Table 3.7)

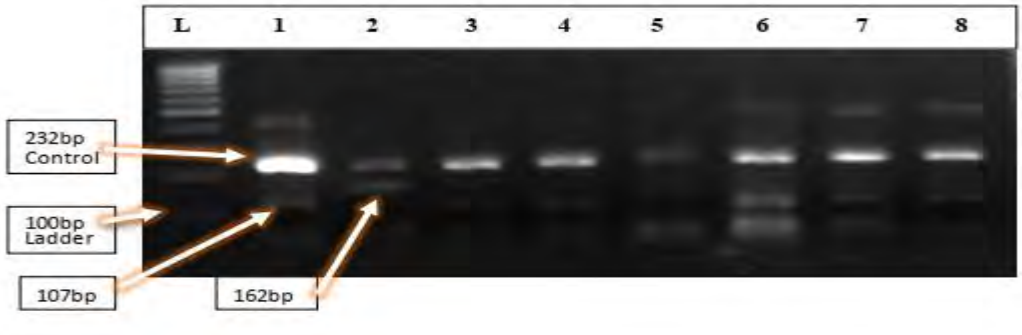


Figure 3.11: Electropherogram for *CYP3A4*22* gene. The control band is 232 bp, the wild-type C-allele band is 107 bp, and the mutated T-allele band is 162 bp. A 100 bp ladder was used for comparison.

Table 3.7. Association of CYP3A4 genotypes with ER Positive breast cancer

Genotype			Controls (410)		Cases (430)			
			n	%	n	%	OR (Upper Limit-Lower Limit)	p-value
CYP3A4*22								
CC	EM [a]	*1/*1	386	94.14	389	90.46	0.5 (0.34-0.99)	0.04
CT	IM [a]	*1/*22	13	3.17	36	8.37	2.79 (1.45-5.34)	0.002
TT	PM [a]	*22/*22	11	2.68	05	1.16	0.42 (0.1- 1.23)	0.11

[a] EM= Extensive Metabolizer , , IM = Intermediate Metabolizer, PM = Poor Metabolizer,

3.3.2. Profiling of Tamoxifen Metabolism

Tamoxifen is metabolized by several enzymes, like *CYP2D6, CYP2C19, CYP2C9, CYP2B6* and *CYP3A* into 4OH-Tamoxifen. *CYP3A4* is a polymorphic gene. One of the alleles, *CYP3A4*22,* has reduced enzyme activity. To determine the association of *CYP3A4*22* in the tamoxifen metabolism, plasma analysis of 430 breast cancer patients was done by HPLC Agilent 1100 UV system.

Out of 430 collected samples, most of the samples had *CYP3A4*1* (wild type) genotypes according to the determined allele frequency (Table 3.7). Therefore, an association between *CYP3A4*22* allele frequency and plasma concentration of tamoxifen and its metabolites was determined. Tamoxifen and its metabolite concentration were also compared between the **1/*1, *1/*22,* and **22/*22* genotypes.

The tamoxifen and three metabolites' median plasma concentrations were calculated in patients in each genotypic group depicted in Table 3.8 and pairwise P value in Table 3.9. No significant difference was observed for the median plasma tamoxifen, 4-OH-Tam, NDM, and endoxifen concentrations among the three different genotypes of patients. (Table 4; Figure 3.12).

Table 3.8. Effects of *CYP3A4* polymorphisms on median metabolic ratios of Tamoxifen and its analytes

Parameters ***CYP3A4*22*** **(430 C>T)**	**Genotype *1/*1 (EM)[a1]**	**Genotype *1/*2 (IM)[a1]**	**Genotype *2/*2 (PM)[a1]**
Tamoxifen	113.00	118.49	118.87
4-OHT	228.08	231.28	233.40
NDM	4.08	3.80	3.93
Endoxifen	25.64	26.41	27.51
[a] EM= Extensive Metabolizer , , IM = Intermediate Metabolizer, PM = Poor Metabolizer, **[1] for NM n = 273, IM n = 103, and PM n = 54** **[2] for NM n = 128, IM n = 126, and PM n = 176**			

Table 3.9. Pairwise P value comparison between different genotypes

Parameters ***CYP2C9*2*** **(430 C>T)**	**Pairwise P values**	**Pairwise P values**
	***1/*1 vs *1/*22**	***1/*1 vs *22/*22**
Tamoxifen	0.88	0.143
4-OHT	0.06	0.139
NDM	0.73	0.55
Endoxifen	0.82	0.39

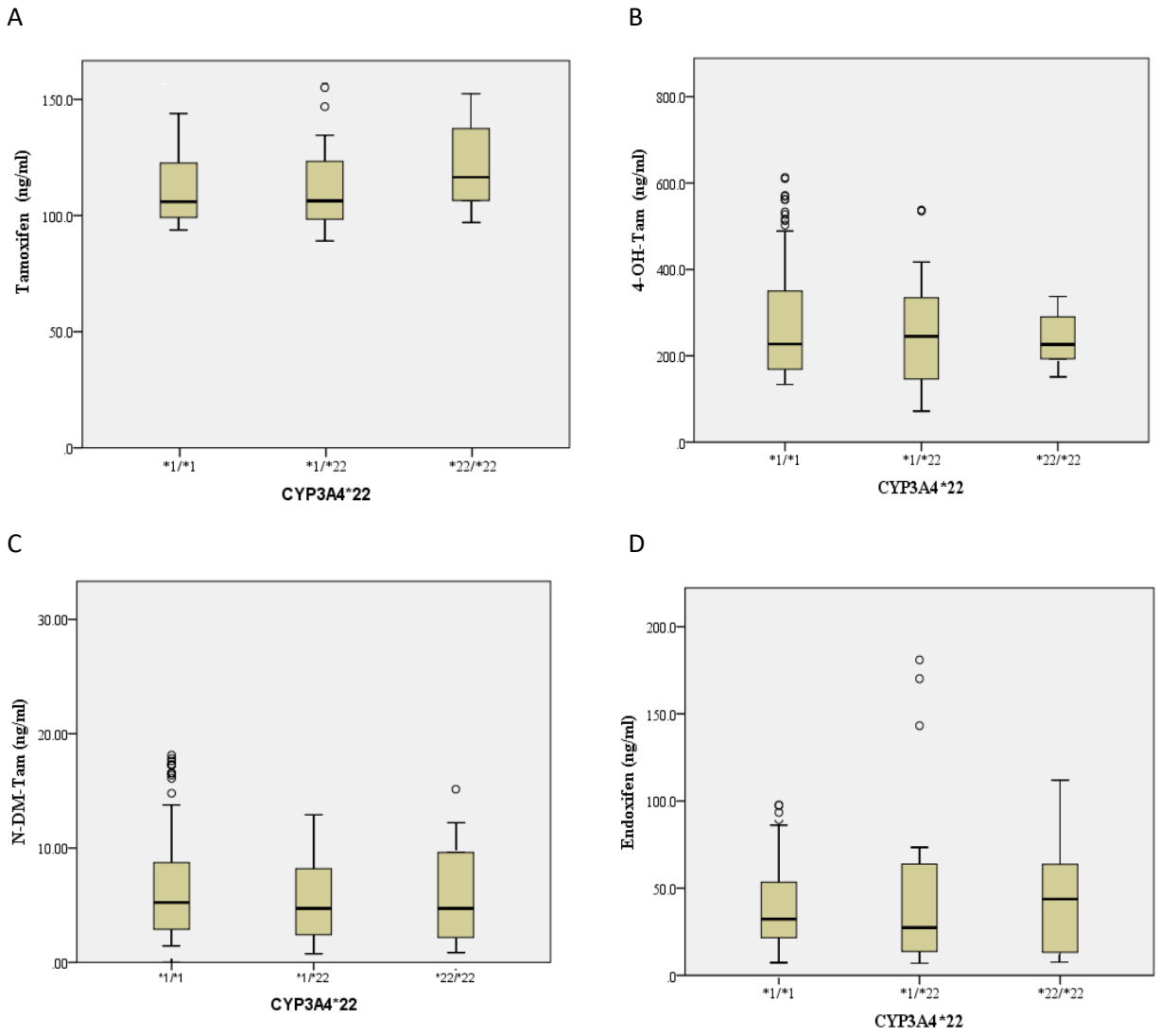


Figure 3.12. Association between CYP2C19*2 genotypes and steady-state median plasma concentrations of (A) Tamoxifen, (B) 4-OH-Tam, (C) N-desM-Tamoxifen, and (D) Endoxifen

In the present study, 410 unrelated healthy individuals and 430 ER-positive breast cancer patients made up the total sample size. The genotype frequencies for *CYP3A4*22* were calculated between the two groups. The allele frequencies of the *CYP3A4* variant in breast cancer patients with ER-Positive status and healthy controls are shown in Table 3.7. Our findings (Table 3.7) demonstrated the genotyping results for unrelated healthy individuals and estrogen-positive breast cancer patients from Pakistan. No noteworthy difference was detected between the allele frequencies of *CYP3A4*22*.

The genotype frequency of *CYP3A4*1/*1*, *CYP3A4*1/*22* and *CYP3A4*22/*22* determined in four hundred thirty breast cancer subjects taking estrogen adjuvant monotherapy were 90.46%, 8.37%, and 1.16%, respectively. In the present results, the percentage frequency of *CYP3A4*1/*1* was high in estrogen receptor-positive BC patients, which can be associated with increased disease progression. CYP3A4 plays an important part in the tamoxifen and its more efficient metabolites' metabolism (Zhou *et al*., 2009). The tamoxifen and its three metabolites median plasma concentrations in *CYP3A4*1/*1*, *CYP3A4*1/*22*, and *CYP3A4*22/*22* genotype shown in Table 3.8. The median plasma concentration of tamoxifen indicates that *CYP3A4*1/*1*, *CYPP3A*1/*22* and *CYP3A4*2/*22* have the almost same concentration of tamoxifen. The significant value determined for Tamoxifen was higher than 0.05. The significant value (determined by Mann-Whitney U test). These results are comparable with Baxter et al., (2014), according to which CYP3A4*22 genetic variation leads to reduced enzymatic activity.

3.4. Study IV

3.4.1. Genotyping of CYP3A5

A total of 430 ER Positive breast Cancer patients and 400 unrelated healthy individuals were incorporated in the study, which aimed to determine the frequency of *CYP3A5*3* and *CYP3A5*6* gene polymorphism and their impact on metabolism of tamoxifen and its metabolites. Allele Specific PCR was performed to determine genotypes and PCR products were visualized on 2% agarose gel electrophoresis.

3.4.1.1. *CYP3A5*3* SNP in Intron 3 at 6986A>G

Two sets of AS-PCR were performed for *CYP3A5*3* genotyping. Normal "A" allele band of 285 bp and mutant "G" specific band of 285 bps were amplified and viewed on gel and are shown in **Figure 3.13** . Homozygous normal and homozygous mutant allele showed band in single lane while heterozygous showed bands in both lanes. *CYP3A5*3* heterozygous (OR: 0.8; 95% CI: 0.64–1.12; P = 0.25), Statistical analysis revealed that *CYP2C9*3* plays no significant role in the development of breast cancer in the current study population, whereas homozygous mutant (OR: 2.71; 95% CI: 1.90–3.86; P < 0.0001) condition was at breast cancer risk factor in our population (Table 3.10).

Table 3.10 Association of CYP3A5 genotypes with ER Positive breast cancer

Genotype			Controls (410)		Cases (430)			
			n	%	n	%	OR (Upper Limit-Lower Limit)	p-value
CYP3A5*3								
CC	NM [a]	*1/*1	119	27.67	186	46.5	Ref (1)	
CT	IM [a]	*1/*2	183	42.55	160	40.0	0.8 (0.64-1.46)	0.25
TT	PM [a]	*2/*2	128	29.76	54	12..5	2.71 (1.90-3.86)	<0.0001
CYP3A5*6								
AA	NM [a]	*1/*1	343	85.75	319	74.18	Ref (1)	
AC	IM [a]	*1/*3	22	5.5	83	19.30	4.10 (2.51-6.72)	<0.001
CC	PM [a]	*3/*3	35	8.75	28	6.51	0.72 (0.43-1.21)	0.22

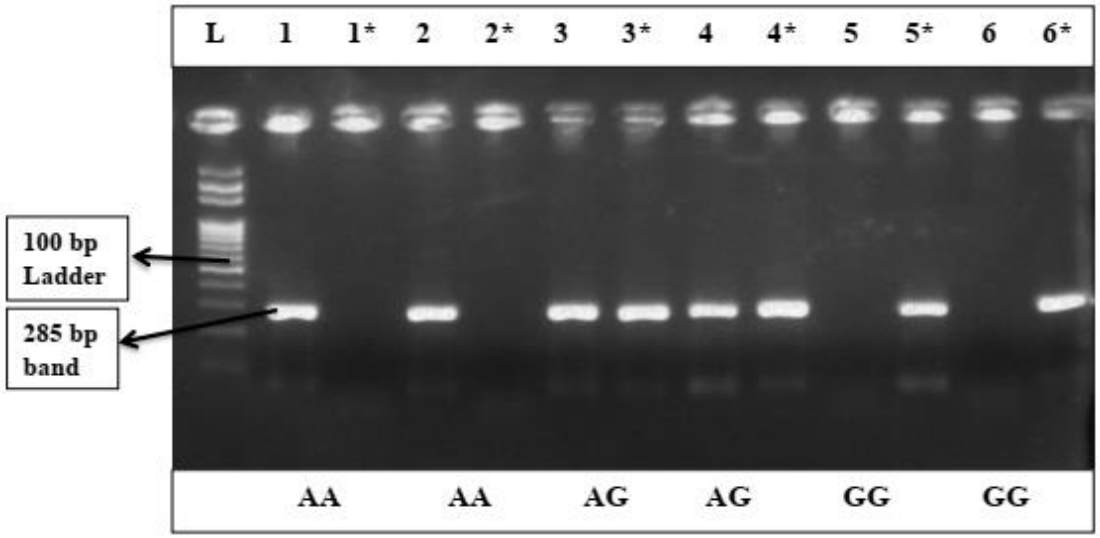


Figure 3.13. Electropherogram of Allele-specific PCR for *CYP3A5*3*. 'L' represents 100 bp ladder which was used for comparison of band length. Asterisk (*) represents mutant allele. Normal 'A' allele and mutant 'G' allele bands are of 285 bp length. and 1* are showing homozygous normal genotype (AA), 3 and 4 represent
heterozygous condition (AG) while, 5 and 6 represent homozygous mutant allele
(GG)

3.4.1.2. *CYP3A5*6* SNP in Exon 7 at 14690G>A

Two sets of AS-PCR were performed for CYP3A5*6 genotyping. Normal 'G' allele band of 150 bp and mutant 'A' allele specific band of 150 bp were amplified and viewed on agarose gel. Results are shown in Figure 3.14. Homozygous mutant and homozygous normal samples showed band in single lane and heterozygous showed were appeared in both lanes.

*CYP3A5*6* heterozygous (OR: 4.10; 95% CI: 2.51 – 6.72; P < 0.25) Statistical analysis revealed the strong association with risk of developing breast cacner. Whereas CYP*3A5*6* homozygous mutant (OR: 0.72; 95% CI: 0.43–1.21; P = 0.22) plays no significant role in the development of breast cancer in the current study population.

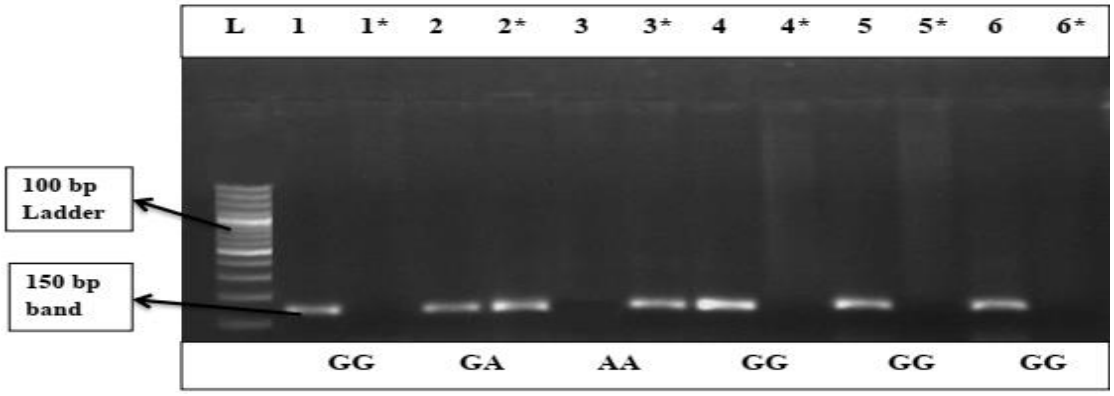


Figure 3.14. Electropherogram of AS-PCR for *CYP3A5*6*. 'L' represents 100 bp DNA ladder which was used for band length comparison. Asterisk (*) represents mutant allele. Normal 'G' and mutant 'A' allele specific band fragments are 150 bp in size. 1, 4, 5 and 6 show homozygous normal genotype (GG) while 2 and 2* show heterozygous genotype (GA) and 3 and 3* show homozygous mutant genotype (AA).

3.4.2. Tamoxifen metabolites profiling

Tamoxifen is metabolized by several enzyme like *CYP2D6, CYP2C19, CYP2C9, CYP2B6* and *CYP3A4/5* in to 4OH-Tamoxifen. Among these, *CYP3A4/5* are accountable for about 50% of tamoxifen metabolism. Among all the CYP enzymes, CYP3A4/5 and CYP2D6 play prominent roles in Tamoxifen metabolism. CYP3A5 play a substantial role in tamoxifen conversion to its primary metabolite, N-desmethyl tamoxifen and in the metabolism of 4-OH TAM to a secondary metabolite, endoxifen (Charoenchokthavee *et al*., 2016).

Table 3.11. Effects of *CYP3A5* polymorphisms on median plasma ratios of Tamoxifen and its analytes

Parameters *CYP2C9*2* (430 C>T)	**Genotype *1/*1 (NM)[a1]**	**Genotype *1/*3 (IM)[a1]**	**Genotype *3/*3 (PM)[a1]**
Tamoxifen	132.24	131.50	132.48
4-OHT	228.57	231.08	231.55
NDM	4.43	3.38	3.87
Endoxifen	23.88	29.41	26.95
Parameters *CYP2C9*3* (1075 A>C)	**Genotype *1/*1 (NM)[a2]**	**Genotype *1/*6 (IM)[a2]**	**Genotype *6/*6 (PM)[a2]**
Tamoxifen	142.93	141.13	143.45
4-OHT	244.34	263.50	288.95
NDM	5.92	4.52	4.46
Endoxifen	32.58	31.87	27.51

[a] NM= Normal Metabolizer , , IM = Intermediate Metabolizer, PM = Poor Metabolizer,
[1] for NM n = 186, IM n = 160, and PM n = 54
[2] for NM n = 319, IM n = 83, and PM n = 28

Polymorphism in the *CYP3A5* genotype accounts for variability in concentrations of Tamoxifen metabolites individually as well as inter-ethnically thus affecting drug response (Lee *et al*., 2003; Charoenchokthavee *et al*., 2017). Only *CYP3A5*1* is functional allele and the individual possessing it's both alleles is normal or extensive metabolizer of tamoxifen. Remaining all other *CYP3A5* allele variants like *CYP3A5*3* and **6* is non-functional and refer to poor metabolizer phenotype (Lamba *et al*., 2012). *CYP3A5* is highly polymorphic.

Its two alleles *CYP3A5*3* and *CYP3A5*6* represent reduced enzyme activity. In order to find association between *CYP3A5*3* and *CYP3A5*6* in metabolism of tamoxifen and its metabolites plasma analysis of 430 ER positive breast cancer patients was done by HPLC Agilent 1100 UV system.

CYP3A5 polymorphisms impact on the plasma concentrations of tamoxifen and its metabolites.

The median plasma concentrations of tamoxifen and three metabolites measured in patients in each genotypic group shown in Table 3.11, Figure 3.15. The associations of tamoxifen and its derivatives with the *CYP3A5*1/*1* (wild type), *CYP3A5*1/*3* (heterozygous), and *CYP3A5*3/*3* (mutant) genotypes are depicted in Figure 3.15

No momentous difference was observed for the median plasma concentration of tamoxifen, among the three genotypes *CYP3A5*1/*1* (132.24 ng/ml) *CYP3A5*1/*3* (131.50 ng/ml) and mutant *CYP3A5*3/*3* (132.48 ng/ml).

No association was observed between 4-OH- Tam median plasma concentration and the three genotypes *CYP3A5*1/*1* (wild type), *CYP3A5*1/*3* (heterozygous), and *CYP3A5*3/*3* (mutant) respectively (228.57ng/ml, 231.08 ng/ml and 231.55 ng/ml) and NDM plasma median concentration (4.43 ng/ml, 3.38ng/ml and 3.87ng/ml). However, insignificant increase was observed in median plasma concentration of Endoxifen in the subjects having heterozygous (**1/*3*) (29.41ng/ml) (P = 0.161) and mutant (**3/*3*) (26.95) (P = 0.658) genotypes in comparison with the subjects having wild-type genotype (**1/*1*) (Table 3.11; Figure 3.2b) .

Table 3.12. Correlation between CYP2C19*17 and ER Positive Breast Cancer patient's Demographic Characteristics.

		Demographic Characters (Ethnicity, Gender, Marital Status, Weight)	Cyp2C19*17
Demographic Characters	Pearson Correlation	1	-.150**
	Sig. (2-tailed)		.003
	N	400	400
Cyp2C19*17	Pearson Correlation	-.150**	1
	Sig. (2-tailed)	.003	
	N	400	410

**. Correlation is significant at the 0.01 level (2-tailed).

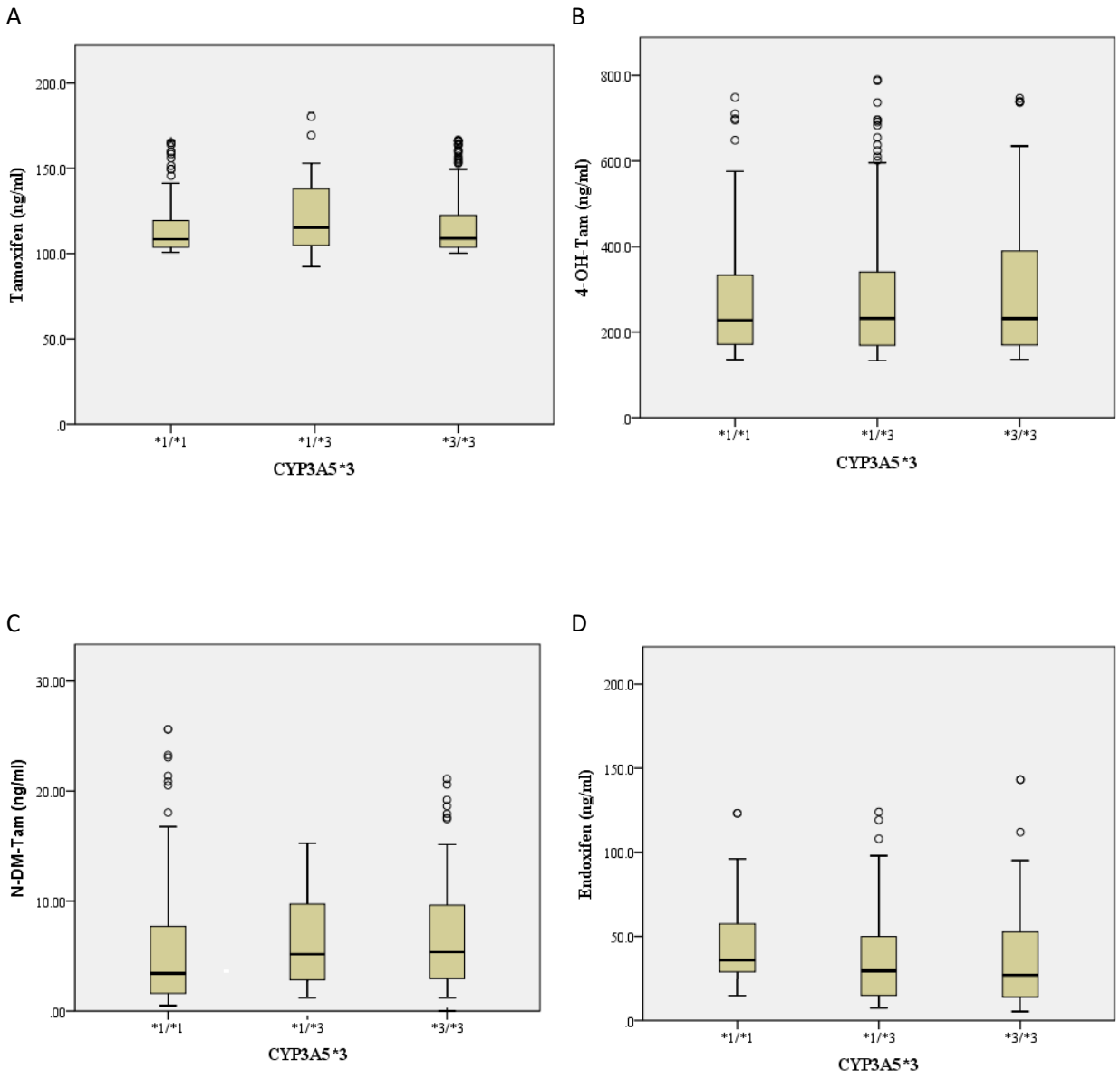


Figure 3.15. Association between CYP3A5*3 genotypes and steady-state median plasma concentrations of (A) Tamoxifen, (B) 4-OH-Tam, (C) N-desM-Tamoxifen and (D) Endoxifen

Figure 3.16 shows the associations of tamoxifen and its derivatives with *CYP3A5*1/*1* (wild type), *CYP3A5*1/*6* (heterozygous), and *CYP3A5*6/*6* (mutant). No significant association was observed for tamoxifen, endoxifen and N-DesM-Tam among the wild-type, heterozygous and mutant genotypes for *CYP3A5*6* locus. However, an insignificant increase was observed in the median plasma concentrations of 4-OH-Tam in the heterozygous (263.5 ng/ml) (*P*=0.242) or mutant (288.95 ng/ml) (*P*=0.363) genotypes, compared to the wild-type (244.34 ng/ml) genotypes at this locus. (Table 3.11, Fig 3.16B).

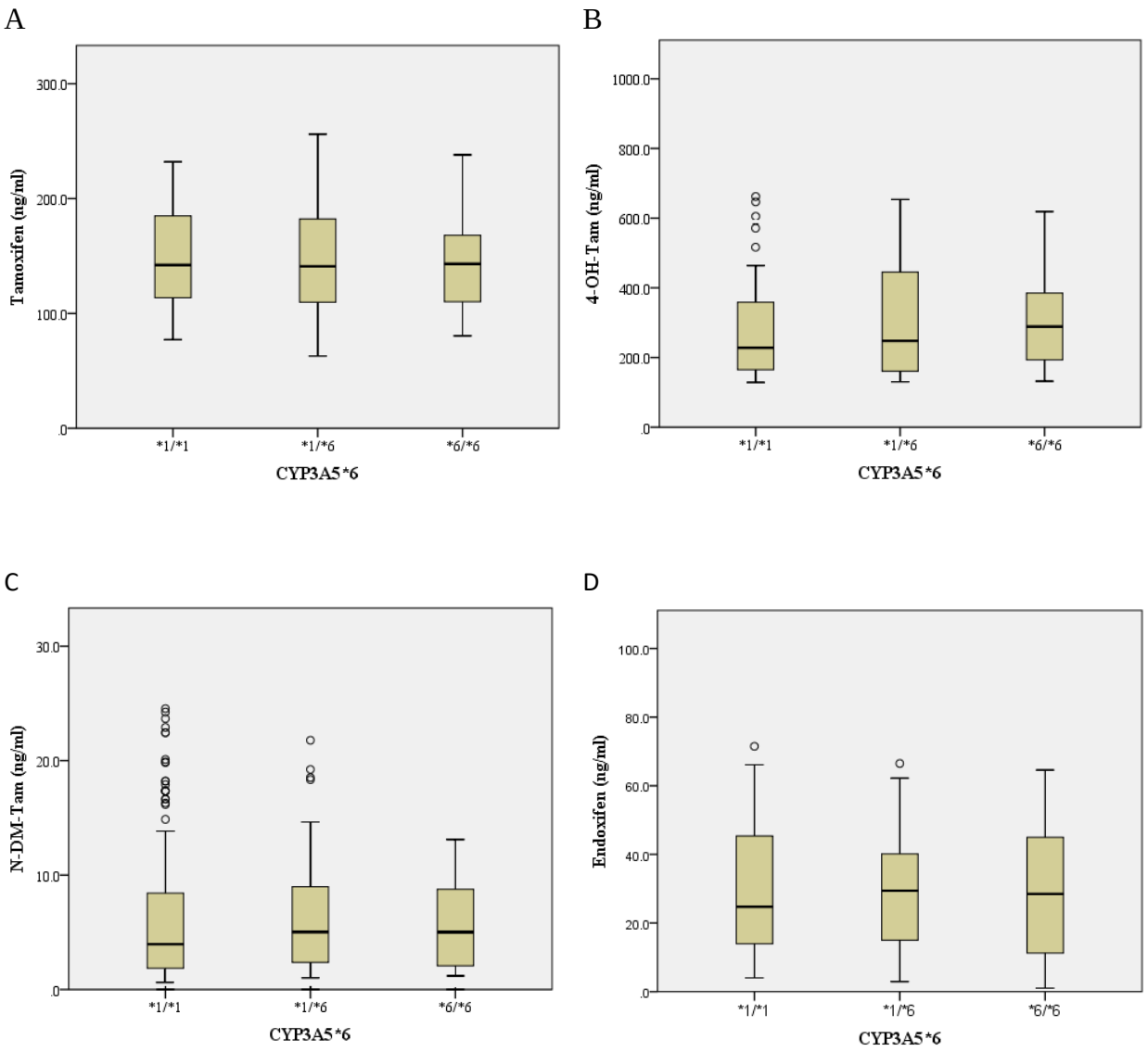


Figure 3.16: Association between CYP3A5*3 genotypes and steady-state median plasma concentrations of (A) Tamoxifen, (B) 4-OH-Tam, (C) N-desM-Tamoxifen and (D) Endoxifen

Tamoxifen is employed as an adjuvant hormonal therapy for ER positive breast cancer patients. It is also used as a preventive drug for those females who are at high risk of developing breast cancer. Tamoxifen is an anti-estrogen drug which competitively binds to ER and inhibits cell proliferation and growth in cancer tissues.

Tamoxifen is metabolized in liver by the activity of CYP enzymes to more potent metabolites like 4-OH TAM and Endoxifen which show greater anti-estrogenic properties. About 75% of all drug metabolism occurs through CYP enzymes (Iyanagi,

2007).

Among all the CYP enzymes, CYP3A4/5 are chief enzymes and are responsible for 30% of entire drug metabolism and constitute 50% hepatic CYP content (Zanger and Schwab, 2013). CYP3A5 is also expressed in other organs of body besides liver and contribute to drug metabolism. CYP3A5 is highly polymorphic and has many variants which are accountable for inter-individual and inter-ethnic variability in CYP3A5 expression (Lamba *et al*., 2012). Two allelic variants *CYP3A5*3* and *CYP3A5*6* represent poor metabolizer phenotype.

In the present study, the total sample was 410 unrelated healthy individuals and 430 ER-positive breast cancer patients. The genotype frequencies for *CYP3A5*3* and *CYP3A5*6* were calculated between the two groups and allele frequencies of the *CYP3A5* variants in control and ER Positive breast cancer patients were expressed, as shown in Table 3.10.

Our findings (Table 3.10) demonstrate the genotyping results for unrelated healthy individuals and estrogen-positive breast cancer patients in Pakistani population. No noteworthy difference was detected between the allele frequencies of *CYP3A5,* but conditional logistic regression shows that *CYP3A5*3* homozygous condition OR: 2.71; 95% CI: 1.90–3.86; P < 0.0001) was a risk factor for breast cancer. The current study strongly implicates the statistical contribution of *CYP3A5*3/*3* homozygous variant in the breast cancer development. Majority of the samples belonged to extensive metabolizer (EM) group in unrelated healthy individuals and ER-positive breast cancer patients.

Genotyping results of *CYP3A5*6* (Table 3.10) showed that most of the breast cancer patients and unrelated healthy individuals belonged to extensive metabolizer group, which indicates the least association of *CYP3A5*6* in development and progression of breast cancer in our population. But in contrary to this, those individuals carrying *CYP3A5*6* heterozygous ((OR: 4.10; 95% CI: 2.51 – 6.72; P < 0.25) are at risk of developing breast cancer. Statistical analysis revealed the strong association with risk

of developing breast cancer. Whereas *CYP3A5*6* homozygous mutant (OR: 0.72; 95% CI: 0.43–1.21; P = 0.22) plays no considerable role in the breast cancer developme]nt in the current study population.

CYP3A5 plays role in the conversion of tamoxifen to its metabolites like 4-OH TAM and endoxifen. Plasma concentrations of Tamoxifen and 4-OH TAM were

determined. The median plasma concentrations of tamoxifen in *CYP3A5*1/*1*, *CYP3A5*1/*3* and *CYP3A5*3/*3* genotype was 132.248 ng/ml, 131.50ng/ml and 132.48ng/ml.

The median plasma concentration of tamoxifen reflects that *CYP3A5*1/*1*, *CYP3A5*1/*3* and *CYP3A5*3/*3* have approximately same concentration of tamoxifen. The significant value was determined which indicated that there is no significant variation in concentration of tamoxifen among *CYP3A5*1/*1*, *CYP3A5*1/*3* and *CYP3A5*3/*3* genotype.

The median plasma concentrations of 4-OH TAM in *CYP3A5*1/*1*, *CYP3A5*1/*3* and *CYP3A5*3/*3* genotype were 228.57ng/ml, 231.08ng/ml and 231.55ng/ml respectively. No significant difference was observed among the three genotypes.

No association was detected between the median plasma concentration of N desmethyl tamoxifen and the three genotypes *CYP3A5*1/*1*, *CYP3A5*1/*3* and *CYP3A5*3/*3.*

The associations of tamoxifen and its derivatives with the *CYP3A5*1/*1* (wild type), *CYP3A5*1/*3* (heterozygous), and *CYP3A5*3/*3* (mutant) genotypes are depicted in Figure 3.15. No significant disparity was observed for the median plasma concentration of tamoxifen, among the three genotypes *CYP3A5*1/*1* (132.24 ng/ml) *CYP3A5*1/*3* (131.50 ng/ml) and mutant *CYP3A5*3/*3* (132.48 ng/ml).

No association was observed between 4-OH- Tam median plasma concentration and the three genotypes *CYP3A5*1/*1* (wild type), *CYP3A5*1/*3* (heterozygous), and *CYP3A5*3/*3* (mutant) respectively (228.57ng/ml, 231.08 ng/ml and 231.55 ng/ml) and NDM plasma median concentration (4.43 ng/ml, 3.38ng/ml and 3.87ng/ml). However, insignificant increase was observed in median plasma concentration of Endoxifen in the subjects having heterozygous (**1/*3*) (29.41ng/ml) (P = 0.161) and

mutant (**3/*3*) (26.95) (P = 0.658) genotypes in comparison with the subjects having wild-type genotype (**1/*1*). Our study demosttrates that *CYP3A5* do not have any significant impact on the metabolism of tamoxifen and its metabolites. Our results are comparable to other studies conducted Jin *et al*. (Jin *et al.*, 2005) formerly observed a non-significant rise in endoxifen concentrations in patients harbouring **1/*1* and **1/*3* genotypes than patients carrying **3/*3* genotype, the plasma concentrations of

endoxifen or other analytes were not found to differ considerably across different genotypes in our study population. Correspondingly, Tucker *et al* (Tucker *et al.*, 2005) also did not find any significant association between *CYP3A5*3* (6986A>G; rs776746) and the plasma concentrations of tamoxifen and its metabolites. It is likely that the functional impact of *CYP3A5*3* (6986A>G; rs776746) on the tamoxifen metabolism is nullified by the presence of other enzymes (CYP3A4, CYP1A2 and CYP2C9) which catalyze the N-demethylation of tamoxifen and 4-OHT (CYP3A4). Another study conducted by Lim and coworkers also did not find any substantial association between *CYP3A5* and Tamoxifen metabolism (Lim *et al.*, 2011) Hence, further investigations on the influence of these polygenic determinants on the pharmacokinetics of tamoxifen are warranted.

3.5. Study V

3.5.1. Effects of Estrogens, Tamoxifen and Metabolites, and Endoxifen on Cell Proliferation

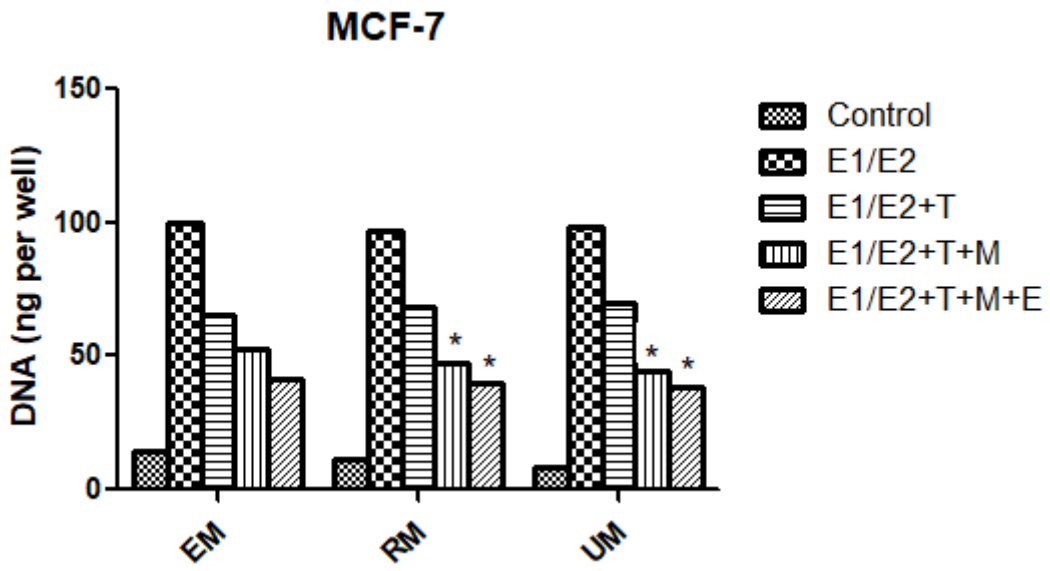
To evaluate the biological effect of the diverse treatments on the panel of9ER-positive breast cancer cell lines (MCF-7, T47D), we used a DNA quantification-based assay as described in the Methods section. To pretend the premenopausal environment we used E1 and E2 concentrations at average circulating levels measured in premenopausal women taking tamoxifen (Jordan *et al.*, 1991). The calculated concentrations for E1 and E2 were 4 and 2 nM, respectively, for luteal phase, which corresponds to the average levels of oestrogens throughout the 30-day menstrual cycle in patients taking tamoxifen. The tamoxifen and its metabolites concentrations grouped by CYP2C19*17 genotypes portrayed in Table 2.15. Estrogens were stimulate the growth of all cells lines ($P < 0.05$ by one-way ANOVA with Tukey's pair-wise comparisons) (Figure 3.17a) and the addition of tamoxifen (labelled as T in the figures) and the combined primary metabolites 4OHT and NDMTAM (labelled as M in the figures) were able to only partially but significantly inhibit the estrogen action in all the cell lines ($P < 0.05$ by one-way ANOVA with Tukey's pair-wise comparisons) (Figure 3.17a). Endoxifen (labelled as E in the figures) in EM concentration in combination with tamoxifen, 4OHT and NDMTAM further inhibit estrogen action and reduce proliferation further ($P < 0.05$ by one-way ANOVA with Tukey's pair-wise comparisons) (Figure 3.17a). Addition of 4OHT and NDMTam at the RM (Rapid Metabolizer) concentration produced more of an anti-estrogenic effect when compared with EM concentration, furthermore endoxifen E addition in M (4OHT and NDMTAM) concentration in combination with tamoxifen, was able to further inhibit estrogen action and reduce proliferation further ($P < 0.05$ by one-way ANOVA with Tukey's pair-wise comparisons) when compared to EM concentration (Figure 3.17a). Treatment with UM (Ultra Raid Metabolizer) concentration produced similar results to RM (Rapid Metabolizer) but showed more anti-estrogenic effect when compared to EM concentration in MCF-7 cell lines ($P < 0.05$ by one-way ANOVA with Tukey's pair-wise comparisons).

T47D cell line was also treated with same compounds shown in table 2.15. Results suggest that T47D is less responsive towards estrogens when compared to MCF-7 cell lines. However, similar sort of results was obtaining with T47D cells when treated with

different concentration of CYP2C19*17 genotypes (EM, RM and UM). Significant difference was observed in proliferation between the EM, RM and UM concentrations (Figure 3.17b).

It should be observed that the endoxifen addition to tamoxifen and its primary metabolites did not completely hinder the effects of estrogens to vehicle control levels in any of the cell lines, in any of the genotype groups ($P < 0.05$ by one-way ANOVA with Tukey's pair-wise comparisons), MCF-7 cell line is the most estrogen responsive .There is a noteworthy difference in cell numbers between vehicle and endoxifen with tamoxifen and primary metabolites treatment ($P < 0.05$ by one-way ANOVA with Tukey's pairwise comparisons) (Figure 3.17a). We decided to focus in our further experiments on the MCF-7 cell line since it is the most estrogen-responsive and most difficult to prevent growth.

A



B

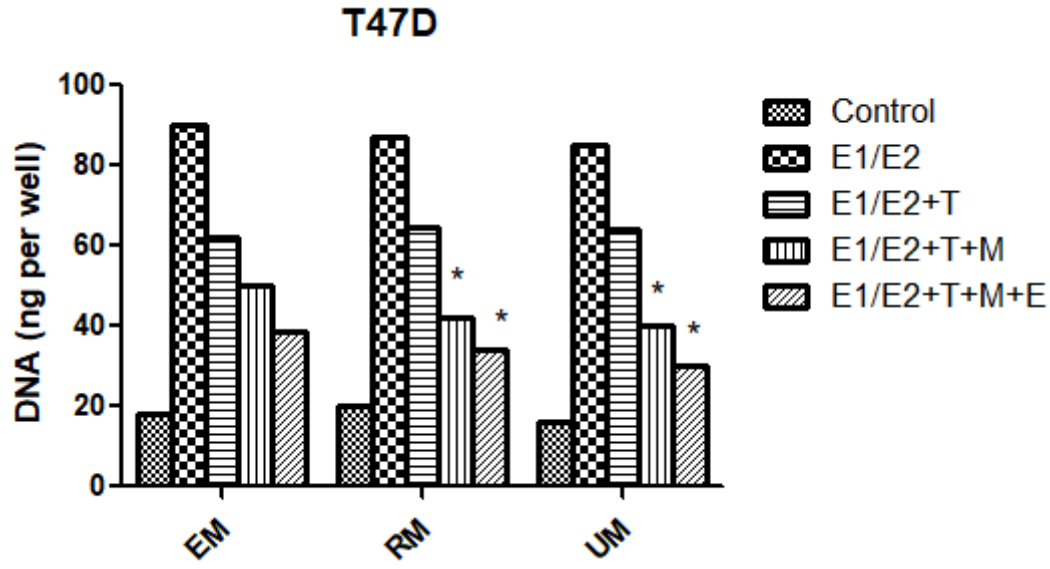


Figure 3.17: Results of ER-positive breast cancer cell proliferation assays: (A) MCF-7, (B) T47D. Treatments were made as follows: Control; E1/E2 9premenopausal estrogens (E1 4 nM, E2 2 nM); E1/E2 + T + M, estrogens at premenopausal levels with tamoxifen (T) and its primary metabolites (NDMTAM and 4OHT) (M) at different CYP2C19*17 genotype concentrations. E1/E2 + T + M + E, estrogens9at premenopausal levels; tamoxifen (T), primary metabolites (M) and endoxifen (E) (Table 2.15. Asterisk indicates statistically significant change in treatment from addition of 4OHT and endoxifen.

3.5.2. Effect of 4-OH- Tamoxifen Endoxifen on Estrogen-Stimulated Genes

To consider the estrogenic and antiestrogenic effect on the transcriptional activity of ER and estrogen-responsive gene expression in MCF-7 cells, we focused on the contribution to the overall effect of estrogen and the antiestrogenic effect of either or both active metabolites 4OHT and endoxifen. We executed RT-PCR as illustrated in the methods section and used primers for *GREB1* and *pS2* estrogen-responsive genes. Results for all genes were similar. We explained that *GREB1* (Figure 3.18), a regulator of hormone response in breast cancer (Rae *et al.*, 2005), was activated 333.42±58.18-fold by estrogens compared with vehicle control ($P = .005$, Student's t test). Addition of EM (Extensive metabolizer) concentrations (Table 2.15) of tamoxifen and NDMTAM reduced the estrogenic effect by 23±5.3% of E1/E2 treatment but was statistically significantly different from vehicle control ($P = .007$). Addition of 4OHT in EM concentration reduced the fold change in *GREB1* mRNA levels even more when compared with the tamoxifen and NDMTAM combination treatment (down to 35±1.08% of E1/E2 treatment, $P = .022$). Addition of endoxifen to tamoxifen and its primary metabolite mix (TPM) further inhibited the estrogenic effect. Additionally, we studied the impacts of RM (Rapid metabolizer) and UR (Ultra Rapid metabolizer) treatments on *GREB1* gene. Results shows that RM concentration (Table 2.15) of Tamoxifen, NDMTAM reduced the estrogenic effect, but addition of 4OHT along with Endoxifen further decreased the estrogenic effect (Figure 3.18b). Similar results were found with UM concentrations (Table 2.15) of Tamoxifen and the three metabolites are shown in Figure 3.18c.

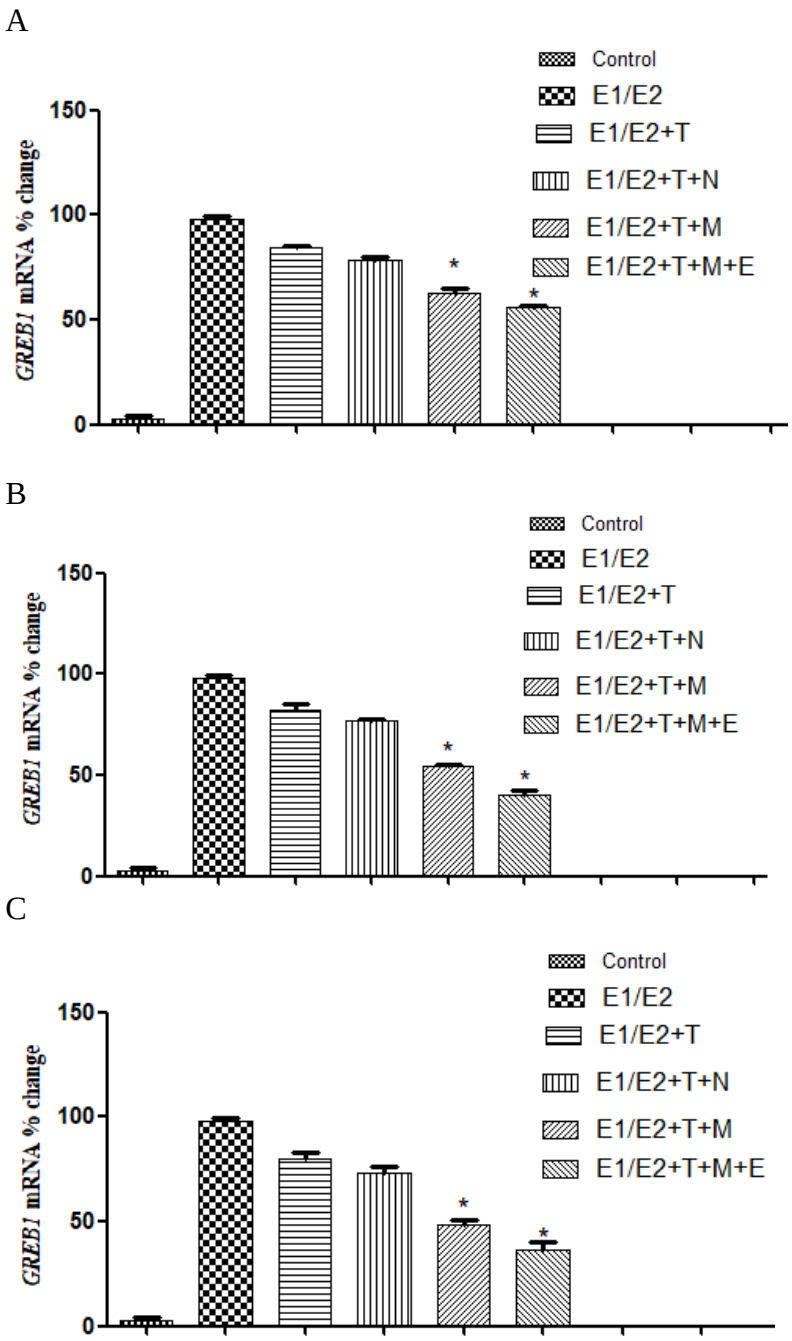
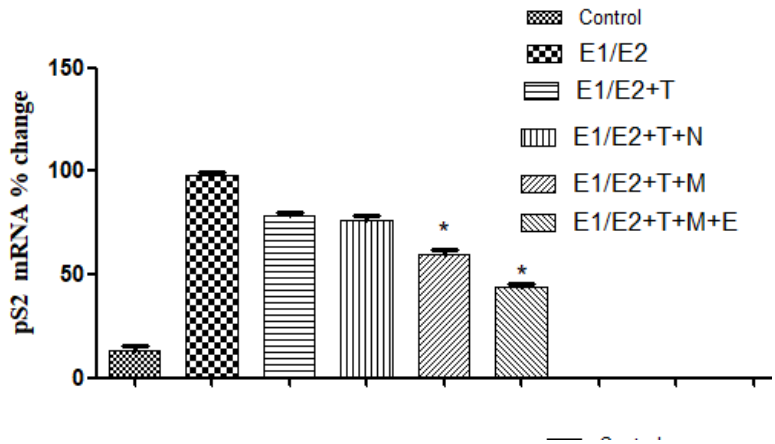


Figure 3.18. Pharmacological effect of tamoxifen and its metabolites with or without 4-OH-Tamoxifen and endoxifen at concentrations corresponding to (A) EM genotype on estrogen-responsive *GREB1* gene expression , (B) RM genotype on estrogen-responsive *GREB1* gene expression.(C) UM genotype on estrogen-responsive *GREB1* gene expression. mRNA expression measurement by real-time PCR were chosen. The results show that 4-OH-Tamoxifen and endoxifen is vital for inhibition of premenopausal estrogen-stimulated gene expression.

Additionally, we studied the effects of treatments of the three metabolizers of CYP2C19*17 on other estrogen responsive genes, such as *pS2* (Figure 3.19). Results validate that tamoxifen and its primary metabolites in EM concentrations are unable to significantly reduce the estrogen-induced RNA production (Figure 6A) ($P > 0.05$ by Student's *t*-test), However, endoxifen addition in EM, RM and UM concentration was able to significantly reduce the estrogen-induced *GREB1* mRNA expression by an average of 50% ($P < 0.05$ by Student's *t*-test for both genes) (Figure 3.19).

A



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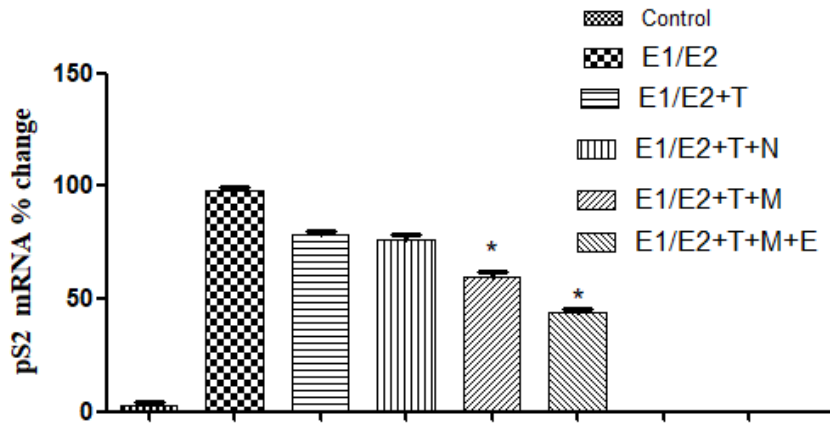

C

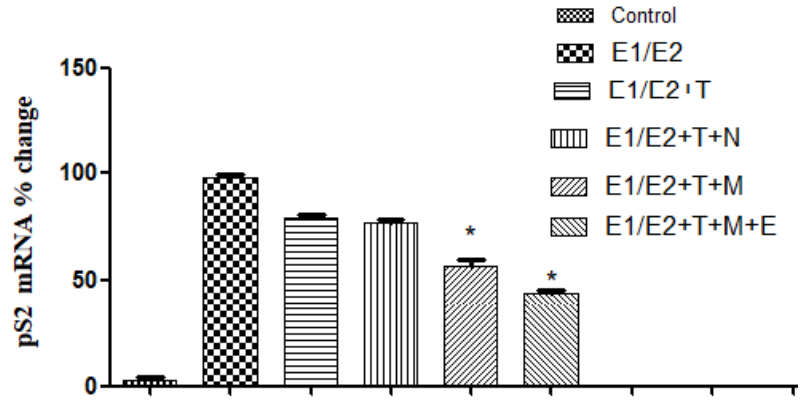

Figure 3.19. Pharmacological effect of tamoxifen and its metabolites with or without 4-OH-Tamoxifen and endoxifen at concentrations corresponding to (A) EM genotype on estrogen-responsive *pS2* gene expression, (B) RM genotype on estrogen-responsive *pS2* gene expression (C) UM genotype on estrogen-responsive *pS2* gene expression. mRNA expression measurement by real-time PCR were chosen. The results show that 4-OH-Tamoxifen and endoxifen is crucial for inhibition of premenopausal estrogen-stimulated gene expression.

3.5.3. Effect of Tamoxifen and Metabolites on ERα Protein Stability

Effect of EM (Extensive metabolizer) Concentration on Tamoxifen and Metabolites on ERα Protein Stability

We measured the effect of EM concentration of tamoxifen and metabolites on ERα protein stability after 24 h of treatment in MCF-7 cell investigated total cell lysates by Western blotting. Our results indicated that MCF-7 cells in a premenopausal estrogen (E1/E2) environment reduced the levels of Erα protein control (Figure 5A, lane 2).

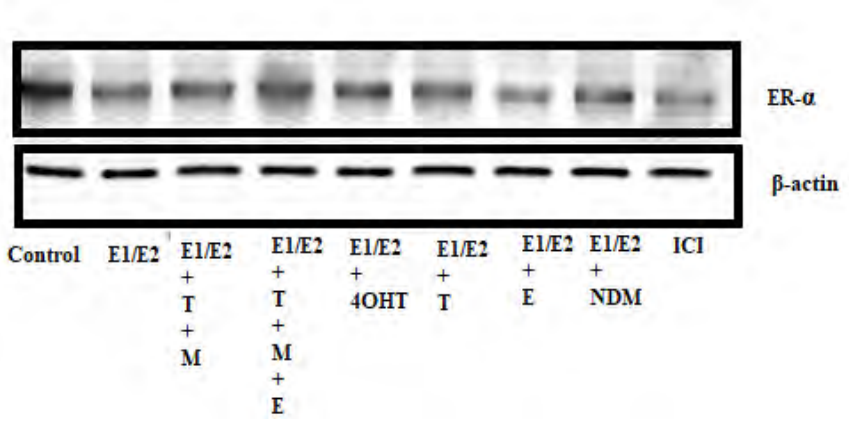

Figure 3.20. Western blotting in MCF-7 cells to exhibit the effects of different 24 h treatments on ERα protein levels. Treatments were made as follows: control; E1/E2, the premenopausal average oestrogen concentrations; E1/E2 + T + M, estrogens with tamoxifen (T) and its primary metabolites (NDMTAM and 4OHT) (M) at EM genotype concentrations (Table 1); E1/E2 + T + M + E, estrogens; tamoxifen (T), primary metabolites (M) and endoxifen (E) at EM genotype concentrations (Table 1). Fulvestrant (ICI) was used as a positive control for ERα protein degradation.

Tamoxifen plus primary metabolites NDMTAM and 4-OH-Tam (TPM) at EM concentrations stabilized the ERα levels when compared with control (Figure 3.20, lane 3). In combination with E1/E2, TPME (Tamoxifen, NDMTAM, 4-OH-Tam and Endoxifen) reversed the estrogens effect on ERα, consistent with the antiestrogenic tamoxifen effect (Figure 3.20, lane 4) and stabilized the ERα protein levels when compared with control.

Effect RM (Rapid metabolizer) Concentration on of Tamoxifen and Metabolites on ER Protein Stability

We evaluated the effect of RM (Rapid Metabolizer, *1/*17) concentration of tamoxifen and metabolites on ERα protein stability after 24 h treatment in MCF-7 cell investigated total cell lysates by Western blotting. Our results indicated that MCF-7 cells in a premenopausal estrogen (E1/E2) environment reduced the ERα protein levels as compared to control (Figure 3.21, lane 2). Tamoxifen plus primary metabolites NDMTAM and 4-OH-Tam (TPM) at EM concentrations stabilized the levels of ERα when compared with control (Figure 3.21, lane 3). In combination with E1/E2, TPME (Tamoxifen, NDMTAM, 4-OH-Tam and Endoxifen) reversed the effect of estrogens on ERα, consistent with the antiestrogenic effect of tamoxifen (Figure 3.21, lane 4) and stabilized the ERα protein levels when compared with control.

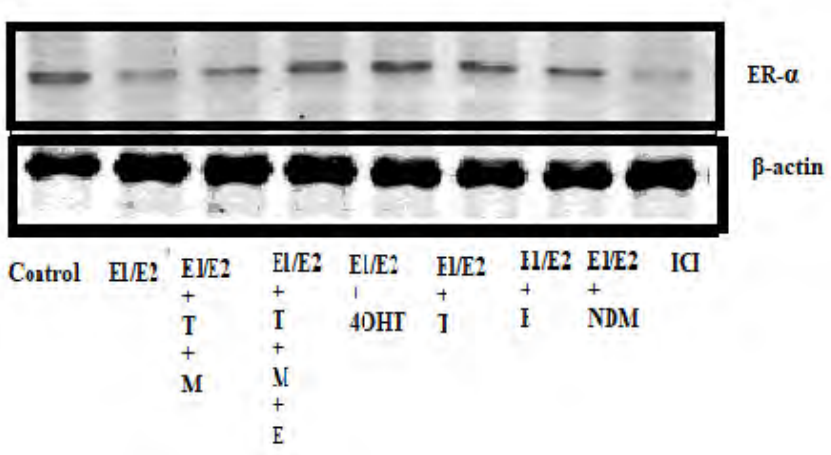

Figure 3.21. Western blotting in MCF-7 cells to show the effects of different 24 h treatments on ERα protein levels. Treatments were made as follows: control; E1/E2, the premenopausal average estrogen concentrations; E1/E2 + T + M, estrogens with tamoxifen (T) and its primary metabolites (NDMTAM and 4OHT) (M) at EM genotype concentrations (Table 1); E1/E2 + T + M + E, estrogens; tamoxifen (T), primary metabolites (M) and endoxifen (E) at RM genotype concentrations (Table 1). Fulvestrant (ICI) was used as a positive control for ERα protein degradation.

Effect UM (Ultra Rapid metabolizer) Concentration on of Tamoxifen and Metabolites on ER Protein Stability

We calculated the effect of UM (Ultra Rapid Metabolizer, *17/*17) concentration of tamoxifen and metabolites on ERα protein stability after 24 h of treatment in MCF-7 cell investigated total cell lysates by Western blotting. Our results indicated that MCF-7 cells in a premenopausal estrogen (E1/E2) environment reduced the ERα protein levels as compared to control (Figure 3.22, lane 2). Tamoxifen plus primary metabolites NDMTAM and 4-OH-Tam (TPM) at UM concentrations stabilized the ERα levels when compared with control (Figure 3.22, lane 3). In combination with E1/E2, TPME (Tamoxifen, NDMTAM, 4-OH-Tam and Endoxifen) reversed the effect of estrogens on ERα, consistent with the tamoxifen's antiestrogenic effect (Figure 3.21, lane 4) and stabilized the ERα protein levels when compared with control.

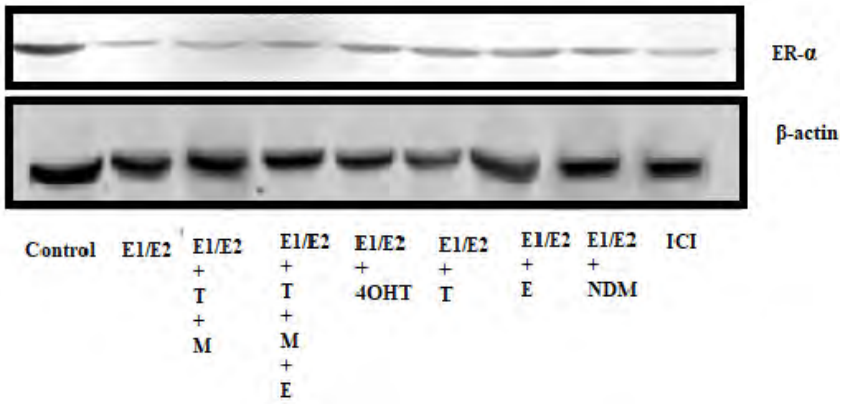

Figure 3.22: Western blotting in MCF-7 cells to show the effects of different 24 h treatments on ERα protein levels. Treatments were made as follows: control; E1/E2, the premenopausal average estrogen concentrations; E1/E2 + T + M, estrogens with tamoxifen (T) and its primary metabolites (NDMTAM and 4OHT) (M) at EM genotype concentrations (Table 1); E1/E2 + T + M + E, estrogens; tamoxifen (T), primary metabolites (M) and endoxifen (E) at RM genotype concentrations (Table 1). Fulvestrant (ICI) was used as a positive control for ERα protein degradation.

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To our knowledge, this study is the first to employ the actual circulating tamoxifen and its metabolites concentrations measured in tamoxifen-treated premenopausal patients and used to block estrogen action from clinically derived plasma levels by a panel of human breast cancer cell lines. We found that tamoxifen and its primary

metabolites in concentrations found in three CYP2C19*17 genotypes (EM, RM, and UM) were found to be involved in reduction of estrogen-induced replication in the pre-menopausal setting. We also established the importance of 4OHT as the active primary metabolite in the antiestrogenic action of tamoxifen in the postmenopausal setting. The estrogenic steroids would be anticipated to accumulate in the cell by binding to the ER. However, at premenopausal estrogens levels, we show an association between the antiestrogenic effect and the levels of endoxifen corresponding to various *CYP2C19*17* genotypes (Maximov, McDaniel, Fernandes, Korostyshevskiy, *et al.*, 2014). Estrogens activate the ER and induce its transcriptional activity by interaction with the estrogen-responsive gene promoters. Estrogen receptor protein turnover is required (Lonard *et al.*, 2000) to retain the continuous mRNA transcription. This turnover is achieved by proteosomal degradation of the ER protein. Binding of antiestrogens, in particular 4OHT, blocks the ER and promotes stabilization of the ER protein (Wijayaratne *et al.*, 2001). In contrast, Wu et al. (Wu *et al.*, 2009) reported that endoxifen targets ERα protein for degradation in MCF-79and T47D cells at higher concentrations (100 and 1000 nM) (Wu *et al.*, 2009). However, these concentrations are not comparable with circulating endoxifen concentrations in patients. The same authors used (Wu *et al.*, 2009) circulating EM and PM genotype concentrations of endoxifen with circulating concentrations of tamoxifen and its primary metabolites in MCF-7 cells and demonstrated ERα degradation only at EM levels of endoxifen. Endoxifen is biologically very similar to 4OHT in breast cancer cells (Johnson *et al.*, 2004b).

Tamoxifen and its metabolites regulate the transcriptional activity of estrogen-responsive genes. *GREB1* plays a considerable role in breast cancer cell hormone-dependent proliferation (Rae *et al.*, 2005). We demonstrated that tamoxifen and NDMTAM partially inhibit *GREB1* estrogen-induced mRNA synthesis; however, addition of 4OHT and endoxifen statistically significantly enhances the antiestrogenic activity of the tamoxifen metabolite pool (Figures 3.18, A, B, and C). These results show that endoxifen, unlike the short-term cell growth end point, plays a substantial role in inhibiting the estrogen-mediated activation of responsive genes. We further examined the endoxifen role in the estrogen regulation of *pS2* gene expression (Figures 3.19, A, B and C). Although these genes are not important for proliferation, they

illustrate the diversity of responsiveness to tamoxifen and its metabolites. Adding the other tamoxifen metabolite 4OHT partially decreased gene expression of *pS2,* but endoxifen suppressed gene expression further. It may be that these observations are

relevant during prolonged adjuvant therapy (Davies *et al.*, 2013) as incomplete gene function suppression may lead to estrogen-stimulated proliferation, development of resistance, and, ultimately, recurrence. These conclusions are supported by a recent report by Hawse et al. (Hawse *et al.*, 2013).

Our immunoblotting results also suggests that tamoxifen and its primary metabolites in concentrations found in three CYP2C19*17 genotypes (EM, RM, and UM) were found to be involved in stabilization ERα protein levels. Furthermore, the addition of 4-OH-Tamoxifen at EM, RM and UM concentration to the anti-estrogenic mix (Tamoxifen, NDM Tamoxifen and), not just completely reversed the estrogen action, but also increased the ERα protein levels, enhancing the anti-estrogenic effect.

It should be noted that the addition of 4-OH-Tamoxifen and endoxifen to tamoxifen and its primary metabolites was not able to completely inhibit the effects of estrogens to control levels in any of the cell lines, in any of the genotype groups, which suggest that the circulating levels of Tamoxifen and its metabolites in our ER positive breast cancer population are not sufficient enough to completely inhibit the estrogen actions.

Conclusion

Breast cancer (BC) is the most commonly occurring malignancy and the leading cause of cancer related death in women both among developed and developing countries while in men it is rarely diagnosed at rate only 1% (Medina *et al.*, 2022). The incidence rate of breast cancer is actually higher in developed countries but its mortality rate is higher in less developed countries as compared to prior ones due to the improved therapeutic, diagnostic methods and breast cancer management in developed countries (Rivera-Franco *et al.*, 2018). Demographic, hereditary, reproductive, breast related, lifestyle and hormonal factors are the types of risk factors for breast cancer (Momenimovahed *et al.*, 2019)

In this study, the relation of breast cancer with different risk factors including age, gender, ethnic groups, family history, educational status, lifestyle, weight, marital status, menopausal status, stages of breast cancer and smoking was determined in 370 Pakistani BC patients.

This study shows that most of the patients 155 (41.89%) were of the age 36-50 while those between 51 and 65 years were 129 (34.86%), 51 (13.78%) were in age group 20-35 and less number of patients aged 66-80 years accounted for 35 (9.46%). According to Helmrich *et al,* 1983, the average age for BC diagnosis was 40 years and 60 years in some countries also while a case control study by Mahouri *et al*, 2007 showed that incidence rate of BC was high at age >50 years. Our data indicated that breast cancer is the disease of women 364 (98.38%) with rare case in men such as 6 (1.62%) out of 370 patients that is in accordance with Giordano *et al*, 2002 due to long-term exposure of women to female sex hormones (estrogen and progesterone). Our findings also exposed that in Pakistan, the breast cancer development is higher in younger women with average age ranging between 36 and 50 years.

The ethnic group distribution of patients reflected that majority of patients 283 (76.49%) were Punjabi followed by 34 (9.19%) Kashmiri, 28 (7.57%) Pashtoon, 9 (2.43%) Saraiki while minimum as 5 (1.35%), 5 (1.35%) and 5 (1.35%) were Sindhi, Hazarvi and Hindkoh correspondingly which might reflect the easy access of patients with high incidence rate of BC while lack of access for those with low frequency and moreover our findings might also show that Punjabis have higher risk of breast cancer as compared to other ethnic groups.

Family history is among the most well-known factors that majorly impact breast cancer risk, with an odds ratio of 1.71 (95% CI: 1.59–1.84) (Engmann *et al.*, 2017). Another study using a large patient cohort exhibited that women with9two or more relatives having a history of breast cancer have a 2.5-fold (95% CI: 1.83–3.47) increased risk of developing breast cancer (L. Liu *et al.*, 2021). The family history data revealed that 86 patients (20%) had a family history of cancer, while 344 patients (80%) had sporadic cancer. Women with a family history of breast cancer have an increased risk of developing the disease. As cousin marriages are prevalent in Pakistan, they have the potential to influence genetic factors.

Our data about educational status showed the majority of patients 325 (87.84%) as illiterate supporting the statements that in Pakistan, the breast cancer's incidence is about 2.5 folds higher as compared to other neighboring countries like Iran and India by Shaukat *et al,* 2013 and Malik, 2002 and the reason behind high frequency of BC in Pakistan is lack of awareness and illiteracy. While remaining 2 (0.54%), 7 (1.89%), 19 (5.14%), 5 (1.35%) and 12 (3.24%) patients were primary, middle, matric, intermediate and graduate respectively.

According to our data, patients were classified into professionals and housewives on base of their lifestyle among which only 15 (4.05%) patients were professional and the rest 355 (95.95%) serving as housewives. Reason behind the high risk of BC in housewives may be their sedentary lifestyle after 40 years of age as reported by Siddiqui, 2000. Similarly, the results of a cohort study by Mctiernan *et al,* 2003 also showed significance of increased physical activity in postmenopausal women aged 50-79 years by reducing their risk for BC development.

Weight data showed that only small number of patients 78 (21.08%) was obese having weight range between 71-100 kg while large number of patients such as 200 (54.05%) were of normal weight (56-70 kg) and 92 (24.86%) of underweight (34-55 kg). Thus, our data was not in accordance with several studies showing the direct link of obesity with BC risk due to conversion of more androgenic precursors into estrogen hormone in adipose tissues and stimulation of the cancer cells growth because of more insulin and insulin like growth factors (Chen *et al*., 2017).

According to our marital status data, a greater number of patients 353 (95.41%) was married followed by less number of unmarried 8 (2.16%), 2 (0.54%) divorced and 7 (1.89%) widowed patients. A study by Jeong *et al,* 2017 exposed that the risk of BC is reduced to 50% in married women due to the combined effects of breast feeding and two or more childbirths. Our findings suggest that marital status has no significant role in decreasing BC risk.

Menopausal status data stated that most of the patients (88.74%) were post-menopausal followed by less (8.24%) pre and lesser (3.02%) peri-menopausal patients. So our findings are in accordance with findings of Thakur *et al,* 2017 according to which the breast cancer is linked with the late age of menopause (post-menopausal status>50 years).

Cancer has various stages on basis of metastasis and severity of disease. Malik, 2002 has reported that about one third of the patients were at stage II of BC. Our findings such as 15 (4.05%) at stage I, 195 (52.70%) stage II, 117 (31.62%) at III and 43 (11.62%) at stage IV correspondingly were also comparable to that of Malik, 2002.

Out of total 400 study subjects, only a smaller number of patients 16 (4.32%) was smokers and the rest 354 (95.68%) were non-smokers. According to literature, smoking is considered to have both negative and positive association with BC due to presence of carcinogens in tobacco and its anti-estrogenic effects respectively.

The tamoxifen metabolism is complex and is mediated by multiple cytochrome P450 enzymes and polymorphisms in genes encoding these enzymes may influence the plasma concentrations of tamoxifen and its metabolites. In this exploratory study, we investigated the effects of previously reported polymorphisms in genes encoding the major enzymes involved in the metabolism of tamoxifen and its metabolites in ER Positive Breast cancer patients.

Tamoxifen was the first and remained among the most significant targeted cancer therapies. Tamoxifen is widely used for breast cancer risk reduction in high-risk populations and adjuvant and metastatic treatment of estrogen receptor-positive breast cancer (Davies *et al.*, 2013). A minor pathway for the tamoxifen conversion to endoxifen is believed to occur by Tamoxifen hydroxylation by *CYP*2D6, *CYP*2B6,

*CYP*2C9, *CYP*2C19, or *CYP*3A4/55,6; this is followed by conversion of 4-hydroxytamoxifen to endoxifen (Brauch *et al.*, 2009). Multiple cytochrome P450 enzymes metabolize tamoxifen, and polymorphisms in the genes encoding these enzymes may affect plasma concentrations of tamoxifen and its metabolites. Tamoxifen's efficacy is dependent on CYP enzymes' conversion to active metabolites. Tamoxifen metabolites can also be transported out of cells, and transporter proteins should be considered in pharmacogenetics studies (Cronin-Fenton *et al.*, 2014). Tamoxifen metabolites can also be transported.

It was reported that tamoxifen and its primary metabolites in concentrations found in three CYP2D6 genotypes (EM, IM, and PM) are sufficient to inhibit estrogen-induced replication in the postmenopausal setting. they established the importance of 4OHT as the active primary metabolite in the antiestrogenic action of tamoxifen in the postmenopausal setting. The estrogenic steroids would be anticipated to accumulate in the cell by binding to the ER (Maximov, McDaniel, Fernandes, Korostyshevskiy, *et al.*, 2014).

Estrogens activate the ER and induce its transcriptional activity by interaction with the estrogen-responsive gene promoters (Maximov, McDaniel, *et al.*, 2014a). Estrogen receptor protein turnover is required (Lonard *et al.*, 2000) to maintain the continuous transcription of mRNA. This turnover is achieved by proteasomal degradation of ER protein. Binding of antiestrogens, in particular 4OHT, blocks the ER and promotes stabilization estrogens activate the ER and induce its transcriptional activity by interaction with the estrogen-responsive gene promoters (Maximov, McDaniel, *et al.*, 2014a).

In the present study, 410 unrelated healthy individuals and 430 ER-positive breast cancer patients made up the total sample size. The genotype frequencies for *CYP2C9 (*2,3, CYP2C9(*2, *3, *17), CYP3A4(*22) and CYP3A5(*3,*6)* were calculated between the two groups.

Study I

Our findings for *CYP2C9* are especially significant considering tamoxifen's efficacy in treating breast cancer in women with hormone receptor-positive tumors. The findings of this study indicate that *CYP2C9* does not significantly influence the plasma concentrations of tamoxifen, its three metabolites, or the metabolic ratios of tamoxifen; however, a non-significant decrease in the mean plasma concentration of 4-OH-tamoxifen was observed in a group of Pakistani women with breast cancer.

Study II

In the this study, CYP2C19 (*2, *3 and *17) variants were genotyped. The *CYP2C19*17* (-806C>T; rs12248560) allele was found to be strongly associated with higher plasma metabolic ratios of 4-OH-tamoxifen in the plasma, while the association with N-DesM-Tam was not found to be significant. This suggests an accumulation of 4-OH-tamoxifen and tamoxifen in plasma when the metabolic alteration of 4-OH-tamoxifen to endoxifen is reduced. Total metabolic ratios also suggest an impaired conversation of 4-OH-tamoxifen to endoxifen. A significant increase has also been observed in the plasma concentration of 4-OH-Tam in patients carrying CYP2C19 **1/*2* and **2/*2* genotype.

The plasma concentration of N-DesM-Tam was comparable between patients with **1/*2, *1/*17* and **2/*2, *17/*17* genotypes. There was minor escalation in the endoxifen plasma concentration in patients with genotype *CYP2C19*1/*1, *1/2 *2/*2,* and **1/*17, *17/*17.* A decrease was observed in the plasma concentration of 4-OH-tamoxifen in patients with **1/*3* and **3/*3* genotype compared with those with **1/*1* genotype.

An amplified gene expression of the *CYP2C19*17* alleles results in a putative ultra-rapid (UM) phenotype (Sim *et al.*, 2006). *CYP2C19* is responsible for tamoxifen metabolism to anti-estrogenic metabolite 4-OH-tamoxifen, exhibiting *in vitro* activities similar to *CYP2D6* (Desta *et al.*, 2004) (Coller *et al.*, 2002). Our data suggest that *CYP2C19*17* has a significant role in the plasma concentration of 4-OH-tamoxifen. An active form of *CYP2C19*17* can cause significant benefits toward the reduction of

breast cancer recurrence, as reported earlier by Schroth and his co-workers (Schroth *et al.*, 2007). However, our studies contradict the study of Joanne S. L. Lim et al., who reported no correlation between *CYP2C19* polymorphism and the pharmacokinetics of tamoxifen (Lim *et al.*, 2011).

Our findings are predominantly significant in light of the efficacy of tamoxifen for women having hormone receptor-positive breast cancer. In conclusion, the present study indicates that *CYP2C19*17* is an essential factor that influences the plasma concentrations of tamoxifen, its three metabolites, and metabolic ratios of tamoxifen in a breast cancer population in Pakistan.

Study III

In the current study genotype frequency of *CYP3A4*1/*1*, *CYP3A4*1/*22* and *CYP3A4*22/*22* in four hundred thirty breast cancer subjects and four hundred ten unrelated healthy individuals as control was determined. In the present results, the percentage frequency of *CYP3A4*1/*1* was high in estrogen receptor positive BC patients as well as in controls. The results of this study suggest that CYP3A4*22 has least effect on metabolism of tamoxifen and its metabolites, which leads to the conclusion that metabolism of tamoxifen can also be influenced by number of other factors including genetics. As number of enzymes are involved in tamoxifen metabolism, further screening of genotypes is required in breast cancer patients to

determine that how polymorphisms (in genes encoding enzyme) can influence tamoxifen metabolism.

Study IV

In this study, CYP3A5 (*3 and *6) variants were genotyped. The results of this study suggest that individuals having CYP3A5*3 homozygous, and CYP3A5*6 (heterozygous) are at risk of developing breast cancer. Our findings of *CYP3A5*3* and *CYP3A5*6* genotyping were contrary to the conclusions of different studies (Coller *et al.*, 2002; Desta *et al.*, 2004; Charoenchokthavee *et al.*, 2016). The important factors resulting in such variations may include genetic and physiological factors. As many enzymes play role in metabolism of tamoxifen, further screening of different genotype is required in cancer subjects to

sort out which genotype is responsible for tamoxifen metabolism among breast cancer subjects of Pakistan. Besides 4-OH tamoxifen, other metabolites such as N-desmethyl tamoxifen and nor-endoxifen could be analyzed in plasma samples of the breast cancer patients to check the effect of the tamoxifen therapy.

Study V

CYP2C19*17 is involved in the production of higher amount of 4-OH-Tamoxifen as demonstrated in study II. In this study simulation Tamoxifen treatment in premenopausal breast cancer patients with different CYP2C19*17 genotypes (EM, RM and UM) was simulated *in vitro* using cells. Results of different experiments showed that higher amount of 4-OH-Tamoxifen inhibits estrogen action but not completely which suggest that the circulating levels of Tamoxifen and its metabolites in our ER positive breast cancer population are not sufficient to completely inhibit the estrogen actions.

Future Prospective

The observation opens up a new line of inquiry to further assess the impact of tamoxifen and its metabolites. Animal studies are necessary to further confirm the impact of circulating tamoxifen and its metabolites. According to the research, 4-OH- Tamoxifen is being produced in greater amounts and is not sufficiently converted to Endoxifen, resulting in an accumulation in the body. Investigating the impact of the body's accumulation of 4-OH- Tamoxifen will require studies. Additionally, correlation studies between breast cancer patients with and without treatment are needed for various genotypes. This research will help clinicians cope with inappropriate drug prescriptions for South Asian and Pakistani patients.

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Cons

ent

form

I am willingly volunteering to provide blood sample for this research. I have been briefed about the research and have been assured that my personal information will be kept confidential & completely anonymous at all times. I am also free to leave this research at any time, at will.

_____

Patient Signature

میں اِس تحقیق کے لیئے رضا کارانہ طور پر اپنے خون کا نمونہ دینے کیلیئے تیار ہوں۔ مجھے اِس تحقیق کے متعلق بنیادی معلومات دی گئی ہیں اور یہ یقین دلایا گیا ہے کہ اِس تحقیق کے دوران یا بعد میں میرے ذاتی کوائف صیغہء راز میں رہیں گے۔ مجھے اِس تحقیق سے کسی بھی وقت الگ ہونے کی مکمل آزادی ہے۔

Annexure II

BREAST CANCER QUESTIONAIRE

***Name: ----------**

***Date of Birth: ----------**

--

***Age: ----------**

***Ethnicity: ----------**

-

***Marital Status: ----------**

-

***Family history of Breast Cancer: ----------**

***Occupation: ----------**

--

***Educational Status: ----------**

--

*** Weight: ----------**

-

***contact no: ----------**

-

***Address: ----------**

-

*** Stage, type, grade and size of tumor/ Cancer: ----------**

*** Time since diagnosis: ----------**

-

*** Taking Tamoxifen : Y or N.**

If yes then for how long----------

Currently taking which treatment: ---------- ------

*** Have you ever smoked cigarettes, including hand-rolled ones, piped/cigar?**

*** Have you been treated before any of the condition below?**

Lung disease	Yes	No	Any medications currently in use
Liver Disease	Yes	No	
Heart dieases	Yes	No	
Blood pressure	Yes	No	
Diabetes	Yes	No	

* Treatment for cancer yet?

Surgery	Yes	No
Chemotherapy	Yes	No
Radiotherapy	Yes	No
Others	Yes	No

*Menopausal status

Pre-menopausal (Regular Periods)	Yes	No
Peri-menopausal (Irregular Periods)	Yes	No
Post-menopausal status (no longer having periods)	Yes	No

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Shu-Feng Zhou. "Polymorphism of Human Cytochrome P450 2D6 and Its Clinical Significance : Part II", Clinical Pharmacokinetics, 12/2009

Abstract Abstract Breast cancer remains the most prevalent cancer among women worldwide. Tamoxifen or aromatase inhibitors are recommended in endocrine therapy because most newly diagnosed breast cancer cases are estrogen-receptor positive. After two or three years of endocrine therapy, tamoxifen has been recommended as either a monotherapy or in combination with an aromatase inhibitor. When administered adjunctively, tamoxifen decreases breast cancer mortality and relapse rates; however, when combined with other metastatic breast cancer treatments, it has been shown to increase survival durations. It is unfortunate that tamoxifen's effectiveness varies significantly. The study objective was to investigate the CYP2C9(*2,*3), CYP2C19(*2,*3 &*17) CYP3A4(*22) and CYP3A5(*3,*6) frequency between Pakistani estrogen positive-breast cancer patients and unrelated healthy controls, and also effect of gene variants on the pharmacogenetics and pharmacokinetics of tamoxifen in patients with estrogen-positive breast cancer. ER-positive breast cancer patients receiving 20 mg/day of tamoxifen (n = 430) and healthy, unrelated individuals (n = 410; control group) comprised the study population. High-Performance Liquid Chromatography was utilized to determine the steady-state plasma concentrations of tamoxifen and its three metabolites in the patients. DNA was extracted and analyzed by ARMS PCR and AS-PCR followed by RFLP. Five phenotypes observed are as follows, extensive metabolizer (EM), poor metabolizer (PM), intermediate metabolizer (IM), Rapid Metabolizer (RM) and ultra- rapid metabolizer (UM). Further statistical analysis were performed on median plasma concentration of tamoxifen and derivatives. Plasma metabolic ratio and total metabolic ratio were determined and correlated with each genotype. In our population, CYP2C9*2 heterozygous (OR: 0.4; 95% CI: 0.53–0.56; p = 0.0001) and homozygous mutant (OR: 3,12; 95% CI: 1.80–5.43; P = 0.0001) condition were identified as breast cancer risk factors. The CYP2C9*2 gene variant had no significant effect on the metabolic ratio between tamoxifen and its three metabolites. However, an insignificant decrease was recorded in the median plasma concentration of 4-OH- Tam in the subjects having heterozygous (*1/*2) (P = 0.747) and mutant (*2/*2) (P = 0.223) genotypes. x Abstract More than 65% of healthy individuals were extensive metabolizers (*1/*1) for CYP2C19, whereas more than 70% of ER-positive BC patients were rapid and ultra- rapid metabolizers (*1/17*, *17/17*). The polymorphism CYP2C19*17 is significantly associated with higher 4-OH-Tamoxifen. Patients with the *17/*17 genotype exhibited 1 to 1.5-fold higher 4-OH-Tamoxifen, which was also high in patients with the *1/*2 and *2/*2 genotypes. Which suggests that CYP219*17 has a significant effect on the higher production of 4-OH-Tamoxifen. The allele9frequencies of the CYP3A4*22 variant in ER positive breast9cancer patients and healthy controls, demonstrated no noteworthy difference between the allele frequencies of CYP3A4*22. Plasma metabolic ratios of tamoxifen and its metabolites indicates that CYP3A4*22 does not have any significant effect on the metabolism of tamoxifen and metbolities. CYP3A5*3 and *6 genotyping results for unrelated healthy individuals and ER positive breast9cancer patients in Pakistani population indicates that most of the individuals in both the groups belongs to extensive metabolizer. The frequencies of the CYP3A5 alleles did not differ noticeably from one another. Our study demosttrates that CYP3A5 do not have any significant impact on the metabolism of tamoxifen and its metabolites. Tamoxifen and its metabolites were tested for their biological effects with different CYP2C19*17 genotypes (EM, RM and UM) on cell proliferation and estrogen-responsive gene regulation in the MCF-7 breast cancer cell line. Real clinical levels of tamoxifen metabolites in breast cancer patients, as well as actual amounts of estrogens reported in premenopausal individuals receiving tamoxifen, were employed in vitro. Interestingly, tamoxifen and its primary metabolites were not able to fully inhibit the estrogen-stimulated expression of estrogen-responsive genes in MCF-7 cells (P < .05 for all genes), but the addition of endoxifen was able to produce additional antiestrogenic effects on these genes. Our results suggest that the circulating levels of tamoxifen and its metabolites are not sufficient to completely block the estrogen stimulated growth in cell line. Role of Cytochrome P450 Polymorphism in Activation and Metabolism of Tamoxifen in ER Positive Breast Cancer Patients xi Abstract Role of Cytochrome P450 Polymorphism in Activation and Metabolism of Tamoxifen in ER Positive Breast Cancer Patients xi Chapter 1 1. Introduction A succession of molecular processes that profoundly disrupt the typical cell features causes cancer. These modified cells replicate and increase in the existence of signals that ordinarily limit cell growth; hence, they no longer need unique signals to trigger cell division and growth. As these cells multiply, they acquire new properties, such as alterations in cell shape, reduced cell adhesion, and the synthesis of novel enzymes. These heritable alterations let the cell and its descendants divide and develop even in the existence of normal cells, which normally limit the proliferation of neighboring cells. Such alterations let the cancer cells spread and infect

surrounding tissues. Figure. 1.1, illustrates the characteristics of cancer. Only a small percentage of the roughly 35,000 human genes have been linked to cancer. Alterations in the same gene are frequently related to many cancer types. These defective genes can be roughly split into three classifications. The first class, proto-oncogenes, generates protein products that promote cell proliferation or prevent natural apoptosis. These genes' mutated variants are known as oncogenes. The second class, tumor suppressors, produces proteins that ordinarily inhibit cell proliferation or induce cell death. The third group comprises DNA repair genes that prevent cancer- causing mutations. 1.1. Breast Cancer Diverse genetic and chromosomal differences and clinical outcomes make breast cancer more difficult to treat (Vargo-Gogola et al., 2007). Most breast cancers are invasive or infiltrating. Most commonly, breast cancer originates in breast lobules (milk production site) and the ducts connecting these lobules to the nipple. At the same time, some breast cancers are also associated with fatty, lymphatic, and connective tissue (Alteri et al., 2015). Several speculated factors are identified to accelerate the possibility of developing breast cancer, including null parity, estrogen (hormone) replacement therapy, post- menopausal obesity, use of oral contraceptives, and intake of high-energy products (Dossus et al., 2014). Nevertheless, recognized breast cancer risk factors comprise gender, age, race/ethnicity, menarche (and post-menopausal status), breast cancer history, benign breast illness, genetic factors, late age at first pregnancy (Helmrich et al., 1983), and exposure to ionizing radiation (Brenner et al., 2003) in early life. Figure 1.1: Hallmarks of cancer (Hanahan, 2022) 1.1.1. Breast Cancer Global Prevalence and Mortality Rate Breast cancer is the highly frequent cancer among women. In 2008, there were reported 1,4 million cancer sufferers (Bray et al., 2012)1.6 million in 2010 (Forouzanfar et al., 2011), 1.67 million were diagnosed in 2012 (25% of all cancers). It is expected that by 2030 this number will rise to 2.1 million (Bray et al., 2012). Developed countries have high prevalence rates, where one out of nine women develops breast cancer. Lifestyles such as smoking, less physical activity, poor diet, and high rate of birth control are causing an increase in breast cancer incidence by up to 5% every year in the western population. Its incidence rate varies 10-fold globally (Jemal et al., 2010). Among 1.67 million breast cancer patients in 2012, 522,000 died (Ferlay et al., 2013), and massive casualties arose in underdeveloped areas (324,0009deaths, 14.3% of the total). Breast cancer is a leading death cause triggered by cancer (after lung cancer), causing 198,000 deaths. Mortality rates, however, may differ among different ethnic groups. Korean women have the lowest mortality rate, while the black women in United States have the maximum mortality rate. According to the-International-Agency for Research-on Cancer-(IARC), in 2012, around 690,000 new cases were diagnosed annually in developed and less developed nations. The death rates in different regions of the world are not as high as the improved breast cancer survival rate in urbanized regions. 1.1.2. Breast Cancer Prevalence in Pakistan Pakistan has the greatest breast cancer incidence rate among Asian countries. One in every nine women in the Pakistani population has breast cancer. The occurrence of breast cancer malignancies in Pakistan is almost 25 times greater than in India and Iran (Garwood et al., 2010). Each year at minimum, 90,0009women (in Pakistan) have breast cancer. From 1998-2002, the rate of breast cancer reported cases in Karachi was 697 per 100,000. Among the Asian nations, specifically in the Pakistani populace, there has been a disconcerting increase in breast cancer cases (I. Malik, 2002). Even though in Pakistan, there is rarely national breast disease occurrence, mortality, or hazard figure information available yet it was reported as the highly common malignancy, representing 34.65% of female diseases (Shamsi et al., 2013). 1.1.3. Treatment approaches for breast cancer Several advances for the breast cancer treatment involve surgery, radiotherapy and chemotherapy, targeted therapy, and the hormone therapy. Radiations halt the growth of the cancerous cells. Radiations have a significant effect on the rapidly dividing cancer cells, as they cannot reverse the damage easily. While radiotherapy is only confined to a specific area; chemotherapy can work throughout the body. Chemotherapy treats cancer using drugs that are given orally as well as intravenously. Chemotherapy is accompanied by many side effects and therefore is not suitable for some breast cancer patients. Adjuvant therapy is the therapy that is given post-primary treatment to facilitate the initial treatment and lessen the chances of recurrence of cancer. It may include chemotherapy, radiotherapy and hormonal therapy. Nastrozole and lentrozole are FDA-approved aromatase inhibitor adjuvants (Network, 2003). 1.2. Tamoxifen Women with estrogen receptore(ER)-positive malignant breast cancer are cured with Tamoxifen, a Selective Estrogen Receptor Modulator-(SERM-TAM). U.S. Food and Drug-Administration (FDA) approved this drug as adjuvanttherapy for breast cancer patients in 1977 (Osborne, 1998). Tamoxifen (TAM), a synthetic anti-estrogen, is a well-known therapeutic drug for treating ER-positive BC patients at any stage of this disease (Group, 1998). It is commonly employed as a chemopreventive drug in women at greater hazard for developing breast cancer (Fisher et al., 1998b). In "early" breast cancer, the tumor is localized in the breast tissue or may present in the nearby lymph nodes in females. Therefore surgery can be done for the removal of breast tumors. However, once the disease progresses to an advanced stage, micrometastatic deposits of cancer, which initially are not detected, may develop into a clinically detectable form over time and ultimately result in the patient's death (Group, 1998). 1.2.1 Tamoxifen Function and Role in Adjuvant Therapy Tamoxifen is a non-steroidal anti-estrogen pro drug, having antagonist activity against estrogen receptors. Tamoxifen is a pioneering therapy for curing and inhibiting ER positive breast cancer. Tamoxifen has been claimed to reduce disease relapse and mortality rates by up to 50 and 30%, respectively (Fisher et al., 1998a). Tamoxifen action is conditional on spreading estrogen quantities, which are lower in post-menopausal women and higher in pre-menopausal women. Tamoxifen was originally thought an antagonist and is presently categorized

as a SERM which is a mixture demonstrating tissue-specific Estrogen Receptor (ER) antagonist activity. It competitively binds to the ERs, thus preventing E2-dependent tumor growth, gene transcription, and cell-proliferation (Maximov, McDaniel, et al., 2014b; Osborne, 1998). Tamoxifen binds to ER having lower affinity than estrogen and detaches the HSP90 protein from the receptor. This tamoxifen-estrogen receptor combination homo or hetero dimerizes, then translocate into the nucleus, activating the AF1 domain and inhibiting the activation factor 2 (AF2) domain of ER. This tamoxifen-ER dimer then binds to DNA at specific Estrogen Response Element (ERE) sequences in the promoter region of E2 genes. Transcription of the E2 gene is reduced because the AF2 (ligand-dependent domain) is dormant, and ER co-activator attaching ability is diminished by the AF1 domain, which persists active in the tamoxifen-ER complex, contributes to partial agonist action (Howell et al., 2000). Figure 1.2: The action mechanism of Tamoxifen in the estrogen genes' promotor region inactivation of the AF2 domain results in diminished gene activity. (Johnson et al., 2004a). Tamoxifen-estrogen receptor complex triggers the AF1 domain and prevents the AF2 domain stimulation. This tamoxifen-estrogen receptor complex then attaches to DNA at 13 bp specific palindromic sequence (ERE sequence) in the estrogen genes' promotor region—inactivation of the AF2 domain results in diminished gene activity (Figure 1.2). Tamoxifen and its primary metabolites combine to the estrogen receptors with various binding affinities. They prevent the estrogen binding to estrogen receptors, inhibiting conformational alterations required to recruit co-activators. The binding of tamoxifen metabolites also leads to the co-repressors (including NCOR1) recruitment. The reduction in estrogen receptors transcriptional action, affects tumor growth, as estrogen-regulated genes are involved in proliferation and angiogenesis (Johnson et al., 2004a). Endoxifen, a key metabolite, is responsible for the in vivo action of tamoxifen. Endoxifen acts differently on the two estrogen receptors. It steadies ERβ and promotes receptor heterodimerization. In addition, its inhibitory effects on the target genes expression are increased. In breast cancer cells, however, endoxifen targets ERα for proteasomal destruction. It has been reported in several studies that tamoxifen is responsible for halting the cell cycle and can induce apoptosis by modifying some growth factors, e.g., by down- regulating expression of TGFα, stromal TGFβ1 expression induction and reduction in the production IGF-1 which is a powerful mitogen (Notas et al., 2015). Several studies demonstrate that tamoxifen can also induce cell cycle detention in the G0/G1 phase, hence limiting cellular growth. Tamoxifen can also accelerate cellular proliferation by acting on numerous signalling pathways(consist of c-MYC and MAPKs) (Ariazi et al., 2011; Miller et al., 1999; Mizutani et al., 2004). In premenopausal and postmenopausal women having estrogen receptor-positive breast cancer, adjunct tamoxifen treatment has been proven to cut yearly recurrence rates and decrease death by one-third (Group, 2005). Tamoxifen is also qualified to avoid breast cancer in women at elevated risk (Fisher et al., 2005). 1.2.2 Tamoxifen Metabolism by CYP Family Tamoxifen is mostly processed by the liver, with minimal local breast metabolism. The Cytochrome P450 (CYP) enzyme system in the liver metabolizes tamoxifen into multiple main and secondary metabolites (Figure 1.3). Some of these metabolites have greater anti-estrogenic activities than tamoxifen in breast cancer cells (Desta et al., 2004; Mürdter et al., 2011). Polymorphisms in many CYP enzymes implicated in tamoxifen metabolism influence metabolites' relative amount and availability and, subsequently, their effects on E2-dependent cell proliferation of breast cancer. The metabolic pathway directly transforms tamoxifen to N-desmethyl tamoxifen by CYP3A4/5,5followed by change to endoxifen. This N-desmethyl tamoxifen conversion to endoxifen is catalyzed by CYP2D6. Moreover, some tamoxifen is initially metabolized to the 4-hydroxytamoxifen (4OH-TAM) by CYP2D6. This 4- hydroxy tamoxifen can be degraded or converted to endoxifen by CYP3A4/5. Endoxifen is a very efficient metabolite of the tamoxifen metabolizing routes (4- hydroxylation and N-demethylation) that are widely observed. The initial focus was on the 4-hydroxylation pathway, mediated by many CYPs, including CYP2D6, because the immediate resultant metabolite, 4-hydroxy-tamoxifen, was 30 to 100 times more effective as an antiestrogen than tamoxifen itself (Desta et al., 2004). However, this pathway only provides nearly 7% of the total tamoxifen metabolism. Tamoxifen's N-demethylation to N-desmethyl tamoxifen is mainly catalyzed by CYP3A4/5 with small influences by CYP2D6, CYP1A2, CYP2C9, and CYP2C19 and contributes roughly 92% of tamoxifen metabolism (Desta et al., 2004; Kiyotani et al., 2012; Stearns et al., 2003). N-desmethyl tamoxifen is oxidized to various tamoxifen- active metabolites, the most remarkable of which is endoxifen (Kiyotani et al., 2012; Stearns et al., 2003). Figure 1.3: Role of several cytochromes in tamoxifen metabolism. The Cytochrome P450 (CYP) enzyme system in the liver metabolizes tamoxifen into multiple main and secondary metabolites (Desta et al., 2004) 1.2.3. Xenobiotics metabolism and Cytochrome P450 function In most breast cancer cases, chemotherapy as adjuvant or neo-adjuvant treatment is most effective when combining numerous drugs. Most anticancer drugs used in this cocktail of drugs are xenobiotic. Some anthracycline (doxorubicin), taxanes, e.g., paclitaxel and docetaxel, 5-fluorouracil, cyclophosphamide, and carboplatin common drugs used as a combination of two or three drugs in neo and adjuvant chemotherapy. Tamoxifen, also known as nolvadex, is an important xenobiotic drug employed as hormonal adjuvant therapy to prevent the reoccurrence of breast cancer. Xenobiotics (including anti-cancer medications) are digested by a series of chemical processes following absorption in the organism. Metabolism of a xenobiotic is ensured through two discrete successive phases known as phase I-and II, though this is not a complete order. Phase I does not always follow Phase II, and Phase II does not constantly follow Phase I (Iyanagi, 2007). Cytochrome P450 is a distinct group of hepatic catalytic enzymes engaged in the drugs biotransformation. This enzyme allows the xenobiotic to be transformed into forms that are easily excreted and are less toxic. Cytochrome

P450 is a phase I biotransformation enzyme, which helps in hydrolysis, reduction, and oxidation reactions (Monk et al., 2014). About 1/4th of CYPs are involved in mammalian drug metabolism which include CYP3A4/5, CYP1A2, CYP2B6, CYP2D6 and CYP2C. They convert lipophilic endogenous substances and drugs into easily excretable hydrophilic metabolites (Wolf et al., 1999). The CYP2C subfamily plays an active role in 24% of mammalian drug metabolic reactions, followed by CYP2D6, which accounts for 20% of metabolic drug reactions (Ye et al., 2014). 1.3 Cytochrome P450 Cytochrome P450 (CYP) is an enzymes superfamily that performs a specialized function in fat-soluble vitamin metabolism, eicosanoid production, and xenobiotic metabolism (Hasler et al., 1999). Cytochrome P450's involvement in the drug metabolism system has appeared as an important determining factor in various drug- drug interactions resulting in drug toxicities, diminished pharmacological effects, and Adverse Drug Reactions (ADRs). About 57 different CYP proteins, called isozymes, are encoded by the human genome that plays crucial roles by catalyzing different reactions (Guengerich, 2005; N. Lewis et al., 2004). Out of these, six CYP isozymes play a major character in drug metabolism, including CYP3A4/5, CYP1A2, CYP2E1,9CYP2D6, CYP2C90and CYP2C19 (Ogu et al., 2000). CYP enzymes account for over 75% of the total drug biotransformation via oxidation (Guengerich, 2005). They convert the lipid-soluble drug into a hydrophilic metabolite. In addition to detoxifying harmful xenobiotic, CYPs activate some medicines into reactive species via biotransformation (Ortiz de Montellano, 2015; Parkinson et al., 2001). Clinical data of 248 drugs shows that CYP3A4/5 is involved in 30% of drug metabolism, accounting for 29% of CYP 450 concentration in the liver (Zanger et al., 2013). CYP2C sub-gene family is vital in 24% of metabolic drug reactions and accounts for 18% CYPs concentration in the liver, followed by CYP1A2, which is involved in 9% of drug metabolic reactions and accounts for about 13% of total CYPs concentration in the liver as shown in Figure 1.4 (Chang, 2014). Figure 1.4: Role of CYP3A4/5, CYP2B6, CYP1A2, CYP2D6, CYP2C with further breakdown into CYP2C19, CYP2C9 AND CYP2C8 in xenobiotic metabolism. CYP2C sub-gene family is vital in 24% of metabolic drug reactions and accounts for 18% CYPs concentration in the liver. (Chang, 2014). The CYPs are a heme group containing membrane proteins located in the gut and liver and bound on the smooth endoplasmic reticulum and inner membrane of mitochondria in various tissues in the body (Ogu et al., 2000). Biotransformation and drug metabolism are mainly catalyzed in the hepatocytes by CYPs and other drug- metabolizing enzymes. During drug metabolism, the oily soluble compounds are converted into less toxic water-soluble compounds that are easily expelled out of the body by urine. Once the drug enters the liver cells, it moves through the smooth endoplasmic reticulum wall, where it gets involved in the CYP metabolic pathway (Coleman, 2010). 1.3.1. Sites of Cytochrome P450 Most Cytochrome P450 enzymes are in the liver and are hydrophobic. CYP450 enzymes are also in the gut, kidney, and small intestine. However, most of them are in hepatocytes bounded by the endoplasmic reticulum and inner mitochondrial membrane, as they are membrane-associated proteins (Guengerich, 2005). When entering hepatocytes, a drug or any other xenobiotic molecule passes through the wall of the smooth endoplasmic reticulum and enters the CYP metabolic pathway, as described in Figure 1.5 (Coleman, 2010). Figure 1.5: CYP Location and their redox partners in hepatocytes. Drug or any other xenobiotic molecule passes through the wall of the smooth endoplasmic reticulum and enters the CYP metabolic pathway (Coleman, 2010). 1.3.2. CYP450 Structure Structurally, CYP450 enzymes are classified into two types. Type 1 includes mitochondrial and bacterial enzymes. The mitochondrial enzyme system has 3 components; FAD, NADH containing flavoprotein, FeS protein comprising Fe, S, and phytochrome 450. Type II comprises microsomal enzymes, which have a eukaryotic nature and comprises of two components, NADH and phytochrome 450 (Smith et al., 1994). CYP450 proteins have a 3-dimensional globular structure with a beta (β) sheet at the N terminal and helix-rich C terminal. The C terminal of these enzymes is reported to be highly conserved. The signature motif "FXXGXXXCXG" is present in all, where X indicates non- specific amino acid. The heme-binding region, consisting of 50 amino acids, is located at the protein's C-terminal (Smith et al., 1994). The structural components of bacterial and mitochondrial CYP450 differ from that of fungi, plants, and animals. It has been reported that prokaryotic CYP450 has three components: a Heme domain, ferredoxin with clusters of Iron (Fe) and Sulfur (S), and NADH-dependent reductase. While microsomal eukaryotic enzymes only contain the heme domain and reductase unit (Smith et al., 1994). 1.3.3. Nomenclature of Cytochrome P450 In 1987, a nomenclature scheme for CYP isoforms was first proposed. As a result of the extent of similarities among primary amino acid sequences determined via gene sequencing, the CYPs are categorized into families and subfamilies (Nebert et al., 1987). The term cytochrome P450 is originated as the human loci for Cytochrome P450 are designated as CYP. At the same time, P450 represents "pigment," which exhibits a spectrophotometric peak at 450 nm when complexed with carbon monoxide and a reduced state. Usually, amino acid sequences with 40% or more resemblance are classified in the identical family, labeled by a digit (e.g., CYP1), but those having match more than 55% are placed in the similar subfamily, labeled by an alphabet (e.g., CYP1A), and the ones with 97% homology, specify the gene by again labeling with a number after the letter (e.g., CYP1A1). Distinct alleles are labeled by adding an asterisk or a numeral and, in particular cases, an alphabet letter-(e.g., CYP1A1*1A), with *1 or *1A representing the wild-type allele (Danielson, 2002) as shown in Figure 1.6. Therefore, this nomenclature system for CYP450 is not dependent on the enzyme's function and reaction that they catalyze. Figure 1.6: Nomenclature of CYP450 superfamily. Cytochrome P450 is labeled with the abbreviation "CYP," resulting in a number representing the gene's family, an alphabet representing the subfamily, and a further number representing an individual gene (isoform). The allele variant is represented by a number before a

star symbol (www.cypalleles.ki.se). 1.3.4. Factors Affecting Enzymatic Activity of CYP Family Members In general, the phenotypic expression of all genes is a manifestation of both genetic and physiological parameters. The existence or deficiency of exogenous compounds, certain hormones, and cytokines determine the expression of respective CYP enzymes. The catalytic activity is deeply influenced by variation in copy number and variant alleles, either present in considerable proportion or rare in a population. Almost all drug-metabolizing cytochrome P450s are polymorphic. Alleles variation from normal to mutant are due to substitution or change in amino acids, base deletions, frameshift mutation, missense mutation, defective splicing, and whole gene deletion (Ingelman-Sundberg, 2004). The genetic variability gives rise to four major phenotypes when both alleles are defective or non-functional, producing a truncated or null protein incapable of catalyzing a reaction and stated as having no enzyme activity. The term used for such a condition is poor metabolizer (PM). PMs have an increased risk of ADRs due to the slow rate of drug metabolism and lack of therapeutic efficacy of prodrugs to produce their active compounds, e.g., tamoxifen. Therefore, alternative medication is advised to lessen the adverse effects of PMs (Zanger and Schwab 2013). The term intermediate metabolizer (IM) is stated for heterozygous for a defective allele or when one of the alleles is functional and translating normal protein. In contrast, the second allele is defective, decreasing enzyme activity. IM needs a lower drug dose than normal for the best treatment response (Sundberg et al., 2007). Extensive metabolizers (EM) possess 2 normal alleles resulting in a normal phenotype. Therefore, a normal drug quantity is required to achieve the best results (Zanger and Schwab, 2013). Ultra-rapid metabolizer (UM) phenotype is defined when due to certain defects increase in enzyme production or enzyme activity is observed or carrying more than two active gene copies (Scott et al., 2011). Ultra- rapid metabolism is due to gene duplications, which cause proportionally increased enzyme expression and regulatory elements which control the transcription. Ultra- rapid metabolizers require higher doses of medicine than normal to achieve the required response. 1.4. CYP2C Sub gene family The CYP2C subfamily metabolizes 20 to 25% of clinically used medicines and several endogenous compounds, including arachidonic acid. CYP2C sub-gene family comprises four gene clusters in a sequence of CYP2C8, CYP2C9, CYP2C19, and CYP2C18 from telomere to the centromere (Zanger et al., 2013). These four gene clusters are 80% identical at the amino acid level, accounting for 20% of the liver's encoded proteins of all cytochrome P450 content. All CYP2C sub-gene families express genetic polymorphism. CYP2C19, CYP2C9, and CYP2C18 have more clinical value among these enzymes. Generally, CYP2C enzymes have overlapping and broad-spectrum substrate specificities. Some enzymes exhibited significant activity against steroids such as testosterone (F. J. Gonzalez et al., 1990). CYP2C9 have high expression level than any other CYP2Cs member (Naraharisetti et al., 2010; Ohtsuki, 2012), followed by CYP2C19 and CYP2C8. The CYP2C9 expression is 10-fold greater than CYP2C19, but CYP2C19 shows broad-spectrum activity in the metabolism of widely prescribed drugs as a result of its polymorphic expression (Danielson, 2002). 1.4.1 CYP2C9 The CYP2C9, a key isozyme of the CYP2C family, represents about 20% of the total liver CYP P-450 content. The cDNAs of CYP2C9 code for a protein of 490 amino acids, all of which have a molecular mass of 55.6 kDa. The CYP2C9 gene' transcribed protein is identified as mephenytoin 4-hydroxylase. The substrate or drug catalyzed by CYP2C9 enzymes is called a CYP2C9 substrate. CYP2C9 metabolizes around 15% of therapeutically effective medications, including anti-diabetic, angiotensin receptor blockers, and anticonvulsant treatments (Table 1.1) (H.-G. Xie et al., 2002). More than 60 drugs are identified to be metabolized by the CYP2C9 enzyme. Most known substrates for CYP2C9 are either lipophilic or weakly acidic, with pKa values that range from 3.8 to 8.1. The electropositive group on the enzyme and the electronegative group on the substrate give rise to electrostatic interaction, which appears to be of key significance in determining the affinity of these medications. (Miners et al., 1998). It has been determined that CYP2C9 is accountable for approving up to 15% of all medicines through Phase I metabolism. "Probe Drug" or "Marker Drug" is a term that was introduced in the early 1970s. A probe drug is formulated to provide information about important enzyme activity issues. Warfarin, an anti-coagulant, is extensively oxidized in humans by the P450 enzyme. Several studies indicate the predominant affinity of warfarin towards CYP2C9. Therefore Warfarin is suggested as a promising probe drug in order to determine CYP2C9 activity in vivo (Pelkonen et al., 2008) Diclofenac, Phenytoin, and Tolbutamide are other important drugs used as probe drugs for CYP2C9. Several important drugs are the substrate of CYP2C9, which include hydroxylation of tolbutamide and hexobarbital, as well as phenytoin and warfarin. Table 1.1: Therapeutic class frequency of CYP2C9 substrates (Miners et al., 1998) Class Anti Inflammatory agents Anticancer agents Endogenous compounds Anticonvulsants Oral hypoglycaemics Substrates flurbiprofen Cyclophosphamide (Tamoxifen) Arachidonic acid Phenytoin (Phenobarbital) (Thimethadione) Tolbutamide Diclofenac 5-hydroxy tryptamine Glyburide Naproxen Linoleic acid Glipizide 1.4.1.1. CYP2C9 Gene Transcriptional regulation The CYP2C9 gene is in the 10q24.33 region of the chromosome and comprises of nine exons. CYP2C9 gene codes a protein constituting 490 amino acid residues. The expressed gene product of the CYP2C9 gene is 92% homologous to the gene product of its neighboring gene CYPC19, varying only by 43 amino acids. However, these two enzymes have entirely distinct substrate specificities. Primarily, gene expression is regulated at the transcription level. Within a 2.2-kkbbregion of the CYP2C9 gene, there are many consensus sequences for glucocorticoid response element (GREs) and identified binding sequences for transcription factors including TATA box, CAAT box, HNF-1, AP-1, and C/EBP. This area is extremely polymorphicsin humans, with at least seven single-nucleotide polymorphisms, a few of which coexist to provide six sequence patterns (Demorais et al., 1993). Some of these polymorphisms

are correlated with decreased enzyme/activity in vitro relative to the wild type. A luciferase reporter gene study revealed that pattern 2 had roughly 40% of the activity of pattern 1 (called wild-type), and those carrying pattern 2 demonstrated a 38% drop in the obvious intrinsic phenytoin clearance relative to persons carrying pattern 1 (Shintani et al., 2001). 1.4.1.2. Genetic Polymorphism of CYP2C9 Polymorphism is the term used to explain a trait that exists in a population as two or more9phenotypes. Vogel and Motulsky (1986) stated: A polymorphism is a Mendelian or monogenic characteristic that occurs in a population in no less than two phenotypes and likely at least two genotypes, none of which are uncommon and none of which occur with a frequency of less than 1-2% CYP2C9, like other members of the 2C subfamily, is vastly polymorphic. CYP2C9 polymorphism was first reported, in the early 1970s, in the metabolism of phenytoin and tolbutamide (J. Scott et al., 1978). Tolbutamide polymorphism was reported due to a rare allele that carries an Ile359Leu mutation, also known as CYP2C9*3 (Sullivan-Klose et al., 1996). Two homozygous poor6metabolizers (PMs) of losartan reported the exact problem. One of these individuals was phenotyped for tolbutamide and proven to be homozygous for the CYP2C9*3 allele; thus, he or she was determined to be a tolbutamide-sensitive individual (Kidd et al., 1999). Another patient with the CYP2C9*3 genotype was shown to have a worsened response to warfarin and a lower ability to remove S-warfarin (Steward et al., 1997). 1.4.1.3 CYP2C9 VARIANTS In recent years, several CYP2C9 single nucleotide polymorphisms have been documented (Bhasker et al., 1997). Due to single nucleotide polymorphism (SNPs), allelic variants have been produced, resulting in a difference in amino acid (Figure1.7). They differ in a few positions in the gene' coding region (Goldstein et al., 1994). Among 24 CYP2C9 variants, CYP2C9*1 is also called a wild-type allele. Both alleles are functional and produce a normal protein (Bhasker et al., 1997). In CYP2C9*2, substituting T (C430T) on exon 3 occurs, resulting in Arg144Cys conversion and reduced activity enzyme (Crespi et al., 1997). CYP2C9*3 variant allele results on exon 7 by A1075C substitution, leading to altered protein with the substitution of Leucine residue (Ile359Leu). Such protein has reduced enzyme activity compared to CYP2C9* (Surendiran et al., 2011). CYP2C9*4 variant result due to (1076T>C) conversion on exon 7, resulting in the isoleucine replacement by threonine residue at 359 position (Imai et al., 2000). Recently, the CYP2C9*5 variant allele has been identified. It is characterized by the transversion of C1080G on exon 7, resulting in Asp360Glu conversion (Dickmann et al., 2001). CYP2C9*6, a new null polymorphism, has been identified in which deletion of adenine nucleotide at 818 positions on exon 5 occur, resulting in protein inactivation (Kidd et al., 2001). CYP2C9*11 is due to the substitution of 1003C>T in exon 7, which results in the substitution of Arg335Trp (Higashi et al., 2002). Figure 1.7: Functionally important allele of the human CYP2C9 gene. To date, more than 15 variant alleles (*1B to *16) and a series of subvariants of the CYP2C gene have been identified (S.-F. Zhou et al., 2010)(S.-F. Zhou et al., 2010) 1.4.1.4 Phenotypes of CYP2C9 The CYP2C9 gene, which codes for the CYP2C9 enzyme, is extremely polymorphic and comprises functional variants of key pharmacogenetic significance (Table 1.2). Genetic variation in CYP2C9 leads to changes in metabolic activity, which is critical in pathogenesis due to ADRs. An inclusive literature review has identified two common SNP variants inside the CYP2C9 gene, Arg144Cys and Thr359>Leu, which leads to poor metabolism (PM) phenotype. Individuals identified with *2 and *3 genotype exhibit the most noticeable pharmacokinetic effects for several drugs, particularly in the heterozygous carrier of both variants. It is reported that patients who are identified as a carrier of *2 and *3 variants, which are associated with poor metabolism, need lower dosages of warfarin to obtain the same anticoagulant effect as those with the wild-type1(CYP2C9*1)ogenotype (Aithal et al., 1999; Lindh et al., 2009). Table 1.2: Mutation and resulting phenotypes of CYP2C9 variant Allele. Allele Mutation Effect Enzyme activity on Phenotype Reference CYP2C9*1 None None Extensive Metabolizer (UM) (Leishman, 1991) CYP2C9*2 430C>T Decrease activity in Poor metabolizer (PM) (García-Martín et al., 2001) CYP2C9*3 1075A>C Decrease activity in Poor metabolizer (PM) (García-Martín et al., 2001) CYP2C9*5 1090C>G Decrease activity in Poor metabolizer (PM) (Dickmann et al., 2001) CYP2C9*6 818delA Decrease activity in Poor metabolizer (PM) (Kidd et al., 2001) CYP2C9*13 269T>C Decrease activity in Poor metabolizer (PM) (Si et al., 2004) 1.4.1.5. Frequencies of CYP2C9 Allelic Variants in the Population CYP2C9*2 is lacking in the East Asian populations, including Chinese, Korean, and Japanese, according to reports. However, the American, African, and Ethiopian populations have reported a general allele frequency of 3.2% for CYP2C9*2. Substantial CYP2C9*2 allele frequency heterogeneity occurs among several Caucasian populations, ranging from 8 to 19%. In the case of Hispanics, CYP2C9*2 has an allele frequency of 12.0%, relative to Caucasians but greater than blacks. In the case of CYP2C9*3, an allele frequency of 1.1%9in Koreans, 2.2%8in Japanese, and 3.3% in Chinese is reported (Inoue et al., 1997) (Leung et al., 1999; Odani et al., 1997). However, blacks have an allele frequency of 1.3% for CYP2C9*3; in the case of Caucasians, for CYP2C9*3 allele frequency, significant heterogeneity is revealed. Hispanics have exhibited an allele frequency of 3.4%, comparable to Chinese 3.3%. CYP2C9 *5, first identified in African-Americans, has an allelic frequency of90.2– 1.7% (Dickmann et al., 2001; Strom et al., 2000; H.-G. Xie et al., 2002) For Hispanic-Americans, the frequency for CYP2C9 *5 ranges from 0–0.5% (Strom et al., 2000; H.-G. Xie et al., 2002) and was found absent or extremely scarce in Caucasians, Chinese, and Japanese population (H.-G. Xie et al., 2002). CYP2C9 *6, a rare and uncommon allele, has an allele frequency of 0.6 to 1.5% in African-American (Kidd et al., 2001). 1.4.1.6. Interethnic Variability Substantial ethnic differences are found in the CYP2C9 allelic variants frequency. Several studies have been designed and implemented to report the phenotypic character and frequency of poor metabolizers in different populations. The CYP2C9*2

variant is most prevalent in the Caucasian population, where 1% are homozygous7(carriers) and about 22% are found to be heterozygous (Sullivan-Klose et al., 1996). CYP2C9*2 and *3 are either rare or absent in the Asian population (Kirchheiner et al., 2005). Patients with *2 and *3 genotypes have been reported to have shown reduced intrinsic clearance of oral anticoagulants and were more susceptible to bleeding events. Patients who were a carrier for *2 or *3 genotype have shown a higher frequency of bleeding complications during warfarin therapy initiation (Aithal et al., 1999; Sullivan-Klose et al., 1996) (Takahashi et al., 2001) Furthermore, it has been reported that the East Asian population has a higher sensitivity to warfarin due to the absence of the CYP2C9*2/*3 variant compared to Caucasian, indicating poor metabolizer status. CYP2C9*5 is assessed to be inherited in 3%9of the African-American population as a C1080>G mutation6(Allabi et al., 2005; Allabi et al., 2004). Additionally, African Americans have fundamentally lower CYP2C9*2 and *3 inheritance rates than the Caucasian population, with an allele frequency of 2.5% and 1.25%, respectively. In the case of the African American-population, the presence of *5 and *6 alleles may be vital in ADRs caused by CYP2C9 substrate drugs. Table 1.3: Interethnic variability of CYP2C9 variant in different populations. Ethnic group No of subjects Allele frequency % *1 *2 *3 References Russian 290 83.9 9.1 7.0 (Gaikovitch et al., 2003) Turkish 499 79.4 10.6 10.0 (Aynacioglu et al., 1999) Ethiopian 150 94.0 4.0 2.0 (Scordo et al., 2005) Chinese 376 96.7 0 3.3 (Hong et al., 2005) Japanese 218 97.9 0 2.1 (Nasu et al., 1997) Korean 358 93.4 0 6.0 (Bae et al., 2005) 1.4.1.7. Role of CYP2C9 in Breast cancer Estrogen is an important growth factor that can stimulate cancer cell growth by interacting with ER and triggering the cellular proliferation pathway. In addition to having a crucial role in the metabolism of anticancer medicines and carcinogens, cytochrome p450 are powerful enzymes that enable estrogen metabolism (Husbeck et al., 2002). Expression of various CYPs has been reported in breast cancer (Forrester et al., 1990). Although several CYP2C have been reported in breast cancer studies, the frequency of CYP2C9 protein expression as measured by western blotting ranged from 0% (Albin et al., 1993) to 100% (Forrester et al., 1990). However, a recent study reported weak CYP2C9 protein expression in all tested tumor samples (Schmidt et al., 2004). CYP2C9 metabolizes estrone sulfate to 16-αhydroxy sulfate metabolite, often linked with increased breast cancer risk. 16α-hydroxy estradiol and 16α-hydroxy estrone are both metabolites that have a strong affinity for estrogen receptors which can lead to the activation of ER. Evidence suggests that there is significant involvement of 16-OH product, a product of CYP2C9 metabolism, in the progression of breast carcinoma (Zhu et al., 1998). 1.4.1.8. Phenotype of CYP2C9 for Tamoxifen Metabolism Among >30 variations of CYP2C9, *2 and *3 have been reported to be associated with a large yet substrate-dependent drop in intrinsic clearance (C. R. Lee et al., 2002). The *3 allele has a greater effect than7*2, with a decrease in enzyme activity of up to 90% for some specific drugs (King et al., 2004). Numerous studies fail to find any link between CYP2C9 polymorphisms and tamoxifen pharmacokinetics (Jin et al., 2005). A recent study suggested that CYP2C9 is vital in the endoxifen formation by the metabolic transformation of tamoxifen into 4-hydroxytamoxifen, which is the ancestor of endoxifen (Mürdter et al., 2011). In contrast to CYP2C9*17(Wild type), expressed CYP2C9*24and CYP2C9*3 variant proteins showed a reduction in the tamoxifen's intrinsic clearances to 4- hydroxytamoxifen. The study also indicated that this reduction would be greater in patients having CYP2C9*3/*37genotype than those with CYP2C9*2/*2 or CYP2C9*2/*3 genotypes (Coller et al., 2002). 1.4.2. CYP2C19 CYP2C19 is the most clinically important form of the CYP2C subfamily. It is a 490 amino acid enzyme with a heme moiety at the active site. Neutral substrate molecules, or slightly alkaline molecules, bind. The substrates or drug molecules catalyzed by CYP2C19 enzymes are termed CYP2C19 substrates. Depending upon the reaction, the CYP2C19 substrate can either act as an inhibitor or an inducer (D. F. Lewis, 1999). A vast category of medicines can be metabolized by CYP2C19 enzymes, such as antidepressants like Citalopram, certain proton pump inhibitors (PPIs) like Omeprazole, antiepileptic drugs like Mephenytoin and Diazepam, antiplatelet drugs like clopidogrel, antimalarial drugs, e.g., proguanil and B-adrenoceptor blocker, e.g., propranolol (Sim et al., 2006). Chromosomal location for CYP2C19 gene cluster at 10q24, spanning a region of 90kb with 9 exons (Riddell et al., 1987). According to an FDA report in 2012, certain inhibitors are listed, but none is specific to the CYP2C19 enzyme. CYP2C19 enzyme is inhibited by several inhibitors that reduce the rate of CYP2C19 substrate turnover. Two inhibition mechanisms are classified as reversible and irreversible (Bjornsson et al., 2003; Yueh et al., 2005). The probability of inhibition was restricted, as in vitro experimental conditions lack true physiological cell state. Therefore, clinical evaluation of inhibition is a powerful approach when studies are held with context to clinical aspects. For CYP2C19 inhibition in humans, Omeprazole might be regarded as a9diagnostic probe. However, it is listed as a moderate inhibitor by FDA. The CYP2C19 gene encodes the CYP2C19 enzyme with a vital role in estrogen catabolism, as it is responsible for 16 α-hydroxylation and 17 β-hydroxy dehydrogenation catalysis of estradiol (Cheng et al., 2001). CYP2C19 is vital in the tamoxifen metabolism during BC therapy, as it is involved in the 4-hydroxylation of tamoxifen (Crewe et al., 2002) . Reduced CYP2C19 activity via haploinsufficiency might be linked to the rise in BC risk, possibly through life-long greater estrogen (Tervasmäki et al., 2014). The nature of CYP2C19 is inductive. The increased intrinsic metabolic clearance is projected if the enzyme concentration or activity is increased in hepatocytes. 1.4.2.1. History of CYP2C19 Polymorphism In the late 1940's, S-Mephenytoin (racemic drug) was marketed as an anticonvulsant drug. Adrian Kupfer, a Ph.D. student at the university of Brene in the 1970s, started research on the enantiomer project and reported stereo-specific mephenytoin metabolism in dogs (Küpfer et al., 1984). Kupfer and his co-worker discovered stereo specific metabolism of S-mephenytoin in

humans. One of the healthy participants in his study suffered from severe sedation after intake a low dosage of Mephenytoin, whereas a normal response was shown by the other individuals. To find the reason, radiolabeled Mephenytoin was analyzed through urine. Results revealed a marked reduction in 4-hydroxy mephenytoin concentration in the patient's urine sample. These findings suspected polymorphism of mephenytoin hydroxylation (Küpfer et al., 1984). A familial study involving 28 families unveiled that CYP2C19 is strongly involved in this poor metabolism of (Inaba et al., 1986). Later on, the immunoblot assay performed by Wrighton and his co-workers provided strong evidence supporting the poor metabolism of mephenytoin by CYP2C19 enzymes (Wrighton et al., 1993). Due to CYP2C19's role in converting S-mephenytoin to 4-OH mephenytoin, CYP2C19 polymorphism is also called "S-mephenytoin polymorphism." Expression analysis of cDNA showed that all CYP2C9 variants produced negative results toward S-mephenytoin. Goldstein and Wrighton's group separately identified CYP2C19 as a key enzyme accountable for the production of 4-OH Mephenytoin using the cDNA expression system of yeast and immunoblot analysis (De Morais et al., 1994) 1.4.2.2. Clinical Important Allele Variants of CYP2C19 At present, 34 genetic CYP2C19 variants have been catalogued (http://www.CYPalleles.ki.se). Most mutations are unique and have no clinical significance. CYP2C19*1, a wild-type allele, has normal enzyme function and no SNP. CYP2C19*2 genetic variant arises due to a single base alteration 681G>A, rs4244285 located in exon five. This mutation is responsible for the premature stop codon, which is twenty amino acids downstream of the amino acid at position 215. As a result, truncated or non-functional protein fails to conduct any function (De Morais et al., 1994) Exon 4 mutation 636G>A, rs4986893, produced premature8stop codon and shortened inactive protein product, forming the CYP2C19*3 allele variant (Morais et al., 1994a). CYP2C19*4 is characterized by C>T, rs28399504 situated at the first base of exon 1, producing initiation codon, and hence no protein product is formed (Ferguson et al., 1998) CYP2C19*5 shows a mutation 1297C>T, rs56337013 in exon 9. This mutation is responsible for Arg433Trp amino acid substitution. Hence, enzyme activity is reduced (Ibeanu et al., 1999). Exon 3 of CYP2C19*6 has the mutation 395G>A. This mutation causes amino acid substitution Arg132Gln. As a result of this mutation, protein structure and stability are affected (Ibeanu et al., 1998) CYP2C19*7 mutation is caused by T>A inversion at the 5´ splice site, producing a splicing flaw in intron 5. Therefore protein synthesis is affected (Ibeanu et al., 1998). CYP2C19*80is associated with 358T>C mutation in exon83. This mutation is responsible for amino acid Try120Arg substitution, causing enzyme activity to decrease (Ibeanu et al., 1999). CYP2C19*17 variant has 2 linked mutations in total non-disjunction equilibrium at - 806C>T and -3402C>T6in the 5´ regulatory region. This mutation increases enzyme activity and expression (Sim et al., 2006). 1.4.2 .3. CYP2C19 Genetic Polymorphism CYP2C19 is a gene with high polymorphism. CYP2C19 genetic variation and its molecular mechanism have been studied through various methodologies. An initial study on liver biopsy reported a reduced concentration of CYP450 isozyme in extensive and poor metabolizers (Meyer et al., 1986). The first mutation in CYP2C19 was identified using liver samples, which were designated as CYP2C19m1 (CYP2C19*2) using reverse transcription and mRNA amplification techniques. Then the second mutation was identified in the Japanese poor metabolizer (PM) subject, and it was designated as CYP2C19m2 (CYP2C19*3) (De Morais et al., 1994). In 2006, Sim and his co-worker found a novel CYP2C19*17 allele as an ultrarapid metabolizer (UM) phenotype, which has tremendous CYP2C19 activity by increasing CYP2C19 gene expression. 1.4.2.4. Transcription Regulatory Mechanism of CYP2C19 CYP2C19 regulatory mechanism involves nuclear receptors ERα (NR3A1), which are activated after the binding of a ligand ((Mwinyi, 2010)), constitutive androstane receptor (CAR), Estrogen response element (ERE) (Wortham et al., 2007) and pregnane X5receptor-(PXR) (Tirona et al., 2005). The functional activity of the constitutive androstane receptor response element (CAR-RE) in the CYP2C19 promoter region is shown. Binding of the ligand to this site is hindered (Chen et al., 2003). In humans, rifampicin, a classic ligand that binds to PXR, is a strong inducer of CYP2C19, starts the transcription process, and synthesizes functional enzymes. As the level of induced enzyme activity is less than the induced mRNA level, the induced enzyme activity is concentration-dependent (Dixit et al., 2007; Xing et al., 2012). When compared with CYP3A4, the induction activity of CYP2C19 is moderate. 1.4.2.5. Phenotypes of CYP2C19 CYP2C19 is one of the main xenobiotics metabolizing enzymes in humans and is polymorphically expressed. Individuals are categorized into four types when polymorphism is implicated in drug metabolism.: normal or extensive, poor, intermediate, and ultra-rapid metabolizer for a specific drug; this classification is based on Phenotyping results. The phenotype of CYP2C19 is measured by comparing the drug metabolites via urine or plasma samples. CYP2C19 phenotypic evaluation is done by giving mephenytoin drug 150mg (Küpfer et al., 1984) and omeprazole drug 20 mg. FDA has recommended omeprazole for phenotypic evaluation (H. M. Gonzalez et al., 2003). Extensive Metabolizer (EM) CYP2C19*1/*1 allele is referred to as a normal or extensive metabolizer when both alleles are functional and produce 490 amino acid proteins. Phenotypic analysis by probe mephenytoin and omeprazole drug for CYP2C19*1/*1 has shown the estimated concentration of metabolites in urine when the enzyme is fully functional (Chang et al., 1995). Intermediate Metabolizer (IM) It is a phenotype in which one allele is not functional. In comparison, just one single allele produces a functional protein (Ingelman-Sundberg et al., 2020), and the phenotypic expression of the protein is less than the EM. The possible allele combination for IM is *1/*2-*8; here, one allele is non-function (S. Scott et al., 2011). Poor Metabolizer (PM) When both alleles are non-functional and produce truncated or null protein, such a phenotype is termed PM. Allele combinations, e.g., *2-*8/*2-*8, that show no or very reduced enzyme activity are included in this class. CYP2C19*2 and CYP2C19*3 are mainly regarded as poor metabolizers of

drugs (H. M. Gonzalez et al., 2003) Ultra-rapid Metabolizer (UM) It is a phenotype with more than one gene, or the enzyme function is enhanced due to a certain defect. CYP2C19*17 is the only allele that is responsible for rapid xenobiotic metabolism. The allele combination *1/*17 plays a part as UM for some drugs (S. Scott et al., 2011) 1.4.2.6. Inter-individual Variability Inter-individual variations in the different drug metabolisms within the human body result from genetic polymorphism. Clinical data suggests that because of these widespread inter-individual differences, the TAM and its metabolites' amount in the plasma differ broadly among patients, affecting the therapeutic outcome. It can be due to CYP450 variable activity that may modify the pattern of TAM metabolism within the body causing variations in TAM and its active metabolites' systemic concentration (Desta et al., 2004). 1.4.2.7. Interethnic Variability Different ethnic groups show differences in the metabolism of CYP2C19. Mutations in a gene encoding a drug-metabolizing enzyme can produce enzyme variants with no activity, moderate activity, or even high activity. Frequency of UM i-e. CYP2C19*17 variant was identified in different ethnic groups and is found to be 18% in the Ethiopian and Swedish populations. In comparison, 1.3% of its allele frequency is found in the Japanese and Chinese populations (Sim et al., 2006). CYP2C19*2 poor metabolizer allele frequency is relatively higher in Chinese, Korean, and Japanese populations (Roh et al., 1996), but it is quite low in Arabs, African, and Caucasian ethnic groups (Persson et al., 1996; H. Xie et al., 1999) whereas, in Cuna Indians of Panama, frequency of poor metabolizer was so low that it could not be detected (Inaba et al., 1988). However, 79% of the inhabitants of the Pacific Ocean islands of Vanuatu are poor metabolizers (Kaneko et al., 1997). 1.5. CYP3A Subgene Family The CYP3A enzyme family consists of enzymes that have a part in metabolizing 50% of therapeutically used drugs and several xenobiotics. These also metabolize important endogenous compounds like retinoic acid, bile acids, and steroid hormones. The CYP3A family consists of four genes, i.e., CYP3A43, CYP3A7, CYP3A5, and CYP3A4. In addition, three pseudogenes are also present in the family, i.e., CYP3AP1, CYP3AP2, and CYP3AP3. All these genes are on human chromosome 7 at position q21-q22.1(Ingelman-Sundberg et al., 2020). The CYP enzymes expression varies with the tissue type. In the fetal stage, the maximum expression of hepatic CYP3A7 and CYP3A4 is not present, while in adults, the case is vice versa, but some adults might have CYP3A7 expression (Saiz-Rodríguez et al., 2020). CYP3A5 expression is the same at all stages of development. The three enzymes, CYP3A4, CYP3A5, and CYP3A7, share a comparable affinity for their respective substrates (X. Liu et al., 2019). There is more than 70% homology present in amino acids sequences of the members of the CYP3A family, (Plant, 2007). 1.5. 1. CYP3A4 The CYP3A4 gene is present on human chromosome no. 7, having 27,592 base pairs and comprising 13 exons. CYP3A4 is known to involve the metabolism of therapeutic drugs and various xenobiotics ; it also participates in the oxidation of various endogenous substrates like fatty acids, steroids, and sex hormones. CYP3A4 involves in the conversion of testosterone into 15β-hydroxy testosterone, 2β-hydroxy testosterone, and 6β- hydroxy testosterone by oxidation reactions (Saiz-Rodríguez et al., 2020) CYP3A4 is the highly present hepatic protein of the CYP superfamily, constituting about 60% of total CYP enzymes in the liver. CYP3A4 is engaged in the metabolism of >120 different therapeutic drugs and is known to metabolize approximately 60% of currently used drugs (Danielson, 2002). Many compounds have molecular weight variations, i.e., cyclosporine (MW = 1203 Dal) to metyrapone (MW = 226 Dal), metabolized by CYP3A4. The enzyme can oxidize massive substrates because of a large binding cavity for a substrate such as statins, cyclosporine, macrolide antibiotics, and taxanes. CYP3A4 catalyzed reactions include about 7% O-dealkylation, 27% N- dealkylation, and 43% aromatic or aliphatic hydroxylation reactions (X.-Y. Zhou et al., 2019). CYP3A4 also metabolizes various drugs with anticancer activity, like paclitaxel, docetaxel, tamoxifen, cyclophosphamide, vinblastine, and irinotecan (Bozina et al., 2009). In addition to drugs, CYP3A4 is also known to metabolize numerous environmental and dietary chemicals like sterigmatocystin, pesticides, aflatoxin B1, G1, mycotoxins, PAH-diols, flavonoids, and a variety of food additives (Bozina et al., 2009) 1.5.1.1. CYP3A4 Substrates, Inducers, and Inhibitors CYP3A4 protein comprises 502 amino acids having 57 kDa molecular weight (S.-J. Lee et al., 2005). Many therapeutic medications, including calcium channel blockers, immunosuppressants, cancer chemotherapeutic agents, sedatives, antihistamines, and synthetic estrogens, are metabolized by the CYP3A4 enzyme. Certain endogenous steroids like cortisol, estradiol, and testosterone are metabolized by the CYP3A4 protein (X.-Y. Zhou et al., 2019). There are a large number of CYP3A4 enzyme substrates, some of them are as follows (Li et al., 1995) Various chemicals (dietary and drugs) present as an inducer for CYP3A4 protein, such as rifampicin, glucocorticoids, and anticonvulsants like carbamazepine. The components of Hypericum perforatum, especially hyperforin, are known as potent CYP3A4 enzyme inducers. In the inhibition of CYP3A4 protein, different chemicals and drugs are involved, such as the azole antifungal agents including ketoconazole, inhibitors of HIV protease like saquinavir, macrolide antibiotics like troleandomycin and antidepressants like as fluoxetine. The probes that are used as a substrate for the study of CYP3A4 protein include erythromycin, midazolam, testosterone, and cortisol (Bozina et al., 2009) 1.5.1.2. CYP3A4 Transcriptional Regulation The CYP3A4 gene is placed on chromosome 7; its promoter region consists of basal transcription elements (from -35 to -50). AP-3 binding site, Estrogen Response Element (ERE), glucocorticoid response element, p53 binding motif (DNA sequence that acts as a binding site for p53 protein). HNF-48(hepatocyte nuclear7factor-4 element) and two HNF-5 are appeared in the 5' UTR (untranslated region). CYP3A4 protein formed is also known as P-450-PCNI, nifedipine oxidase, and NF-25 (Albertsen, 2005). In CYP3A4 gene regulation, various signaling pathways are involved. In constitutive transcription regulation, both regulators,

i.e., negative, and positive, are involved, especially CCAAT/Enhancer Binding Protein (C/EBPα and C/EBPβ), USF, HNF1α, HNF3γ, and HNF4α. A range of xenobiotics and cis-acting modules accountable for inducible-transcriptional control of genes are the proximal PXR (Pregnane X Receptor) Responsive Element prPXRE, the Constitutive6Liver Enhancer Module 45(CLEM4) (-11.4 to -10.5 kb) and Xenobiotic-Responsive Enhancer Module (XREM) (-7.2 to -7.8 kb). The drug ligands bind, the xenosensors CAR (Constitutive7Androstane Receptor) and PXR translocate to the nucleus, and then heterodimerization of Retinoid X Receptor (RXR) occurs and thus transcription enhanced to several folds. The ligand-dependent regulation of gene transcription is driven by the bile acid receptor7FXR, glucocorticoid receptor, oxysterol receptor LXR, and the vitamin D receptor. PPARα is also involved in gene's constitutive and inducible transcriptional regulation (Zanger et al., 2013) 1.5.1.3. CYP3A4 Genetic Polymorphism CYP3A4 enzyme exhibit variation in its activity in different individuals, leading to its unpredictable role in xenobiotic (especially drug) metabolism within a population. This variation arises from gene expression regulation, genetic polymorphism, and xenobiotics interactions with the enzyme. It was reported that genetic polymorphism is accountable for 90% of the variation in CYP3A4 activity (Sukprasong et al., 2021). The variation in nucleotide sequence is termed polymorphism only if its frequency is ≥ 0.01 in a population; otherwise (frequency is < 0.01), it is known as mutation (Albertsen, 2005). Genetic polymorphism is a persistent alteration in the nucleotide sequence at a particular gene locus. SNPs (single-nucleotide polymorphisms)7are the most prevalent genetic variants in human cytochrome P450 genes. The nonsynonymous SNPs are present in the gene's coding region and are very important as these are involved in amino acid sequence change of the relative CYP protein. On average, 14.6 nonsynonymous SNPs exist for each cytochrome P450 gene (Sukprasong et al., 2021). CYP3A4 polymorphism is not only because of variation in the exonic region of the gene but also involves variation in the non-coding, i.e., intronic region. According to the Human Cytochrome P450-(CYP)7Allele Nomenclature Database (http://www.cypalleles.ki.se), 19 subtypes of wild type CYP3A4, i.e., CYP3A4*1 having 18 different SNPs but these do not affect the expression of mRNA and the complementary sequence of DNA. The SNPs in exonic regions are reported to be 24 (Werk et al., 2014). 1.5.1.4. Allelic Variants of CYP3A4 The null allele for CYP3A4 has not been reported yet, unlike other enzymes of the CYP family. Variations in the functions and level of protein due to genetic change in the intronic, exonic, or flanking region are possible. However, full-length mRNA has been found in all adult individuals studied until now (Lin et al., 2019). Some of the important alleles of CYP3A4 are mentioned in the Table 1.4 given below (Werk et al., 2014) http://www.cypalleles.ki.se/cyp3a4.htm) 1.5.1.5. CYP3A4 Allele Frequency CYP3A4 shows a vast inter-individual variation in its activity, having a difference of about 40-fold. One of the most common and frequent variants is CYP3A4*1B. It was found in 1998 that the CYP3A4*1B variant is coupled with prostate cancer in relevance to its higher grade and stage. In the CYP3A4*1B variant, the point mutation, i.e., SNP (A392G), exists in a 5' flanking region, i.e., 392 base pairs upstream from the translational initiation site (Werk et al., 2014). The CYP3A4*1B variant frequency was estimated as 53% in the African American population, 9% in the Caucasian population, and 0% in the Taiwanese population. The polymorphism is known to alter NFSE (Nifedipine Oxidase Specific Element), an important regulatory element in the transcription process and essential for CYP3A4 normal expression. (Hsieh et al., 2001; Seredina et al., 2012; Wojnowski et al., 2006; Zanger et al ., 2013). CYP3A4*2 allele encodes the protein that has kinetics depending on the substrate. It was found that CYP3A4*2 frequency is 2.7% in Caucasians, but Chinese and blacks lack the variant (Hu et al., 2017). The CYP3A4*9, CYP3A4*5, and CYP3A4*66 frequency was determined to be 1.5, 0.98, and 0.5%, respectively, in the Chinese population (Hu et al., 2017). In the Asian population, CYP3A4*18 polymorphism is the most frequent. The frequency reported in the Chinese, Japanese, Korean, and Malaysian populations to be 0.008~0.01, 0.013, 0.012~0.017, and 0.021, respectively (J. S. Lee et al., 2013). CYP3A4*22 is known to have decreased enzyme activity and has been reported in the Caucasian population. The allelic frequencies of important CYP3A4 variants in Caucasians, African and Asian populations are as follows (Vichi et al., 2021): Table 1.4: CYP3A4 allelic frequencies variants in different populations. CYP3A4 Allele Allelic frequency Caucasians (%) Africans (%) Asians (%) Caucasian (%) CYP3A4*1B 5.5 76.2 0.0 CYP3A4*2 0.3 0.0 0.0 CYP3A4*3 1.6 0.0 0.0 CYP3A4*4 0.0 0.0 0.2 CYP3A4*5 0.0 0.0 0.3 CYP3A4*7 0.5 0.0 0.0 CYP3A4*11 0.1 0.2 0.0 CYP3A4*12 0.3 1.0 0.0 CYP3A4*15 0.0 1.6 0.0 CYP3A4*16 0.0 0.0 0.5 CYP3A4*18 0.0 0.0 1.7 CYP3A4*19 0.0 0.0 2.3 CYP3A4*22 5.3 0.0 0 1.5.1.6. CYP3A4 and Breast Cancer Estrogen hormones interact with the estrogen receptor7(ER) and trigger cell proliferation, functioning as a cancerous cell growth development element. Cytochrome P450 members are critical in the metabolism of estrogen, anti-cancerous drugs, and carcinogens (Husbeck et al., 2002). CYP3A4 contributes to the 4- and 16α- hydroxylation process of estrogen, especially estrone, most commonly present in post- menopausal women. CYP3A4 and CYP1A2 (catalyze 2-hydroxy estrone formation) play roles in determining 2-hydroxy estrone: 16α- hydroxy estrone ratios related to breast carcinoma (Keshava et al., 2004). The correlation between CYP3A4 overexpression and breast cancer is also described in certain studies. In a study, the expression of mRNA of CYP family members was determined in normal and tumor tissues of breast cancer, and CYP3A4 mRNA expression was reported in 70% normal and 18% tumor tissues (Huang et al., 2004). In China (Shanghai), a population-based study in a women subgroup determined the association of urinary cortisol ratios with breast carcinoma risk. It was found that urinary cortisol: 6β-hydroxy cortisol ratio was correlated with breast cancer risk, and this ratio is also the measure of enzymatic activity of CYP3A4. It has also been shown that breast cancer risk increases dose-dependently (Zheng et

al., 2001). 1.5.1.7. CYP3A4 and Tamoxifen Metabolism Tamoxifen, a pro-drug, is metabolized by CYP family proteins into more potent metabolites. These metabolites have different binding affinities for ER. CYP3A4/5 and CYP2D6 are the major enzymes implicated in these metabolic reactions. N- desmethyl tamoxifen is a primary and major metabolite formed by CYP3A4/5 catalyzed reaction, and CYP2D6 mediates the development of 4-OH-tamoxifen. In secondary metabolic reactions, these metabolites, i.e., SULT1A1, *CYP2C19* and disease-free survival in early breast cancer patients receiving tamoxifen", Pharmacogenomics, 10/03/2011">N-desmethyl tamoxifen and SULT1A1, *CYP2C19* and disease-free survival in early breast cancer patients receiving tamoxifen", Pharmacogenomics, 10/03/2011">4- OH-Tamoxifen, are converted into SULT1A1, *CYP2C19* and disease-free survival in early breast cancer patients receiving tamoxifen", Pharmacogenomics, 10/03/2011">4-OH-SULT1A1, *CYP2C19* and disease-free survival in early breast cancer patients receiving tamoxifen", Pharmacogenomics, 10/03/2011">N-desmethyl-tamoxifen (likewise called Endoxifen) SULT1A1, *CYP2C19* and disease-free survival in early breast cancer patients receiving tamoxifen", Pharmacogenomics, 10/03/2011">by CYP2D6 and CYP3A4/5 respectively. Norendoxifen is also produced during metabolic reactions of tamoxifen by CYP3A4/5 activity, inhibiting CYP19A1. The most important and active metabolites of tamoxifen are 4-OH-tamoxifen and endoxifen. They have similar binding affinity for ER, i.e., 33 times more than tamoxifen, but plasma endoxifen concentration is 5-10 times more than 4-OH- tamoxifen. The other enzymes of the CYP family involved in tamoxifen metabolism are CYP1A1, 1B1, 2B6, 2C9, and 2C19 (Fig 5). The enzymes Sulfotransferases 1A1 (SULT1A1) and UDP glucuronosyltransferases (UGT) are responsible for the inactivation of tamoxifen and its metabolites. Certain xenobiotics induce or inhibit various CYP proteins (Singh et al., 2011). 1.5.2. CYP3A5 CYP3A5 is the CYP3A predominant extra-hepatic isoform expressed in the prostate, kidneys, breast, lungs, and small intestine besides the liver (J. K. Lamba et al., 2002). CYP3A5 causes the cortisol to 6β-hydroxy cortisol metabolism in the kidneys, which is the Na+ transport regulator in kidney epithelium and causes its retention. Thus any variation leading to increased CYP3A5 expression can lead to salt-sensitive hypertension (A. R. Khan et al., 2020). CYP3A5 is also vital in the estrogen and 16α-hydroxy estrogen metabolism to 2 and 4- hydroxy estrogens in prostate and breast tissues. These metabolites, specifically 4- hydroxy estrogen, are carcinogenic and thus responsible for disease risk in these organs (J. K. Lamba et al., 2002). A study by Kuehl et al. (2001) indicates that 50% hepatic content of CYP3A is contributed by CYP3A5 in individuals possessing both functional alleles of the gene (Kuehl et al., 2001). CYP3A5 shows highly overlapping substrate specificity with CYP3A4, so it also metabolizes many CYP3A4 substrates in the liver. The metabolic activity of CYP3A5 is lower than CYP3A4 (Lolodi et al., 2017). The CYP3A5 gene is located at 7q22.1 on the negative strand of the long arm of chromosome 7. The gene is ~33 kb in length, has thirteen exons and twelve introns, and encodes a protein with 502 amino acid residues and a molecular mass of 57 kDa (Rodriguez-Antona et al., 2022). 1.5.2.1. Transcriptional regulation of CYP3A5 All CYP3A genes are clustered on the chromosome, but their regulation is quite different. In order to analyze the transcriptional regulation for CYP3A5 polymorphism, (Lolodi et al., 2017) CYP3A5 promoter was analyzed by (Jounaıdi et al., 1996), and two mutations were found to be responsible for CYP3A5 variations. However, later it was revealed through sequencing that these SNPs were present in CYP3AP1(pseudogene) promoter, which shows great homology with the CYP3A5 gene, thus, not responsible for CYP3A5 polymorphism (Kuehl et al., 2001). The transcription regulation of CYP3A5 is different from CYP3A4 in that no nuclear hormonal inducers or distant enhancers are involved in its regulation (Lolodi et al., 2017). In CYP3A5 transcriptional regulation, the CCAAT box and Basal Transcription Element (BTE) cis-elements are involved, located at the -90 to -40 region of the CYP3A5 gene. Moreover, transcription factors implicated in transcriptional regulation are Nuclear Factor Y proteins (NF-Y)9and Specificity-proteins7(SP family), including SP1 and SP3 nuclear factors (Iwano et al., 2001). Both these classes are constitutively active transcription factors and are the reason for the vast expression of CYP3A5 in different body tissues (Kolell et al., 2002). 1.5.2.2. CYP3A5-mediated drug metabolism More than half of clinically treated drugs are metabolized by the CYP3A subfamily, where CYP3A48and CYP3A5 are predominant. These enzymes have a huge metabolic diversity because of their large active site, which can bind to several substrates, often more than one (Guo et al., 2020). CYP3A5 cDNA shows 90% sequence similarity with CYP3A4. Thus both share many substrates, inducers, and inhibitors (Y.-T. Liu et al., 2007). Thus, it must be noted that it is uncertain whether the alteration in metabolism is because of CYP3A5 alone or because both enzymes have equal efficiencies for CYP3A substrates metabolism and work competently together. However, some careful studies indicated a substantial function of CYP3A5 in the metabolism of certain drugs; saquinavir for HIV-I infections treatment (Fröhlich et al., 2004; Mouly et al., 2005), quinine for malarial infections (Mirghani et al., 2006) haloperidol and chlorpromazine for depression and mental illness, Tacroliums which is used after an organ transplant (Y.-T. Liu et al., 2007). 1.5.2.3. CYP3A5 inter-individual and inter-ethnic variability CYP3A5 was first discovered by Aoyama and Schuetz in 1989, who noted that CYP3A5 protein levels were not the same in every individual. Instead, some individuals express extremely high levels, while others show reduced or negligible levels (Huang et al., 2004). Such observations suggest variable expression of CYP3A5. Inter-individual differences might be due to previous exposure to drugs, CYP inhibition by other drugs, first-pass effect after oral intake, dietary factors, or due to genetic mutations (J. S. Lee et al., 2013; H.-G. Xie et al., 2004). It was suggested that about 70-95% of inter-individual variability of CYP3A is due to genetic variations (Oleson et al., 2010). Further studies indicated that in addition to inter-individual variability, inter-ethnic differences in CYP3A5 expression are also

present, which can be termed as 'Polymorphism.' Interethnic variability ranges from 6 to 99% in ethnic groups (Roy et al., 2005). According to research, almost 10-25% of Europeans, 55- 95% of African Americans, and 30-50% of Asians and South Americans showed considerable levels of CYP3A5 protein in the liver (J. K. Lamba et al., 2002). The CYP3A5 variant frequency, which causes normal metabolism, is 5% in Europeans, 27%8in Chinese, 30% in Koreans, 29% in Japanese, and 73%5in African-Americans (Hustert et al., 2001). These variations are because of underlying genetic reasons on the one hand, while environmental factors and drug-drug interactions can also be the source of polymorphisms on the other hand (Roy et al., 2005). Genetically, SNPs are responsible for formation of different alleles with variable phenotypes (Xie et al., 2004). About 25 allele variants of CYP3A5 have been recognized. Four alleles,6CYP3A5*1,8CYP3A5*3,7CYP3A5*6, and6CYP3A5*7, occur most commonly and are majorly accountable for CYP3A5 variable expression (Lamba et al., 2012). Environmental factors like selection pressure on alleles might also cause such variations, e.g., CYP3A5 genetic distribution in industrialized countries differs from that of developing countries (Roy et al., 2005). 1.5.2.4. CYP3A5 Genetic Polymorphism Genetic polymorphism is the manifestation of two or more alleles of the same gene with a minimum 1% frequency in a population. CYP3A5 shows a highly polymorphic expression than any other CYP3A isoform. Consequently, it is accountable for inter-individual and inter-ethnic variation in CYP3A-mediated drug metabolism (Lee et al., 2003). There are about 25 allelic variants of CYP3A5 narrated by different researchers. Seven alleles are present in the exonic region of the gene: CYP3A5*2, CYP3A5*7, CYP3A5*4, CYP3A5*6, CYP3A5*8,8CYP3A5*9 and CYP3A5*10. In addition, some variants are also present in intronic regions, including CYP3A5*3 and CYP3A5*5 (Xie et al., 2004) Positions of these alleles in the CYP3A5 gene locus are shown in (Figure 1.8) CYP3A5*1, CYP3A5*3,3CYP3A5*6, and CYP3A5*7 occur most commonly and are chiefly responsible for all this inter-individual and inter-ethnic CYP3A5 polymorphism (Lamba et al., 2012). CYP3A5*1 is a fully operational wild-type allele, while the remaining three exhibit SNPs in their sequences and result in nonfunctional alleles. Figure 1.8. Localization of genetic polymorphisms in the human CYP3A5 gene and Positions of these alleles in the CYP3A5 gene locus (J. Lamba et al., 2012).. 1.5.2.5. Clinically important variants of CYP3A5 To date, 25 CYP3A5 allelic variants have been stated (http://www.cypalleles.ki.se/cyp3a5.htm). Only CY3A5*1 is functional, and the remaining are non-operational forms. CYP3A5*1 is the only allele that forms a full-length functional CYP3A5 protein. It is a wild type of allele with no SNP. CYP3A5*2 was identified by Jounaidi et al. in 1996 in Caucasian liver samples. CYP3A5*2 shows a substitution mutation at 27289C>A, rs28365083 in exon 11, thus forming a non-functional protein. CYP3A5*3 is the most prevalent non-functional allele with an SNP at 6986A>G, rs776746. The change from A to G occurred at this position, creating a cryptic splice site in intron three, ultimately leading to modified mRNA splicing and truncated protein formation. According to a study by (Kuehl et al., 2001), individuals expressing CYP3A5*3 allele showed a reduced level of CYP3A5 protein in the liver than those possessing wild type allele. CYP3A5*3 allele is present in all ethnic groups with varying frequencies, and in many populations, it is more widespread than wild type allele (J. K. Lamba et al., 2002). The CYP3A5*3 frequency is 71-75% in East Asians, 59.8% in South Asians,829-35% in Black Americans, 60-66% in Hispanics, and 92-94% in Caucasians (H.-G. Xie et al., 2004). CYP3A5*4 is an SNP in exon seven at 14665A>G, rs56411402 position. CYP3A5*4 protein shows a non-expresser phenotype (Lim et al., 2011) CYP3A5*5 transitions from T→A at the 5'-end of exon number 5, causing frameshift and formation of non-functional protein (Chou et al., 2001). CYP3A5*6, also a non-functional allele, shows SNP at 14690G>A, rs10264272 in exon 7, skipping this exon and changing the reading frame and truncated non- functional protein forms (J. Lamba et al., 2012). CYP3A5*6 allele is comparatively common in black individuals8(7-17%) but rare in Asian and Caucasian populations. CYP3A5*7 reflects an insertion of T nucleotide at exon 11 (rs41303343; 27125_27126insT). This insertion causes the formation of premature termination codons and immature, non-functional proteins (J. Lamba et al., 2012). Like CYP3A5*6, CYP3A5*7 is also comparatively frequent in Black Population (5-12%) and comparatively rare in Asian and American populations (H.-G. Xie et al., 2004). CYP3A5*8 is a substitution at 3699C>T, rs55817950 in exon 2, triggering an amino acid shift at codon 28 and non-functional protein formation (S.-J. Lee et al., 2003) CYP3A5*9 results from SNP at 19386G>A, rs28383479 in exon 10 triggering amino acid shift and non-functional protein (H.-G. Xie et al., 2004) CYP3A5*10 shows a point mutation in exon 12 at 29753T>C, rs41279854 resulting in an amino acid change (Oleson et al., 2010) 1.5.2.6. Phenotypes of CYP3A5 CYP3A5 is important in drug metabolism and has about 25 allelic variants. When genetic polymorphism is implicated in drug metabolism, individuals are categorized as normal or extensive poor, intermediate, and ultra-rapid metabolizers according to the phenotype of their expression polymorphism. The phenotype of CYP3A5 is measured by comparing the polymorphism with drug metabolite concentration in blood serum and urine. The phenotypic evaluation was done for CYP3A5 polymorphism in different studies. Extensive Metabolizer (EM) CYP3A5*1/*1 variant is an extensive or normal metabolizer, where both alleles are functional and yield full-length CYP3A5 protein. Individuals with the CYP3A5*1/*1 genotype were observed to have higher Tacrolimus drug clearance than any other genotype (Passey et al., 2011). Intermediate Metabolizer (IM) Intermediate metabolizer phenotype refers to the heterozygous CYP3A5 genotype, where one allele is functional, i.e., CYP3A5*1, and the other allele is non-functional, e.g., CYP3A5*3, *6, *7 (Ingelman-Sundberg et al., 2020). Poor Metabolizer (PM) When both alleles are non-functional and create truncated or null protein, such phenotype is called Poor Metabolizer (PM) phenotype. Allelic combinations for PM are CYP3A5*2/CYP3A5*2-*10, which all show very reduced or no enzymatic activity (Floyd et al.,

2003). Among them, CYP3A5*3 non-functional allele is very common. 1.5.2.7. Role of CYP3A5 in Tamoxifen Metabolism Among all the CYP enzymes, CYP3A4/5 and CYP2D6 play prominent roles in Tamoxifen metabolism. CYP3A5 is significant in tamoxifen conversion to its primary metabolite, N-desmethyl tamoxifen, and in the metabolism of 4-OH TAM to a secondary metabolite, endoxifen (Charoenchokthavee et al., 2016). Polymorphism in the CYP3A5 genotype accounts for variability in concentrations of Tamoxifen metabolites individually and inter-ethnically, thus affecting drug response (Charoenchokthavee et al., 2017). Only CYP3A5*1 is a functional allele, and the individual possessing both alleles is a normal or extensive metabolizer of tamoxifen. All other CYP3A5 allele variants, like CYP3A5*3 and *6, are non-functional and refer to poor metabolizer phenotype (J. Lamba et al., 2012). 1.6. CYP2D Subfamily The subfamily of cytochrome P450 called CYP2D comprises three genes: an active or functional gene, CYP2D61, and two pseudogenes, CYP2D7P and CYP2D8P (Yasukochi et al., 2011) 1.6. CYP2D Subfamily The subfamily of cytochrome P450 called CYP2D comprises three genes: an active or functional gene, CYP2D61, and two pseudogenes, CYP2D7P and CYP2D8P (Yasukochi et al., 2011). 1.6.1. CYP2D6 It is present on the long arm of chromosome 22 in band 13 and sub-band 1 (González et al., 2008)). Among all other liver enzymes, the percentage of CYP2D6 is 1-4%. This enzyme hydrolyzes about 25% of drugs, making it the most well-known (Owen et al., 2009). The gene comprises 9 exons, traversing almost 4400 nucleotides (Yang et al., 2017). CYP2D6 is an enzyme consisting of 497 amino acids or is a CYP2D family member of the superfamily cytochrome P450 for which the highly polymorphic gene called CYP2D6 is positioned on the q arm of chromosome 22 at position 13.1 in a region of 45kb near to the two pseudogenes (CYP2D7P and CYP2D8P) having similar nucleotide sequences to that of CYP2D6. This gene consists of 12 exons in the liver, central nervous system, and small intestine (Kimura et al., 1989; Zanger et al., 2004) It is among the widely studied CYPs due to its clinical importance. CYP2D6 has elaborated in the metabolism and removal of about 25% drugs presently available in clinics and phase 1 reactions of xenobiotics. CYP2D6 is also known as xenobiotic or microsomal monooxygenase and debrisoquine 4-hydroxylase. Moreover, it can also be represented as CYP2D, CYD6, CYP2DL1, CYP2D7P2, CYP2D7BP, CYP2D7AP, P450-DB1, P450C2D, CYP2D8P2 and CYP11D6 (Gopisankar, 2017). 1.6.1.1. Polymorphism and Phenotypes of CYP2D6 CYPs have 18 families, 43 subfamilies, 57 active, and 58 pseudogenes in humans. The 18 families are represented as CYP1, CYP2, 3, 4, 5, 7, 8, 11, 17, 19, 20, 21, 24, 26, 27, 39, 46, and 51, out of which 2, 3 and 4 have more genes than remaining. The 57 active genes are classified on a functional basis into two types denoted by D, and B-types called Detoxification type (35 out of 57 genes belong to this type) and Biosynthesis type (22 genes lie in this category), having more conserved sequences than D-type. Among 18 families, only four, 1, 2, 3, and 4 belong to D and the remaining to B-type (Katsumura et al., 2014; Song et al., 2015). The vastly polymorphic genes 2D6, 2C9, and 2C19 together perform 40% of the oxidative metabolism of the drug (Sadee, 2011). Until now, highly polymorphic 2D6 genes possess >100 variants comprising duplications, SNPs, frameshift mutations, or deletions/insertions of the gene (S.-F. Zhou, 2009). These variants have different frequencies and influence drug response in different groups. Alleles are classified into 4 types on the rate of metabolism of 2D6 substrates basis termed as extensive metabolizer or EM alleles, poor metabolizer (PM) or null alleles, intermediate metabolizer or IM alleles, and ultrarapid metabolizer or UM alleles (Bradford, 2002; Gaedigk et al., 2007; Ingelman-Sundberg, 2005). 2D6 is the only CYP with copy number variants (CNVs) represented by CYP2D6*(variant) XN reported for, while XN is for no copies of the gene (Lam, 2019). As a result of 2D6 polymorphism, there exist four phenotypes differing from each other CYP2D6 in patients with aripiprazole-related extrapyramidal symptoms: a case–control study", Personalized Medicine, 07/2008">based on the number of active alleles as individuals with PM phenotype have no active 2D6 alleles but rather possess both inactive alleles and have either no production of 2D6 enzyme or kind of 2D6 enzyme produced with no activity. In contrast, those having either one wild type and other inactive allele or two active alleles with decreased function or an allele with decreased activity along with inactive allele are IMs, and EMs have two wild type alleles. UMs contain multiple copies of the 2D6 gene due to duplication or multiplication of the gene (Gaedigk et al., 1991). Table 1.5 shows the 2D6 classification ((Gopisankar, 2017; Lam, 2019). Table 1.5: Classification of CYP2D6 Alleles Types CYP2D6 alleles Names Null/PM alleles *3 *4 *5 *6 *7 *8 *11 *12 *13 *14 *15 *16 *18 *19 *20 *21 *38 *40 *42 *44 *48 *56 *62 *68 *92 *10 *101 Partial function/IM alleles *10 *14 *17 *18 *36 *41 *47 *49 *50 *51 *52 *54 *55 *57 *59 *69 *72 Normal function/EM alleles *2A, *17 x 2, *27, *35, *39, *41 x 2, *48 Increased function/UM alleles *1 x N > 2, *2 x N > 2 1.6.1.2. Prevalence of CYP2D6 Phenotypes The frequency of the 2D6 phenotypes gene vastly differs concerning different racial groups. At the same time, on a broad spectrum, a larger proportion of the population (nearly 72-80%) consists of EM phenotype, and the remaining is composed of nearly 1-20% PM phenotype along with UM or increased function phenotype by only 1-10% proportion. EM phenotype is more common than PM and UM phenotypes in the general population, both of which differ in various ethnic groups by their frequency (Dean, 2012). According to a study by Alva´n et al., 1990, 5-10% of Caucasians have PM phenotype, 3-5% have UM phenotype, while a larger proportion of Caucasians with EM phenotype and 10-17% are IMs. On the other hand, PM and UM phenotypes were also observed in the populations like PM up to 19% in African Americans, about 1% in Chinese and 0.6% South Indians, and the UM phenotype in black Ethiopians up to 16% while up to 2% in Swedish Caucasians (Naveen et al., 2006). In short, UM phenotype is commonly observed in Oceania and North America, the PM phenotype mostly in Europe, and the IM phenotype in Asia (Gopisankar, 2017). Table 1.6: Different variants of CYP2D6 with their characteristics

CYP2D6 Alleles Allele Designation Enzyme Activity *1, *2, *33, *35 Normal (C, C) Normal *3-*8, *11-*16, *18-*21, *36, *38, *40, *42, *44, *56, *62. Null (T, T) Negligible *9, *10, *17, *29, *41, *59 Reduced activity (C, T) Decreased Duplicated Alleles Allele multiplication Enzyme activity *1xN, *2xN, *35xN Normal (C, C) Increased *10xN, *17xN, *29xN, 41xN Reduced function (C, T) Decreased *4xN, *6xN, *36xN Null alleles (T, T) Negligible A major biological factor in the CYP2D6 polymorphism is ethnicity. Previous studies reported that the CYP2D6*10 allelic variant is common in almost 50% of the Asian population, while in the Caucasian population, it is less prevalent (3%) (Chin et al., 2016; Yin et al., 2012). In CYP2D6*10, the enzyme's function is greatly reduced, as is the concentration of active metabolites of Tamoxifen in the blood ((Areepium et al., 2013; Lan et al., 2018). 1.6.1.3. CYP2D6*6 and Breast Cancer CYP2D6*6 is one of the PM or defective alleles of a gene (2D6) that codes for the 2D6*6 enzyme with no function and so has poorer or no contribution in tamoxifen metabolism in case of breast cancer. It is mostly found in European Caucasians and two other PM alleles, such as 2D6*3 and 2D6*4. 2D6*6 is identified by deleting the single nucleotide T in the third exon at position 1707 (1707delT) near the deletion site, such as CAG.TGG.GTG, a stop codon represented by red (CAG.GGG.TGA), is formed due to the deletion of T located at the 1707 nucleotide position (Bradford, 2002) . Subtypes of 2D6*6 are given in the following Table 1.7) (Rasmussen et al., 2011). Table 1.7: Subtypes of CYP2D6*6 Variant Subtype Similarity Difference (Change in Nucleotide) 2D6*6A 1707delT 2D6*6B 1707delT Having G>A at position 1976 2D6*6C 1707delT G>A at 1976 and G>C at 4180 2D6*6D 1707delT G>A at position 3288 Novel Variant 1707delT A>G at 1749, CT>AG at 1754-1755 and G>A at 1976 Table 1.8: Distribution of different CYP2D6 allelic variants in different populations Population Most recurrent alleles Africans and African Americans CYP2D6*5/*17a Alaska Natives CYP2D6*1b American Indians CYP2D6*1b Caucasians CYP2D6*4c Chinese CYP2D6*10a,d Malaysian Malay CYP2D6*10a Malaysian Indians CYP2D6*4/*10a (a: (Chin et al., 2016) b: (B. A. Khan et al., 2018) c: (Moyer et al., 2011) d: (Lan et al., 2018). 1.6.1.4. Role of CYP2D6 Genetic Polymorphism in Tamoxifen Metabolism and Breast Cancer Various studies have been done to find the correlation between CYP2D6 polymorphism and the metabolism of TAM. CYP2D6*48and CYP2D6*10 are the most common CYP2D6 allelic variants, and their prevalence was found to be associated with the prevalence of BC (Thota et al., 2018). In other studies, it was shown that CYP2D6 polymorphism (most commonly *5 and *10) has an impact on plasma endoxifen level (active metabolite) and TAM/endo metabolic ratio (B. A. Khan et al., 2018; Lim et al., 2011). Other studies demonstrated that CYP2D6*10/*10 (homozygous) and other CYP2D6 heterozygous null alleles are associated with a low endoxifen level, corresponding to lower TAM hydroxylation. Thus, ethnic groups carrying PM have an improved risk of BC relapse (Motamedi et al., 2012; Teh et al., 2012). Zembutsu et al., in 2017, carried out a study on this topic by identifying Ki-67 expression as a clinical response to TAM therapy. He found that patients having a wild-type genotype (or a minimum of one wild-type allele) underwent higher Ki-67 expression than homozygous mutant alleles (Zembutsu et al., 2017). Table 1.9: CYP2D6 polymorphism role in breast cancer and therapeutic recommendations accordingly CYP2D6 Metabolizer Status Activity Score Plasma Endoxifen Concentration/Implications Therapeutic Recommendations Ultrarapid Normal >2.0 1.5-2.0 Normal therapeutic Endoxifen level Strong recommendation to start treatment with 20mg TAM/day (Standard dose) and avoid any CYP2D inhibitor.a Intermediate 0.5-1.0 Lower Endo level/ increased Risk of BC relapse Post-menopausal women are moderately recommended to take estrogen inhibitors (AI), while premenopausal women should carry ovarian function suppression treatment.b Another option is to use a high dose of 40mg TAM/day (FDA approved)c if AI use is contraindicated. Poor 0 Very low to none Endo Conc/ higher chances of BC recurrence. AI therapy and ovarian function suppression therapy are strongly recommended for premenopausal women, but AI alone is needed in the case of postmenopausal women.b If AI use is contraindicated, TAM (40mg/day)c can be used, but it is noted that this concentration increases but cannot normalize endo concentration.c a: (Horn et al., 2017) b:(Group, 2015); (Pagani et al., 2014) c: (Hertz et al., 2016) Chapter 2 2. Materials and Methods 2.1. Study Population The sample size was assessed with the formula provided {n = Zα/22pq / (MOE)2} using the breast cancer frequency from literature as 12–15%. Therefore, applying an estimated population size of 15% with a 5% error margin, the sample size was 195 (S. S. Malik et al., 2020), where Zα/2 is a statistical constant, p is prevalence, and MOE is the error margin relative precision. After taking patients' and controls' written informed consent, sampling was carried out. This study enrolled 430 subjects (425 female and five male patients). Clinically diagnosed ER-positive breast cancer9patients at any disease stage and taking tamoxifen as adjuvant therapy with a day-to-day dosage of 20 mg for at least three months prior to sampling were included in this study. Demographic characteristics, including age, race, weight, marital status, family history, and smoking, were compiled using a specially designed questionnaire and are shown in Table 2.1. In addition, we recruited patients' reports and hospital records or clinical information such as surgery, chemotherapy, radiotherapy, tumor size, and cancer stage. Patients with liver, kidney, heart, or neurological disorders or those diagnosed with diabetes mellitus or on any other medication except tamoxifen for the last seven days were excluded from the study. To compare the genotyping results with the control population, 410 age and gender-matched healthy individuals from the same cities and socioeconomic backgrounds were also enrolled and are shown in Table 2.2. Study I Investigated the allelic frequency of CYP2C9*27and CYP2C9*3-variants and their association with tamoxifen and metabolites' metabolism among ER-positive breast cancer patients. Study II Genotypic allelic frequencies of CYP2C19*2, CYP2C19*3,7and CYP2C19*17 were determined, and their impact on the metabolism of tamoxifen and its metabolites among Pakistani ER Breast Cancer

Patients. Study III Impact of CYP3A4 polymorphism on tamoxifen6metabolism in the ER positive breast cancer patients. Study IV Role of CYP3A5 variants was determined in tamoxifen7metabolism in ER- Positive breast7cancer patients. Study V In vitro simulation of tamoxifen therapy in breast cancer7patients with distinct CYP2C19 genotypes. Table 2.1. Demographic Characters Percentage (%) Percentage and frequency of demographic characters of ER Positive BC patients. Age 20-35 15.11 36-50 40.69 51-65 32.55 Demographic Characte6r6s-80 Percentage11(.b) Ethnic Groups Punjabi 57.2 Saraiki 2.55 Pushtoon 16.04 Sindhi 10.46 Kashmiri 8.6 Hazarvi 1.16 Hindkoh 3.95 Marital Married 91.16 Status Un-married 4.41 Divorced 2.79 Widow 1.62 Weight Under-weight 24.18 Normal weight 52.32 Obese 23.48 Educational Status Illiterate 82.79 Primary 1.62 Middle 3.02 Matric 6.04 Inter-mediate 2.55 Graduate 3.95 Life Style House Wives 92.7 Professional 7.2 Family History Sporadic 80 Familial 20 Smoking non-smokers 92.5 smokers 7.44 Stage Of Breast Stage 1 5.34 Cancer Stage 2 50.93 Stage 3 31.86 Stage 4 11.86 Menopausal Pre-menopause 49.3 Status Peri-menopause 10.01 Post-menopause 40.69 Table 2.2. Percentage and frequency of demographic characters of Healthy individuals. Age 20-35 17.07 36-50 52.19 51-65 26.09 66-80 4.63 Ethnic Groups Brahui 19.51% Kashmiri 12.20% Pashtoon 19.02% Punjabi 17.07% Saraiki 15.12% Sindhi 17.07% Marital Status Married 64.39 Un-married 30.24 Divorced 4.63 Widow 0.73 Weight Under-weight 10.48 Normal weight 78.78 Obese 10.73 Educational Status Illiterate 36.09 Primary 11.21 Middle 20.97 Matric 17.07 Inter-mediate 6.09 Graduate 8.533 Life Style House Wives 65.83 Professional 34.14 2.2. Ethical Approval The Bioethical Committee6(BEC) of Quaid-i-Azam University Islamabad accepted the current study, as shown by protocol number BEC-FBS-QAU-40. Samples were collected from Sir Ganga Ram Hospital (SGRH), Lahore, Pakistan; Institute of8Radiotherapy and Nuclear Medicine, Peshawar; Centre for6Nuclear Medicine and Radiotherapy8(CENAR), Quetta; and Nuclear Oncology Research Institute (NORI), Islamabad, Pakistan. 2.2. Sample Collection Blood and Plasma samples of ER-positive breast cancer patients taking tamoxifen monotherapy were collected in vacutainers Syringe (BD 0.6mm × 25mm 23G × 1, Becton Dickinson, Pakistan). The sampling criteria were that the patient had undergone surgery, chemotherapy, and radiotherapy before treatment with adjuvant tamoxifen taken as monotherapy for at least 3 months at 20 mg daily. Samples were taken 48 h after the last dosage taken by the patients and stored at 4 ºC till DNA and Plasma extraction. An equal number of healthy individuals were enrolled in this study as a control, and 3 mL of blood was collected in a vacationers Syringe (BD 0.6mm × 25mm 23G × 1, Becton Dickinson, Pakistan). The participants filled out the consent form and were informed about the study. 2.2.1. DNA Extraction from Whole Blood DNA was extracted by the procedure described by Sambrook et al., 1989. The blood sample (5 mL) was vortexed (VWR Scientific; vortex Genie 2TM) and transferred to the labeled 5 mL falcon tube (50 mL polypropylene conical tube 30 × 115, BD Franklin Lakes, NJ. USA). Lysis buffer (3 times the blood volume) was added to the blood sample into a falcon tube and placed on ice for 30 min to assist the red blood cells lysis. After incubation, the mixture was centrifuged (MSe: Mistrex 3000i, centrifuge) at 12000rpm for-10 min at 4 ºC. The supernatant was discarded, and theepellet was resuspended by tapping the tube. Again 10 mL of lysis buffer was put into the sample and centrifuged at 1200 rpm (MSe: Mistrex 3000i, centrifuge) at 4 ºC for 10 min. The supernatant was eliminated, and the pellet was resuspended correctly. Briefly,4.75 mL of STE buffer was put into the pellet, adding 10% SDS while vortexed (VWR Scientific; vortex Genie 2TM). Briefly,10 uL of Proteinase K (20 mg/mL, broad-spectrum serine proteinase) was added to the sample and incubated in a water bath at 55 ºC overnight. On the following day, to get rid of proteins, DNA was extracted with equal volume (5 mL) of equilibrated phenol (8.0 pH) and shaken for 10 min by inverting 50 mL falcon tube (30 × 115, BD Franklin Lakes, NJ. USA) and put on ice for 10 min. After centrifugation at 3200 rpm, t h e aqueous layer was separated by cut tips to another labeled 15 mL falcon tube (8400 × G Maximum RCF, Biologix. USA). To this aqueous solution, 5 mL of chilled Chloroform-Isoamyl7 alcohol (24:1) was added, mixed for 10 min, and placed on ice for 10 min. The sample was centrifuged (MSe: Mistrex 3000i, centrifuge) at 3200 rpm for530 min at 4 ºC, and the aqueous7 layer was separated by cut tips into labeled 15 mL falcon tube (8400 × G Maximum RCF, Biologix. USA). To this aqueous layer, 10 uL of RNase (10 mg/ml) was added a n d incubated in a shaker water bath (US Patent No. DES 288; 600) at 37 ºC for 2 h; optimum for enzyme action. After incubation, 250 uL of 10%8SDS and 10 uL of proteinase-K (20 mg/mL) were added and incubated in a shaker water bath at (US Patent No. DES 288; 600) 55ºC for 1 hour. After this, 5 mL of equilibrated Phenol (8.0 pH) was added to the sample, shaken for 10 min, placed on the ice for 10 min, and later centrifuged at 32008rpm for 30 min at 4 ºC. After centrifugation, the upper aqueous layer was separated into another labeled 15 mL falcon tube. To this aqueous layer, 5 mL of chilled chloroform-isoamyl alcohol7(24:1) was added, shaken for 10 min, placed on ice for 10 min, and afterwards centrifuged (MSe: Mistrex 3000i) at 3200 rpm for 306min at 4 ºC. Then upper aqueous layer was separated by cut tips into separate labeled 15 mL falcon tube (8400 × G Maximum RCF, Biologix. USA). Briefly, 500 uL of Ammonium7acetate and 3 mL of chilled isopropanol were added and mixed to this aqueous layer until DNA was precipitated into visible white threads by inverting the falcon tube many times and then placed overnight at -20 ºC. The sample was centrifuged at 3200 rpm for 60 min at 40ºC to deposit the precipitated DNA at the base of a 15 mL falcon tube (8400 × G Maximum RCF, Biologix. USA). The pellet was resuspended, and the supernatant was removed. For washing, 5 mL of 70% ethanol was added and centrifuged0 at 3200 rpm for 40 minnat 4 ºC. After centrifugation supernatant was discarded. Th e pellet was dried by inverting a falcon tube on tissue paper in a laminar flow hood for 3 h. When t h e pellet was completely dried, 300 uL of Tris-HCl (pH 8.0) was added and incubated at 55 ºC in a water bath

shaker. Tapping and incubation were repeated periodically in a day so that DNA dissolved efficiently. The optical density of t h e DNA sample was taken by Nanodrop (2000c; Thermo Scientific, USA) at 260 and 280 nm. Now DNA sample was shifted to a labeled Eppendorf and stored at 4 ºC. This method will take 2 days if step (xiii) is modified by keeping t h e sample for 15 min at 70 ºC. 2.2.2. Quantitative Analysis of Extracted DNA Using Nanodrop technology (2000c; Thermo Scientific, USA), the DNA concentration was determined. The extracted DNA's absorbance was measured at both 260 and 280 nm. The O.D. of genomic DNA less than 2 and around 1.8 are considered good quality. The DNA dilutions of 100 ng/µl were made for a polymerase chain reaction. The PCR result was tested on a 2% agarose gel after DNA amplification to confirm the patients' genotype. Theoretically, the phenotype was deduced from the genotype. 2.2.3. Amplified product Analysis by Gel Electrophoresis The amplified product was checked by resolving on 2% agarose gel. By suspending 7 g of agarose into 350 mL of 0.5 TAE buffer, 2% agarose gel was prepared. Then the solution was heated in a microwave oven until (approx. 7-10 min) a clear solution was obtained, then 59µL of Ethidium Bromide was added to this solution. The solution was kept for cooling down in a water-bath at 55 ºC for 15 min. The solution was then poured into a gel casting tray, three combs of 40 wells were adjusted, and the gel was left to solidify. After polymerizing the gel, the combs were removed delicately without disturbing the wells. The gel was cut down according to the required wells and transferred to a gel tank (Maxi cell: EC 360M, USA) containing 0.5 TAE buffer. After amplification, 5 µL of the Orange G dye containing 0.125% Orange G, 20% Ficol, and 100 Mm EDTA was put into each sample. The samples were then loaded on the 2% agarose gel. The 100 bp ladder (Fermentas cat# SM0403) was also stuffed into a well to locate the fragment size of the PCR product. The electrophoresis was conducted at 120 volts for thirty 35 min. The gel was then placed on the Gel Doc system (UVITEC, Cambridge UK) to visualize gel bands of PCR product, and an image was taken. 2.2.4. Reagent Preparation for DNA Isolation and Gel Electrophoresis a. Cell-lysis buffer (1L) Briefly, 1 g potassium bicarbonate and 8.29 g ammonium chloride were dissolved in deionized H2O. A 200 µL of 0.5M EDTA was added, and pH was adjusted by combining 1M HCL and 1M NaOH. The solution was centrifuged at 2500 rpm for 10 min, autoclaved, and stored at room temperature. b. 1M Tris (1L, pH 8.0) Approximately 121.1g of Tris Base was added to 800 mL of water, and the pH was corrected to 8.0 by adding concentrated HCl. The volume was brought to 1L by adding deionized water. The solution was filtered through 0.4 µM filter paper and stored at room temperature. c. 0.5M EDTA (1L, pH 8.0) A total of 186.15g of EDTA was mixed in 700 mL of deionized water, and pH 8.0 was maintained by adding 4M NaOH. The final volume of 1L was made by adding more deionized water autoclaved and saved at room temperature. d. TE (Tris EDTA) (1L, pH 8.0) Briefly, 33.3 mL (100 mM) of 3M NaCl was mixed with 50 mL (50 mM) of 1M Tris (pH 8.0) and 2 mL (1 mL)0of 0.56M EDTA8(pH 8.0). The ultimate volume of 1L was achieved by adding deionized water and storing it at room temperature. e. 10% SDS Solution (250 mL) A total of 25 g of SDS was carefully weighed at a weighing balance, and 150 mL of deionized water was added. The solution was allowed to stir at a low speed to avoid the formation of foam. The volume was up to 250 mL, filtered through filtration assembly, and stored at room temperature. f. Chloroform-Isoamyl alcohol8(24:1, 500 mL) A total of 480 mL of Chloroform8and 20 mL of Isoamyl-alcohol were mixed at the ratio of 24:1 and stored at 40 ºC. g. Iso Propanol Isopropanol was transferred from stock in another bottle, wrapped with aluminum foil, and placed in the refrigerator for future use. h. Equilibrated Phenol Usually, distilled phenol is kept at -20°C. When needed, it is melted in a 55 °C water bath (The phenol's melting temperature is 42 °C). To 1 kg melted phenol, 1g of 8-hydroxyquinoline (0.1%) and an equal volume of 1 M Tris were added and mixed by shaking in a fume hood for 2-3 min. The bottle was left in the hood for shaking on a shaker for 30 min. The bottle was then kept stationary in the hood for a few minutes to separate the Tris and the phenol layer; the upper Tris layer was removed. 0.1M Tris was added, and the bottle was stirred for 2-3 min, left still for a few minutes, and removed from the upper Tris layer. The procedure was repeated until the pH was stable at 8.0. Finally, 100 mL of 0.1M Tris-HCl containing 0.2% β- mercaptoethanol (2 mL) was included and stored at 4 °C. i. 70% Ethanol (chilled, 500 mL) 500 ml of 70% ethanol was prepared by mixing 350ml of absolute ethanol with 150 mL deionized water and stored at -20 °C. j. Ammonium Acetate (1L) By suspending 770 g of ammonium8acetate in 800 mL of deionized water, a 1 L solution with a concentration of 10 M was produced. The total volume of 1L was made up by adding dH2O and sterilized by filtration through a 0.227µm filter. The solution was in a sealed bottle in a freezer at 4°C. k. 0.5X TAE buffer A total of 5 L 0f 10X TAE buffer was made by adding 2429g Tris base, 57.18glacial acetic acid, and 100 mL of EDTA, and a final volume of up to 5 L was made up by adding dH2O. This 10XTAE buffer was diluted to 0.5XTAE buffer, which was used for the gel electrophoresis. l. Loading dye A total of 25 mg8bromophenol blue, 25 mg xylene cyanol, and 49g sucrose were into deionized water, making a total volume of 10 mL, and used as tracking dye. Bromophenol blue always moves forward to xylene cyanol as its size is smaller. m. Ethidium Bromide Dissolve 400 mg into 20 mL of deionized water, and 5 µL was used to locate DNA bands on the gel; EtBr molecules intercalate with DNA and glow under UV rays. 2.3. Primer Design Reference SNP-4244285, SNP-12248560, SNP-1799853, and SNP-1057910 sequences were taken from the National Centre of Biotechnology Information8(NCBI). Primers were created using the primer designing software Primer3 (Rozen and Skaletsky, 2000). The sequence of primers used is mentioned in8Table 2.3. T a b l e 2 . 3 : P r i m e r s f o r C Y P 2 C 9 (* 2 , * 3) , C Y P 2 C 1 9 (* 2 , * 3 & * 1 7) , C Y P 2 D 6 (* 6 , * 1 0) , C Y P 3 A 4 (* 2 2) , C Y P 3 A 5 (* 3 , * 6) G e n o t y p i n g . No SNP ID mer ID Sequence (5'-3') Lengt h Tm Size (bp) Type of PCR Product 2C9*2-F AATAGTAACTTCGTTTGCTGTTATCTC 27 62.2 C-allele 2C9*2R-C GGGCTTCCTCTTGAACACG

19 59.9 493 specific 1 rs1799853 2C9*2F-T GGAAGAGGAGCATTGAGGACT 21 61.3 T-allele 2C9*2-R CAGTAGAGAAGATAGTAGTCCAGTAAGGT 29 67.6 127 specific 2C9*3-F GCCATTTTTCTCCTTTTCCAT 21 55.5 C-allele 2C9*3 R-C TGGTGGGGAGAAGGTCAAG 19 59.9 177 specific 2 rs1057910 2C9*3F-A GCACGAGGTCCAGAGATACA 20 60.5 A-allele 2C9*3-R GGAGAACACACACTGCCAGA 20 60.5 295 specific 2C19*2-F CAGAGCTTGGCAATATTGTATC 22 57.1ºC 291 Control 2C19*2-R ATACGCAAGCAGTCACATAAC 21 57.4ºC 3 rs42442852C19*2-A GTAATTTGTTATGGGTTCCT 20 52.3ºC A-allele 2C19*2-F CAGAGCTTGGCAATATTGTATC 22 57.1ºC 169 specific 2C19*2-G ACTATCATTGATTATTTCCCG 21 55.6ºC 202 G-allele 2C19*2-R ATACGCAAGCAGTCACATAAC 21 57.4ºC specific rs4986893 2C19*3R AACTTGGCCTTACCTGGATC 20 58.4 ºC C19*3F2 TATTATTATCTGTTAACAAATATGA 25 52.7ºC 253 Control 2C19*3F1 TATTATTATCTGTTAACAAATATG 24 51.6ºC 253 T-Allele 2C19*3R(T) AACTTGGCCTTACCTGGATT 20 56.4ºC fragment 2C19*17-F AAGAAGCCTTAGTTTCTCAAG 21 55.5 2C19*17-R AAACACCTTTACCATTTAACCC 22 56.6 507 Control 5 rs12248560 2C19*17-T TGTCTTCTGTTCTCAAAGTA 20 52.3 218 T-allele 2C19*17-R AAACACCTTTACCATTTAACCC 22 56.6 specific 2C19*17-F AAGAAGCCTTAGTTTCTCAAG 21 55.5 C-allele 2C19*17-C ATTATCTCTTACATCAGAGATG 22 54.7 330 specific CYP3A5*6 NF(G) CTTTGTGGAGAGCACTAAG 19 55.2°C 150 G-Allele Specific 6 rs10264272 CYP3A5*6 R1 AGTGGATGAATTATACGATATGT 23 55.7°C CYP3A5*6 MF(A) CTTTGTGGAGAGCACTAAA 19 53.0°C 150 A-Allele Specific CYP3A5*6 NF(G) CTTTGTGGAGAGCACTAAG 19 55.2°C CYP3A5*3 F1 CAGATGACACAGCTCTAGATGTCC 24 65.3 °C 285 T-Allele 7 rs776746 CYP3A5*3 NR(T) ATATGTGGTCCAAACAGGGAAGAGATAT 28 65.7 °C Specific CYP3A5*3 F2 CAGATGACACAGCTCTAGATGTCC 24 65.3 °C 285 C-Allele CYP3A5*3 F1 CAGATGACACAGCTCTAGATGTCC 24 65.3 °C Specific 3A4*22 F TTGTCTGATAGTGGGTCTCTGTCTT 25 64.2 232 Control 3A4*22 R CTTCCTCTATGCATGCAACAGG 22 62.1 8 3A4*22 F TTGTCTGATAGTGGGTCTCTGTCTT 25 64.2 107 C Allele rs35599367 3A4*22 C ATGCAGCTGGCCCTACG 17 57.2 Fragment 3A4*22 T TGAATCTCCATCACACCCAGT 21 59.4 3A4*22 R CTTCCTCTATGCATGCAACAGG 22 62.1 162 T Allele Fragment rs5030655 Cyp2D6*6F TCCCAGCTGGAATCCGGTGTCG 22 59.9 Cyp2D6*6R GGAGCTCGCCCTGCAGAGACTCC 24 61.1 9 Cyp2D6*6F TCCTCGGTCACCCA 14 56.3 Cyp2D6*6F Tmut GTCGCTGGAGCAGG 14 54.8 rs1065852 2D6*10F TCAACACAGCAGGTTCA 17 50oC 433 Control 10 2D6*10R CTGTGGTTTCACCCACC 17 54.8oC (followe d by RFLP) 2.4. Genotyping 2.4.1. Genotyping for CYP2D6 variants. Amplification Refractory7Mutation System (ARMS) Polymerase Chain Reaction6(PCR) was utilized to genotype CYP2D6*6 and CYP2D6*10 in breast cancer patients and unrelated healthy individuals via thermal cycler Bio-rad T100TM. For performing each PCR, 25 µL (final volume) of the reaction mixture for each sample was prepared by using 1X PCR buffer8(2.5 µL) (Fermentas), 1.5 mM7MgCl2, 0.2 mM dNTPs, 0.5 µM each of forward and reverse primer, 0.2 units of Taq DNA polymerase8(Fermentas) and DNA sample of 100 ng was used. Optimized conditions for amplification for CYP2D6*6 and CYP2D6*10 allele amplification are shown in Tables 2.4 and 2.5. Table 2.4: PCR8Conditions CYP2D6*6 amplification Steps Sub-Cycles Conditions Temperature Time PCR Cycles Initial9Denaturation 94 oC 10 min PCR Cycles Denaturation Primer annealing Primer extension 94 oC 63.0 oC 72 oC 30 s 30 s 1 min 42 Cycles Final Extension 72 oC 7 min Hold 4 oC ∞ Table 2.5: PCR conditions for CYP2D6*10 amplification Steps Sub-Cycles Conditions Temperature Time PCR Cycles Initial Denaturation 95 oC 5 min 35 PCR Cycles Denaturation Primer annealing Primer extension 95 oC 52.8 oC 72 oC 1 min 1 min 2 min Cycles Final Extension 72 oC 7 min Hold 4 oC ∞ The amplified7products were detected on 2% agarose gels for genotyping. 2.4.2. Genotyping for CYP2C9 variants Sequence-specific polymerase chain9reaction (PCR) and refragment length polymorphism7(RFLP) was utilized to genotype CYP2C9*2 and CYP2C9*3 in breast cancer patients and unrelated healthy individual via thermal cycler Bio-Rad T100TM. For performing PCR, 25 µL (final volume) of the reaction mixture for each sample was prepared by using 1X PCR buffer7(2.5 µL) (Fermentas), 0.2 mM dNTPs,71.5 mM MgCl2, 0.5 µM each of forward8and reverse primer, 0 .29units of Taq DNA polymerase (Fermentas) and 1009ng of DNA sample was used. The optimized conditions for amplification for CYP2D6*6 and CYP2D6*10 allele amplification are shown in Tables 2.6 and 2.7. Table 2.6: Optimized conditions for CYP2C9*2 amplification Steps Sub-Cycles Conditions Temperature Time PCR Cycles Initial Denaturation 95 oC 5 min 35 PCR Cycles Denaturation Primer annealing Primer extension 95 oC 60 oC 72 oC 1 min 40 s 45 s Cycles Final Extension 72 oC 10 min Hold 4 oC ∞ The amplified products were detected on 2% agarose gels for genotyping. 2.4.2.1 Restriction Fragment Length6Polymorphism (RFLP) Selection of Enzyme The nucleotide position Arg144Cys was selected using the Ensemble tool based on reference site rs1799853. The mutation site was confirmed via Mutation Taster and dbSNP tool. Restriction enzyme against Arg144Cys was selected from a single base pair cutter using NEB cutter V2.0. The restriction enzyme AvaII having restriction site GGACC, was selected. a. Digestion of the CYP2C9*2 by Ava II The CYP2C9*28PCR 396 bp product was digested with 800 U Ava II. 32µl RFLP reaction mixture for each sample was prepared, which consisted of 1 µl restriction enzyme Ava II (10 U/µL), 2 µL8of 10X Buffer R9(10 mM Tris-HCli(pH 8.5),1100 mM KCl,10mM MgCI2 and BSA (0.l mg/mL)), 10 µL of PCR product and 187µL of nucleases free water. After adding the reaction mixture to the samples, they were centrifuged at 3000 rpm for 10 s and incubated at 37 °C for 16 h. The enzyme was inactivated by incubating the reaction7mixture at 65 °C for 20 mins. Following incubation, restriction enzyme digests were separated on a 2% agarose gel at 90-120 volts for 45 min. Patients 'genotype were analyzed based on the number and size of the band. Table 2.7: Optimized conditions for CYP2C9*3 amplification Steps Sub-Cycles Conditions Temperature Time PCR Cycles Initial Denaturation 95 oC 10 min 35 PCR Cycles Denaturation Primer annealing Primer extension

95 oC 53 oC 72 oC 1 min 1 min 45 s Cycles Final Extension 72 oC 10 min Hold 12 oC ∞ 2.4.2.2. Restriction Fragment8Length Polymorphism (RFLP) a. Selection of Enzyme KpnI The nucleotide sequence A1075C was selected using an ensemble tool via Genome assembly based on reference site rs1057910. The restriction enzyme KpnI having restriction site was 5 "GGTACC3" selected. b. Kpn I, restriction enzyme digestion, and PAGE of the CYP2C9*3 digests The 12 µL CYP2C9*38PCR product was digested with 10 U/µl Kpn1. The restriction enzyme cocktail comprised of 1 µL restriction enzyme Kpn1 (l0 U/ µl), 3.0), 2 µL of 10X Buffer, 15 µL DEPC treated water for each PCR product sample. The reactionnmixture was incubated at 378°C for 16 h. The digested products and size marker were separated by PAGE at 1408V for 3 h on the polyacrylamide (l0% T, 2.9□) gel and visualized. The patient's genotype was analyzed based on the band's size. RFLP product against rs1057910 for 1075A>C generate bands of various size. In case of two bands of size 85 and 20 bp, the subject was designated as a homozygous mutant (+/+), in the case of 3 bands of size 105 bp, 85 bp, and 20 bp subject was considered as heterozygous mutant(+/-) and band of 105 bp subject was regarded as normal (-/-). c. Polyacrylamide Gel Electrophoresis (PAGE) of restriction enzyme digests. The 105 bp CYP2C9*3 PCR7digests were evaluated by polyacrylamide7gel electrophoresis (PAGE). A loading buffer of 10 µL (70%8sucrose comprising 0.01 % BPB) was add up to each sample. Each was blended, centrifuged, and loaded onto an 8% PAGE gel and divided at room temperature on a GIBCOBRL Vertical Gel Electrophoresis Apparatus in 18X TBE buffer at 140 V for 3 h. A 30% acrylamide stock solution: methylene bisacrylamide, was prepared. 8% PAGE gels were formulated by adding 13.4 mL of 30%9polyacrylamide and 5 mL 10 x8TBE buffer, and the volume was raised to 50 mL by adding distilled H20 in a tiny beaker. A 400 µL aliquot of a 10% ammonium persulphate (APS) solution and 25 µL TEMED was included, and the solution was blended well. Combs were adjusted to create wells. At room temperature, the gels were let to polymerize for 20 min. The combs were separated, and the wells washed with lX TBE gels were transferred from the casting device to the GIBCOBRL Vertical Gel Electrophoresis Apparatus. After loading the samples onto the gels, they were operated at 140 V, 42 mA, and 6W. A 20 bp ladder was included in each electrophoretic run. The gels were taken from between the glass plates and immersed for 4 min in a 0.0001% EtBr7staining solution produced from a 10 mg/EtBr8stock solution. The bands were seen with ultraviolet (UV) light and photographed using an ultraviolet (UV) transilluminator (Biometra, Gottingen, Germany) Gel Documentation System. 2.4.3. Genotyping for CYP2C19 Variants Amplification Refractory8Mutation System (ARMS) Polymerase Chain Reaction (PCR) was utilized to genotype CYP2C19*2,8CYP2C19*3 and CYP2C19*17 in breast cancer patients and unrelated healthy individuals via thermal cycler Bio-Rad, T100TM. For PCR, 25 µL (final volume) of the reaction mixture was prepared for each sample using 1X PCR buffer7(2.5 µL) (Fermentas), 1 .57mM MgCl2, 0.2 mM dNTPs, 0.56µM of each forward and reverse primer, 0.27units of Taq DNA polymerase6(Fermentas) and2100 ng of a DNA sample. Optimized Conditions for amplification for CYP2D6*6 and CYP2D6*10 allele amplification are shown in Tables. 2.8, 2.9, and 2.10. Table 2.8: Optimized conditions for CYP2C19*2 amplification Steps Sub-Cycles Conditions Temperature Time PCR Cycles Initial Denaturation 95 oC 3 min 35 PCR Cycles Denaturation Primer annealing Primer extension 95 oC 53 oC 72 oC 1 min 40 s 45 s Cycles Final Extension 72 oC 10 min Hold 4 oC ∞ Table 2.9: Optimized conditions for CYP2C19*3 amplification Steps Sub-Cycles Conditions Temperature Time PCR Cycles Initial Denaturation 95 oC 5 min 35 PCR Cycles Denaturation Primer annealing Primer extension 95 oC 54 oC 72 oC 1 min 40 s 1 min Cycles Final Extension 72 oC 10 min Hold 4 oC ∞ Table 2.10: Optimized conditions for CYP2C19*17 amplification Steps Sub-Cycles Conditions Temperature Time PCR Cycles Initial Denaturation 95 oC 10 min 35 PCR Cycles Denaturation Primer annealing Primer extension 95 oC 53 oC 72 oC 1 min 1 min 45 s Cycles Final Extension 72 oC 10 min Hold 12 oC ∞ The amplified products were detected on 2% agarose gels for genotyping. 2.4.4. Genotyping for CYP3A4 Variants Amplification Refractory5Mutation System (ARMS) Polymerase Chain7Reaction (PCR) was utilized to genotype CYP3A4*22 in breast cancer patients and unrelated healthy individuals via thermal cycler Bio-Rad T100TM. For PCR, 25 µL (final volume) of the reaction mixture was prepared for each sample using 1X PCR buffer (2.5 µL) (Fermentas), 1.57mM MgCl2, 0.2 mM7dNTPs, 0.5 µM of each forward and reverse primer, 0 .27units of Taq DNA polymerase7(Fermentas) and 1006ng of a DNA sample. The optimized conditions for amplification for CYP2D6*6 and CYP2D6*10 allele amplification are shown in Table 2 .11. Table 2.11: Optimized conditions for CYP3A4*22 amplificationSteps Sub-Cycles Conditions Temperature Time PCR Cycles Initial Denaturation 94 oC 10 min 34 PCR Cycles Denaturation Primer annealing Primer extension 94 oC 63 oC 72 oC 1 min 1 min 1 min Cycles Final Extension 72 oC 5 min Hold 4 oC ∞ The amplified products were detected on 2% agarose gels for genotyping. 2.4.5. Genotyping for CYP3A5 Variants Allele-specific PCR (AS-PCR) was performed for genotyping and was utilized for genotyping CYP3A5*3 and CYP3A5*6 in breast cancer patients and unrelated healthy individuals by thermal cycler Bio-Rad T100TM. For PCR, 25 µL (final volume) of the reaction mixture was prepared for each sample using 1X PCR buffer (2.5 µL) (Fermentas), 1.5 mM MgCl2, 0.2 mM dNTPs, 0.5 µM of each forward and reverse primer, 0.2 units of Taq DNA polymerase (Fermentas) and 100 ng of a DNA sample. The optimized conditions for amplification for CYP2D6*6 and CYP2D6*10 allele amplification are shown in Tables 2.12 and 2.13. Table 2.12 Optimized conditions for CYP3A5*3 amplification Steps Sub-Cycles Conditions Temperature Time PCR Cycles Initial Denaturation 94 oC 5 min 34 Cycles Denaturation 94 oC 1 min PCR Cycles Primer N(A) annealing 63.5 oC 45 s Primer M(C) annealing 62.2 oC 45 s Primer extension 72 oC 1 min Final Extension 72 oC 5 min Hold 4 oC ∞ To determine the genotype of CYP3A5*3, two sets of PCR were performed. In the the first set of PCR, forward (F1) primer and wild-type allele "A" specific reverse

primer (R) were utilized to amplify the wild-type6allele fragment of 285 bps. The presence of wild-type allele genotype "AA" was confirmed by 2% agarose gel electrophoresis. In the second PCR, the same sample was used to check the presence of the mutant allele. In allele-specific PCR reaction, the control forward (F2) primer and "G" allele-specific primer R (G) were used to amplify the mutant allele fragment of 285 bps and confirmed by 2% agarose gel electrophoresis. Table 2.13: Optimized conditions for CYP3A4*22 amplification: Steps Sub-Cycles Conditions Temperature Time PCR Cycles Initial Denaturation 94 oC 5 min 34 PCR Cycles Primer annealing N(G) 53 oC 45 s Denaturation 94 oC 1 min Cycles Primer annealing M(A) 54 oC 45 s Primer extension 72 oC 1 min Final Extension 72 oC 5 min Hold 4 oC ∞ second PCR, the same sample was used to check the presence of the mutant allele. In allele-specific PCR reaction, the control forward (F2) primer and the "G" allele- specific primer R (G) were used to amplify the mutant allele fragment of 285 bps and confirmed by 2% agarose gel electrophoresis. 2.5. Tamoxifen and its metabolites quantification in plasma 2.5.1. High-Performance Liquid6Chromatography (HPLC) For determining the tamoxifen and its metabolites in the biosamples, High- Performance Liquid6Chromatography (HPLC) is the easily available and cost- effective approach (Antunes et al., 2012). Chemicals for HPLC The list of chemical reagents required for HPLC is given in Table 2.14. Table 2.14 List of chemicals for HPLC No. Chemicals 1. Tamoxifen (TAM) (Sigma) 2. Endoxifen (Sigma) 3. N-desmethyl tamoxifen (Sigma) 4. 4-hydroxytamoxifen 5. Tris ammonium methane (Sigma) Verapamil (Sigma) 6. Methanol 7. Phosphoric acid (85%) 8. Triethyl-ammonium phosphate buffer (pH=3.0) 9. Acetonitrile 10. N-propanol, n-hexane (60%) 11. HPLC water 2.5.2. Chromatographic conditions. To determine the association of CYPs in the metabolism of tamoxifen and its metabolites, we employed HPLC Agilent 1100 UV system for the plasma analysis of 430 ER-positive breast cancer patients at a 280nm wavelength. The separation was conducted on a CNW Athena C18 columnn(150 mm × 4.69 mm, particle diameter 5.0 um). The flow rate was adjusted at 1.0 mL min-1 until the end of the analysis. The column temperature was set at 30 ◦C. The overall run timeewas 16 min. The retention time was 2.05 min for verapamil (IS), 4.14 min for Endoxifen, 4.5 min for 4-OH- Tamoxifen, 11.13 min for N-DesM-Tam, and 13.7 min for tamoxifen Figure 2.1, and chromatogram of sample is shown in figure 2.2. Figure 2.1:7Chromatogram of standards: wavelength= 280 nm, the retention time of Verapamil (IS)= 2.05, endoxifen = 4.2, the retention time of 4-hydroxy tamoxifen= 4.5 min, the retention time of N-desmethyl tamoxifen = 11.0 and retention time of tamoxifen= 13.7 min. Figure 2.2: Chromatogram of sample: Wavelength = 280 nm, retention time of endoxifen = 4.3, retention time of 4-hydroxy tamoxifen= 4.6 min, retention time of N- desmethyl tamoxifen = 11.1 and retention time of tamoxifen= 13.3 min 2.5.3. Mobile Phase Buffer Preparation for HPLC 500µl of triethyl-ammonium phosphate buffer was diluted with 1000 mL of ultrapure water to formulate mobile phase buffer for HPLC. It was then filtered through a 0.2 µm cellulose acetate membrane, and its pH was adjusted to 3.3 by using triethyl- ammonium phosphate as a base while phosphoric acid as an acid. Before using the mobile phase buffer, it was sonicated for about 15 minutes. To prepare the mobile phase, 5 mM of triethyl-ammonium phosphate buffer6(pH = 3.3) and acetonitrile were mixed in a ratio of 45:55, respectively. A total of 95 mL9hexane and 5 mL8n- propanol were combined to create an extraction solvent (Antunes et al., 2013). 2.5.4. Solutions and Standards Preparation for HPLC Separate solutions for stock of Tam (tamoxifen),44-OH tamoxifen9(4-OH-Tam), N- desmethyl tamoxifen hydrochloride-(N-Des-Tam), andiN-desmethyl-4 hydroxytamoxifen (Endoxifen/Endo) were composed, at the concentration of 1mg/ml for Tam, the concentration of 1 mg/mL for 4-OH-Tam, 1 mg/mL for N-Des-Tam and 1mg /ml for Endo, by dissolving them in methanol. Verapamil (1 mg/mL) was employed as an internal standard (IS). Working solutions of Tam,84-OH-Tam,-N- Des-Tam, and Endo were prepared by combining standard solution and proper volume of methanol to gain the following concentration of Tam, 4-OH-Tam, N-Des- Tam, and Endo; 40 ng/mL,820 ng/mL,710 ng/mL, and 5 ng/mL respectively. Mobile phase buffer was composed by diluting 500 uL triethylammonium phosphate buffer into ultrapure water (1000 mL). The mobile phase buffer was filtered afterward by using a Millex Syringe-driven unit. 2.439g of Tris-hydroxymethyl9aminomethane was dissolved in ultrapure7water (100 mL) to prepare Tris buffer. Using 0.1M NaOH, the pH was properly adjusted to 10.0. A solvent for extraction was set up by combining 95 mL hexane with n-propanol (5 mL) (Antunes et al., 2013). 2.5.5. Plasma Sample6Preparation A volume of 2 mL of the patient's plasma sample was mixed in Tris buffer (0.7 mL) with pH 10.0 and 5.2 mL extraction solvent in a 15 mL falcon tube. The samples were gently mixed for 10 min anddcentrifuged at 2000 g for 10 min. The organic layer was shifted into another falcon tube, and 200 µL of phosphoric acid 0.1% (v/v) was added. After homogenization, it was again centrifuged at 4000 rpm for 15 min. A fresh tube was used to collect the liquid layer, and 20 µL of the aqueous layer was injected into Agilent HPLC 1100 system. The following formula was used for tamoxifen and8metabolites quantification in the plasma sample: Concentration of Sample = Concentration of Standard/Area of Standard × Purity of Standard/100 × Area of Sample 2.6. Cell Culture9Studies 2.6.1. Effects of9Estrogens, Tamoxifen and8Metabolites on Cell Proliferation We tried to investigate the effect of CYP2C19 EM (extensive metabolizers), Rapid Metabolizers and UM (Ultra Rapid Metabolizers) metabolites concentration on estrogens on cell proliferation. ER-positive8human breast cancer cell line MCF7 and T47D was employed in this study. All cells were attained from the American8Type Culture Collection9ATCC, Manassas, VA, USA) and were kept in phenol red8RPMI 1640 medium, comprising 10% FBS9(HyClone Laboratories , Logan, UT, USA), 2 mM glutamine,8penicillin at 1008units·mL-1, streptomycin8at 1008µg·mL-1, 1× non-essential9amino acids (all from Life Technologies, Grand Island, NY, USA), and bovine7insulin at 6 ng·mL-1 (Sigma-Aldrich,9St. Louis, MO, USA). All

cells9were cultured in T185 culture flasks5(Thermo Scientific,8Pittsburgh, PA, USA) and passaged9twice a week in 1:38ratio. All cultures were grown in75% CO2 at 737°C. Cells were8cultured in oestrogen-free medium8[phenol red-free RPMI 1640 media supplemented with 10 % charcoal7stripped FBS8(SFS)] for three days before the beginning of the8proliferation assay. On day60 of the experiment, cells were9seeded in oestrogen-free RPMI media comprising 10% SFS at a density of 10000 cells per well, respectively, in a 24-well cell8culture plates (Corning, Tewksbury,9MA, USA). After 24 h, cells were9treated with combinations of oestrogens, tamoxifen, and its metabolites in different CYP2C19 EM, RM and UM concentrations (Tables 2.15) set in estrogen-free RPMI. All treatments were accomplished in triplicate. The medium comprising the test compounds was replaced on days94 and 7, and the experiment was halted on day 8. Cells were splashed with cold9PBS (Life Technologies) at least twice and analyzed with a fluorescent DNA quantification8kit (Bio-Rad, Hercules,9CA, USA) according to the9manufacturer's instructions, and the samples were read9on a Mithras LB5408fluorometer/luminometer (Berthold Technologies ,8Oak Ridge, TN, USA) in black wall 96-well plates9(Thermo9Scientific). 2.6.2. Real Time8PCR MCF-79cells were cultured in9oestrogen-free medium for three days before9seeding and treatment.9Cells were seeded after9oestrogen deprivation in96-well cell culture plates (Corning) at a density of9300 000 cells per well. Cells were handled with test compounds for 488h. Total9RNA was isolated9using TRIzol reagent (Life Technologies) and an9RNeasy RNA purification kit0(Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. Real-time PCR9was performed by first9synthesizing cDNA by the reverse transcribing 19µg of total RNA by a9high-capacity5cDNA reverse9transcription kit (Life9Technologies) as per9the manufacturer's guidelines and consequently diluted to85008µL with nuclease8free water.9The real-time PCR9was accomplished in a 209µL reaction, which involved 1× SYBR8green PCR master mix8(Life Technologies), 1259nM each of forward9and reverse primers and 59µL of diluted cDNA9using an ABI Prism 7900 HT9Sequence Detection System9(Life Technologies). Primers sequences that were used for human pS2 cDNA amplification are: 5'-CAT CGACGTCCCTCCAGAAGA-3' sense, and 5'-CTCTGGGACTAATCACCGTGCTG-3'9anti-sense; human9GREB1 gene: 5'-CAAAGAATAACCTGTTGGCCCTGC-3'sense, 5'- GACATGCCTGCGCTCTCATACTTA-3'9anti-sense; human progesterone receptor the reference9gene 36B4: 5' GTGTTCGACAATGGCAGCAT-3'7sense, 5 '-GACAC CCTCCAGGAAGCGA-3' anti-sense. All primers were acquired from Integrated DNA Technologies7Inc. (Coralville,IA). Experiments, each containing of three replicates, were executed at least thrice. 2.6.3. Immunoblotting MCF-7 Cells were kept7in oestrogen-free8media (oestrogen-starved) for 39days before seeding. Cells were seeded on 106cm Petri dishes0(Corning) at a density of93 million cells per7plate and were8incubated overnight. The cells were8treated for 248h with the actual9tamoxifen metabolites' clinical levels in breast cancer7patients (Table 2.15) along with actual levels of estrogens examined in postmenopausal patients9taking tamoxifen. After media aspiration, cells were rinsed with cold PBS8(pH 7.4), trypsinized and pelleted in 158ml falcon tubes. The cells8were lysed with8freezing lysis buffer with fresh protease8inhibitors (Protease Inhibitor9Cocktail Set7III, Calbiochem, La Jolla, CA) incubated9over ice for 20 min. The cells9were centrifuged at6140008g for 30 min at94 °C. From supernatant930 µg of protein was resolved on84-12% polyacrylamide gel. After7shifting to8a nitrocellulose membrane9probed with primary9antibodies anti-Erα0(clone G-20; Santa Cruz Biotechnology,0Santa Cruz, CA, USA),8and with anti-β-actin9(clone AC-15; Sigma- Aldrich)8diluted in blocking9buffer at ratios recommended9by the suppliers at94°C overnight. The membranes9were washed thrice (109min each) the next day9with buffer and subsequently9incubated with9appropriate HRP-linked9secondary antibodies9(anti-mouse9or anti-rabbit from Cell Signaling9Technology, Danvers, MA, USA) diluted9in blocking buffer9for 1 h at9room temperature and detected9by chemiluminescence9autoradiography. Intensity of9protein band was measured9from scanned images with9Image J software. The9intensity of control9band was normalized to9actin and considered9as unity (1.0)9whereas the intensity of9treated band9was determined relative9to control and data9was presented as9mean ± SD on0three replicates. Chapter 3 3. Results and Discussion Blood samples of four hundred and thirty patients were collected. Every Participant filled and signed the consent form (Annexure I), and these forms were kept as a record. The participants were interviewed with the help of a designed questionnaire (annexure II) and information regarding various parameters and risk factors such as age, ethnic group, marital status, stage of breast cancer,6family history, treatment undertaken by breast cancer patients, and presence of any other disease, were obtained (Table 2.1). To compare the genotyping results with the control population, 410 age and gender-matched healthy individuals from the same cities and socioeconomic backgrounds were also enrolled (Table 2.2). DNA was8extracted by using the phenol- chloroform extraction method. The results were visualized by using 2% agarose gel electrophoresis. 3.1 Study I A total of 430 ER Positive breast Cancer8patients and 400 unrelated healthy individuals were included in the study, which aimed to verify the CYP2C9*2 and *3 gene polymorphism frequency and their impact on the metabolism of tamoxifennand its metabolites. 3.1.1 Genotyping of CYP2C9 Variants 3.1.1.1 CYP2C9*2 SNP at 430C > T in Exon 3 A polymerase chain7reaction was conducted to amplify the CYP2C9*2. The product size was 396 bp, as shown in Figure 3.1a. The amplified fragment was further digested using the AvaII enzyme and generated different fragments. Restricted fragments show wild-type homozygous C allele with two fragments size; 223 and 173 bp. The heterozygous C allele and T allele exhibit three bands of size; 396, 223, and 173 bp, while the mutant T allele did not undergo restriction and showed a band of 396 bp, as shown in Figure 3.1b. CYP2C9*2 heterozygous (OR: 0.4; 95□I: 0.53– 0.56; P1= 0.0001) and homozygous mutant (OR:

3,12; 95% CI: 1.80–5.43; P = 0.0001) condition was at breast cancer risk factor in our population (Table 3.1). 3.1.1.2 CYP2C9*3 SNP at 1075A > C in Exon 7 The CYP2C9*3 gene7was amplified using a polymerase chain reaction. As depicted in Figure 3.1c, a 105-bp-long product was generated. This product was further digested by the Kpnl enzyme, which generates fragments of varying sizes. Homozygous A allele did not undergo restriction, whereas the mutant homozygous CC allele genotype displays two bands of 85 and 20 bp sizes. As shown in Figure 3.1d, heterozygous A and C alleles exhibit three sizes 105, 85, and 20 bp bands. Statistical analysis revealed that CYP2C9*3 plays no significant role in the breast cancer development in the current study population (Table 3.1). Figure 3.1: 3.1a: Electropherogram of PCR for CYP2C9*2 allele. Band size: 396bp and 100bp ladder is used for comparison. 3.1b: Electropherogram of RFLP for CYP2C9*2 allele. Homozygous mutant type (TT)= 396bp, heterozygous mutant (CT)= 396bp, 223bp & 173bp and homozygous wild type (CC)= 223bp & 173bp. 3.1c: Electropherogram of PCR for CYP2C9*3 gene of 410 individuals. Band size: 105bp and 100bp ladder is used for comparison. 3.1d: Electropherogram of RFLP for CYP2C9*3 gene of 410 individuals. Homozygous wild type (AA)= 105bp, heterozygous mutant (AC)= 85bp, 20bp & 105bp and homozygous mutant type (CC)= 85bp & 20bp. Table 3.1 Association of CYP2C9 genotypes with ER Positive breast cancer Controls (410) Cases (430) Genotype n % n % OR (Upper Limit-Lower Limit) p-value CYP2C9*2 CC NM a *1/*1 229 55.85 273 63.48 1.37 (1.04-1.81) 0.02 CT IM a *1/*2 163 39.75 103 23.95 0.47 (0.35-0.64) <0.0001 TT PM a *2/*2 18 4.39 54 12..55 3.12 (1.80-5.43) 0.0001 CYP2C9*3 AA NM a *1/*1 140 34.1 128 29.76 0.81 (0.61-1.09) 0.17 AC IM a *1/*3 111 27.2 126 29.30 1.11 (0.82-1.50) 0.47 CC PM a *3/*3 159 38.7 176 40.93 1.09 (0.82-1.44) 0.52 a NM = Extensive9Metabolizer, IM = Intermediate6Metabolizer, PM = PoorrMetabolizer 3.1.2 Tamoxifen and its Metabolites Analysis Tamoxifen is converted to 4-OH-Tamoxifen by enzymes including CYP2D6, CYP2C19, CYP2C9, CYP2B6,9and CYP3A. CYP2C9 is the utmost abundant and polymorphic CYP2C enzyme. CYP2C9 plays a minor role in the tamoxifen metabolic pathway (Antunes et al., 2013). Mutants CYP2C9*2 and7CYP2C9*3 have low enzyme activity. To assess CYP2C9*2 and CYP2C*3 role in forming hydroxytamoxifen from tamoxifen and N-desmethyl tamoxifen from Tamoxifen, plasma analysis was performed on 430 ER-positive breast cancer patients using the Agilent 1100 UV HPLC system. CYP2C9 polymorphisms impact the tamoxifen and its metabolites' plasma concentrations. The median plasma concentrationssof tamoxifen and three metabolites measured in patients in each genotypic9group (Table 3.4, Figure 3.2). The associations of tamoxifen and its derivatives with the CYP2C*1/*1 (wild type), CYP2C9*1/*2 (heterozygous), and CYP2C9*2/*2 (mutant) genotypes are depicted in Figure 3.2. No substantial difference was detected for the median plasma8concentration of tamoxifen, 4-OH- Tam, NDM, and endoxifen concentrations among the three different genotypes of patients. (Table 3.2; Figure 3.2 a,b,c,d). However, an insignificant decrease was observed in the median plasma8concentration of 4-OH- Tam in the subjects having heterozygous (*1/*2) (P = 0.747) and mutant (*2/*2) (P = 0.223) genotypes in comparison with the subjects having wild-type genotype (*1/*1) (Table 3.2; Figure 3.2b). a b c d Figure 3.2. Association between CYP2C9*2 genotypes and steady-state8plasma concentrations of (A) Tamoxifen, (B) 4-OH-Tam, (C) N-desmethyl tamoxifen (D) Endoxifen. Table 3.2. Effects of CYP2C9 polymorphisms on8(median) plasma concentrations (ng/ml) of tamoxifen and its analytes. Parameters CYP2C9*2 (430 C>T) Genotype *1/*1 (NM)a1 Genotype *1/*2 (IM)a1 Genotype *2/*2 (PM)a1 Tamoxifen 145.43 142.50 140.32 4-OHT 252.57 228.79.65 234.40 NDM 3.96 4.13 3.95 Endoxifen 29.49 30.20 32.66 Parameters CYP2C9*3 (1075 A>C) Genotype *1/*1 (NM)a2 Genotype *1/*3 (IM)a2 Genotype *3/*3 (PM)a22 Tamoxifen 141.93 144.13 140.45 4-OHT 255.34 237.50 238.95 NDM 3.92 4.29 3.91 Endoxifen 27.47 28.46 27.51 a NM= Normal0Metabolizer , , IM = Intermediate0Metabolizer, PM = Poor Metabolizer, 1 for NM n = 273, IM n = 103, and PM n = 54 2 for NM n = 128, IM n = 126, and PM n = 176 The associations of tamoxifen and its derivatives with the CYP2C9*1/*18(wild type), CYP2C9*1/*3 (heterozygous), and CYP2C9*3/*3 (mutant)8genotypes are shown in Table 3.2 and Figure 3.3. No substantial association was examined for tamoxifen, 4- OH-Tam, or NDM among the wild-type, heterozygous and mutant genotypes for the CYP2C9*3 locus. a b c d Figure 3.3. Association between CYP2C9*3 genotypes and9steady-state plasma9concentrations of (A) Tamoxifen, (B) 4-OH-Tam, (C) N-desmethyl tamoxifen (D) Endoxifen 3.1.3. CYP2C9 polymorphisms impact on Total Metabolic Ratio of tamoxifen9and its metabolites For the CYP2C9*2 locus, the median Plasma Metabolic Ratio of MR NDM-TAM was marginally higher in the heterozygous (1.78) (P = 0.85) and mutant (1.80) (P = 0.19) genotypes compared to the wild-type (1.68) genotypes (Table 3.5, Figure 3.4a). No significant difference was detected in Plasma metabolic ratio MR End-4-OHT between the three genotypes (Table 3.3, Figure 3.4b). An insignificant decrease was observed in the total metabolic ratio of 4-OH-Tam in the subjects having heterozygous (*1/*2) (P = 0.36) and mutant (*2/*2) (P = 0.51) genotypes in comparison with the subjects having wild-type genotype (*1/*1) (Table 3.3, Figure 3.4c). No substantial difference was observed in the median TMR NDM ratio among the three genotypes (Table 3.3, Figure 3.4d). CYP2C9*3 locus had no considerable effect on the median plasma metabolic ratio of MR NDM-TAM and MR END-4-OHT (Table 3.3, Figure 3.5a, 3.5b) and the median total metabolic ratio of TMR NDM and TMR 4-OHT (Table 3.3, Figure 3.5c, 3.5d). Table 3.3. Outcomes of CYP2C9 polymorphisms on median metabolic ratios of Tamoxifen and its analytes Parameters Genotype Genotype Genotype CYP2C9*2 (Exon 3 at 430C>T)) *1/*1 (NM)a1 *1/*2 (IM)a1 *2/*2 (PM)a1 Plasma metabolic ratios (MRs) MR NDM-TAM 2.01 2.88 2.80 MR END-4-OHT 14.54 14.34 14.42 Total metabolic ratios (TMRs) TMR NDM 2.52 2.43 2.63 TMR 4-OHT 13.96 11.21 12.84 Parameters CYP2C9*3 Genotype Genotype Genotype CYP2C9*3(Exon 7 at 1075A>C) *1/*1

(NM)a2 *1/*3 (IM)a2 *3/*3 (PM)a2 Plasma metabolic ratios (MRs) MR NDM-TAM 2.32 2.35 2.31 MR END-4-OHT 15.20 15.21 15.20 Total metabolic ratios (TMRs) TMR NDM 2.82 2.56 2.11 TMR4-OHT 14.63 14.14 14.75 a NM= Normal Metabolizer , , IM = Intermediate Metabolizer, PM = Poor Metabolizer, 1 for NM n = 273, IM n = 103, and PM n = 54 2 for NM n = 128, IM n = 126, and PM n = 176 a b c d Figure 3.4. CYP2C9*2 genotype association with plasma metabolic ratio of (a) NDM-Tam (b) End-4OHT and total metabolic ratios of (c) 4-HydroxyTamoxifen and (d) N-desmethyl tamoxifen a b c d Figure 3.5. CYP2C9*3 genotype association with plasma metabolic ratio of (a) NDM-Tam (b) End- 4OHT and total metabolic ratios of (c) 4-HydroxyTamoxifen and (d) N-desmethyl tamoxifen. Tamoxifen was8the first and among most effective targeted cancer therapies. Tamoxifen is9widely used for9breast cancer risk reduction in high-risk9populations and adjuvant and metastatic treatment of estrogen receptor-positive breast cancer (Davies et al., 2013). A minor9pathway for the tamoxifen conversion to endoxifen is believed to occur by Tamoxifen hydroxylation by CYP2D6,9CYP2B6, CYP2C9, CYP2C19,0or CYP3A4/55,6; this is followed8by conversion of84-hydroxytamoxifen to endoxifen (Brauch et al., 2009). Multiple9cytochrome8P450 enzymes metabolize tamoxifen, and polymorphisms in the genes9encoding9these enzymes may affect tamoxifen9and its metabolites' plasma9concentrations. Tamoxifen's efficacy is dependent on CYP enzymes' conversion to active metabolites. Tamoxifen metabolites9can also be transported9out of cells, and transporter proteins should be9considered in pharmacogenetics9studies (Cronin-Fenton et al., 2014). Tamoxifen metabolites9can also be transported. It was reported that tamoxifen9and its primary9metabolites in concentrations9found in three CYP2D69genotypes (EM, IM, and PM) are9sufficient to constrain estrogen- induced9replication in the9postmenopausal setting. They recognized the importance9of 4OHT as the9active primary metabolite9in the antiestrogenic9action of tamoxifen in the postmenopausal9setting. The estrogenic steroids9would be anticipated to accrue in the cell by binding to the ER (Maximov, McDaniel, Fernandes, Korostyshevskiy, et al., 2014). Estrogens activate9the ER and induce its9transcriptional activity by interaction with the9estrogen-responsive9gene promoters (Maximov, McDaniel, et al., 2014a). Estrogen receptor9protein turnover is essential (Lonard et al., 2000) to provide the continuous mRNA transcription. This turnover is accomplished by proteosomal8degradation of the ER protein. The binding9of antiestrogens, particularly 4OHT, blocks the ER and promotes9stabilization. Estrogens activate the ER and induce its9transcriptional activity by interaction with9the estrogen-responsive gene promoters (Maximov, McDaniel, et al., 2014a). In this9exploratory study, we examined the effects of formerly reported polymorphisms in the CYP2C9 gene, which is involved in tamoxifenmand its metabolites' metabolism, in ER-positive breast9cancer patients in Pakistan. Additionally, the genotype frequencies for CYP2C9*2 and CYP2C9*3 were assessed between the two groups, and the allele frequencies of the9CYP2C9 variants in healthy controls and ER-positive breast are shown in Table 3.1. Our findings demonstrated the genotyping results for unrelated healthy individuals and estrogen-positive breast cancer patients from Pakistan. No noteworthy difference was detected between the allele frequencies of CYP2C9*2, but conditional logistic regression shows that CYP2C9*2 heterozygous (OR: 0.4; 95↑I: 0.35–0.64; P9< 0.0001) and homozygous mutant (OR: 3.1; 95%iCI: 1.80–5.43; P = 0.0001) condition was at risk of developing8breast cancer. The cytochrome9P450 variants CYP2C9*2 and9CYP2C9*3 encode9proteins with lowered enzymatic activity.9Individuals carrying9these variants metabolize9drugs more slowly than individuals9with wild- type CYP2C9*1, potentially altering their response9to drugs and their disease risk (Sausville et al., 2018). No significant difference was detected between the allele frequencies of CYP2C9*3 in unrelated healthy and estrogen-positive breast cancer patients. CYP2C9 contributes to forming the principal tamoxifen metabolites94-hydroxy- tamoxifen and9N-desmethyl-tamoxifen (Antunes et al., 2013), though to a smaller extent than the isoforms (CYP2D6 and CYP3A5) (Coller et al., 2002). This study explored the effect of previously reported polymorphisms in genes encoding the enzymes responsible for tamoxifen and three8metabolites' metabolism in Pakistani breast cancer patients. There was no variation in the tamoxifennor itssmetabolites median plasma concentrations between6patients with two wild-type alleles and those with heterozygous or homozygous CYP2C9*2 and CYP2C9*3 variant alleles. However, a minor or insignificant decrease was observed in the median9plasma concentrations of 4-OH-tamoxifen patients with *1/*2 and *2/*2 genotypes. Our findings were consistent with those of Teft et al.9(no p-values stated) and Jin et al. (p- values 0.05) in that there was no significant difference in tamoxifen9or its metabolites plasma concentrations between9patients involving two9wild-type alleles or carriers9of heterozygous or homozygous9CYP2C9*2 and CYP2C9*3 variant alleles. Lim et al. also found comparable results concerning CYP2C9*3 and its impact on the concentrations of tamoxifen and metabolites (Lim et al., 2011). However, homozygous9wild-type carriers and carriers of the CYP2C9*2 and *3 alleles demonstrated significantly different rates of 4-hydroxy-tamoxifen production from tamoxifen (p = 0.007) and significantly lower plasma levels of 4-hydroxy-tamoxifen (Saladores et al., 2015). 3.2 Study II 3.2.1. Genotyping of CYP2C19 3.2.1.1. Genotyping frequency for CYP2C19*2 SNP in Exon 5 at 681 G>A: An-amplificationsrefractoryamutation system (ARMS) was used for DNA amplification at this locus, producing three band sizes. 291 bp (positivescontrol for thee locus), 202 bp (wild type), and 169 bp (mutant). The subject was considered heterozygous (CYP2C19*1/*2) in case three bands of size 2911bp, 202 bp,9and 169 bp were detected; homozygousawild type (CYP2C19*1/*1) if two bandssof size 291 bp and 202 bp wereedetected; and homozygous mutant s(CYP2C19*2/*2) if two bands of size 2911bp and 1699bp were observed. CYP2C19*22heterozygous condition was aa breast9cancer risk factor (OR: 0.6; 95□I: 0.43–0.84; P9= 0.003) in our population,n whereas

nooassociation was found forrthe homozygous mutant ccondition. (Fig 3.6 A, Table 3.4) 3.2.1.2. Genotyping9frequency for CYP2C19*3 SNP in Exon 4 at 636 G>A Allele-specific PCRaamplified CYP2C19*3 gene.eNormal "C" alleleeband of 253bp anddmutant "T" alleleeband of 253bp werewere amplified too determine genotype. The allele-specificcband marked theepresence offrespective alleles innthe CYP2C19 gene. Homozygousswith majorrallele 'C' and homozygousswith minor allelee'T' have shown one band, whereassheterozygous has shownnbands with both primer. Statistical analysis unveiled a substantial provision of the CYP2C19*3 heterozygous variantt(OR: 0.34; 95% CI: 0.24–0.48; P<0.001) towards breast cancer development in the current study population. (Fig 3.6 B, Table 3.4) 3.2.1.3. Genotyping frequency for CYP2C19*17 (-806C>T; rs12248560) PCR technique ARMS was employed for implication of CYP2C19*17. Three bandss produced inn this case were 507 bp (control), 3300bp (wild type), and 2188bp (mutant). If all three bands detected, the subjecttwas considered as heterozygous (CYP2C19*1/*17). The subject wassdesignated asshomozygous wild type (CYP2C19*1/*1) if 22 bands of 5077bp and 3300bp were detected and homozygouss mutant (CYP2C19*17/*17) if bands of the sizes 5077bp and 2188bp were observed. Heterozygous individualss(OR: 7.177; 95% CI: 5.088–10.10; P<0.001) and homozygous mutant individual (OR: 5.366; 95% CI: 3.180–9.011; P<0.001) were at significant risk off breast cancer progression. (Fig 3.6 C, Table 3.4) Figure 3.6. Genotyping of CYP2C19 SNPs. a) Electropherogrammof ARMS PCR for CYP2C19*2 allele. The control band is 2911bp, the wild-type G-allele band is 2022bp, and the mutatedAA-allele band is 169bp. b) Electropherogrammof PCR for CYP2C19*3 allele. Asterisk (*) representssmutant allele. Wild type CCallele band= 253bp, mutant type T allele= 253bp. 1 &1* lane showing heterozygous major C and minor T allele, 2* and 3* showing homozygous minor T allele, while 5 and 6 show homozygous major C allele. c) Electropherogram of ARMS PCR for CYP2C19*17. Control band of 507 bp, wild-type C- allele band of 330 bp, and mutated T-allele band of 218 bp. 100 bp ladder is used for comparison. Table 3.4. Association of CYP2C19 genotypes with ER Positive breast cancer Genotype Controls (410) Cases (430) n % n % OR (95% C.I) P-value CYP2C19*2 GG NM a *1/*1 210 51.2 214 49.8 1.0 (Ref.) GA IM a *1/*2 180 43.9 190 44.2 0.6 (0.43 to 0.84) 0.003 AA PM a *2/*2 20 4.9 26 6 0.81 (0.39 to 1.71) 0.594 CYP2C19*3 GG NM a *1/*1 152 37.1 159 37.0 1.0 (Ref.) AG IM a *1/*3 214 52.2 205 47.7 0.34 (0.24 to 0.48) >0.001 AA PM a *3/*3 44 10.7 66 15.3 0.68 (0.39 to 1.16) 0.165 CYP2C19*17 CC CT TT NM a RM a UM a *1/*1 *1/*17 *17/*1 7 265 107 38 64.6 26.1 9.3 80 281 69 18.6 65.3 16.1 1.0 (Ref.) 7.17 (5.08 to 10.1) 5.36 (3.18 to 9.01) >0.001 >0.001 a EM = ExtensiveyMetabolizer, IM = IntermediateuMetabolizer, PM = PoortMetabolizer, RM=Rapid Metabolizer and UM = Ultra Rapid Metabolizer 3.2.2. Analysis of Tamoxifennand its metabolites Tamoxifen is metabolizedsby enzymes comprisinggCYP2D6, CYP2C19, CYP2C9, CYP2B6, and CYP3A into 4-OH-Tamoxifen. CYP2C19 is a polymorphic gene. CYP2C19*2 anddCYP2C19*3 mutantsshave low enzyme action, whereass CYP2C19*17 heterozygotes and mutants have enhanced enzyme activity. CYP2C199polymorphisms impact the plasma9concentrations and Total Metabolic Ratio of tamoxifen9and its metabolites Median plasmaa concentrationsgof the tamoxifen and the three9metabolites measuredd in patients offeach genotypic groups are shown in Table 3.5. Figure 3.7 displays thee associations off tamoxifen and itss derivatives with CYP2C19*1/*1 (wild-type), CYP2C19*1/*17 (heterozygous) CYP2C19*17/*177 (mutant)ggenotypes. No significantt difference wass observed for the the median plasmattamoxifen and endoxifen concentrationss among the threee different genotypes off patients (Table 3.5; Fig 3.7A & 3.7D). However, median palsaama showed a strong association with this locus . concentrations off 4-OH-tamoxifens, which wassrecorded too be 288.59 ng ml/L in the wild-typeegenotypes, 354.533 ng ml/L (P<0.0010) in the heterozygous genotypes (62.0%) increased thannthe wild-type patients), and 378.255 ng ml/L (P<0.001)9for the mutants genotypes (75.0 % increase compare to the wild-type patients) (Table 3.7; Fig 3.7B). A slighttdecline (P= 0.3620) for median concentrations of N-DesM-Tamm was observed in heterozygous & mutant patient (Table 3.5; Fig. 3.7C). Figure 3.8 shows theeassociations off tamoxifen along with derivatives CYP2C19*1/*11 (wild-type), CCYP2C19*1/*39(heterozygous), & CYP2C19*3/*33 (mutant) genotype. No significantt association wassobserved for tamoxifen & N- DesM-Tam amongst the wild-type, heterozygous as well as mutant genotypes for the CYP2C19*33 locus. However, an insignificant decreases was observedsin the median plasmas concentrations9off 4-OH-Tam for heterozygous (240.634 ng ml/L) (P=0.420) or mutants (276.293 ng ml/L) (P=0.879) genotype, compared too the wild- types (288.437 ng ml/L) genotypes for this locus. (Table 3.5, Fig 3.8B). Conversely, an increment for the median plasmas concentrations were noted for endoxifen in the heterozygouss (24.737 ng ml/L) and mutants (35.408 ng ml/L) genotype, compares to the wild-type (19.733 ng ml/L) genotype (Table 3.5, Fig 3.8D). Figure 3.9 depicts the associations of tamoxifen and also the derivatives with CYP2C19*1/*17(wild-type), CYP2C19*1/*229(heterozygous), and CYP2C19*2/*22 (mutant) genotype. Weak association for three types of genotypes were noted for the median plasma’s9concentration of tamoxifen & N-DesM-Tam (Fig. 3.9A & 3.9C). However, an increased for median plasmas concentration of 4-OH-Tam was noted in the heterozygouss (272.657 ng ml/L) (P<0.021) and mutants (305.250 ng ml/L) (P<0.004) genotype, in comparison with the concentrations of the wild-type (255. 753 ng ml/L) genotypes (Table 3.5, Fig 3.9B). Similarly, there was stepwise increase noted for median plasma concentration off endoxifen: 19.433 ng ml/L in the wild- type, 27.173 ng ml/L for heterozygouss, and 32.501 ng ml/L in the mutant genotype (Table 3.50, Fig 3.9D). Table 3.5. Effects of CYP2C19 polymorphisms on (median) plasma concentrations (ng/ml) of tamoxifen and its analytes. Parameters CYP2C19*2 (681G>A; rs4244285) Genotype *1/*1 (NM)a1 Genotype *1/*2 (IM)a1 Genotype *2/*2 (PM)a1 Tamoxifen 116.96 119.68 118.49 4-

OH- Tamoxifen 255.75 272.65 305.25 N-DesM-Tam 3.96 4.10 4.91 Endoxifen 19.34 27.15 32.50 Parameters CYP2C19*3 (636G>A; rs4986893) Genotype *1/*1 (NM)a2 Genotype *1/*3 (IM)a2 Genotype *3/*3 (PM)a22 Tamoxifen 116.87 116.99 117.26 4-OH- Tamoxifen 288.43 240.63 276.29 N-DesM-Tam 3.02 3.13 1.68 Endoxifen 19.34 24.73 35.40 Parameters CYP2C19*17 (-806C>T; rs12248560) Genotypes *1/*1 (NM)a3 Genotype *1/*17 (RM)a3 Genotype *17/*17 (UM)a3 Tamoxifen 118.97 120.15 121.88 4-OH- Tamoxifen 288.59 354.53 378.25 N-DesM-Tam 4.78 3.47 3.20 Endoxifen 29.49 27.04 31.40 a EM = Extensive8Metabolizer, IM = IntermediateiMetabolizer, PM = Poor9Metabolizer, RM= Rapid Metabloizer and UM = Ultra Rapid Metabolizer 1 for RM nn = 214, IM n = 190, and PMM n = 26 2 for RM nn = 159, IM n = 205, and PMM n = 66 3 for RM nn= 69, UM n= 299, and UMM n= 62 Figure 3.7. Association between CYP2C19*171(*1/*1, *1/*17 & *17/*171) genotype and9steady- state median plasmaaconcentrations of (A) Tamoxifen, (B) 4-OH-Tam, (C) N-desM-Tamoxifen & (D) Endoxifen. No significant9difference for median plasma9concentration of Tamoxifen and Endoxifen between the three genotypes. A extensively raised amount of 4-OH-Tam concentrations in *17 genotype compared with wild type (P= 0.001). Non-significant reduction in median plasmaaconcentration of N-desM-Tamoxifen (P= 0.3621) in *17 genotype compared with wild type. Figure 3.8. Association b/w CYP2C19*33(*1/*1, *1/*3 and *3/*3) genotype and9steady-state median plasma concentration off (A)9Tamoxifen, (B) 4-OH-Tam, (C) N-desMTamoxifen & (D) Endoxifen. No measurable change was noted for median plasma concentration of Tamoxifen and N-desM- Tamoxifen between the genotype. Non-significant reduce in concentration 4-OH-Tam in *3 genotype to wild type (P=0.4210), whereas there was a little boost in the median plasma concentrations of Endoxifen in *3 genotypes to *1/*1. Figure 3.9. Correlation between CYP2C19*2 genotypes and9steady-state median plasma concentrations of (A)9Tamoxifen, (B) 4-OH-Tam, (C) N-desM-Tamoxifen, & (D) Endoxifen. No recordable difference in Tamoxifen and N-desM-Tamoxifen concentration for the three genotypes, a substantial increment in the median plasma9concentration of 4- OH-Tam (P= 0.0212) for *1/*2, and (P= 0.004) for *2/*2 compared to wild type. Endoxifen concentration depicts an non-significant increase in *1/*22and *2/*2 comparedd to *1/*1. For the CYP2C19*17 locus, the mediann Total Metabolic Ratio of 94-OH-Tam (TMR-44-OH-Tam) was suggestively higher for heterozygous (187.91) (P<0.0011) and mutant (210.900) (P<0.001) genotype, compared to the wild-type (122.464) genotype (Tablee 3.6, Fig 6A). Correspondingly, TMRR-N-desM-Tamoxifen median amounts also depict an increasing trend: 7.668 in the wild-type, 8.920 in heterozygous, and 9.213 in the mutant genotype (Table 3.6, Fig 6B). Although no considerable correlation examined between the CYP2C19*22 polymorphisms and the TMRR-4-OH-Tam (Table 3.6, Fig 6C), patients sheltering the *1/*22 heterozygous (P<0.001) and *2/*2 homozygous mutants genotype (P=0.2272) displayed increases median TMRR-4-OH-Tam. In contrast, TMRE-N-desM- TTamoxifen were parallel for the patients *1/*1, *1/*2 and *2/*2 (Table 3.6, Fig 6D). Non-significant relationship was noted for the median TMRr-4-OH-Tam and TMRE- N- desM-Tamoxifen in the patients bearing *1/*1, *1/*3, and *3/*3 genotypes (Table 3.6, Fig 6 E, F). Table 3.6. Effects of CYP2C19 polymorphisms on median metabolic ratios of Tamoxifen and its analytes Parameters Genotype Genotype Genotype CYP2C19*2 (681G>A; *1/*1 (NM)a1 *1/*2 (IM)a1 *2/*2 (PM)a1 rs4244285) Plasma metabolic ratios (MRs) MR NDM-TAM 0.21 0.26 0.37 MR END-4-OHT 7.87 7.46 12.31 Total metabolic ratios (TMRs) TMR NDM 6.64 7.30 8.76 TMR 4-OHT 143.78 183.79 213.7 Parameters CYP2C19*3 (636G>A; rs4986893) Genotype *1/*1 (NM)a2 Genotype *1/*3 (IM)a2 Genotype *3/*3 (PM)a2 Plasma metabolic ratios (MRs) MR NDM-TAM 0.32 0.32 0.27 MR END-4-OHT 13.72 12.67 12.01 Total metabolic ratios (TMRs) TMR NDM 7.88 7.81 8.34 TMR4-OHT 93.81 82.04 87.27 Parameters Genotypes Genotype Genotype CYP2C19*17 (-806C>T; *1/*1 (NM)a33 *1/*17 (RM) a3 *17/*17 (UM) a3 rs12248560) Plasma metabolic ratios (MRs) MR NDM-TAM 0.28 0.28 0.26 MR END-4-OHT 0.26 0.16 0.19 Total metabolic ratios (TMRs) TMR NDM 6.68 8.98 9.23 TMR 4-OHT 122.48 187.91 210.90 a EM = ExtensiveuMetabolizer, IM = IntermediatejMetabolizer, PM = PoorhMetabolizer, and UM = Ultra Rapid Metabolizer 1 for EMM n = 214, IM n = 190, and PMNn = 26 2 for EMM n = 159, IM n = 205, and PMNn = 66 3 for EMM n= 69, RM n= 299, and UMOn= 62 Figure 3.10. CYP2C19 genotypes and metabolic ratios of N-desM-Tamoxifen and 4- HydroxyTamoxifen. (A) The median TMR of 4-OH-Tam (TMRR- 4-OHT) was significantly higher in *1/*17 (P<0.001) and *17/*17 (P<0.001) genotypes than *1/*11 genotype. (B) An insignificant increase in the median TMR N-desM-Tamoxifen (TMR-N-des) median values with genotype *1/*17 and *17/*177 compared to *1/*1. (C) Significantly higher median TMR of 4-OH-Tam (TMR- 4-OH-Tam) between the CYP2C19*2 genotypes *1/*2 (P<0.001) and *2/*2 genotype (P=0.2327). (D) The median TMR N-desM-Tamoxifen (TMR-N-desM-Tam) was similar among *1/*1, *1/*2, and *2/*2 genotypes. (E) No substantial association was detected in median TMR of 4-OH-Tam (TMR- 4-OH-Tam) between *1/*1, *1/*3 and *3/*3 genotypes. (F) No significant association was detected for the median TMR N-desM-Tamoxifen (TMR-N-desM- Tam) between *1/*1, *1/*3, and *3/*3 genotypes. The CYP2C19 gene plays crucial role in majority Proton Pump Inhibitors (PPI) and antiepileptic drug metabolism. The metabolic pathways have not been completely understood so far. Cytochrome P450 enzymes, major participants in the metabolism of xenobiotics. Breast cancer has been extensively diagnosed cancer in women9worldwide (Jemal et al., 2010). All women, apart from their ethnic or racial origin or familial history, are prone to breast cancer, whereas males are at a decreased risk9of developing breast cancer (Naeem et al., 2008). Major influencers in breast carcinoma progression are hormones (endogenous and exogenous) in females, genetic factors, environmental factors. We earlier reported the frequency of CYP2C19*2 & CYP2C19*17 alleles in different racial groups of Pakistan. Moreover, we reportedd that the overalllratio of the expected poor smetabolizer (PM)

(*2/*2) allele was 29.10% in contrast to the ultra- rapid metabolizer (UM) allele (*17/*17) 23.70% (Riaz et al., 2019). A German study could not find any relation between9CYP2C19*22polymorphism & breast cancer risk (Justenhoven et al., 2009). Iraqi population suggests that CYP2C19*2 polymorphisms with the proliferation of breast cancer (Jabir et al., 2018). CYP2C19*2 heterozygous mutants breast cancer patients taking tamoxifen have greater opportunities of survival (Ruiter et al., 2010) (Bai et al., 2014). Furthermore, the CYP2C19*3 allele was related to expanded breast cancer. Chinese studies revealed, arachidonic acid's metabolisms can be regulated by CYP2C19's anti-apoptotic effect, subsequently causing breast cancer (Gan et al., 2011). Another study by A. B. Sanchez-Spitman concludes that CYP2C19 has no notable effect on tamoxifen metabolism9or breast8cancer relapse (Sanchez-Spitman et al., 2021). CYP2C19*177 allele has been linked to the sublime in breast cancer in a German cohort, indicating that CYP2C19*17 causes increase in estrogen catabolism, declining the risk for9breast cancer (Justenhoven et al., 2009). The study's total sample was 410 unrelated healthy individuals and 430 ER-positive breast cancer patient. The genotypes frequency for CYP2C19*22, CYP2C19*337and CYP2C19*17 were calculated for the two groups, and allele frequenciessof the CYP2C19 variants in control and ER Positive8breast cancer patient was present, as shown in Table 02.0. Our result findings (Tablee3.4) demonstrates the genotyp resultss for controlled individuals & estrogen-positive breast cancer patient in the Pakistan. No noticeable change was found among the allele frequencies of CYP2C19*2; conditional logistic regressions show that CYP2C19*22 heterozygous conditions were a risk reason for breast cancer9(OR: 0.56; 095%iCI: 0.413–00.84; P=0.003). The current findings strongly encourages the statistical contribution of the CYP2C19*33 heterozygous variant in the formation of breast cancer8(OR: 90.34; 95% CI: 0.224–0.418; P<0.001). Mostly samples were from to the extensive metabolizer (EM) group in normal individual and ER-positive breast9cancer patients. Tablee3.4 demonstratess the allelics frequency of CYP2C19 variants, but a notable difference was recorded for unrelated healthy individual and ER-positive9breast cancer patients for CYP2C19*177 allele frequencies. Over 65.0% of unrelated healthy individuals were extensive metabolizers (*1/*1) in CYP2C19. On contrary, results were very different for ER-positive breast cancer patients, representing more than 70.0% of them were ultra-rapid metabolizers (*1/17*, *17/17) (OR: 7.717; 95% CI: 5.108–10.11). Multiple cytochrome9P450 enzymes and polymorphisms in the genes producing these enzymes facilitates the multichannel metabolism of tamoxifen, exerting a influential impact on tamoxifen and its various metabolites6plasma concentration. In the present research, we emphasized to elucidate the influence of earlier identified polymorphisms in genes coding for the enzymes catalyzing the tamoxifen's metabolism and its metabolites in Pakistani patients with breast cancer. In the light of the current findings, the CYP2C19*177 (-806C>T; rs12248560) allele was corelated with higher plasma metabolic ratio of 4-OH-tamoxifen for plasma, whereas the linkage with N-DesM-Tam was non-significant. Thus indicates that, there is accumulation of 4-OH-tamoxifen & tamoxifen in plasma when9the metabolic conversion of 4-OH-tamoxifen to endoxifen is not sufficient. Impaired conversation of 4-OH-tamoxifen to endoxifen was also suggested by total metabolic ratios. There was substantial increment was noted for 4-OH-Tam plasma concentrations in patients carrying9CYP2C19 *1/*2 & *2/*22 genotype. N-DesM-Tam plasma concentrations were similar for the patient with *1/*2, *1/*17, and *2/*2, *17/*177 genotypes. Minor fluctuation for endoxifen plasma concentrations in patient having genotype CYP2C19*1/*11,*1/22 *2/*2, and *1/*17,*17/*177. Plasma concentrations of 4-OH-tamoxifen in patient with *1/*3 and *3/*3 genotype was found to be lower than those have *1/*1 genotype. An expanded gene expression9of the CYP2C19*17 allele resulting in a putative9ultra- rapid (UMm) phenotype (Sim et al., 2006). CYP2C19 carries the tamoxifen metabolism to anti-estrogenic metabolite 44-OH-tamoxifen, conceiving in vitro activities9similar to CYP2D66 (Desta et al., 2004) (Coller et al., 2002). Our data9suggest that CYP2C19*17 has a crucial and significant part in the formation of plasma concentrations of 4-OH-tamoxifen. An active type of CYP2C19*177can produce significant results toward the control of breast cancer recurrence, previously published by Schroth and his co-workers (Schroth et al., 2007). But, our study oppses the findings off Joanne S. L. Lim et al., suggested no relationship between CYP2C19 polymorphisms and tamoxifen's pharmacokinetics (Lim et al., 2011) 3.3 Study III A total of 430 ER-positive breast cancer9patients and 400 unrelated healthy individuals were included in the study, which aimed to regulate the frequency of CYP2C9*2 and *3 gene polymorphism and their impact on the metabolism of tamoxifen9and its metabolites 3.3.1. Genotyping Genotyping of four hundred breast cancer patients for CYP3A4*1/*1, CYP3A4*22/*22, and CYP3A4*1/*22 was done with the help of Amplification refractory mutation system (ARMS) by thermocycler Biorad T100TM. The results were visualized on 2% agarose gel. 3.3.1.1 CYP3A4*22 SNP in Intron 6 at 15389C>T By ARMS PCR, the CYP3A4*22 gene was amplified. The control band of 232 bp was amplified, as shown in figure 19. Three bands produced in this case were 232 bp (control), the `C` allele (wild type) 107 bp, and the `T` allele (mutant) 162 bp. In the case of detecting all three bands, the subject was considered heterozygous (CYP3A4*22/*22). The subject was designated as homozygous wild type (CYP3A4*1/*1) if two bands of 232 and 107 bp were detected and homozygous mutant (CYP2C19*17/*17) if two bands of the sizes 232 bp and 162 bp were observed. Heterozygous individuals9(OR: 2.79; 95% CI: 1.45 –5.34; P=0.43) and homozygous mutant individuals (OR: 0.42; 95% CI: 0.18–1.23; P=0.11). Statistical analysis revealed that CYP3A4*22 plays no significant role in the breast cancer development in the current study population. (Figure 3.11, Table 3.7) Figure 3.11: Electropherogram for CYP3A4*22 gene. The control band is 232 bp, the wild-type C- allele band is 107 bp, and the mutated T-allele band is 162 bp. A 100 bp ladder was used for comparison. Table

3.7. Association of CYP3A4 genotypes with ER Positive breast cancer Controls (410) Cases (430) Genotype n % n % OR (Upper Limit-Lower Limit) p- value CYP3A4*22 CC EM a *1/*1 386 94.14 389 90.46 0.5 (0.34-0.99) 0.04 CT IM a *1/*22 13 3.17 36 8.37 2.79 (1.45-5.34) 0.002 TT PM a *22/*22 11 2.68 05 1.16 0.42 (0.1- 1.23) 0.11 a EM= Extensive9Metabolizer , , IM = Intermediate0Metabolizer, PM = Poor9Metabolizer, 3.3.2. Profiling of Tamoxifen Metabolism Tamoxifen is metabolized by several enzymes, like CYP2D6, CYP2C19, CYP2C9, CYP2B6 and CYP3A into 4OH-Tamoxifen. CYP3A4 is a polymorphic gene. One of the alleles, CYP3A4*22, has reduced enzyme activity. To determine the association of CYP3A4*22 in the tamoxifen metabolism, plasma analysis of 430 breast cancer patients was done by HPLC Agilent 1100 UV system. Out of 430 collected samples, most of the samples had CYP3A4*1 (wild type) genotypes according to the determined allele frequency (Table 3.7). Therefore, an association between CYP3A4*22 allele frequency and plasma concentration of tamoxifen and its metabolites was determined. Tamoxifen and its metabolite concentration were also compared between the *1/*1, *1/*22, and *22/*22 genotypes. The tamoxifen and three metabolites' median plasma9concentrations were calculated in patients in each genotypic group9depicted in Table 3.8 and pairwise P value in Table 3.9. No significant difference was observed for the median plasma tamoxifen, 4-OH- Tam, NDM, and endoxifen concentrations among the three different genotypes of patients. (Table 4; Figure 3.12). Table 3.8. Effects of CYP3A4 polymorphisms9on median metabolic ratios of Tamoxifen and its analytes Parameters CYP3A4*22 (430 C>T) Genotype *1/*1 (EM)a1 Genotype *1/*2 (IM)a1 Genotype *2/*2 (PM)a1 Tamoxifen 113.00 118.49 118.87 4-OHT 228.08 231.28 233.40 NDM 4.08 3.80 3.93 Endoxifen 25.64 26.41 27.51 1 for NM n = 273, IM n = 103, and PM n = 54 2 for NM n = 128, IM n = 126, and PM n = 176 a EM= Extensive9Metabolizer Poor9Metabolizer, , , IM = Intermediate0Metabolizer, PM = Table 3.9. Pairwise P value comparison between different genotypes Parameters CYP2C9*2 (430 C>T) Pairwise P values *1/*1 vs *1/*22 Pairwise P values *1/*1 vs *22/*22 Tamoxifen 0.88 0.143 4-OHT 0.06 0.139 NDM 0.73 0.55 Endoxifen 0.82 0.39 A B C D Figure 3.12. Association between CYP2C19*2 genotypes and steady-state median plasma concentrations of (A) Tamoxifen, (B) 4-OH-Tam, (C) N-desM-Tamoxifen, and (D) Endoxifen In the present study, 410 unrelated healthy individuals and 430 ER-positive breast cancer patients made up the total sample size. The genotype frequencies for CYP3A4*22 were calculated between the two groups. The allele9frequencies of the CYP3A4 variant in breast9cancer patients with ER-Positive status and healthy controls are shown in Table 3.7. Our findings (Table 3.7) demonstrated the genotyping results for unrelated healthy individuals and estrogen-positive breast cancer patients from Pakistan. No noteworthy difference was detected between the allele frequencies of CYP3A4*22. The genotype frequency of CYP3A4*1/*1, CYP3A4*1/*22 and CYP3A4*22/*22 determined in four hundred thirty breast cancer subjects taking estrogen adjuvant monotherapy were 90.46%, 8.37%, and 1.16%, respectively. In the present results, the percentage frequency of CYP3A4*1/*1 was high in estrogen receptor-positive BC patients, which can be associated with increased disease progression. CYP3A4 plays an important part in the tamoxifen and its more efficient metabolites' metabolism (Zhou et al., 2009). The tamoxifen9and its three metabolites median plasma concentrations in CYP3A4*1/*1, CYP3A4*1/*22, and9CYP3A4*22/*22 genotype shown in Table 3.8. The median plasma concentration of tamoxifen indicates that CYP3A4*1/*1, CYPP3A*1/*22 and CYP3A4*2/*22 have the almost same concentration of tamoxifen. The significant value determined for Tamoxifen was higher than 0.05. The significant value (determined by Mann-Whitney U test). These results are comparable with Baxter et al., (2014), according to which CYP3A4*22 genetic variation leads to reduced enzymatic activity. 3.4. Study IV 3.4.1. Genotyping of CYP3A5 A total of 430 ER Positive breast Cancer9patients and 400 unrelated healthy individuals were incorporated in the study, which aimed to determine the9frequency of CYP3A5*3 and CYP3A5*6 gene polymorphism and their impact on metabolism of tamoxifen and its metabolites. Allele Specific PCR was performed to determine genotypes and PCR products were9visualized on 2% agarose gel9electrophoresis. 3.4.1.1. CYP3A5*3 SNP in Intron 3 at 6986A>G Two sets of AS-PCR were performed for CYP3A5*3 genotyping. Normal "A" allele band of 285 bp and mutant "G" specific band of 285 bps were amplified and viewed on gel and are shown in Figure 3.13 . Homozygous normal and homozygous mutant allele showed band in single lane while heterozygous showed bands in both lanes. CYP3A5*3 heterozygous (OR: 0.8; 95% CI: 0.64–1.12; P = 0.25), Statistical analysis revealed that CYP2C9*3 plays no significant role in the development of breast cancer in the current study population, whereas homozygous mutant (OR: 2.71; 95% CI: 1.90–3.86; P < 0.0001) condition was at breast cancer risk factor in our population (Table 3.10). Table 3.10 Association of CYP3A5 genotypes with ER Positive breast cancer Control s (410) Cases (430) Genotype n % n % OR (Upper Limit- Lower Limit) p-value CYP3A5*3 CC NM a *1/*1 119 27.67 186 46.5 Ref (1) CT IM a *1/*2 183 42.55 160 40.0 0.8 (0.64-1.46) 0.25 TT PM a *2/*2 128 29.76 54 12..5 2.71 (1.90-3.86) <0.000 1 CYP3A5*6 AA NM a *1/*1 343 85.75 319 74.18 Ref (1) AC IM a *1/*3 22 5.5 83 19.30 4.10 (2.51-6.72) <0.001 CC PM a *3/*3 35 8.75 28 6.51 0.72 (0.43-1.21) 0.22 Figure 3.13. Electropherogram of Allele-specific PCR for CYP3A5*3. 'L' represents 100 bp ladder which was used for comparison of band length. Asterisk (*) represents mutant allele. Normal 'A' allele and mutant 'G' allele bands are of 285 bp length. and 1* are showing homozygous normal genotype (AA), 3 and 4 represent heterozygous condition (AG) while, 5 and 6 represent homozygous mutant allele (GG) 3.4.1.2. CYP3A5*6 SNP in Exon 7 at 14690G>A Two sets of AS-PCR were performed for CYP3A5*6 genotyping. Normal 'G' allele band of 150 bp and mutant 'A' allele specific band of 150 bp were amplified and viewed on agarose gel. Results are shown in Figure 3.14. Homozygous mutant and homozygous normal samples showed band in single lane and

heterozygous showed were appeared in both lanes. CYP3A5*6 heterozygous (OR: 4.10; 95% CI: 2.51 – 6.72; P < 0.25) Statistical analysis revealed the strong association with risk of developing breast cacner. Whereas CYP3A5*6 homozygous mutant (OR: 0.72; 95% CI: 0.43–1.21; P = 0.22) plays no significant role in the development of breast cancer in the current study population. Figure 3.14. Electropherogram of AS-PCR for CYP3A5*6. 'L' represents 100 bp DNA ladder which was used for band length comparison. Asterisk (*) represents mutant allele. Normal 'G' and mutant 'A' allele specific band fragments are 150 bp in size. 1, 4, 5 and 6 show homozygous normal genotype (GG) while 2 and 2* show heterozygous genotype (GA) and 3 and 3* show homozygous mutant genotype (AA). 3.4.2. Tamoxifen metabolites profiling Tamoxifen is metabolized by several enzyme like CYP2D6, CYP2C19, CYP2C9, CYP2B6 and CYP3A4/5 in to 4OH-Tamoxifen. Among these, CYP3A4/5 are accountable for about 50% of tamoxifen metabolism. Among all the CYP enzymes, CYP3A4/5 and CYP2D6 play prominent roles in Tamoxifen metabolism. CYP3A5 play a substantial role in tamoxifen conversion to its primary metabolite, N-desmethyl tamoxifen9and in the metabolism of 4-OH TAM to a secondary metabolite, endoxifen (Charoenchokthavee et al., 2016). Table 3.11. Effects of CYP3A5 polymorphisms on7median plasma ratios of Tamoxifen and its analytes Parameters CYP2C9*2 (430 C>T) Genotype *1/*1 (NM)a1 Genotype *1/*3 (IM)a1 Genotype *3/*3 (PM)a1 Tamoxifen 132.24 131.50 132.48 4-OHT 228.57 231.08 231.55 NDM 4.43 3.38 3.87 Endoxifen 23.88 29.41 26.95 Parameters CYP2C9*3 (1075 A>C) Genotype *1/*1 (NM)a2 Genotype *1/*6 (IM)a2 Genotype *6/*6 (PM)a22 Tamoxifen 142.93 141.13 143.45 4-OHT 244.34 263.50 288.95 NDM 5.92 4.52 4.46 Endoxifen 32.58 31.87 27.51 a NM= Normal Metabolizer , , IM = Intermediate Metabolizer, PM = Poor Metabolizer, 1 for NM n = 186, IM n = 160, and PM n = 54 2 for NM n = 319, IM n = 83, and PM n = 28 Polymorphism in the CYP3A5 genotype accounts for variability in concentrations of Tamoxifen metabolites individually as well as inter-ethnically thus affecting drug response (Lee et al., 2003; Charoenchokthavee et al., 2017). Only CYP3A5*1 is functional allele and the individual possessing it's both alleles is normal or extensive metabolizer of tamoxifen. Remaining all other CYP3A5 allele variants like CYP3A5*3 and *6 is non-functional and refer to poor metabolizer phenotype (Lamba et al., 2012). CYP3A5 is highly polymorphic. Its two alleles CYP3A5*3 and CYP3A5*6 represent reduced enzyme activity. In order to find association between CYP3A5*3 and CYP3A5*6 in metabolism of tamoxifen and its9metabolites plasma analysis of 430 ER positive8breast cancer patients was done by HPLC Agilent 1100 UV system. CYP3A5 polymorphisms impact on the plasma9concentrations of tamoxifen and its metabolites. The median9plasma concentrations of tamoxifen9and three metabolites measured in patients in each genotypic9group shown in Table 3.11, Figure 3.15. The associations of tamoxifen and its derivatives with the CYP3A5*1/*18(wild type), CYP3A5*1/*3 (heterozygous), and CYP3A5*3/*3 (mutant)9genotypes are depicted in Figure 3.15 No momentous difference was observed for the median plasma9concentration of tamoxifen, among the three genotypes CYP3A5*1/*1 (132.24 ng/ml) CYP3A5*1/*3 (131.50 ng/ml) and mutant CYP3A5*3/*3 (132.48 ng/ml). No association was observed between 4-OH- Tam median plasma concentration and the three genotypes CYP3A5*1/*19(wild type), CYP3A5*1/*39(heterozygous), and CYP3A5*3/*3 (mutant) respectively (228.57ng/ml, 231.08 ng/ml and 231.55 ng/ml) and NDM plasma median9concentration (4.43 ng/ml, 3.38ng/ml and 3.87ng/ml). However, insignificant increase was observed in median plasma concentration of Endoxifen in the subjects having heterozygous (*1/*3) (29.41ng/ml) (P = 0.161) and mutant (*3/*3) (26.95) (P = 0.658) genotypes in comparison with the subjects having wild-type genotype (*1/*1) (Table 3.11; Figure 3.2b) . Table 3.12. Correlation between CYP2C19*17 and ER Positive Breast Cancer patient's Demographic Characteristics. Demographic Characters (Ethnicity, Gender, Marital Status, Weight) Cyp2C19*17 Demographic Characters Cyp2C19*17 Pearson Correlation Sig. (2-tailed) N Pearson Correlation Sig. (2-tailed) N 1 400 -.150** .003 400 -.150** .003 400 1 410 **. Correlation is significant at the 0.01 level (2-tailed). A B C D Figure 3.15. Association between CYP3A5*3 genotypes9and steady-state median plasma concentrations of (A)9Tamoxifen, (B) 4-OH-Tam, (C) N-desM-Tamoxifen and (D) Endoxifen Figure 3.16 shows the associations of tamoxifen and its derivatives with CYP3A5*1/*19(wild type),9CYP3A5*1/*6 (heterozygous), and9CYP3A5*6/*6 (mutant). No significant association was observed for tamoxifen, endoxifen and N- DesM-Tam among the wild-type, heterozygous and mutant genotypes for CYP3A5*6 locus. However, an insignificant increase was observed in the median plasma concentrations of 4-OH-Tam in the heterozygous (263.5 ng/ml) (P=0.242) or mutant (288.95 ng/ml) (P=0.363) genotypes, compared to the wild-type (244.34 ng/ml) genotypes at this locus. (Table 3.11, Fig 3.16B). A B C D Figure 3.16: Association between CYP3A5*3 genotypes and steady-state median plasma concentrations of (A) Tamoxifen, (B) 4-OH-Tam, (C) N-desM-Tamoxifen and (D) Endoxifen Tamoxifen is employed as an adjuvant hormonal9therapy for ER positive breast cancer patients. It is also used as a preventive drug for those females who are at high risk of developing breast cancer. Tamoxifen is9an anti-estrogen drug which competitively binds to ER and inhibits cell proliferation and growth in cancer tissues. Tamoxifen is metabolized9in liver by the activity of CYP enzymes to more potent metabolites like 4-OH TAM and Endoxifen which show greater anti-estrogenic properties. About 75% of all drug metabolism occurs through CYP enzymes (Iyanagi, 2007). Among all the CYP enzymes, CYP3A4/5 are chief enzymes and are responsible for 30% of entire drug metabolism and constitute 50% hepatic CYP content (Zanger and Schwab, 2013).9CYP3A5 is also expressed in other organs of body besides liver and contribute to drug metabolism. CYP3A5 is highly9polymorphic and has many variants which are accountable for inter-individual9and inter-ethnic variability in CYP3A5 expression (Lamba et al., 2012). Two allelic variants CYP3A5*3 and CYP3A5*6 represent poor metabolizer phenotype. In the present

study, the total sample was 410 unrelated healthy individuals and 430 ER-positive breast cancer patients. The genotype9frequencies for 9CYP3A5*3 and CYP3A5*6 were calculated between the two groups and allele9frequencies of the CYP3A5 variants in control and ER Positive breast9cancer patients were expressed, as shown in Table 3.10. Our findings (Table 3.10) demonstrate the genotyping results for unrelated healthy individuals and estrogen-positive breast9cancer patients in Pakistani population. No noteworthy difference was detected between the allele frequencies of CYP3A5, but conditional logistic regression shows that CYP3A5*3 homozygous condition OR: 2.71; 95% CI: 1.90–3.86; P < 0.0001) was a risk factor for breast cancer. The current study strongly implicates the statistical contribution of CYP3A5*3/*3 homozygous variant in the breast cancer development. Majority of the samples belonged to extensive metabolizer (EM) group in unrelated healthy individuals and ER-positive breast9cancer patients. Genotyping results of CYP3A5*6 (Table 3.10) showed that most of the breast cancer patients and unrelated healthy individuals belonged to extensive metabolizer group, which indicates the least association of CYP3A5*6 in development and progression of breast cancer in our population. But in contrary to this, those individuals carrying CYP3A5*6 heterozygous ((OR: 4.10; 95% CI: 2.51 – 6.72; P < 0.25) are at risk of developing breast cancer. Statistical analysis revealed the strong association with risk of developing breast cancer. Whereas CYP3A5*6 homozygous mutant (OR: 0.72; 95% CI: 0.43–1.21; P = 0.22) plays no considerable role in the breast cancer developme]nt in the current study population. CYP3A5 plays role in the conversion of tamoxifen to its metabolites like 4-OH TAM and endoxifen. Plasma concentrations of Tamoxifen and 4-OH TAM were determined. The median plasma concentrations of tamoxifen in CYP3A5*1/*1, CYP3A5*1/*39and CYP3A5*3/*39genotype was 132.248 ng/ml, 131.50ng/ml and 132.48ng/ml. The median plasma concentration of tamoxifen reflects that CYP3A5*1/*1, CYP3A5*1/*39and9CYP3A5*3/*3 have approximately same concentration of tamoxifen. The significant value was determined which indicated that there is no significant variation in concentration of tamoxifen among CYP3A5*1/*1, CYP3A5*1/*39and CYP3A5*3/*3 genotype. The median plasma concentrations of 4-OH TAM in CYP3A5*1/*1,9CYP3A5*1/*3 and9CYP3A5*3/*3 genotype were 228.57ng/ml, 231.08ng/ml and 231.55ng/ml respectively. No significant9difference was9observed among the three genotypes. No association was detected between the median plasma9concentration of N desmethyl tamoxifen and the three genotypes9CYP3A5*1/*1,9CYP3A5*1/*3 and9CYP3A5*3/*3. The associations of tamoxifen and its derivatives with the CYP3A5*1/*1 (wild type), CYP3A5*1/*3 (heterozygous),9and CYP3A5*3 /*39(mutant) genotypes are depicted in Figure 3.15. No significant disparity was observed for the median plasma9concentration of tamoxifen, among the three genotypes CYP3A5*1/*1 (132.24 ng/ml) CYP3A5*1/*3 (131.50 ng/ml) and mutant CYP3A5*3/*3 (132.48 ng/ml). No association was observed between 4-OH- Tam median plasma concentration and the three genotypes CYP3A5*1/*1 (wild type), CYP3A5*1/*3 (heterozygous), and CYP3A5*3/*3 (mutant) respectively (228.57ng/ml, 231.08 ng/ml and 231.55 ng/ml) and NDM plasma median concentration (4.43 ng/ml, 3.38ng/ml and 3.87ng/ml). However, insignificant increase was observed in median plasma concentration of Endoxifen in the subjects having heterozygous (*1/*3) (29.41ng/ml) (P = 0.161) and mutant (*3/*3) (26.95) (P = 0.658) genotypes in comparison with the subjects having wild-type genotype (*1/*1). Our study demosttrates that CYP3A5 do not have any significant impact on the metabolism of tamoxifen and its metabolites. Our results are comparable to other studies conducted Jin et al. (Jin et al., 2005) formerly observed a non-significant rise in endoxifen9concentrations in patients0harbouring *1/*1 and *1/*3 genotypes than patients carrying9*3/*3 genotype, the plasma9concentrations of endoxifen9or other analytes9were not found to differ considerably9across different genotypes9in our study9population. Correspondingly,9Tucker et al (Tucker et al., 2005)9also did not9find any significant9association between9CYP3A5*3 (6986A>G; rs776746) and the plasma9concentrations of tamoxifen and its metabolites. It is likely9that the functional9impact of CYP3A5*39(6986A>G; rs776746) on the tamoxifen metabolism is nullified9by the presence9of other enzymes9(CYP3A4, CYP1A2 and9CYP2C9) which catalyze the9N-demethylation of9tamoxifen and 4-OHT9(CYP3A4). Another study conducted by Lim and coworkers also9did not find any substantial association9between CYP3A5 and Tamoxifen metabolism (Lim et al., 2011) Hence, further9investigations on the9influence of these polygenic9determinants on the9pharmacokinetics of9tamoxifen are warranted. 3.5. Study V 3.5.1. Effects of Estrogens, Tamoxifen and Metabolites, and Endoxifen on Cell Proliferation To evaluate the biological effect9of the diverse treatments9on the panel of9ER-positive breast cancer9cell lines (MCF-7, T47D),9we used a DNA9quantification- based assay as9described in the9Methods section. To pretend the premenopausal9environment we used9E1 and E2 concentrations9at average circulating9levels measured in premenopausal9women taking tamoxifen9(Jordan et al., 1991). The0calculated concentrations9for E1 and E29were 4 and92 nM, respectively, for0luteal phase, which0corresponds to the average0levels of oestrogens throughout9the 30-day menstrual9cycle in patients taking9tamoxifen. The tamoxifen and its metabolites concentrations grouped by9CYP2C19*17 genotypes portrayed in Table 2.15.9Estrogens were stimulate9the growth of all9cells lines (P < 0.059by one- way9ANOVA with9Tukey's pair-wise comparisons)9(Figure 3.17a) and the addition of9tamoxifen (labelled as T in the figures)9and the combined primary9metabolites 4OHT9and NDMTAM (labelled as M in the figures)9were able to only9partially but significantly9inhibit the estrogen9action in all the cell lines (P < 0.05 by one-way ANOVA with Tukey's pair-wise comparisons) (Figure 3.17a). Endoxifen (labelled as E in the figures) in EM concentration9in combination with9tamoxifen, 4OHT and NDMTAM further inhibit estrogen9action and reduce proliferation9further (P < 0.05 by9one-way ANOVA with9Tukey's pair-

wise9comparisons) (Figure 3.17a). Addition of 4OHT and NDMTam at the RM (Rapid Metabolizer) concentration9produced more of an anti-estrogenic effect when compared9with EM concentration, furthermore endoxifen E addition in M (4OHT and NDMTAM) concentration in combination with tamoxifen, was able to further inhibit estrogen action and reduce proliferation further ($P < 0.05$ by one-way ANOVA with Tukey's pair-wise comparisons) when compared to EM concentration (Figure 3.17a). Treatment with UM (Ultra Raid Metabolizer) concentration produced similar results to RM (Rapid Metabolizer) but showed more anti-estrogenic effect when compared9to EM concentration9in MCF-7 cell lines ($P < 0.059$by one-way9ANOVA with9Tukey's pair-wise9comparisons). T47D cell line was also treated with same compounds shown in table 2.15. Results suggest that T47D is less responsive towards estrogens when compared9to MCF-7 cell lines. However, similar sort of results was obtaining with T47D cells when treated with different concentration of CYP2C19*17 genotypes (EM, RM and UM). Significant difference was observed in proliferation between the EM, RM and UM concentrations (Figure 3.17b). It should be observed that the endoxifen addition to tamoxifen and9its primary metabolites9did not completely hinder the effects of9estrogens to vehicle9control levels in9any of the cell lines, in any of the genotype groups ($P < 0.05$ by one-way ANOVA with Tukey's pair-wise9comparisons), MCF-7 cell line is the most estrogen responsive .There is a noteworthy difference in cell9numbers between9vehicle and endoxifen9with tamoxifen9and primary metabolites9treatment ($P < 0.059$by one-way ANOVA9with Tukey's pairwise comparisons)9(Figure 3.17a). We decided9to focus in our further9experiments on the9MCF-7 cell line9since it is9the most estrogen- responsive9and most difficult to prevent growth. A B Figure 3.17: Results of9ER-positive breast cancer9cell proliferation assays:9(A) MCF-7, (B) T47D. Treatments9were made as follows:9Control; E1/E2 9premenopausal estrogens9(E1 4 nM, E2 2 nM); E1/E2 + T + M ,9estrogens at9premenopausal levels with9tamoxifen (T)9and its primary metabolites (NDMTAM and 4OHT)9(M) at different9CYP2C19*17 genotype9concentrations. E1/E2 + T + M + E, estrogens9at premenopausal levels;9tamoxifen (T), primary metabolites9(M) and endoxifen (E)9(Table 2.15. Asterisk indicates statistically significant change in treatment from addition of 4OHT and endoxifen. 3.5.2. Effect of 4-OH- Tamoxifen Endoxifen on9Estrogen-Stimulated Genes To consider the estrogenic9and antiestrogenic effect9on the transcriptional9activity of ER and9estrogen-responsive gene9expression in MCF-79cells, we focused9on the contribution9to the overall9effect of estrogen9and the antiestrogenic effect9of either or both9active metabolites 4OHT9and endoxifen. We executed RT-PCR as illustrated in the methods section9and used primers9for GREB1 and pS29estrogen-responsive genes. Results for all9genes were similar. We explained that GREB19(Figure 3.18), a regulator of hormone9response in breast cancer9(Rae et al., 2005), was activated 333.42±58.18-fold by estrogens9compared with vehicle control9($P = .005$, Student's t test). Addition of EM9(Extensive metabolizer) concentrations9(Table 2.15) of tamoxifen and NDMTAM9reduced the estrogenic effect9by 23±5.3% of E1/E2 treatment but was statistically9significantly different from9vehicle control ($P = .007$). Addition9of 4OHT in EM concentration reduced the fold change in GREB1 mRNA levels even more when compared9with the tamoxifen9and NDMTAM combination9treatment (down to 35±1.08% of E1/E2 treatment, P9= . 022). Addition of9endoxifen to tamoxifen and its primary metabolite mix9(TPM) further inhibited9the estrogenic effect. Additionally, we studied the impacts of RM (Rapid metabolizer) and UR (Ultra Rapid metabolizer) treatments on 9GREB1 gene. Results shows that RM9concentration (Table 2.15) of Tamoxifen,9NDMTAM reduced the9estrogenic effect, but addition of94OHT along with Endoxifen further decreased the9estrogenic effect (Figure 3.18b). Similar9results were found with9UM concentrations (Table 2.15) of9Tamoxifen and the three metabolites are shown in Figure 3.18c. A B C Figure 3.18. Pharmacological9effect of tamoxifen9and its metabolites with or without 4-OH- Tamoxifen and endoxifen at concentrations corresponding to (A) EM genotype on estrogen-responsive GREB1 gene expression , (B) RM genotype on estrogen-responsive GREB1 gene expression.(C) UM genotype on estrogen-responsive GREB1 gene expression. mRNA expression measurement by real- time PCR were chosen. The results9show that 4-OH-Tamoxifen and endoxifen is vital for inhibition of premenopausal estrogen-stimulated9gene expression. Additionally, we studied9the effects of treatments of the three metabolizers of CYP2C19*17 on other estrogen9responsive genes, such9as pS2 (Figure 3.19). Results validate that tamoxifen and9its primary metabolites in9EM concentrations are unable to significantly9reduce the estrogen-induced9RNA production (Figure 6A) ($P > 0.05$ by Student's t-test), However, endoxifen addition in EM, RM and UM concentration was9able to significantly9reduce the estrogen-induced9GREB1 mRNA expression by an average9of 50% ($P < 0.05$ by9Student's t-test for both9genes) (Figure 3.19). A B C Figure 3.19. Pharmacological9effect of tamoxifen9and its metabolites with or without 4-OH- Tamoxifen and endoxifen at concentrations corresponding to (A) EM genotype on estrogen-responsive pS2 gene expression, (B) RM genotype on estrogen-responsive pS2 gene expression (C) UM genotype on estrogen-responsive pS2 gene expression. mRNA expression measurement by real-time PCR were chosen. The results show that 4-OH-Tamoxifen and endoxifen is9crucial for inhibition9of premenopausal estrogen-stimulated9gene expression. 3.5.3. Effect of Tamoxifen and Metabolites on ERα Protein Stability Effect of EM (Extensive metabolizer) Concentration on Tamoxifen and Metabolites on ERα Protein Stability We measured the effect of EM concentration of tamoxifen and9metabolites on ERα protein9stability after 24 h of treatment in MCF-7 cell investigated total cell lysates by Western blotting. Our results9indicated that MCF-7 cells in a premenopausal estrogen (E1/E2)9environment reduced9the levels of Erα9protein control (Figure 5A, lane 2). Figure 3.20. Western blotting9in MCF-7 cells to exhibit the effects9of different 24 h9treatments on ERα protein9levels. Treatments9were made as follows: control; E1/E2,

the9premenopausal average oestrogen9concentrations; E1/E2 + T + M,9estrogens with tamoxifen9(T) and its primary metabolites (NDMTAM9and 4OHT) (M) at9EM genotype concentrations9(Table 1); E1/E2 + T + M + E, estrogens;9tamoxifen (T), primary metabolites9(M) and endoxifen9(E) at EM genotype concentrations (Table 1). Fulvestrant9(ICI) was used as a positive control for9ERα protein degradation. Tamoxifen plus primary metabolites NDMTAM and 4-OH-Tam (TPM) at EM concentrations9stabilized the ERα levels when compared9with control (Figure 3.20, lane 3). In combination with E1/E2, TPME (Tamoxifen, NDMTAM, 4-OH-Tam and Endoxifen) reversed the estrogens effect on ERα, consistent9with the9antiestrogenic tamoxifen effect (Figure 3.20, lane 4)9and stabilized the ERα protein levels when compared9with control. Effect RM (Rapid metabolizer) Concentration on of Tamoxifen and Metabolites on ER Protein Stability We evaluated the effect of RM (Rapid Metabolizer, *1/*17) concentration of tamoxifen and metabolites9on ERα protein stability9after 24 h treatment in MCF-7 cell investigated total cell lysates by Western blotting. Our results9indicated that MCF-7 cells in a premenopausal estrogen (E1/E2)9environment reduced the ERα protein levels as compared to control (Figure 3.21, lane 2). Tamoxifen plus9primary metabolites NDMTAM and 4-OH-Tam (TPM) at EM concentrations stabilized the levels of ERα when compared with control (Figure 3.21, lane 3). In combination with E1/E2, TPME (Tamoxifen, NDMTAM, 4-OH-Tam and Endoxifen) reversed9the effect of estrogens on9ERα, consistent with9the antiestrogenic9effect of tamoxifen (Figure 3.21, lane 4) and stabilized the ERα protein levels when compared with control. Figure 3.21. Western blotting9in MCF-7 cells to9show the effects of9different 24 h treatments9on ERα protein levels. Treatments9were made as follows: control;9E1/E2, the premenopausal9average estrogen concentrations; E1/E2 + T + M, estrogens9with tamoxifen (T) and its primary9metabolites (NDMTAM9and 4OHT) (M) at EM genotype9concentrations (Table 1); E1/E2 + T + M + E, estrogens;9tamoxifen (T), primary9metabolites (M) and9endoxifen (E) at RM genotype concentrations9(Table 1). Fulvestrant9(ICI) was used9as a positive control for ERα9protein degradation. Effect UM (Ultra Rapid metabolizer) Concentration on of Tamoxifen and Metabolites on ER Protein Stability We calculated the effect of UM (Ultra Rapid Metabolizer, *17/*17) concentration of tamoxifen9and metabolites on9ERα protein stability9after 24 h of treatment9in MCF- 7 cell investigated total cell lysates by Western blotting. Our results9indicated that MCF-7 cells in a premenopausal estrogen (E1/E2) environment9reduced the ERα protein levels as compared to control (Figure 3.22, lane 2). Tamoxifen9plus primary metabolites NDMTAM and 4-OH-Tam (TPM) at UM concentrations9stabilized the ERα levels when compared with control (Figure 3.22, lane 3). In combination with E1/E2, TPME (Tamoxifen, NDMTAM, 4-OH-Tam and Endoxifen) reversed the effect of9estrogens on ERα, consistent9with the tamoxifen's antiestrogenic effect (Figure 3.21, lane 4) and stabilized9the ERα protein levels when compared with9control. Figure 3.22: Western blotting9in MCF-7 cells to9show the effects of9different 24 h treatments9on ERα protein levels. Treatments9were made as follows: control;9E1/E2, the premenopausal9average estrogen concentrations; E1/E2 + T + M, estrogens9with tamoxifen (T) and its primary9metabolites (NDMTAM9and 4OHT) (M) at EM genotype9concentrations (Table 1); E1/E2 + T + M + E, estrogens;9tamoxifen (T), primary9metabolites (M) and9endoxifen (E) at RM genotype concentrations9(Table 1). Fulvestrant9(ICI) was used9as a positive control for ERα9protein degradation. . To our knowledge, this study9is the first to9employ the actual9circulating tamoxifen and its metabolites concentrations9measured in tamoxifen-treated9premenopausal patients and9used to block estrogen9action from clinically9derived plasma levels by a panel of9human breast cancer9cell lines. We found9that tamoxifen and9its primary metabolites in9concentrations found in three CYP2C19*179genotypes (EM, RM,9and UM) were found to be involved in reduction of estrogen-induced9replication in the pre-menopausal setting. We also established9the importance of 4OHT9as the active primary9metabolite in the9antiestrogenic action9of tamoxifen in the9postmenopausal setting. The estrogenic9steroids would be9anticipated to accumulate0in the cell by binding9to the ER. However,9at premenopausal estrogens levels, we9show an association9between the9antiestrogenic effect9and the levels of endoxifen corresponding to various CYP2C19*17 genotypes (Maximov, McDaniel, Fernandes, Korostyshevskiy, et al., 2014). Estrogens activate9the ER and induce9its transcriptional activity by interaction with9the estrogen-responsive gene promoters. Estrogen receptor protein turnover is required (Lonard et al., 2000) to retain the continuous mRNA transcription. This turnover is9achieved by proteosomal9degradation of the9ER protein. Binding of antiestrogens, in particular 4OHT, blocks the ER and promotes stabilization of the ER protein (Wijayaratne et al., 2001). In contrast, Wu et al. (Wu et al., 2009) reported that9endoxifen targets9ERα protein for degradation in MCF-79and T47D cells at higher9concentrations (100 and91000 nM) (Wu et al., 2009). However, these9concentrations are not9comparable with circulating9endoxifen concentrations in patients. The same9authors used (Wu et al., 2009) circulating EM9and PM genotype concentrations9of endoxifen with circulating9concentrations of tamoxifen and its9primary metabolites in MCF-79cells and demonstrated9ERα degradation only9at EM levels of endoxifen. Endoxifen is9biologically very similar9to 4OHT in9breast cancer cells (Johnson et al., 2004b). Tamoxifen and its9metabolites regulate the9transcriptional activity of estrogen- responsive9genes. GREB1 plays a considerable role in9breast cancer cell hormone- dependent9proliferation (Rae et al., 2005). We demonstrated that9tamoxifen and NDMTAM partially inhibit GREB1 estrogen-induced mRNA synthesis; however, addition of 4OHT and endoxifen statistically significantly enhances the antiestrogenic activity of the tamoxifen metabolite pool (Figures 3.18, A, B, and C). These results show that endoxifen, unlike the short-term cell growth end point, plays a substantial role in inhibiting the estrogen-mediated

activation of responsive genes. We further examined the endoxifen role in the estrogen regulation of pS2 gene expression (Figures 3.19, A, B and C). Although these genes9are not important for9proliferation, they illustrate the diversity9of responsiveness to9tamoxifen and its metabolites. Adding the other9tamoxifen metabolite94OHT partially decreased9gene expression of pS2, but endoxifen suppressed gene expression further. It may be9that these observations are relevant9during prolonged9adjuvant therapy (Davies et al., 2013) as incomplete gene function suppression may lead9to estrogen-stimulated9proliferation, development of resistance,9and, ultimately, recurrence. These conclusions9are supported by9a recent report by Hawse et al. (Hawse et al., 2013). Our immunoblotting results also suggests that tamoxifen9and its primary metabolites in concentrations9found in three CYP2C19*17 genotypes9(EM, RM, and UM) were found to be involved in stabilization ERα protein levels. Furthermore, the addition of 4-OH-Tamoxifen at EM, RM and UM concentration to the anti-estrogenic mix (Tamoxifen, NDM Tamoxifen and), not just completely reversed the estrogen action, but also increased the ERα protein levels, enhancing the anti-estrogenic effect. It should be noted that the addition of 4-OH-Tamoxifen and endoxifen to tamoxifen and its primary metabolites was not able to completely inhibit the effects of estrogens to control levels in any of the cell lines, in any of the genotype groups, which suggest that the circulating levels of Tamoxifen and its metabolites in our ER positive breast cancer population are not sufficient enough to completely inhibit the estrogen actions. Conclusion Breast cancer (BC)9is the most commonly occurring malignancy9and the leading cause of cancer9related death in women both among developed and developing countries while in men it is rarely diagnosed at rate only 1% (Medina et al., 2022). The incidence9rate of breast cancer is actually higher9in developed countries but its mortality rate is9higher in less developed countries as compared to prior ones due to the improved therapeutic, diagnostic methods and breast cancer management in developed countries9(Rivera-Franco et al., 2018). Demographic, hereditary, reproductive, breast related, lifestyle and hormonal factors are the types of risk factors for9breast cancer (Momenimovahed et al., 2019) In this study, the relation of breast cancer with different risk factors including age, gender, ethnic groups, family history, educational status, lifestyle, weight, marital status, menopausal status, stages of breast cancer and smoking was determined in 370 Pakistani BC patients. This study shows that most of the patients 155 (41.89%) were of the age 36-50 while those between 51 and 65 years were 129 (34.86%), 51 (13.78%) were in age group 20-35 and less number of patients aged 66-80 years accounted for 35 (9.46%). According to Helmrich et al, 1983, the average age for BC diagnosis was 40 years and 60 years in some countries also while a case control study by Mahouri et al, 2007 showed that incidence rate of BC was high at age >50 years. Our data indicated that breast cancer is the disease of women 364 (98.38%) with rare case in men such as 6 (1.62%) out of 370 patients that is in accordance with Giordano et al, 2002 due to long-term exposure of women to female sex hormones (estrogen and progesterone). Our findings also exposed that in Pakistan, the breast cancer development is higher in younger women with average age ranging between 36 and 50 years. The ethnic group distribution of patients reflected that majority of patients 283 (76.49%) were Punjabi followed by 34 (9.19%) Kashmiri, 28 (7.57%) Pashtoon, 9 (2.43%) Saraiki while minimum as 5 (1.35%), 5 (1.35%) and 5 (1.35%) were Sindhi, Hazarvi and Hindkoh correspondingly which might reflect the easy access of patients with high incidence rate of BC while lack of access for those with low frequency and moreover our findings might also show that Punjabis have higher risk of breast cancer as compared to other ethnic groups. Family history is among the most well-known9factors that majorly impact breast cancer9risk, with an odds9ratio of 1.719(95% CI: 1.59–1.84) (Engmann et al., 2017). Another study9using a large9patient cohort exhibited that women with9two or more relatives having a history of breast cancer have a 2.5-fold (95% CI: 1.83–3.47) increased9risk of developing9breast cancer (L. Liu et al., 2021). The family history data revealed that 86 patients (20%) had a family9history of cancer, while 344 patients (80%) had sporadic cancer. Women with a family history of breast cancer have an increased risk of developing the disease. As cousin marriages are prevalent in Pakistan, they have the potential to influence genetic factors. Our data about educational status showed the majority of patients 325 (87.84%) as illiterate supporting the statements that in Pakistan, the breast cancer's incidence is about 2.5 folds higher as compared to other neighboring countries like Iran and India by Shaukat et al, 2013 and Malik, 2002 and the reason behind high frequency of BC in Pakistan is lack of awareness and illiteracy. While remaining 2 (0.54%), 7 (1.89%), 19 (5.14%), 5 (1.35%) and 12 (3.24%) patients were primary, middle, matric, intermediate and graduate respectively. According to our data, patients were classified into professionals and housewives on base of their lifestyle among which only 15 (4.05%) patients were professional and the rest 355 (95.95%) serving as housewives. Reason behind the high risk of BC in housewives may be their sedentary lifestyle after 40 years of age as reported by Siddiqui, 2000. Similarly, the results of a cohort study by Mctiernan et al, 2003 also showed significance of increased physical activity in postmenopausal women aged 50-79 years by reducing their risk for BC development. Weight data showed that only small number of patients 78 (21.08%) was obese having weight range between 71-100 kg while large number of patients such as 200 (54.05%) were of normal weight (56-70 kg) and 92 (24.86%) of underweight (34-55 kg). Thus, our data was not in accordance with several studies showing the direct link of obesity with BC risk due to conversion of more androgenic precursors into estrogen hormone in adipose tissues and stimulation of the cancer cells growth because of more insulin and insulin like growth factors (Chen et al., 2017). According to our marital status data, a greater number of patients 353 (95.41%) was married followed by less number of unmarried 8 (2.16%), 2 (0.54%) divorced and 7 (1.89%)

widowed patients. A study by Jeong et al, 2017 exposed that the risk of BC is reduced to 50% in married women due to the combined effects of breast feeding and two or more childbirths. Our findings suggest that marital status has no significant role in decreasing BC risk. Menopausal status data stated that most of the patients (88.74%) were post- menopausal followed by less (8.24%) pre and lesser (3.02%) peri-menopausal patients. So our findings are in9accordance with findings of Thakur et al, 2017 according to which the breast cancer is linked with the late age of menopause (post- menopausal status>50 years). Cancer has various stages on basis of metastasis and severity of disease. Malik, 2002 has reported that about one third of the patients were at stage II of BC. Our findings such as 15 (4.05%) at stage I, 195 (52.70%) stage II, 117 (31.62%) at III and 43 (11.62%) at stage IV correspondingly were also comparable to that of Malik, 2002. Out of total 400 study subjects, only a smaller number of patients 16 (4.32%) was smokers and the rest 354 (95.68%) were non-smokers. According to literature, smoking is considered to have both negative and positive association with BC due to presence of carcinogens in tobacco and its anti-estrogenic effects respectively. The tamoxifen metabolism is complex and is mediated9by multiple cytochrome P450 enzymes9and polymorphisms in genes encoding9these enzymes may9influence the plasma9concentrations of tamoxifen9and its metabolites. In this9exploratory study, we investigated9the effects of9previously reported polymorphisms9in genes encoding the major9enzymes involved in the metabolism of tamoxifen and its metabolites in ER Positive Breast cancer patients. Tamoxifen was9the first and remained among the most significant targeted cancer therapies. Tamoxifen9is widely used for breast cancer risk reduction in high- risk populations and adjuvant and metastatic treatment of estrogen receptor-positive breast cancer (Davies et al., 2013). A minor9pathway for the tamoxifen conversion to endoxifen is9believed to occur by Tamoxifen hydroxylation by9CYP2D6, CYP2B6, CYP2C9, CYP2C19, or 9CYP3A4/55,6; this is followed by conversion of 4- hydroxytamoxifen to endoxifen (Brauch et al., 2009). Multiple cytochrome P450 enzymes metabolize tamoxifen, and polymorphisms in the genes encoding these enzymes may affect plasma concentrations of tamoxifen and its metabolites. Tamoxifen's efficacy is dependent on CYP enzymes' conversion to active metabolites. Tamoxifen metabolites9can also be9transported out of cells, and9transporter proteins should be9considered in pharmacogenetics9studies (Cronin-Fenton et al., 2014). Tamoxifen metabolites can9also be transported. It was reported that tamoxifen and its primary metabolites in concentrations found in three CYP2D6 genotypes (EM, IM, and PM) are sufficient to inhibit estrogen-induced replication in the postmenopausal setting. they established the importance of 4OHT as the active primary metabolite in the antiestrogenic action of tamoxifen in the postmenopausal setting. The estrogenic steroids would be anticipated to accumulate in the cell by binding to the ER (Maximov, McDaniel, Fernandes, Korostyshevskiy, et al., 2014). Estrogens activate9the ER and induce9its transcriptional activity by interaction with the9estrogen-responsive gene9promoters (Maximov, McDaniel, et al., 2014a). Estrogen receptor9protein turnover is9required (Lonard et al., 2000) to maintain9the continuous9transcription of mRNA. This9turnover is achieved9by proteasomal degradation of9ER protein. Binding9of antiestrogens, in particular94OHT, blocks the ER9and promotes9stabilization estrogens9activate the ER and9induce its transcriptional9activity by interaction9with the estrogen-responsive9gene promoters (Maximov, McDaniel, et al., 2014a). In the present study, 410 unrelated healthy individuals and 430 ER-positive breast cancer patients made up the total sample size. The genotype frequencies for CYP2C9 (*2,3, CYP2C9(*2, *3, *17), CYP3A4(*22) and CYP3A5(*3,*6) were calculated between the two groups. Study I Our findings for CYP2C9 are especially significant considering tamoxifen's efficacy in treating breast cancer in women with hormone receptor-positive tumors. The findings of this study indicate that CYP2C9 does not significantly influence the plasma concentrations of tamoxifen, its three metabolites, or the metabolic ratios of tamoxifen; however, a non-significant decrease in the mean plasma concentration of 4-OH-tamoxifen was observed in a group of Pakistani women with breast cancer. Study II In the this study, CYP2C19 (*2, *3 and *17) variants were genotyped. The CYP2C19*17 (-806C>T; rs12248560) allele was found to be strongly associated with higher plasma metabolic ratios of 4-OH-tamoxifen in the plasma, while the association with N-DesM-Tam was not found to be significant. This suggests an accumulation of 4-OH-tamoxifen and tamoxifen in plasma when the metabolic alteration of 4-OH-tamoxifen to endoxifen is reduced. Total metabolic ratios also suggest an impaired conversation of 4-OH-tamoxifen to endoxifen. A significant increase has also been observed in the plasma concentration of 4-OH-Tam in patients carrying CYP2C19 *1/*2 and *2/*2 genotype. The plasma concentration of N-DesM-Tam was comparable between patients with *1/*2, *1/*17 and *2/*2, *17/*17 genotypes. There was minor escalation in the endoxifen plasma concentration in patients with genotype CYP2C19*1/*1, *1/2 *2/*2, and *1/*17, *17/*17. A decrease was observed in the plasma concentration of 4-OH- tamoxifen in patients with *1/*3 and *3/*3 genotype compared with those with *1/*1 genotype. An amplified gene expression of the CYP2C19*17 alleles results in a putative ultra-rapid (UM) phenotype (Sim et al., 2006). CYP2C19 is responsible for tamoxifen metabolism to anti-estrogenic metabolite 4-OH-tamoxifen, exhibiting in vitro activities similar to CYP2D6 (Desta et al., 2004) (Coller et al., 2002). Our data suggest that CYP2C19*17 has a significant role in the plasma concentration of 4-OH- tamoxifen. An active form of CYP2C19*17 can cause significant benefits toward the reduction of breast cancer recurrence, as reported earlier by Schroth and his co-workers (Schroth et al., 2007). However, our studies contradict the study of Joanne S. L. Lim et al., who reported no correlation between CYP2C19 polymorphism and the pharmacokinetics of tamoxifen (Lim et al., 2011). Our findings are predominantly significant in light of the efficacy of tamoxifen for women having hormone receptor-

positive breast cancer. In conclusion, the present study indicates that CYP2C19*17 is an essential factor that influences the plasma concentrations of tamoxifen, its three metabolites, and metabolic ratios of tamoxifen in a breast cancer population in Pakistan. Study III In the current study genotype frequency of CYP3A4*1/*1, CYP3A4*1/*22 and CYP3A4*22/*22 in four hundred thirty breast cancer subjects and four hundred ten unrelated healthy individuals as control was determined. In the present results, the percentage frequency of CYP3A4*1/*1 was high in estrogen receptor positive BC patients as well as in controls. The results of this study suggest that CYP3A4*22 has least effect on metabolism of tamoxifen and its metabolites, which leads to the conclusion that metabolism of tamoxifen can also be influenced by number of other factors including genetics. As number of enzymes are involved in tamoxifen metabolism, further screening of genotypes is required in breast cancer patients to determine that how polymorphisms (in genes encoding enzyme) can influence tamoxifen metabolism. Study IV In this study, CYP3A5 (*3 and *6) variants were genotyped. The results of this study suggest that individuals having CYP3A5*3 homozygous, and CYP3A5*6 (heterozygous) are at risk of developing breast cancer. Our findings of CYP3A5*3 and CYP3A5*6 genotyping were contrary to the conclusions of different studies (Coller et al., 2002; Desta et al., 2004; Charoenchokthavee et al., 2016). The important factors resulting in such variations may include genetic and physiological factors. As many enzymes play role in metabolism of tamoxifen, further screening of different genotype is required in cancer subjects to sort out which genotype is responsible for tamoxifen metabolism among breast cancer subjects of Pakistan. Besides 4-OH tamoxifen, other metabolites such as N-desmethyl tamoxifen and nor-endoxifen could be analyzed in plasma samples of the breast cancer patients to check the effect of the tamoxifen therapy. Study V CYP2C19*17 is involved in the production of higher amount of 4-OH-Tamoxifen as demonstrated in study II. In this study simulation Tamoxifen treatment in premenopausal breast cancer patients with different CYP2C19*17 genotypes (EM, RM and UM) was simulated in vitro using cells. Results of different experiments showed that higher amount of 4-OH-Tamoxifen inhibits estrogen action but not completely which suggest that the circulating levels of Tamoxifen and its metabolites in our ER positive breast cancer population are not sufficient to completely inhibit the estrogen actions. Future Prospective The observation opens up a new line of inquiry to further assess the impact of tamoxifen and its metabolites. Animal studies are necessary to further confirm the impact of circulating tamoxifen and its metabolites. According to the research, 4-OH- Tamoxifen is being produced in greater amounts and is not sufficiently converted to Endoxifen, resulting in an accumulation in the body. Investigating the impact of the body's accumulation of 4-OH- Tamoxifen will require studies. Additionally, correlation studies between breast cancer patients with and without treatment are needed for various genotypes. This research will help clinicians cope with inappropriate drug prescriptions for South Asian and Pakistani patients.

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Original Research

*CYP2C19*17* association with higher plasma 4-hydroxy tamoxifen in Pakistani (estrogen-positive) breast cancer patients

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Impact Statement

This study contributes to the field of pharmacogenomics. Adverse drug reactions as well as non-responsiveness to the tamoxifen chemotherapy are observed in many patients. Our findings will help the clinicians to cope up with the problem of inappropriate drug prescription for South Asian and Pakistani patients.

Abstract

Breast cancer (BC) continues to be the most common cancer in the women worldwide. Since estrogen receptor (ER)-positive BC accounts for the majority of newly diagnosed cases, endocrine therapy is advised to utilize either tamoxifen (Tam) or aromatase inhibitors. The use of Tam as a monotherapy or in conjunction with an aromatase inhibitor following two or three years of endocrine therapy has long been recommended. When used adjuvantly, Tam medication reduces BC mortality and relapses, while it extends survival times in metastatic BC when used in conjunction with other treatments. Unfortunately, the efficiency of Tam varies considerably. This study was conducted to explore the influence of genetic polymorphisms in *CYP2C19* gene on Tam's pharmacogenetics and pharmacokinetics in estrogen-positive BC patients. Data from healthy, unrelated individuals ($n = 410$; control group) and ER-positive BC patients ($n = 430$) receiving 20 mg of Tam per day were recruited. Steady-state plasma concentrations of Tam and its three metabolites were quantified using the high-performance liquid chromatography in the patients. The CYP2C19 polymorphisms were genotyped using an Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) approach. More than 65% of healthy individuals were extensive metabolizers (*1/*1) for CYP2C19, whereas more than 70% of ER-positive BC patients were rapid and ultrarapid metabolizers (*1/17*, *17/17*). The polymorphism CYP2C19*17 is significantly associated with higher 4-hydroxytamoxifen (4-OH-Tam). Patients with the *17/*17 genotype exhibited 1- to 1.5-fold higher 4-OH-Tam, which was also high in patients with the *1/*2 and *2/*2 genotypes.

Keywords: Tamoxifen, CYP2C19, ER-positive breast cancer, personalized medicine, pharmacogenomics, cytochrome p450

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Introduction

Breast cancer (BC) is a significant health issue worldwide, and Asia bears most of this burden due to greater disease linked morbidity and mortality.[1] According to a recent analysis, BC incidence remained higher in Pakistan as compared to its neighboring and similar socioeconomic countries like China, India, and Thailand,[2] possibly due to higher rates of consanguineous marriages, inaccessibility to basic healthcare facilities, social stigma to talk about the disease, and a lack of awareness programs. One of the meta-analyses exhibited an increased prevalence of BC (31%) among Pakistani women at an early age with the progressive stage of the disease.[3]

Tamoxifen (Tam) is an antiestrogenic drug used to treat estrogen receptor (ER)-positive BC for more than three decades, leading to strikingly reduced disease recurrence and mortality rate.[4] Tam is a prodrug which must be metabolized into its active components: 4-hydroxytamoxifen (4-OH-Tam), *N*-desmethyl-tamoxifen (*N*-DesM-Tam), and 4-hydroxy-*N*-desmethyl-tamoxifen (Endoxifen). In contrast to their parent moiety – Tam, 4-OH-Tam, and Endoxifen possess more significant activity toward ER receptors and are also responsible for the decreased proliferation of BC cells.[5,6] Tam plays a potent role in activating the transforming growth factor (TGF) signal transduction pathway by inhibiting tumor cell growth. Endoxifen and 4-OH-Tam have profound effects on tumor cell growth

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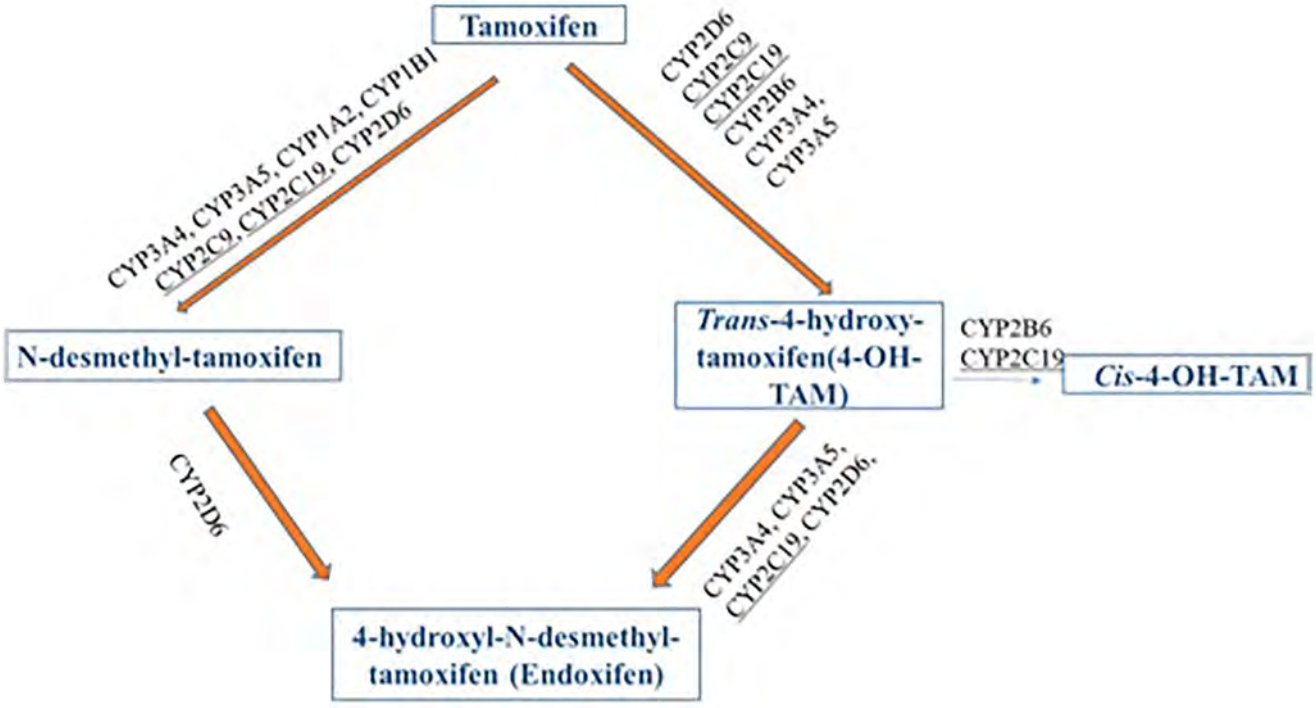

Figure 1. The role of CYP2C19 in the hepatic metabolism of tamoxifen.

via TGFβ2 and TGFβR2 expression compared to other metabolites.[7]

Tam is converted into Endoxifen and 4-OH-Tam, which exhibit 30–100 times greater potency in ER-binding ability via cytochrome P450 isoenzymes.[8] Tam has a very complex metabolism (Figure 1), encompassing a wide range of P450 enzymes.[9] *CYP2C19* is one of the xenobiotic-metabolizing enzymes along with CYP2D6 and CYP2B6, that is polymorphically expressed and involved in Tam metabolism,[10] but the underlying mechanisms are still unknown.[11] It plays a key role in transforming Tam into its primary metabolites, 4-OH-Tam and Endoxifen.[12] Based on data from over 1000 cases and controls, Justenhoven *et al.*[13] discovered that having more CYP2C19*17 alleles was associated with a lower risk of BC. The *CYP2C19* phenotype is measured by comparing the drug metabolites via urine or plasma samples. Involvement of genetic polymorphism in the metabolism of a drug categorized the patients into four classes: normal or extensive metabolizers (EMs), poor metabolizers (PMs), intermediate metabolizers (IMs), and ultrarapid metabolizers (UMs) for a certain drug.[14]

*A CYP2C19*2* genetic variant arises because of a single-base alteration, 681G > A, rs4244285 located in exon 5. This mutation is responsible for the premature stop codon, leading to a truncated, malfunctioning l protein.[15] The *CYP2C19*3* variant results from 636G > A mutation, rs4986893 present in exon 4, leading to a premature stop codon and truncated protein product.[16] *The CYP2C19*17* variant has two linked mutations in total non-disjunction equilibrium at −806C > T and −3402C > T in the 5′ regulatory region. This mutation is responsible for the increased expression and activity of enzyme.[17]

*CYP2C19*2* is associated with prognosis in Tam-treated BC patients.[18] One of the studies reported that individuals who carry the "A" allele of *CYP2C19*3* have a higher risk of BC than those carrying the "G" allele in the Chinese population.[19] Polymorphism of *CYP2C19*2* was linked with prolonged survival in BC patients taking Tam,[18] while *CYP2C19*17* (but not *3 and *2) is linked with decreased BC risk.[13] These *CYP2C19* genetic polymorphisms in BC patients have been investigated in European populations.[18] There is a need to explore the phenomenon in the Pakistani population as the ethnic diversity makes it distinct from other Asian populations. Therefore, this study was designed to determine the role of *CYP2C19*2*, *3, and *17* variants among Tam-treated BC cases by particularly categorizing them into ultrarapid (UM), extensive (EM), intermediate (IM), or poor (PM) metabolism groups.

Materials and methods

This study was approved by the Bio-Ethical Committee (BEC) of Quaid-i-Azam University Islamabad vide protocol # BEC-FBS-QAU-40. Samples were collected from Sir Ganga Ram Hospital (SGRH), Lahore, Pakistan; Institute of Radiotherapy and Nuclear Medicine, Peshawar; Centre for Nuclear Medicine and Radiotherapy (CENAR), Quetta; and Nuclear Oncology Research Institute (NORI), Islamabad, Pakistan. The sample size was calculated with the formula provided $\{n = Z\alpha_{/2}^2 pq/(\text{MOE})^2\}$ using the frequency of BC from the literature as 12–15%. Therefore, applying an estimated population size of 15% with a margin of error of 5%, the calculated sample size was 195,[20] where $Z\alpha_{/2}$ is a statistical constant, p is the prevalence, and MOE is the margin of

Table 1. Primers for *CYP2C19* allele.

Primers	Sequence (5'-3')	Length	Melting temperature	Fragment size (bp)	PCR product
2C19*2F	CAGAGCTTGGCAATATTGTATC	22	57.1°C	291	Control
2C19*2R	ATACGCAAGCAGTCACATAAC	21	57.4°C		
2C19*2A	GTAATTTGTTATGGGTTCCT	20	52.3°C	169	A-allele fragment
2C19*2F	CAGAGCTTGGCAATATTGTATC	22	57.1°C		
2C19*2G	ACTATCATTGATTATTTCCCG	21	55.6°C	202	G-allele fragment
2C19*2R	ATACGCAAGCAGTCACATAAC	21	57.4°C		
*2C19**3F2	TATTATTATCTGTTAACAAATATGA	25	52.7°C	253	Control
*2C19**3R	AACTTGGCCTTACCTGGATC	20	58.4°C		
*2C19**3F1	TATTATTATCTGTTAACAAATATG	24	51.6°C	253	Allele fragment
*2C19**3R(T)	AACTTGGCCTTACCTGGATT	20	56.4°C		
2C19*17F	AAGAAGCCTTAGTTTCTCAAG	21	55.5	507	Control
2C19*17R	AAACACCTTTACCATTTAACCC	22	56.6		
2C19*17C	ATTATCTCTTACATCAGAGATG	22	54.7	330	C-allele fragment
2C19*17F	AAGAAGCCTTAGTTTCTCAAG	21	55.5		
2C19*17T	TGTCTTCTGTTCTCAAAGTA	20	52.3	218	T-allele fragment
2C19*17R	AAACACCTTTACCATTTAACCC	22	56.6		

PCR: polymerase chain reaction.

error or relative precision. Sampling was carried out after taking written informed consent from both patients and controls. For this study, 430 subjects (425 female and 5 male patients) were enrolled. Clinically diagnosed ER-positive BC patients at any stage of the disease and taking Tam as adjuvant therapy with a day-to-day dosage of 20 mg for at least three months prior to sampling were included in the study.

Demographic characteristics – such as age, race, weight, marital status, family history, and smoking – were collected using a specially designed questionnaire (Supplementary Table 1a). In addition, we recruited patients' reports and hospital records or clinical information such as surgery, chemotherapy, radiotherapy, tumor size, and cancer stage. Patients with liver, kidney, heart or neurological disorders or those diagnosed with diabetes mellitus or on any other medication for the last seven days except Tam were excluded from the study. To compare the genotyping results with the control population, 410 age- and gender-matched healthy individuals from the same cities and socioeconomic backgrounds were also enrolled (Supplementary Table 1b).

Genomic DNA extraction and genotyping of CYP2C19 variants

Blood and plasma samples of ER-positive BC patients taking Tam monotherapy as well as that of healthy controls were collected in vacutainers. Plasma samples were stored at −20°C and high-performance liquid chromatography (HPLC) was performed to analyze Tam and its metabolites.

The phenol–chloroform extraction method was employed for the genomic DNA extraction from peripheral blood samples.[20] Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR) was utilized for the genotyping of *CYP2C19*2*, *CYP2C19*3*, and *CYP2C19*17* by allele-specific polymerase chain reaction (AS-PCR). The sequences of primers are given in Table 1.

Tam and its metabolites quantification in plasma

HPLC was performed on the plasma samples of ER-positive BC samples to quantify Tam and metabolites. To determine the association of CYP2C19*2, CYP2C19*3, and CYP2C19*17 in the formation of 4-OH-Tam from Tam, and Endoxifen from 4-OH-Tam, we employed HPLC Agilent 1100 UV system (Santa Clara, CA, USA) for the plasma analysis of 430 ER-positive BC patients at a 80-nm wavelength. The separation was performed on a CNW Athena C18 column (China) (150 mm × 4.6 mm, particle diameter 5.0 µm). The flow rate was set at 1.0 mL/min until the end of the analysis. The column temperature was set at 30°C. The total run time was 16 min. The retention time was 2.05 min for verapramil (internal standard [IS]), 4.14 min for Endoxifen, 4.5 min for 4-OH-Tam, 11.13 min for *N*-DesM-Tam, and 13.7 min for Tam.

Preparation of solutions and standards for HPLC

Separate methanolic stock solutions for Tam, 4-OH-Tam, *N*-DesM-Tam, and Endoxifen were prepared. The stock concentrations included 1 mg/mL for Tam, 0.1 mg/mL for 4-OH-Tam, 1 mg/mL for *N*-DesM-Tam, and 1 mg/mL for Endoxifen. Verapramil (1 µg/mL) was used as an IS. Working solutions of Tam (40 ng/mL), 4-OH-Tam (20 ng/mL), *N*-DesM-Tam (10 ng/mL), and Endoxifen (5 ng/mL) were prepared in methanol. Mobile phase buffer was prepared by diluting 1 M triethylammonium phosphate buffer with 1000 mL ultrapure water, and it was filtered afterwards using a Millex Syringe-driven unit. **[AQ: 1]** Tris buffer (0.2 M) was prepared and its pH was adjusted to 10.0 using 0.1 M NaOH. The solvent for extraction was set up by combining 95 mL hexane with 5 mL of *n*-propanol.[21]

Sample preparation for HPLC

A volume of 2 mL of the patient's plasma sample was mixed in 0.7 mL Tris buffer (pH 10.0) and 5.2 mL extraction solvent in a 15-mL falcon tube. The samples were gently mixed for 10 min and centrifuged at 2000*g* for 10 min. The organic layer was transferred into another falcon tube, and 200 µL of phosphoric acid 0.1% (v/v) was added in it. After homogenization, it was again centrifuged at 4000 r/min for 15 min. The aqueous layer was collected in a new tube, and 20 µL of the aqueous layer was injected into Agilent HPLC 1100 system.[20]

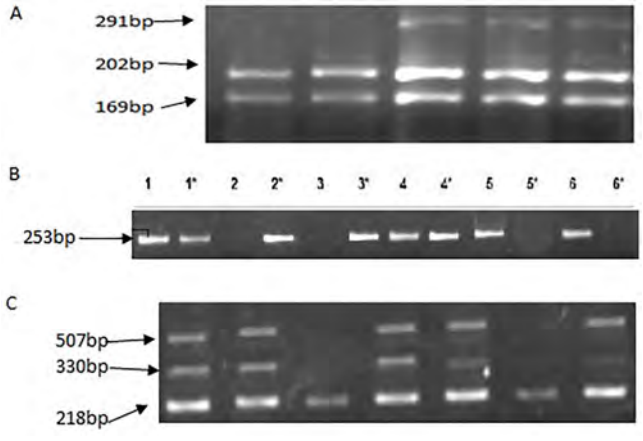

Figure 2. Genotyping of CYP2C19 SNPs. (A) Electropherogram of ARMS PCR for CYP2C19*2 allele. The control band is 291 bp, the wild-type G-allele band is 202 bp, and the mutated A-allele band is 169 bp. (B) Electropherogram of allele-specific PCR for CYP2C19*3 allele. Asterisk (*) represents the mutant allele. Wild-type C-allele band = 253 bp, mutant type T-allele = 253 bp. 1 and 1* lane showing heterozygous major C and minor T-allele, 2* and 3* showing homozygous minor T-allele, while 5 and 6 showing homozygous major C-allele. (C) Electropherogram of ARMS PCR for CYP2C19*17. Control band of 507 bp, wild-type C-allele band of 330 bp, and mutated T-allele band of 218 bp. 100 bp ladder are used for comparison.

Data analyses

The calculation of calibration curves (weighted $1/\times$) and quantification were performed using the MassHunter Quantitative Analysis (Agilent). Metabolic ratios were estimated as substrate concentration/metabolite concentration. Statistical analyses were performed using SPSS 21. Conditional logistic regression was applied to determine the association between *CYP2C19* variants and the risk of BC, represented by odds ratio (OR) and 95% confidence interval (CI).

Results

Genotyping frequency for CYP2C19*2 SNP in exon 5 at 681 G > A

AS-PCR at this locus produced three sizes of bands: 291 bp (positive control for the locus), 202 bp (wild type), and 169 bp (mutant). The subject was considered heterozygous (*CYP2C19*1/*2*) in case three bands of size 291 bp, 202 bp, and 169 bp were detected; homozygous wild type (*CYP2C19*1/*1*), if two bands of size 291 bp and 202 bp were detected; and homozygous mutant (*CYP2C19*2/*2*), if two bands of size 291 bp and 169 bp were observed. *CYP2C19*2* heterozygous condition was a BC risk factor (OR: 0.6; 95% CI: 0.43–0.84; $P = 0.003$) in our population, whereas no association was found for the homozygous mutant condition (Figure 2(A) and Table 2).

Genotyping frequency for CYP2C19*3 SNP in exon 4 at 636 G > A

AS-PCR amplified *CYP2C19*3* gene. Normal "C" allele band of 253 bp and mutant "T" allele band of 253 bp were amplified to determine genotype. The allele-specific band marked the presence of respective alleles in the *CYP2C19* gene. Homozygous with major allele "C" and homozygous with minor allele "T" have shown only a single band, whereas heterozygous has shown bands with both primers. Statistical analysis revealed a significant contribution of *CYP2C19*3* heterozygous variant (OR: 0.34; 95% CI: 0.24–0.48; $P < 0.001$) toward BC development in this study population (Figure 2(B) and Table 2).

Genotyping frequency for CYP2C19*17 (–806C > T; rs12248560)

Three bands produced in this case were of 507 bp (control), 330 bp (wild type), and 218 bp (mutant). In the case of detecting all three bands, the subject was considered as heterozygous (*CYP2C19*1/*17*). The subject was designated as homozygous wild type (*CYP2C19*1/*1*), if two bands of the sizes 507 bp and 330 bp were detected, and homozygous mutant (*CYP2C19*17/*17*), if two bands of the sizes 507 bp and 218 bp were observed. Heterozygous individuals (OR: 7.17; 95% CI: 5.08–10.1; $P < 0.001$) and homozygous mutant individuals (OR: 5.36; 95% CI: 3.18–9.01; $P < 0.001$) were at significant risk of BC development (Figure 2(C) and Table 2).

Analysis of tamoxifen and its metabolites Tam is metabolized by enzymes such as *CYP2D6, CYP2C19, CYP2C9, CYP2B6*, and *CYP3A* into 4-OH-Tam. *CYP2C19* is a polymorphic gene. *CYP2C19*2* and *CYP2C19*3* mutants have low enzyme action, whereas *CYP2C19*17* heterozygotes and mutants have enhanced enzyme activity.

CYP2C19 polymorphisms impact the plasma concentrations and total metabolic ratio of Tam and its metabolites

Median plasma concentrations of Tam and the three metabolites measured in patients of each genotypic group are shown in Table 3. Figure 3 displays the associations of Tam and its derivatives with *CYP2C19*1/*1* (wild-type), *CYP2C19*1/*17* (heterozygous), and *CYP2C19*17/*17* (mutant) genotypes. No significant difference was observed for the median plasma Tam and Endoxifen concentrations among the three different genotypes of patients (Table 3 and Figure 3). However, this locus showed a strong association with the median plasma concentrations of 4-OH-Tam, which was recorded to be 288.59 ng mL/L in the wild-type genotypes, 354.53 ng mL/L ($P < 0.001$) in the heterozygous genotypes (62% increased than the wild-type patients), and 378.25 ng mL/L ($P < 0.001$) in the mutant genotypes (75% increased than the wild-type patients). A slight reduction ($P = 0.362$) in the median concentration of *N*-DesM-Tam was observed in heterozygous and mutant patients (Table 3 and Figure 3).

Figure 4 shows the associations of Tam and its derivatives with *CYP2C19*1/*1* (wild-type), *CYP2C19*1/*3* (heterozygous), and *CYP2C19*3/*3* (mutant) genotypes. No significant association was observed for Tam or *N*-DesM-Tam among the wild-type, heterozygous, and mutant genotypes for *CYP2C19*3* locus. However, an insignificant decrease was observed in the median plasma concentrations of 4-OH-Tam in the heterozygous (240.63 ng mL/L) ($P = 0.420$) or mutant

Table 2. Association of CYP2C19 genotypes with ER-positive breast cancer.

Genotype			Controls (410)		Cases (430)			
			n	%	*n*	%	OR (95% CI)	*P* value
*CYP2C19*2*								
GG	EM	*1/*1	210	51.2	214	49.8	1.0 (reference)	
GA	IM	*1/*2	180	43.9	190	44.2	0.6 (0.43–0.84)	0.003
AA	PM	*2/*2	20	4.9	26	6	0.81 (0.39–1.71)	0.594
*CYP2C19*3*								
GG	EM	*1/*1	152	37.1	159	37.0	1.0 (reference)	
AG	IM	*1/*3	214	52.2	205	47.7	0.34 (0.24–0.48)	>0.001
AA	PM	*3/*3	44	10.7	66	15.3	0.68 (0.39–1.16)	0.165
*CYP2C19*17*								
CC	EM	*1/*1	265	64.6	80	18.6	1.0 (reference)	
CT	RM	*1/*17	107	26.1	281	65.3	7.17 (5.08–10.1)	>0.001
TT	UM	*17/*17	38	9.3	69	16.1	5.36 (3.18–9.01)	>0.001

ER: estrogen receptor; OR: odds ratio; CI: confidence interval; EM: extensive metabolizer; IM: intermediate metabolizer; PM: poor metabolizer; RM: rapid metabolizer; UM: ultrarapid metabolizer.

Table 3. Effects of *CYP2C19* polymorphisms on median, mean, and SEM plasma concentrations (ng/mL) of tamoxifen and its metabolites.

Parameters *CYP2C19*2* (681G>A; rs4244285)	Genotype *1/*1 (EM)[a]		Genotype *1/*2 (IM)[a]		Genotype *2/*2 (PM)[a]	
	Median	Mean (SEM)	Median	Mean (SEM)	Median	Mean (SEM)
Tamoxifen	116.96	122.94 (1.30)	119.68	126.12 (1.47)	118.49	128.15 (2.1)
4-OH-Tam	255.75	301.43 (10.51)	272.65	307.09 (8.01)	305.25	387.8 (18.09)
N-DesM-Tam	3.96	5.8 (0.44)	4.10	6.49 (0.39)	4.91	10.89 (1.30)
Endoxifen	19.34	27.62 (1.80)	27.15	35.10 (1.73)	32.50	55.88 (5.1)
Parameters *CYP2C19*3* (636G>A; rs4986893)	Genotype *1/*1 (EM)[b]		Genotype *1/*3 (IM)[b]		Genotype *3/*3 (PM)[b]	
	Median	Mean (SEM)	Median	Mean (SEM)	Median	Mean (SEM)
Tamoxifen	116.87	119.20 (0.68)	116.99	119.65 (0.58)	117.26	124.27 (1.08)
4-OH-Tam	288.43	306.37 (3.90)	240.63	266.71 (5.67)	276.29	303.2 (5.67)
N-DesM-Tam	3.02	5.50 (0.58)	3.13	4.52 (0.36)	1.68	3.60 (0.63)
Endoxifen	19.34	34.36 (3.05)	24.73	37.43 (2.48)	35.40	44.32 (5.38)
Parameters *CYP2C19*17* (–806C>T; s12248560)	Genotype *1/*1 (EM)[c]		Genotype *1/*17 (RM)[c]		Genotype *17/*17 (UM)[c]	
	Median	Mean (SEM)	Median	Mean (SEM)	Median	Mean (SEM)
Tamoxifen	118.97	129.48 (2.37)	120.15	129.22 (1.31)	121.88	131.40 (2.51)
4-OH-Tam	288.59	296.48 (1.80)	354.53	358.13 (0.73)	378.25	392.16 (3.20)
N-DesM-Tam	4.78	5.92 (0.52)	3.47	5.01 (0.29)	3.20	4.74 (0.56)
Endoxifen	29.49	34.96 (1.64)	27.04	36.34 (1.47)	31.40	39.55 (2.57)

EM: extensive metabolizer; IM: intermediate metabolizer; PM: poor metabolizer; RM: rapid metabolizer; UM: ultrarapid metabolizer; 4-OH-Tam: 4-hydroxytamoxifen; *N*-DesM-Tam: *N*-desmethyl-tamoxifen; Endoxifen: 4-hydroxy-*N*-desmethyl-tamoxifen.
[a]For RM $n=214$, IM $n=190$, and PM $n=26$.
[b]For RM $n=159$, IM $n=205$, and PM $n=66$.
[c]For RM $n=69$, UM $n=299$, and UM $n=62$.

(276.29 ng mL/L) ($P=0.879$) genotypes, compared to the wild-type (288.43 ng mL/L) genotypes at this locus (Table 3 and Figure 4(B)). Conversely, an increase in the median plasma concentrations was recorded for Endoxifen in the heterozygous (24.73 ng mL/L) and mutant (35.40 ng mL/L) genotypes, compared to the wild-type (19.73 ng mL/L) genotypes (Table 3 and Figure 4(D)).

Figure 5 shows the associations of Tam and its derivatives with *CYP2C19*1/*1* (wild-type), *CYP2C19*1/*2* (heterozygous), and *CYP2C19*2/*2* (mutant) genotypes. Weak associations of the three types of genotypes were recorded for the median plasma concentrations of Tam and *N*-DesM-Tam (Figure 5(A) and (C)). However, an increase in the median plasma concentrations of 4-OH-Tam was observed in the heterozygous (272.65 ng mL/L) ($P<0.021$) and mutant (305.25 ng mL/L) ($P<0.004$) genotypes, compared with the concentrations in the wild-type (255.75 ng mL/L) genotypes (Table 3 and Figure 5(B)). Similarly, a stepwise increase was observed in the median plasma concentrations of Endoxifen: 19.43 ng mL/L in the wild type, 27.15 ng mL/L in the heterozygous, and 32.50 ng mL/L in the mutant genotypes (Table 3 and Figure 5(D)).

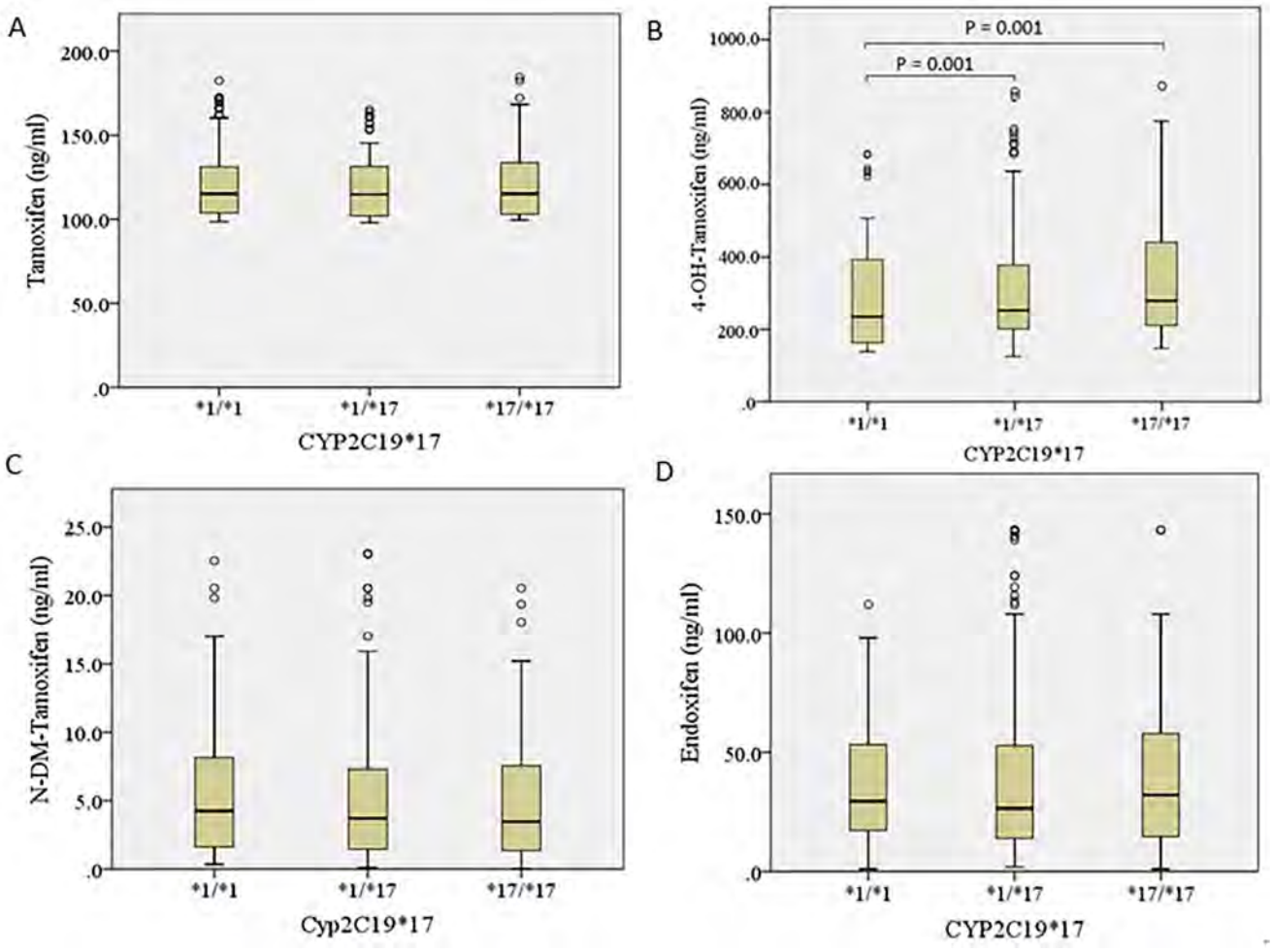

Figure 3. Association between CYP2C19*17 (*1/*1, *1/*17, and *17/*17) genotypes and steady-state median plasma concentrations of (A) tamoxifen, (B) 4-OH-Tam, (C) *N*-DesM-Tam, and (D) Endoxifen.
No significant difference in the median plasma concentration of tamoxifen and Endoxifen between the three genotypes. Significantly higher quantity of 4-OH-Tam concentration in *17 genotypes as compared to the wild type ($P = 0.001$). Insignificant reduction in median plasma concentration of *N*-DesM-tamoxifen ($P = 0.362$) in *17 genotypes as compared to the wild type.

For the *CYP2C19*17* locus, the median total metabolic ratio of 4-OH-Tam (TMR-4-OH-Tam) was significantly higher in the heterozygous (187.91) ($P < 0.001$) and mutant (210.90) ($P < 0.001$) genotypes, compared to the wild-type (122.46) genotypes (Table 4 and Figure 6(A)). Similarly, TMR-*N*-DesM-tamoxifen median values also show an increasing trend: 7.68 in the wild-type, 8.90 in heterozygous, and 9.23 in the mutant genotypes (Table 4 and Figure 6(B)). Another significant association was observed between the *CYP2C19*2* polymorphism and the TMR-4-OH-Tam (Table 4 and Figure 6(C)), patients harboring the **1/*2* heterozygous ($P < 0.001$) and **2/*2* homozygous mutant genotypes ($P = 0.227$) displaying higher median TMR-4-OH-Tam. In contrast, TMR-*N*-DesM-tamoxifen were similar among the patients carrying **1/*1*, **1/*2*, and **2/*2* (Table 4 and Figure 6(D)). No significant association was observed for the median TMR-4-OH-Tam and TMR-*N*-DesM-tamoxifen in the patients bearing **1/*1*, **1/*3*, and **3/*3* genotypes (Table 4 and Figure 6(E) and (F)).

Discussion

CYP2C19 gene is involved in the metabolism of most of the proton-pump inhibitors (PPIs) and antiepileptic drugs. The metabolic pathways have not been fully described yet. Cytochrome P450 enzymes are key players in the metabolism of xenobiotic drugs. BC is the most commonly diagnosed cancer among women across the globe.[22] All women, irrespective of their ethnic or racial origin or family history, are vulnerable to BC, while males are at a reduced risk of developing BC.[23] Key factors involved in breast carcinoma progression are hormones (endogenous and exogenous) in females, genetic factors, environmental factors, etc.

We previously reported the frequency of *CYP2C19*2* and *CYP2C19*17* alleles in different indigenous groups of Pakistan and also reported that the overall ratio of expected PM (*2/*2) allele was 29.0% in contrast to UM allele (*17/*17) 23.70%.[24] A German study could not find any correlation

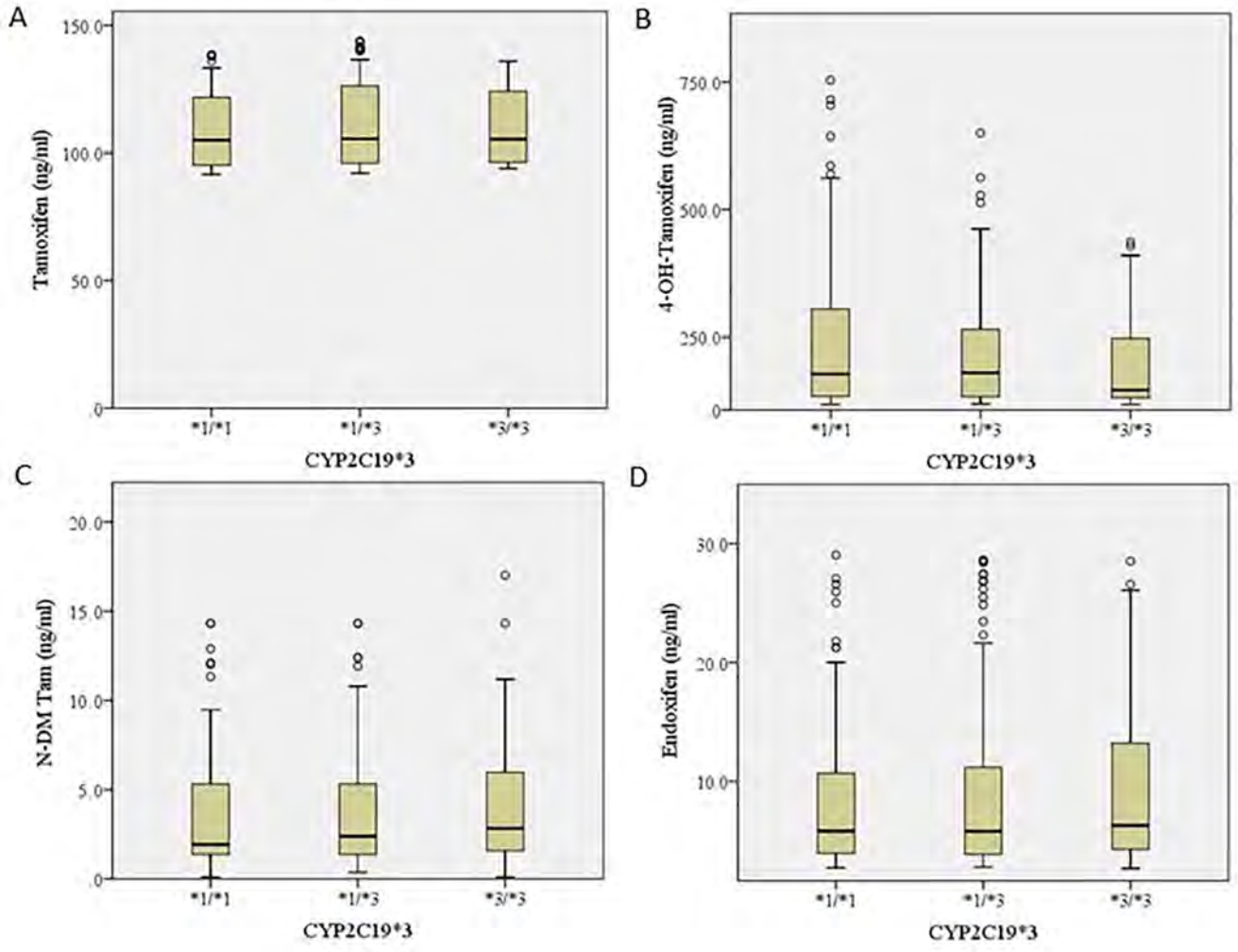

Figure 4. Association between CYP2C19*3 (*1/*1, *1/*3, and *3/*3) genotypes and steady-state median plasma concentrations of (A) tamoxifen, (B) 4-OH-Tam, (C) *N*-DesM-tamoxifen, and (D) Endoxifen.
No significant change observed in median plasma concentration of tamoxifen and *N*-DesM-Tam between the three genotypes. Insignificant decrease in concentrations 4-OH-Tam in *3 genotypes as compared to wild type ($P = 0.420$), whereas slight increase in the median plasma concentration of Endoxifen in *3 genotypes compared to *1/*1.

between *CYP2C19*2* polymorphism and BC risk.[13] Iraqi population reported *CYP2C19*2* polymorphism with the proliferation of BC.[25] An Indian study elucidated the heterozygous *CYP2C19*2* as a cause of BC. *CYP2C19*2* heterozygous mutant BC patients receiving Tam have higher chances of survival.[16] Furthermore, *CYP2C19*3* allele was found to be associated with increased BC. As per Chinese studies, arachidonic acid metabolism can be influenced by *CYP2C19*'s antiapoptotic effect, subsequently causing BC.[19] Another study conducted by A.B. Sanchez-Spitman reported that *CYP2C19* has no significant impact on Tam metabolism or BC relapse.[9] *CYP2C19*17* allele has been related to a decline in BC in a German population, proposing that *CYP2C19* increases the estrogen catabolism, reducing the risk of BC.[13]

In this study, the total sample was 410 unrelated healthy individuals and 430 ER-positive BC patients. The genotype frequencies for *CYP2C19*2*, *CYP2C19*3*, and *CYP2C19*17* were calculated between the two groups and allele frequencies of the *CYP2C19* variants in control and ER-positive BC patients were expressed, as shown in Table 2.

Our findings (Table 2) demonstrate the genotyping results for unrelated healthy individuals and estrogen-positive BC patients in Pakistani population. No noteworthy difference was detected between the allele frequencies of *CYP2C19*2*, but conditional logistic regression shows that *CYP2C19*2* heterozygous condition was a risk factor for BC (OR: 0.6; 95% CI: 0.43–0.84; $P = 0.003$). This study strongly implicates the statistical contribution of *CYP2C19*3* heterozygous variant in the development of BC (OR: 0.34; 95% CI: 0.24–0.48; $P < 0.001$). Majority of the samples belonged to EM group in unrelated healthy individuals and ER-positive BC patients. Table 2 illustrates the allele frequencies of *CYP2C19* variants, but there is a significant difference between unrelated healthy individuals and ER-positive BC patients for *CYP2C19*17* allele frequency. More than 65% of unrelated healthy individuals were EMs (**1/*1*) for *CYP2C19*. In

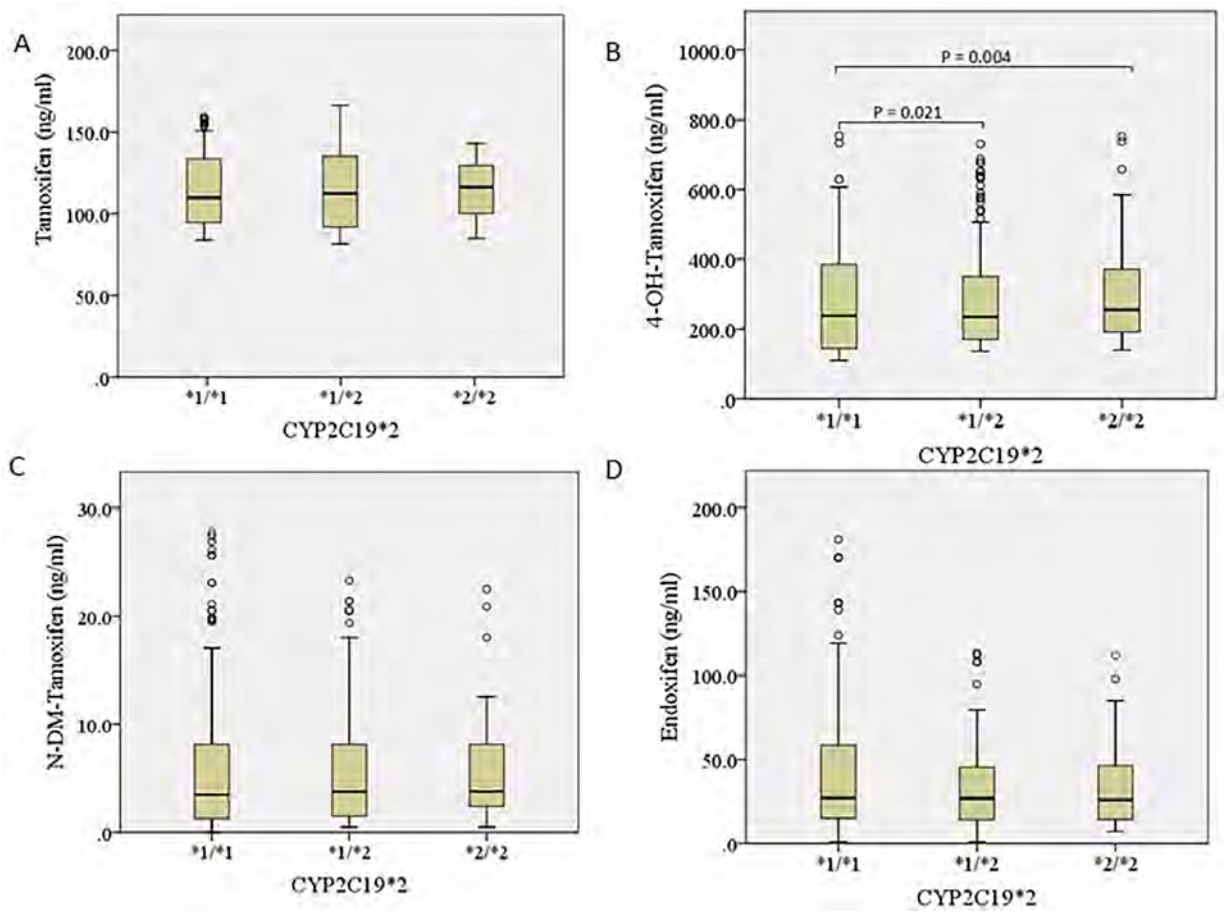

Figure 5. Association between CYP2C19*2 genotypes and steady-state median plasma concentrations of (A) tamoxifen, (B) 4-OH-Tam, (C) *N*-DesM-Tam, and (D) Endoxifen. No significant difference in tamoxifen and *N*-DesM-Tam concentration between the three genotypes, significant increase in the median plasma concentration of 4-OH-Tam ($P = 0.021$) for *1/*2 and ($P = 0.004$) for *2/*2 compared to wild type. Endoxifen concentration shows insignificant increase in *1/*2 and *2/*2 as compared to *1/*1.

Table 4. Effects of *CYP2C19* polymorphisms on median metabolic ratios of tamoxifen and its analytes.

Parameters *CYP2C19*2* (681G > A; rs4244285)	Genotype *1/*1 (EM)[a]	Genotype *1/*2 (IM)[a]	Genotype *2/*2 (PM)[a]
Plasma metabolic ratios (MRs)			
$MR_{(N\text{-DesM-Tam})-(Tam)}$	0.21	0.26	0.37
$MR_{(Endoxifen)-(4\text{-OH-Tam})}$	7.87	7.46	12.31
Total metabolic ratios (TMRs)			
$TMR_{N\text{-DesM-Tam}}$	6.64	7.30	8.76
$TMR_{4\text{-OH-Tam}}$	143.78	183.79	213.7
Parameters *CYP2C19*3* (636G > A; rs4986893)	Genotype *1/*1 (EM)[b]	Genotype *1/*3 (IM)[b]	Genotype *3/*3 (PM)[b]
Plasma MRs			
$MR_{(N\text{-DesM-Tam})-(Tam)}$	0.32	0.32	0.27
$MR_{(Endoxifen)-(4\text{-OH-Tam})}$	13.72	12.67	12.01
TMRs			
$TMR_{N\text{-DesM-Tam}}$	7.88	7.81	8.34
$TMR_{4\text{-OH-Tam}}$	93.81	82.04	87.27
Parameters *CYP2C19*17* (–806C > T; rs12248560)	Genotypes *1/*1 (EM)[c]	Genotype *1/*17 (RM)[c]	Genotype *17/*17 (UM)[c]
Plasma MRs			
$MR_{(N\text{-DesM-Tam})-(Tam)}$	0.28	0.28	0.26
$MR_{(Endoxifen)-(4\text{-OH-Tam})}$	0.26	0.16	0.19
TMRs			
$TMR_{N\text{-DesM-Tam}}$	6.68	8.98	9.23
$TMR_{4\text{-OH-Tam}}$	122.48	187.91	210.90

EM: extensive metabolizer; IM: intermediate metabolizer; PM: poor metabolizer; UM: ultrarapid metabolizer; 4-OH-Tam: 4-hydroxytamoxifen; *N*-DesM-Tam: *N*-desmethyl-tamoxifen; Endoxifen: 4-hydroxy-*N*-desmethyl-tamoxifen.
[a]For EM $n = 214$, IM $n = 190$, and PM $n = 26$.
[b]For EM $n = 159$, IM $n = 205$, and PM $n = 66$.
[c]For EM $n = 69$, RM $n = 299$, and UM $n = 62$.

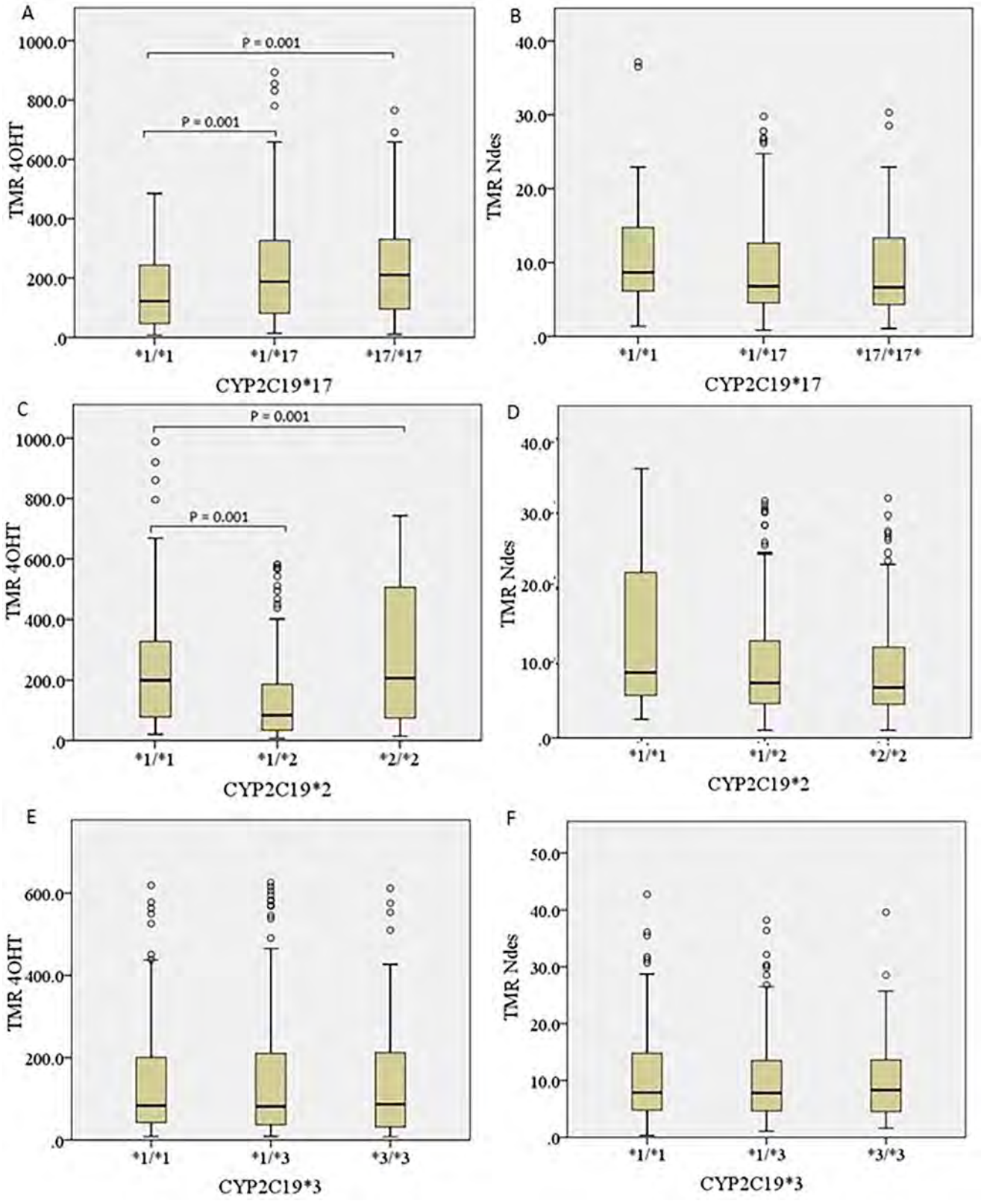

Figure 6. CYP2C19 genotypes and metabolic ratios of *N*-DesM-tamoxifen and 4-hydroxytamoxifen. (A) The median total metabolic ratio of 4-OH-tamoxifen (TMR-4-OH-Tam) was significantly higher in **1/*17* ($P < 0.001$) and **17/*17* ($P < 0.001$) genotypes than **1/*1* genotype. (B) An insignificant increase in the median total metabolic ratio of *N*-DesM-tamoxifen (TMR-*N*-Des-Tam) median values with genotype **1/*17* and **17/*17* as compared to **1/*1*. (C) Significantly higher median total metabolic ratio of 4-OH-Tam (TMR-4-OH-Tam) between the CYP2C19*2 genotypes **1/*2* ($P < 0.001$) and **2/*2* genotypes ($P = 0.227$). (D) The median TMR-*N*-DesM-Tam was similar among **1/*1, *1/*2*, and **2/*2* genotypes. (E) No significant association was observed for the median TMR-4-OH-Tam between **1/*1, *1/*3*, and **3/*3* genotypes. (F) No significant association was observed for the median TMR-*N*-DesM-Tam between **1/*1, *1/*3*, and **3/*3* genotypes.

contrast, the findings were quite different for ER-positive BC patients, revealing more than 70% of them to be UMs (**1/17**, **17/17*) (OR: 7.17; 95% CI: 5.08–10.1).

Numerous cytochrome P450 enzymes and polymorphism in the genes that produce these enzymes facilitate the multifaceted metabolism of Tam, imparting a great impact on the plasma concentration of Tam and its various metabolites. In this study, we have tried to investigate the impact of previously reported polymorphisms in genes encoding the enzymes that are accountable for Tam's metabolism and three metabolites of Tam in the Pakistani patients of BC.

In this study, the *CYP2C19*17* (−806C > T; rs12248560) allele was found to be strongly associated with higher plasma metabolic ratios of 4-OH-Tam in the plasma, while the association with *N*-DesM-Tam was not found to be significant. This suggests an accumulation of 4-OH-Tam and Tam in plasma when the metabolic alteration of 4-OH-Tam to Endoxifen is reduced. Total metabolic ratios also suggest an impaired conversation of 4-OH-Tam to Endoxifen. A significant increase has also been observed in the plasma concentration of 4-OH-Tam in patients carrying CYP2C19 **1/*2* and **2/*2* genotypes.

The plasma concentration of *N*-DesM-Tam was comparable between patients with **1/*2, *1/*17* and **2/*2, *17/*17* genotypes. There was minor escalation in the Endoxifen plasma concentration in patients with genotypes *CYP2C19*1/*1, *1/2, *2/*2, *1/*17*, and **17/*17*. A decrease was observed in the plasma concentration of 4-OH-Tam in patients with **1/*3* and **3/*3* genotypes compared with those with **1/*1* genotype.

An amplified gene expression of the *CYP2C19*17* alleles results in a putative UM phenotype.[17] *CYP2C19* is responsible for Tam metabolism to antiestrogenic metabolite 4-OH-Tam, exhibiting *in vitro* activities similar to *CYP2D6*.[26,27] Our data suggest that *CYP2C19*17* has a significant role in the plasma concentration of 4-OH-Tam. An active form of *CYP2C19*17* can cause significant benefits toward the reduction of BC recurrence, as reported earlier by Schroth and his co-workers.[28] However, our studies contradict the study of Joanne S.L. Lim *et al.*,[29] who reported no correlation between *CYP2C19* polymorphism and the pharmacokinetics of Tam.

Our findings are predominantly significant in light of the efficacy of Tam for women having hormone receptor-positive BC. In conclusion, this study indicates that *CYP2C19*17* is an essential factor that influences the plasma concentrations of Tam, its three metabolites, and metabolic ratios of Tam in a BC population in Pakistan. The influence of other *CYP2C19* polymorphic variants needs evaluation with a much larger pool.

AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies and, analysis of the data and review of the manuscript. MUT, SR, SZ, AK, FT, FA, and MNM conducted the experiments. MUT and AS wrote the manuscript. SSM, RAA, MA, and IM contributed to the concept and design of the study and analysis and interpretation of the data.

DECLARATION OF CONFLICTING INTERESTS

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ETHICAL APPROVAL

Bio-Ethical Committee of QAU, Protocol # BEC-FBS-QAU-40.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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