Republic of Iraq
Ministry of Higher Education and Scientific Research
University of Kerbala
College of Pharmacy

Effect of Genetic Polymorphism of *CYP3A4* and *ABCB1* on Tamoxifen Efficacy in Iraqi Breast Cancer Women

A Thesis
Submitted to the Council of College of Pharmacy/ University of Kerbala as Partial Fulfillment of the Requirements for the Master Degree of Science in Pharmacology and Toxicology

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بِسْمِ اللَّهِ الرَّحْمنِ الرَّحِيمِ

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دَرَجَـٰتٍۚ وَٱللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ﴾

صَدق الله العليُ العظيم

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Dedication

To all the patients who are suffering from Cancer …… I wish them healthy life

To my parents and all my beloved family who have been my source of inspiration

To my faithful husband who constant encouragement, limitless giving and helped me accomplished my degree and chase my dream

To my beloved daughters, who have given to me a life time of love, support and laughter

To all the people in my life who touch of my heart

Arjwan 2024

Acknowledgements

First, I render my gratitude to ALLAH who bestowed me with self-confidence, ability and strength to complete this work.

I owe a sincerest gratitude to the Dean of Pharmacy College at Kerbala University, Asst. Prof. Dr. Mohammed Ibrahim Rasool for giving me the chance to complete my thesis.

I would like to thank everyone at the College of Pharmacy / Department of Pharmacology and Toxicology and Clinical Laboratory Science for their invaluable assistance, advice, and generous support.

With deep sense of gratitude, I express my sincere thanks to my esteemed and worthy supervisor Prof. Dr. Ahmed Salih Sahib whose excellence as a researcher and supervisor has enabled the completion of this thesis.

I would like to thank my co-supervisor Dr. Jamal Ali Ashoor for his support, and for sharing his profound knowledge with me.

I would like to express my heartfelt appreciation and gratitude to Dr. Shaima Jabbar for her invaluable support, assistance and advice.

I am deeply grateful to all doctors, medical staff "pharmacists and nurses" and laboratory staff at Imam AL- Hussein Medical City /Oncology center in Kerbala for their generous help, valuable advice and kindness during the clinical study.

My special thanks go to my beloved friend clinical pharmacist at Imam Hussain Medical City/Oncology Center Ph. Bushra Baroody for her assistance and continuous support.

My deepest appreciation to the patients participating in this study and their families who helped me complete this thesis.

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List of Abbreviations	
Abbreviation	Meaning
4-OH TAM	4-hydroxy tamoxifen
ABCB1	ATP- binding cassette transporter family B member 1
ARMS	Amplification refractory mutation system
AS	Allele specific
BCS	Breast conserving surgery
BMI	Body Mass Index
bp	base pair
CA 15.3	Cancer antigen 15.3
ChE	Cholesterol esterase
CHO	Cholesterol
CHOD	Cholesterol oxidase
CLIA	Chemiluminescent immunoassay
CYP p450	Cytochrome p 450
CYP2D6	Cytochrome P450 Family 2 Subfamily D Member6
CYP3A4	Cytochrome P450 Family 3 Subfamily A Member4
CYP3A5	Cytochrome P450 Family 3 Subfamily A Member5
CYP2C19	Cytochrome P450 Family 2 Subfamily C Member 19
CYP2C9	Cytochrome P450 Family 2 Subfamily C Member 9
DCIs	Ductal carcinoma in situ
DFS	Disease free survival
DNA	Deoxyribonucleic acid
E1	Estrone
E2	Estradiol
EDTA	Ethylenediaminetetraacetic acid
Endoxifen	4-hydroxy-N-desmethyltamoxifene
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERE	Estrogen receptor element
ET	Endocrine therapy
GnRHa	Gonadotropin-releasing hormone agonist
HDL	High density lipoprotein
HER2	Human epidermal growth factor receptor 2
IBC	Invasive breast carcinoma
ICR	Iraqi cancer registry
IGF-1	insulin like growth factor
IF	Inner forward
IPA	Ingenuity pathway analyses
IR	Inner reverse
LCI	Lobular carcinoma in situe
LDL	Low density lipoprotein
MDR1	Multi drug resistance 1
MRI	Magnetic resonance imaging
ND-TAM	N-desmethyl tamoxifen

NST	Invasive breast cancer of no specific type
OF	Outer forward
OR	Outer reverse
PCR	Polymerase chain reaction
P-gp	P- glycoprotein
Pmol/µl	Pico mole per microliter
POD	peroxidase
PR	Progesterone receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SERDs	Selective estrogen receptor degraders
SERMs	Selective estrogen receptor modulators
SNPs	Single nucleotide polymorphisms
SULTs	Sulfotransferase enzyme
TAM	Tamoxifen
TBE	Tris/Borate/EDTA
TNBC	Triple negative breast cancer
TNM	Type, number, metastasis
TG	Triglyceride
UGT	Uridine5- diphosphate glucuronosyl transferase
US	Ultrasonography

Abstract

Background: Breast cancer is the most common type of cancer affecting women. Tamoxifen is a selective estrogen receptor modulator considered the cornerstone in the treatment of hormonal receptor positive breast cancer. Tamoxifen is a prodrug and converted to the more active metabolites by many metabolizing enzymes. Pharmacogenomics has a critical impact on the interindividual variability that affect treatment response, and it is important to study the polymorphic genes included in tamoxifen kinetics. Therefore, the genetic polymorphisms of tamoxifen's important metabolizing enzyme *CYP3A4* and tamoxifen with its metabolites efflux transporter *ABCB1* might have potential impact on therapeutic outcome of tamoxifen.

The study aims to: detect the *CYP3A4*22* G>A (rs35599367) and *ABCB1* C3435T (rs1045642) genetic polymorphisms among breast cancer Iraqi women included in this study and to investigate the influence of these genes' variants on tamoxifen efficacy.

Patients and methods: This is an observational cross-sectional study conducted at Imam Al-Hussein Medical City and Imam AL-Hassan AL-Mujtaba Teaching Hospital /Oncology Center in Kerbala along with laboratories of College of Pharmacy / University of Kerbela carried out between November 2022 to April 2023. One hundred female patients with breast cancer that were estrogen-receptor and / or progesterone-receptor positive were selected. Patients aged 45- and above, being on tamoxifen 20 mg daily dose for at least four months were enrolled in this study. The study also included one hundred healthy women provides a critical comparison group for biochemical analysis (Estradiol level, Tumor marker CA15.3, Calcium and lipid profiles).

Five milliliters of venous blood were drawn from each patient after they are signed written informed consent to measure biochemical parameters such as estradiol, CA15.3, calcium, and lipid profile in addition to genetic analysis. The Amplification Refractory Mutation System and Allele Specific Polymerase Chain Reactions were used for the detection *CYP3A4*22* G>A (rs35599367) and *ABCB1* C3435T (rs1045642) respectively.

Results: The obtained findings demonstrated that there were different genetic variants of *CYP3A4*22* G>A (rs35599367) and *ABCB1* C3435T (rs1045642). For rs35599367 G>A, the wild homogenous (GG) the most common genotype followed by homogenous mutant (AA) and the (GA) heterogeneous genotypes are less common with 39%, 35% and 26% frequencies respectively. But for rs1045642 C>T, the mutant homogenous (TT) is the most frequent genotype while, the heterogeneous (CT) and wild homogenous (CC) genotypes are less with 43%, 29% and 28% frequencies respectively. The levels of estradiol, calcium, and low-density lipoprotein in the serum showed insignificant, while, level of serum tumor marker CA15.3 show significant association between the studied two single nucleotide polymorphisms of *CYP3A4* and *ABCB1* genes with p-value less than 0.05. Furthermore, the present study found that hot flash side effect occurs at high incidence about 82% and low recurrence rate of 5%.

Conclusion: The present study concluded that there be a correlation between genetic variation in *CYP3A4*22* and *ABCB1* genes and the variability in the response to tamoxifen therapy among Iraqi women with breast cancer in which the wild GG genotype of *CYP3A4*22* rs35599367 and the mutant TT genotype of ABCB1 rs1045542 are good responder groups with severe hot flashes occurrence.

Chapter One
Introduction

1. Breast cancer

1.1. Overview

Among all cancers, breast cancer considered the most frequent women disease diagnosed globally also the primary reason to cancer-related deaths in women (Nisar et al., 2024). Usually, breast cancer starts as an overabundance of ductal cell proliferation, which progresses to benign or metastatic tumors. Ageing, smoking, reproductive variables like early menstruation, late menopause, and family history are some of the many risk and causal elements that lead to the development of breast cancer (Britt et al., 2020).

Breast cancer is diverse disease and is characterized by many hallmarks, progesterone receptor (PR), estrogen receptor α (ER), and human epidermal growth factor receptor 2 (HER2) expression which considered indicative of diverse nature of breast cancer. ER-positive (ER+) female breast cancer makes up approximately 70% of all breast cancer cases. One practical strategies for treating ER + ve breast cancer is to target ERs (Almeida et al., 2020) (Yang et al., 2024).

Diagnosis is usually confirmed by a biopsy and histopathological analysis. After diagnosis is made, further tests are done to detect the presence of metastases and to plan for the treatment protocol (Mutebi et al., 2020). Surgery, hormonal therapy, radiotherapy, chemotherapy, and targeted therapy are the different lines of therapy of breast cancer patients (Britt et al., 2020). Tamoxifen, an antiestrogen used to treat breast carcinoma possibly prevent it or halt its progression in high-risk groups (Ottenbourgs & Van Nieuwenhuysen, 2024).

1.2. Epidemiology:

Worldwide, the most frequent cancer to be diagnosed in female is breast cancer, surpassing lung cancer, with a projected more than 2 million newly diagnosed cases representing 11.7 % of overall cancer cases, 684,996 instances of breast cancer are expected to die from the disease. These high incidence and death rates make breast cancer a real health problem worldwide. (Sung et al., 2021) (Arnold et al., 2022).

Since mammography screening became widely used, breast cancer incidence rate has risen. Although this cancerous tumor is becoming more common worldwide, industrialized nations have the highest prevalence of this tumor and developed nations account for about half of all cases worldwide. (Ferlay et al., 2020). The dominant western lifestyle, linked to bad dietary habits, obesity, high levels of stress, and physical inactivity, is mostly responsible for this trend (Chatterjee et al., 2024).

More than 250,000 cases of breast cancer are detected in the United States each year (Giaquinto etal., 2022). The World Health Organization (WHO) predicts that between 2012 to 2030, breast cancer rates in the Middle East would double, reflecting the greatest relative increase of any region in the world (Bray et al., 2018).

Among Iraqi women, breast cancer considered the most widespread malignant tumor and the 2nd most common reason for mortality of Iraqi women after cardiovascular disorders (N. A. S. Alwan, 2016). As shown in figure (1-1), the report of the Iraqi Cancer Registry (ICR) for the year 2019 revealed that, over the past twenty years, breast cancer has risen and being the first of the community's ten most common malignant neoplasm (19.7%), followed by bronchi and lungs. (N. A. S. Al Alwan, 2022).

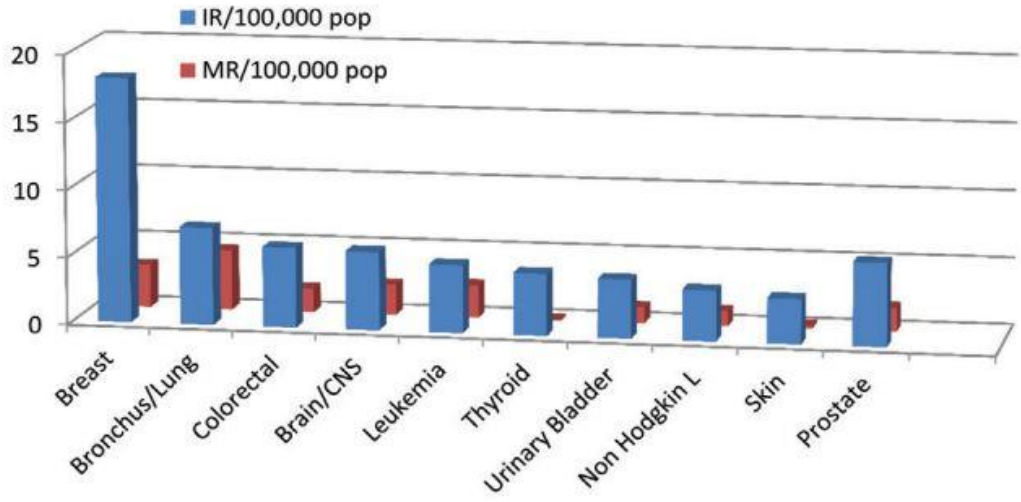

Figure (1-1): Incidence and mortality rates per 100,000 populations of the top ten cancers in Iraq (2019)(N. A. S. Al Alwan, 2022)

1.3. Pathophysiology

Certain genes and signaling pathways control normal breast growth which regulate motility, differentiation, cell death, and division. The development of initial tumors and metastases are caused by gene dysregulation involved in several signaling processes. The multi-step process known as the "metastatic cascade" comprises the local tumor cells invasion the passage to circulation, the elimination of those cancerous cells from the circulation, and ultimately the colonization at distant organs. (Chhichholiya et al., 2021). Normally, cells undergo programmed cell death, also known as cell suicide, as soon as their use has not been needed. However, malignant cells are distinguished by their unregulated growth and avoidance of apoptosis, which prolongs their survival. (Pfeffer & Singh, 2018).

The risk of having a breast tumor is increased by numerous factors, much like other malignancy (Obeagu & Obeagu, 2024).

Damage to the deoxyribonucleic acid (DNA) and genetic alterations have been linked to estrogen exposure, indirectly via its proliferation effect on the tissue of the breast, or directly through its impact on DNA, increasing the risk of genetic mutations that play a role for essential cellular processes such as proliferation, repair of DNA, and apoptosis can guide to breast cancer (Pfeffer & Singh, 2018).

1.3.1. The Impact of Estrogen on the Breast Cancer Progression

Three steroid hormones that make up estrogen are estriol, 17β-estradiol (E2), and estrone (E1). During adolescence and sexual maturity, 17β-estradiol, the main ovarian steroid in circulation, has a role in breast growth. Additionally, it increases the development and spread of target tissue malignancies in the ovaries, colon, lung, breast, endometrial, prostate, and ovary (Yaşar et al., 2017).

Premenopausal women produce estradiol from the ovaries, but postmenopausal women primarily produce estrone from peripheral organs (adrenal glands and adipose tissues). The enzyme that catalyzes the transformation of androgens into estrogens is called aromatase. E1, is created when androstenedione is aromatized in extra glandular tissues. In peripheral tissues, the enzyme 17β-hydroxysteroid dehydrogenase can reversibly convert E1 to estradiol. (Fuentes & Silveyra, 2019).

The two receptors that estrogen uses to communicate are ERα and ERβ. Only ERα is required for breast growth and is the one that starts proliferative signaling in both healthy breast tissue and breast cancer, in breast, ERβ frequently acts in opposite to ERα (Mal et al., 2020).

Because estrogen can promote the expression of genes encoding various growth factors, it can be linked to ER-mediated proliferation and growth and the initiation of cancer (Torres etal., 2021).

Synthesis of reactive oxygen species (ROS) and DNA depurinating adducts from activated metabolites, such as catechol estrogen, semiquinones, quinones, and free radicals, produced during the metabolism of estrogens, could have a critical role in having breast cancer (Starek-Świechowicz et al., 2021).

It was shown that the enzymes required for the conversion of E2 to the estradiol DNA adducts are present in both normal breast tissue in aromatase transfected mice and MCF-7 breast cancer cells (which are widely utilized as a laboratory model to investigate breast cancer biology). Following the animals' E2 injection, genotoxic compounds were found in the breast tissue. (Starek-Świechowicz et al., 2021).

It was observed that in ERα knockout transgenic mice, tumor formation was less common (Rusidzé et al., 2021). These findings confirm the genetically toxic ER-independent as well as ER-dependent impact of E 2 on the development of breast cancer.

1.4. Clinical Manifestation

The primary sign of breast cancer is usually an abnormal mass that is distinct from the tissue around it. Other signs include skin cracking, nipple drainage, breast discomfort in a particular area, swelling under the axilla, changes in the size of one breast and modification in nipples appearance. Early breast cancer typically does not produce any symptoms. It is, therefore, very important for women to follow recommended guidelines for finding breast cancer before symptoms develop (H. Li et al., 2021).

Breast cancer may manifest as a disease with metastases. Metastatic breast cancer is a possible symptom of the disease. The brain, liver, bone, and lung are common places for metastasis. Depending on the metastatic site, symptoms can include fever, loss of appetite, jaundice, painful bones, or neurological problems. (R. Wang et al., 2019).

1.5. Etiological Factors

Breast cancer etiology is complex and the illness is likely caused by a combination of hereditary factors, hormonal impact on the mammary glands as well as lifestyle factors (Obeagu & Obeagu, 2024).

Apart from being female, aging is a major risk factor of breast cancer. Other risk marker reflecting an accumulative exposure in the epithelium of the breast to estrogen are early age of menarche and older age at menopause, not experiencing a full-term pregnancy or having the first pregnancy after the age of 25, not breast feeding, postmenopausal obesity, being physically inactive and exogenous hormonal elements such as oral contraceptives and hormone replacement therapy (Osei-Afriyie et al., 2021).

Additional risk factors include alcohol consumption, radiation therapy to the chest or breasts before the age 30 and previous history of neoplastic disease or hyperplasia in the breast (Michaels et al., 2024).

Hereditary factors account for around 10% of breast cancer cases. A woman’s risk is higher if she has a relatives who have received a diagnosis of breast- or ovarian carcinoma (Sun et al., 2017). Women with BRCA1, BRCA2, and germline pathogenic variations and P53 have an elevated danger of breast cancer. (Consortium, 2021).

1.6. Breast cancer histological classification

1.6.1. Noninvasive breast cancer

A precursor tumor only affects a specific area within breast tissue; This never propagates to neighboring tissues, lobules, or ducts. These atypical cells have the potential to develop into metastatic breast cancer even they cannot propagate outside of the lobules or ducts. (Wullkopf et al., 2017).

Many types such as lobular carcinoma in situ (LCIS) and ductal carcinoma in situ (DCIS) are instances of this form of cancer. DCIS happens when abnormal cells form in lactation ducts inner lining yet fail to disseminate on neighboring tissue. Breast lobules are where LCIS starts to proliferate; it has not yet migrated to other breast tissues (Nicosia et al., 2024).

1.6.2. Invasive breast cancer

Wide spectrum malignancies that exhibit variety in their shape, behavior, and clinical presentation are referred to as invasive breast cancers (IBC). WHO recognizes at least eighteen distinct forms of histopathological breast cancer (Cserni, 2020).

Invasive breast cancer of no specific type (NST) is the most prevalent category (40%-80%), originally referred to as invasive ductal carcinoma. (Nguyen & He, 2021).

Approximately 25% of IBC exhibit unique cytological characteristics and development patterns, leading to their classification as certain categories (neuroendocrine, mucinous A, mucinous B, invasive lobular carcinoma, tubular). (Erber & Hartmann, 2020).

1.7. Intrinsic Molecular Classification of Breast Cancer

According to the levels of messenger RNA gene expression, IBC can be classified molecularly regardless of histopathological subtypes. This allows to identify microarray gene expression data into four molecular categories: Basal-like, luminal, normal breast-like and human epidermal growth factor receptor 2 (HER2)-enriched (Shah et al., 2020)**.** Since normal mammary glands are likely to have contaminated the sample, normal breast like subclass is no longer present.

1.7.1. The Luminal Breast Cancer

Luminal breast cancer, or ER-positive tumors, account for over 70% of all cases of breast cancer in Western countries. (Pandit et al., 2020). Two subtypes of this subclass, Luminal A and luminal B, were identified, each with a distinct clinical prognosis.

The absence of HER2 and the presence of ER and/or PR are characteristics of Luminal A tumors. exhibits a decreased level of gene expression involved in proliferation of cells in this subtype. They often have a good prognosis, are low-grade, and grow slowly (Johnson et al., 2021). Luminal B tumors have a poorer outcome and are of a higher grade than subtype A. They might be HER2-positive and/or PR-negative, in addition to being ER-positive. Moreover, genes linked to proliferation are highly expressed in it. (Raj-Kumar et al., 2019).

1.7.2. Human Epidermal Growth Factor Receptor2

10–15% of breast cancer patients belong to the HER2-enriched group. It is distinguished by HER2 being highly expressed whereas ER and PR are absent. Proteins and genes involved in proliferation, including as HER2, which mostly presented with this subclass. (Raj-Kumar et al., 2019).

The HER2 enriched subtype had a poorer outcome and a faster growth rate than luminal tumors until the introduction of HER2 targeted treatment (H. Chen et al., 2024).

1.7.3. Triple-Negative/Basal-Like Carcinoma

A diverse group of breast tumors with the characteristics of being triple negative breast carcinoma (TNBC) which is negative for PR, ER and HER2. They make up roughly 15% of all cases of breast cancer. (Liman et al., 2022). TNBC is frequently linked to a worse prognosis and has a tendency to be biologically aggressive (da Silva et al., 2020).

1.8. Prognostic and Predictive Factors for Breast Cancer

These factors can be measured during the moment of patient diagnosis often used to predict the tumor's type. In contrast to prognostic biomarkers, predictive biomarkers aid in the early identification of patients who may respond favorably or poorly to a certain treatment.

Breast cancer has set the standard for the application of therapeutic predictive biomarkers. For instance, more than 40 years ago, the assessment of ER and PR for predicting the outcome of hormonal therapy was introduced into clinical practice. Although HER2 testing was standard practice over a decade ago to predict trastuzumab efficacy (Sinn et al., 2019).

1.8.1. Estrogen receptor

Since roughly 70% of breast carcinoma identified by markedly elevated expression of ER, estrogen receptor is a crucial predictive factor. (Y. Li et al., 2020). ER belonged to nuclear steroid receptor class (Acconcia et al., 2021).

The use of ER expression as a diagnostic biomarker for breast cancer is supported by the correlation found among the expression of this gene and a family history of the disease, particularly among situations where there is a family risk. (Tse et al., 2015).

1.8.2. Progesterone receptor

Being specific to progesterone, the progesterone receptor belongs to the nuclear hormone receptor family. ER-positive individuals have significant levels of PR expression (>50%), whereas ER-negative patients rarely have this expression. (Y. Li et al., 2020). Since both PR and ER highly found in cells of breast cancer, they are regarded as important prognostic and diagnostic indicators for the disease (particularly ER+ tumor) (Wu et al., 2020).

1.8.3. The Human Epidermal Growth Factor -2

Tyrosine kinase HER2 receptor is one of epidermal growth factor family. When it forms homodimers or heterodimers with other HER receptors, it activates a number of intracellular signals that are involved in improved survival of cells, growth, and differentiation. (Miligy et al., 2019). About 15–25% of breast tumors are HER2 positive, and the expression of this gene is important for deciding how best to treat patients with breast cancer (Mohanty et al., 2022).

1.8.4. Histological grade

Histological grade classifies breast cancer tumors according to their degree of differentiation and reflects how well the tumor cells resemble normal cells when viewed digitally or under a microscope. Histologic grade is strongly correlated with prognosis. Grade III tumors are poorly differentiated and tend to be more aggressive (Xing et al., 2020).

1.8.5. Breast cancer staging

One significant predictive factor is the tumor stage. Using a TNM staging model, “T” describes largest dimension of primary breast tumor, ”N” stands for number for affected local lymph node, “M” describes if patients has distant metastasis (Ostapenko, 2024). One important predictor of early-stage breast cancer survival is metastasis to local lymph nodes (Fitzgibbons et al., 2000). The latest (8th) edition of the TNM classification has also added other factors, such as tumor grade, proliferation rate, ER-, PR- and HER2-status as well as results from genomic panels (Giuliano et al., 2017).

By comparison, stage 0 refers to the noninvasive stage of a malignancy, meaning that there are both malignant and non-malignant cells inside the breast’s boundaries region where site of tumor initiation to grow. While, Stages one to three can develop throughout breast tissue and/or the primary lymph nodes, but the stage four is thought to be cancer spread to other parts of the body with a unfavorable outcome (Agre et al., 2021).

1.8.6. The tumor marker

Tumor markers are biomarkers present in blood (serum), urine, or bodily tissues that are either released from tumor itself or its host in reaction to a tumor. After initial chemo-and radio therapies, it is mainly utilized to determine the presence of tumors and assess the state of disease progression (Shreevatsa et al., 2024).

Among these markers, the cancer antigen 153 (CA 15.3). Majority of early-stage breast cancer patients and almost all women with advanced-stages breast cancer have elevated blood levels of CA15.3. Levels of CA15.3 typically fall after successful management (Nam et al., 2019).

1.9. Breast Cancer Diagnosis

In women, breast cancer accounts for a large number of cancer-related fatalities, but early diagnosis of the disease play important role to better disease prognosis, therapy planning, and increased survival (Siegel et al., 2024).

1.9.1. History and Physical Examination

It is important to fully investigate both one's personal and family histories. Patients should be checked for specific symptoms such weight loss, breast ache, painful bones, fatigue, and discharge from nipple. Physicians perform physical evaluations which involve examining the breasts, neck region, and axillae (Lohani et al., 2023).

1.9.2. Mammography Screening

A mammogram is regarded as the gold-standard method for early diagnosis of breast cancer in females; In addition to providing 2-dimensional pictures that are well saved and sent to a radiologist for assessment, it aids in the detection of masses in dense tissue (Gilbert & Pinker-Domenig, 2019).

1.9.3. The magnetic Resonance Imaging

For a long time, magnetic resonance imaging (MRI) had been regarded suitable screening technique to identification of breast cancer. It is a magnetic field-based technique. While mammography has a greater specificity, MRI has a higher sensitivity (Jaglan et al., 2019).

1.9.4. Ultrasonography

Ultrasonography (US) is widely utilized for tumor detection, breast cancer screening for other condition and axilla assessment. (Gilbert & Pinker-Domenig, 2019).

Due to its lack of radiation, quicker imaging times, increased sensitivity and accuracy, and reduced cost compared to mammography and MRI, US is now widely used for tumor detection and diagnosis (Iacob et al., 2024).

1.9.5. The Positron Emission Tomography

Positron emission tomography is a molecular image technique for breast cancer. It is an extremely sensitive method that is useful for some therapeutic applications, including the systemic staging of recently discovered locally advanced disease, evaluating treatment response in patients with metastatic cancer, and anticipated disease recurrence recognition. (Ulaner, 2019).

1.9.6. The Breast Biopsy

The gold standard method for detecting breast cancer is through breast biopsy. To improve the accuracy of the diagnosis and avoid false positive finding, diagnosis and assessment of breast, image of the breast, , and breast biopsy together be done (Montezuma et al., 2019).

1.10. Treatment Strategies of Breast Cancer

Methods for treating breast cancer aims at improving quality of life, delay the spread of the disease, and increase patients' survival. The characteristics of the tumor and its clinical stage at diagnosis determine the available treatment choices (N. Alwan & Shawkat, 2020).

Modern therapies used for early breast cancer involves combinations of local modalities including surgery and radiotherapy as well as systemic therapies, including hormonal therapy, chemotherapy and other specialized treatment, in various sequences and combinations (Filetti et al., 2019).

1.10.1. Loco regional treatment

1.10.1.1. Surgery of breast cancer

One important aspect of treating breast cancer is surgery and is for most patients the first step of their multimodal treatment. Nearly half the early stage breast cancer patients will not experience relapse after primary surgery alone, or after surgery followed by radiotherapy. Breast conservation surgery (BCS) and mastectomy are two types of surgical treatment (Christiansen et al., 2022).

Breast conserving surgery makes it possible to remove malignant cells while simultaneously preserving healthy breast tissue. This technique is frequently used in conjunction with oncoplasty, a type of cosmetic surgery. A mastectomy involves removing the entire breast, and it is usually associated with rapid reconstructive surgery.

Better cosmetic results, less psychological impact on the patient, and fewer surgical problems are the main benefits associated with BCS. (Tahmasebi et al., 2022).

1.10.1.2. Radiotherapy

Following surgery and/or chemotherapy, radiotherapy is a common local treatment for breast cancer. Radiotherapy aims to reduce the likelihood of a return of breast cancer by ensuring that all malignant cells are eradicated. Additionally, it is beneficial when breast cancer spreads to other areas or becomes unrespectable (Corradini et al., 2019).

1.10.2. Systemic therapy

1.10.2.1. Preoperative systemic treatment

Neoadjuvant (preoperative) treatment is used to downstage the tumors to reduce the surgical extent, but also yields information on the response to therapy. The neoadjuvant approach is at present also preferred in patients with tumors larger than two cm, with risk factors indicating a benefit of chemotherapy, i.e., in triple negative, HER2-positive, grade III or node positive disease (Montemurro et al., 2020)

1.10.2.2. Adjuvant treatment

The purpose of adjuvant treatment is to eradicate microscopic foci of cancer cells that might remain after breast cancer surgery, or micro-metastases that have get away from the regional lymph nodes and surrounds the breast, as to lower the risk of the distant, local, and recurrent breast cancer-related death (Filetti et al., 2019).

1.10.3. Chemotherapy

Chemotherapy is a cytotoxic medication used to stop the growth and proliferation of malignant cells. Because of its non-selective action, it can also affect healthy cells and cause serious adverse effects like diarrhea, vomiting, nausea, anemia, and thinning hair. It can also damage the kidney, bone marrow, and heart, which increases the risk of the patient's death (Ingole et al., 2024).

Adjuvant chemotherapy is recommended in HER2 positive, luminal –B and triple negative tumors. The most common current regimens include anthracyclines and/ or taxanes.

Postoperative chemotherapy with anthracyclines and taxanes reduces the relative risk of mortality of breast cancer by roughly one-third within the initial ten years, compared to no chemotherapy (Group, 2023).

Recent data suggest that the addition of carboplatin and the taxanes paclitaxel to anthracyclines is an effective alternative in triple negative disease (Yu et al., 2020). Adjuvant capecitabine improves disease-free survival (DFS) and yields an absolute gain of around 4 % in overall survival in patients with remaining cancer cells after neoadjuvant therapy with anthracyclines and taxanes (Joensuu et al., 2022).

1.10.4. Biological treatment

At every step of breast therapy, biological treatment can be given in two ways: either as neoadjuvant therapy before surgery or as adjuvant therapy after surgery. The standard course of treatment for patients diagnosed with HER2-positive breast cancer is biological therapy, which often includes the use of trastuzumab, deruxtecan,.lapatinib, and neratinib (Marra et al., 2024).

1.10.5. Hormonal (Endocrine) therapy

Estrogen influences tumor growth and oncogenesis in many cancer types. In breast carcinogenesis, it has been demonstrated that endocrine therapy (ET), which includes ovarian suppression in premenopausal women, and selective estrogen degraders (SERDS), inhibits the steroid hormone’s ability to promote tumor growth, through ER (Huang et al., 2023) (Woolpert et al., 2024).

ET works to inhibit the spread of breast cancer cells by lowering estrogen levels. However, during such treatment, half of cases of hormonoreceptor-positive breast cancers show increased resistance to hormonal treatment (Łukasiewicz et al., 2021).

Hormonal therapy is highly effective in both pre- and postmenopausal patients and possibly utilized as a neoadjuvant or adjuvant treatment for those with breast cancer who have the luminal-molecular subtype. It is employed effectively in situations where breast cancer has metastasized or recurred (Rej et al., 2024).

Patients with breast cancer who receive ET in addition to chemotherapy seem to have lower death rates (Sestak et al., 2019).Although ET has been demonstrated to increase breast cancer patients' chances of survival; nevertheless, long-term adherence to endocrine therapy is still a significant challenge, in part because to the adverse effects of the medication, which have an impact on a patient's quality of life, social function, and adherence to therapy (Y.-K. Lee et al., 2020).

1.10.5.1. Estrogen-depleting hormonal (endocrine) therapy

1.10.5.1.A. Gonadotropin releasing hormone agonist

Goserelin, a monthly depot injection of gonadotropin releasing hormone agonist (GnRHa), and radiation therapy to the ovaries or surgical ovarian excision are two methods used to suppress estrogen in premenopausal women, goserelin act: by reducing the gonads' production of sex hormones and gonadotropins, which lowers the generation of endogenous estrogen (Lu et al., 2021).

Ovarian suppression in conjunction with tamoxifen compared to tamoxifen alone improves survival rate, with an absolute improvement of around 2% (Francis et al., 2023).

1.10.5.1.B. Aromatase inhibitor

After menopause, the ovary no longer releases estrogen, and the enzyme aromatase, which is present in numerous tissues including fat, liver, muscle, and breast cancer cells, is primarily responsible for synthesizing estrogen from nonglandular sources (Sahu et al., 2023); Thus, aromatase inhibitors will lower endogenous estrogen levels and lower the prognosis of breast cancer (Mohammed Alwan et al., 2022). Right now, third-generation aromatase inhibitors are being used, including letrozole, exemestane, and anastrozole (Bertelsen et al., 2024).

1.10.5.2. Endocrine treatment that directly target estrogen receptors

1.10.5.2.A. Selective estrogen receptor degrader

Selective estrogen receptor degraders are antiestrogens that attach to the ER to promote its breakdown in order to disrupt the ER's signaling pathway and prevent dimerization, hence destabilizing the function of the estrogen receptor(Memon & Patel, 2022).

1.10.5.2.B. Selective estrogen receptor modulator

Because ERα is primarily responsible for the development and progression of breast cancer, selective estrogen receptor modulators (SERMs) clearly regulate ERs and slow down the growth of breast malignancy. They work as both antagonists of the transcription process in the breast cancer cell and agonist in other tissues, such as the endometrium and bone; On the other hand, prolonged use of SERMs is linked to a number of adverse effects, including the incidence of endometrial cancer and other conditions (Das et al., 2022). The class of anti-estrogen medications most frequently prescribed for breast cancer are (tamoxifen, toremifene and raloxifene) (Patel et al., 2023).

1.11. Tamoxifen

Tamoxifen is a selective estrogen receptor modulator has been used as adjuvant therapy either before surgery or after surgery for the treatment of both early-stage and metastatic breast cancer for more than 40 years. Tamoxifen is one of the most commonly utilized hormonal therapy for treating ER-positive breast cancer (Rodriguez et al., 2024).

Adjuvant tamoxifen significantly lowers mortality and relapse rates in premenopausal and postmenopausal breast cancer women (Basmadjian et al., 2024). It is one of the safest and most effective medications needed in a health system, according to the WHO List of Essential Medicines (Jenei et al., 2022). It was first identified in 1960, and the FDA authorized it in 1977 for use in treating patients with metastatic breast cancer. Later, it was allowed for use as an adjuvant treatment for early breast cancer (Hu et al., 2024). Despite its effectiveness, tamoxifen adjuvant therapy is ineffective for 30 to 50% of individuals. While some of them shown no response, others displayed unfavorable reactions or a recurrence of cancer and even death (X. Chen et al., 2024).

1.11.1. Mode of action of Tamoxifen

When estradiol binds to a target cell's ER, a series of events happen. When the estrogen-ER complex homodimers, it binds to certain DNA sequences in the so-called estrogen response element (ERE), which is regulatory area found in estrogen-sensitive gene. The two estrogen-ER complex transcriptional activation activities, AF1 and AF2, communicate with other proteins known as transcriptional coactivators which stimulate ribonucleic acid (RNA) polymerase II effects so control gene activity (Hohl & Marcelli, 2023).

Tamoxifen completely prevents estrogen from binding to ER. Additionally, homodimerization and binding of the tamoxifen-ER complex to the ERE of estrogen sensitive genes. Furthermore, only AFl remains active, and AF2 inactivation decreases coactivator binding and the transcriptional activity of the estrogen-responsive gene. As a result, tamoxifen restricts cell growth by preventing the Gl phase of the cell cycle (Clemons et al., 2002).

It increases the inhibitory growth factor transforming growth factor β's synthesis, which could have a negative paracrine effect on breast cancer cells. Tamoxifen inhibits synthesis of IGF-1(insulin like growth factor), which functions as a strong mitogen for breast cancer(Antunes et al., 2015).

1.11.2. Pharmacodynamics of tamoxifen

Tamoxifen binding opposes the effects of estrogen binding, forming a nuclear complex that lowers DNA synthesis and stops estrogen from acting on breast cancer cells. As a result, estrogen is unable to bind to the cancer cell, preventing it from receiving signals from estrogen to divide and growth (Miziak et al., 2023). Different genetic variants may result in different responses from the individuals to the medication (Golubenko et al., 2024).

1.11.3. Pharmacokinetic of tamoxifen

After being taken orally, tamoxifen is rapidly absorbed and peak plasma concentrations of roughly 40 µg/L were seen in 4–7 hours whereas steady state level reached after three to four weeks of therapy. Over 99% of tamoxifen is bound to plasma proteins, primarily albumin (Shahbaz, 2017). Following an oral dose of 20 mg of tamoxifen, there was a high concentration in breast tissue and lymph nodes, above the serum ratio and 50–60 L/kg is the volume of distribution (Furlanut et al., 2007).

Tamoxifen undergoes extensive liver metabolism, approximately 65% of the dosage is eliminated from the body within two weeks, with fecal excretion as the main route of excretion. Primarily, tamoxifen is eliminated as polar conjugates, along with unconjugated metabolites and unaltered drug responsible for fewer than 30% of fecal excretion (Helland et al., 2017). Tamoxifen exhibits a biphasic fall in plasma levels, with a final elimination half-life of roughly 5 to 7 days (Cyrus et al., 2010).

Disposition of tamoxifen and its metabolites may also be influenced by a transmembrane transport protein that acts as an adenosine triphosphate-dependent (ATPase) efflux transporter pump (P-glycoprotein), since tamoxifen, N-desmethyltamoxifen and 4-hydroxytamoxifen have been reported to be substrates for P-glycoprotein, based on ATPase stimulation (Tazzite et al., 2016).

1.11.3.1. Metabolism of tamoxifen

Because of its weak ER binding affinity, tamoxifen is considered a prodrug. After ingestion, some metabolism takes place in the small intestine, but the main metabolism takes place in the liver (Helland et al., 2021). Phase I and II enzymes, including cytochrome p450 (CYPs), sulfotransferases (SULTs), and uridine 5'-diphospho-glucuronosyltransferase (UDP-glucuronosyltransferase, UGT), catalyzed the comprehensive metabolism of tamoxifen into a variety of metabolites. CYPs enzymes initially demethylate or hydroxylate tamoxifen after it enters the liver through the hepatic portal vein (Sidibe et al., 2024). CYP3A4 (Cytochrome P450 Family 3 Subfamily A Member4) and CYP3A5 (CYP Family 3 Subfamily A Member5*)* -mediated metabolism of tamoxifen to N-desmethyltamoxifen (ND-Tam) is responsible for 92% of tamoxifen metabolism (Desta et al., 2004).

Subsequently, the metabolite ND-Tam continues to be catalyzed by CYP2D6 (Cytochrome P450 Family 2 Subfamily D Member6) and generates 4-hydroxy-Ndesmethyltamoxifen (endoxifen) as represented in figure (1-2) (Jin et al., 2005).

CYP2D6 metabolizes the minor route from tamoxifen to 4-hydroxy tamoxifen (4-OH-Tam), with small roles from *CYP3A4*, *CYP3A5*, *CYP2C19*, and *CYP2C9* (Cytochrome P450 Family 2 Subfamily C Member 19 and 9 respectively). 4-OH-Tam is additionally converted to endoxifen. 4-OH-Tam and endoxifen are the two most major therapeutically active metabolites (Mulder et al., 2021). Endoxifen has an average plasma concentration approximately six times greater than 4-OH-Tam in breast cancer patients receiving tamoxifen (20 mg/day) (Stearns et al., 2003). According to these results, endoxifen is the most potent tamoxifen metabolite (Souwer et al., 2023)

Following phase I metabolism, the parent drug and its metabolites are subjected to further sulfation and glucuronidation, which are facilitated by *SULT* and *UGT* enzymes. Ultimately, these byproducts are eliminated through bile and urine (Ahern et al., 2011). Some research have revealed that 4-OH-tam and endoxifen have similar efficacy in terms of ER-binding affinity, as well as have comparable anti-estrogenic effects on the prevention of proliferation of breast cancer cell lines MCF-7, T47D, and BT474(Hoskins et al., 2009).

An important enzyme in tamoxifen metabolism, *CYP2D6* accounts for as much as 39% of the variation in endoxifen and 4-OH-tam plasma concentration (Fohner et al., 2013). Each pathway's enzymes are controlled by polymorphic genes, and changes in the activity of enzymes cause inter-individual variability in the plasma levels of main metabolites, which may account for individuals' response variations.

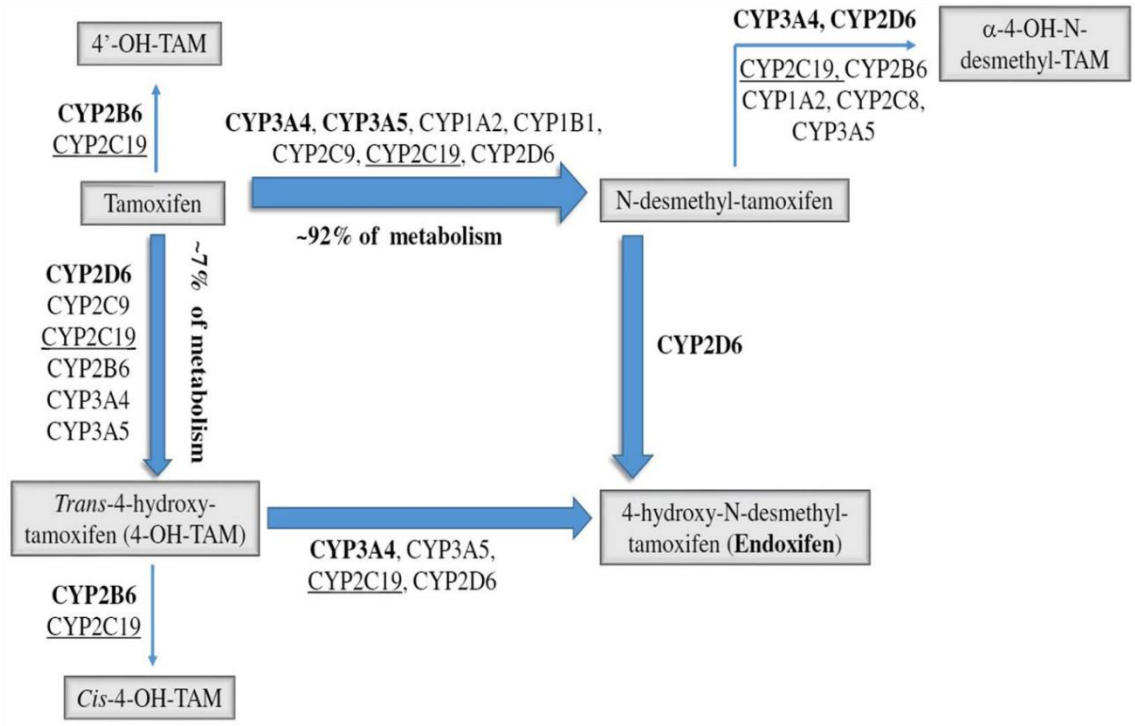

Figure (1-2): Metabolic Pathways of Tamoxifen (Boocock et al., 2002)

1.11.4. Medical uses of tamoxifen

1.11.4.1. Breast cancer

Tamoxifen is frequently used in pre- and postmenopausal female to treat ER+ breast cancer, regardless of its stage. (Rugo et al., 2016). Tamoxifen use is recommended for five to ten years (Gupta et al., 2018).

Tamoxifen frequently induces remission in men with metastasis whose tumors express estrogen receptors. For male breast cancer patients, it is now the gold standard endocrine therapy (Sonia et al., 2017).

1.11.4.2. Infertility condition

50%-90% of infertile women with anovulatory disorders are prescribed tamoxifen to induce ovulation; 30%-50% of these women end up pregnant (Jie et al., 2018). It helps women when clomiphene treatment fails. Neither multiple pregnancies nor ovarian hyperstimulation are occurs with tamoxifen (Jie et al., 2018).

These results might be associated with enhanced corpus luteum activity and an enhanced cervical mucus score (Dhaliwal et al., 2011).

1.11.4.3. The gynecomastia

Tamoxifen can be used to avoid or cure gynecomastia. It is used as a prophylactic strategy in small doses, or used as soon as one notice any signs of discomfort, including nipple sensitivity or pain, (Arya et al., 2016).

1.11.5. Adverse effects of tamoxifen

Estrogen receptors are expressed not only in breast tissue, but also in the uterus, ovaries, the musculoskeletal-, cardiovascular-, central nervous- and the immune systems (Gustafsson, 2003).

Side effects may occur due to binding of tamoxifen to ER in other organs besides the breast. Tamoxifen is an antagonist in breast tissue. In contrast, it has agonistic effects in the endometrium and bone tissue, resulting in endometrial hyperplasia as well as increased bone mineralization (P. Chen et al., 2022).

The most common side effects are postmenopausal symptoms, such as hot flashes and sweats, affecting up to 80% of the patients. Hot flashes are characterized by the sudden onset of an intense warmth that begins in the chest and may progress to the neck and face. They are often accompanied with anxiety, palpitations, profuse sweating, and red blotching of the skin. Hot flash symptoms can affect a woman’s ability to work, her social life, her sleep pattern, and her general perception of health. Hot flash is a common symptom and is often encountered by numerous cancer patients, which led to have captured the attention of oncology as they may be linked to cancer progression and reactions to medications such as tamoxifen (Shanafelt et al., 2002).

The pathophysiology of hot flashes is not entirely understood, but several theories have been proposed. One theory suggests that a decline in estrogen levels causes a change in the thermoregulatory set point in the anterior portion of the hypothalamus. The thermoregulatory nucleus initiates perspiration and vasodilation to keep core body temperature within a well-regulated range called the thermoregulatory zone. Researchers have demonstrated a narrowing of the zone between sweating and shivering in symptomatic women, so that small elevations within the zone cause a change in hormones or neurotransmitters, producing a hot flash (Jager et al., 2013) (Hussain et al., 2023).

Other common adverse effects include sleep disturbances, mood swings, lowered libido, vaginal dryness, joint pain and weight gain (Engström et al., 2024).

Serious, although rare, adverse effects are a slightly higher chance of endometrial carcinoma mainly for postmenopausal women, who have a cumulative risk at around 3% with extended treatment, as well as a small risk of venous thrombosis and pulmonary embolism (Iatrakis et al., 2024).

1.11.6. Drug – drug interaction

Endoxifen and other active forms of tamoxifen are primarily produced in the liver by *CYP2D6* and *CYP3A4/5* (Antunes et al., 2015). The use of tamoxifen in conjunction with drugs that inhibit *CYP2D6*, such as selective serotonin reuptake inhibitors like paroxetine and fluoxetine, which are powerful *CYP2D6* inhibitors, whereas moderate/weak inhibitors such as escitalopram, cimetidine, amiodarone, ticlopidine, and haloperidol would significantly lower plasma level of endoxifen (Goetz et al., 2008).

Furthermore, the effectiveness of tamoxifen is reduced with *CYP3A4* inhibitors (Antunes et al., 2015).

Tamoxifen bioavailability was decreased by rifampin because of *ABCB1* induction, an efflux transporter, or by stimulating *UGTs*, thereby increasing tamoxifen metabolism (Hansten, 2018).

1.11.7. Tamoxifen pharmacogenomics

Pharmacogenomics has an essential role in the medical area since it anticipates potential drug reactions, which helps to reduce adverse effects and optimize prescribed doses.

Single nucleotide polymorphism (SNP) is a genetic sequence variant from the wild type or common gene that is frequently generated by changes in single nucleotide in DNA (Pirmohamed, 2023).

A number of host, environmental, and tumor variables may affect response or resistance to tamoxifen. One of the resistance mechanisms identified was variation in ER expression and function. Genetic variations in drug-metabolizing enzymes that play a role in tamoxifen metabolism are considered host variables that can influence clinical efficacy. These variations happening in DNA sequences are mostly SNPs that create heterogeneity in systemic exposure to the major active metabolite, endoxifen (Uslu et al., 2023)

Genetic diversity in the *CYP2D6* gene causes variations in enzyme activity, which results in the categorization of patients into several phenotypes: *CYP2D6* ultrarapid, extensive, intermediate, and poor metabolizers, so this accounts for a considerable amount of the variation in plasma level of endoxifen (Teft et al., 2013)(Souwer et al., 2023). More than a hundred different *CYP2D6* allelic variations exist, each with its own unique metabolic pattern. Serum levels of 4OHTAM and endoxifen are elevated in patients who possess *CYP2D6* genotypes that are predicted to have high enzymatic activity (Yurchenko et al., 2024).

However, concerning the association between endoxifen concentration and drug response, the risk of recurrence and overall survival is unclear (Mulder et al., 2021). Therefore, other variables contributing to the variability of endoxifen concentration need to be considered.

The rate-limiting enzyme in tamoxifen metabolism is believed to be *CYP2D6,* but other CYP enzymes, especially *CYP3A4,* may potentially play a significant role. Therefore, patients' endoxifen levels and the possible benefits of tamoxifen therapy can be predicted by polymorphisms in *CYP2D6* and *CYP3A4* (Teft et al., 2013).

Other significant enzymes involved in tamoxifen metabolism are *CYP2C* family members that contributes less than 3% to endoxifen synthesis (Mürdter et al., 2011). *CYP2C9* enzymes can exhibit different metabolic activities depending on their polymorphic state.

Clearance of 4-hydroxy tamoxifen was found to be respectively 48% and 49% lower in *CYP2C9*2* and *CYP2C9*3* variants than in wild-type homozygotes (Sidibe et al., 2024). On the contrary, other studies such as Teft et al. reported no association between metabolite levels in homozygotes and the two variants (Teft et al., 2013).

Tamoxifen and its metabolites are mostly eliminated and rendered inactive by glucuronidation and sulfation, processes that are performed by UGTs and SULTs (Mürdter et al., 2011).

As *UGT2B15*2* has been reported to have an elevated glucuronidation activity, the variant allele *UGT2B7*2* has been linked to lower activity. Whereas *UGT1A8*3* show no activity (Ahern et al., 2011). SULTs or sulfotransferases are hepatic phase II enzymes that increase water solubility for better excretion (Helland et al., 2021).

Less information about the function of drug transporters with respect to effectiveness, levels of tamoxifen and its metabolites that have been measured, or the severity of adverse medication reactions. Cellular excretion of tamoxifen or endoxifen has been associated with a variety of transporters, including efflux transporters like permeability glycoprotein (P-gp) and drug uptake transporters like *OATP1B1* gene (Gao et al., 2017).

Patients of Asian origins who carry drug transporters genetic variant have been found to have a lower overall survival rate from breast cancer (T. Wang et al., 2021).

1.11.7.1. The impact of *CYP3A4* gene variants on tamoxifen

Endogenous and exogenous substances are metabolized by CYP enzymes (Danielson, 2002). Only one subfamily, *CYP3A*, is included in the human *CYP3* gene family; it consists of the following four genes: *CYP3A4*, *3A5*, *3A7*, and *3A43*, and it is located on chromosome 7 (Zanger & Schwab, 2013). The most prevalent CYP enzyme, *CYP3A4*, is accountable for the metabolism of over 40% of all drugs that are presently in use (Zhao et al., 2021). Although *CYP3A4* is most abundant in the liver, it is also present in numerous organs and tissues that play a crucial metabolic function (Zanger & Schwab, 2013).

Variability in *CYP3A4* metabolic indices (10 fold - 100 fold) between individuals influences drug response and toxicity due to genetic and/or environmental factors, including ultraviolet exposure, drug interactions, and dietary patterns (Carr et al., 2021). Polymorphisms in genes encoding for *CYP3A* have been related to tamoxifen metabolism (Sim et al., 2013). *CYP3A4* is involved in the enzymatic conversions of tamoxifen to 4-hydroxy-tamoxifen, endoxifen from 4-hydroxytamoxifen, and tamoxifen to NDM-tamoxifen (Lin et al., 2002).

A polymorphism (*CYP3A4*22*) located in intron 6 of *CYP3A4*, as shown in figure (1-3), was recently discovered to be associated with reduced mRNA expression and enzyme activity (D. Wang et al., 2011).

CYP3A4 genetic polymorphisms that influence tamoxifen metabolism to some degree have been identified. (Tseng et al., 2014). It has been reported that the *CYP3A4*22* allele is a functional *CYP3A* allele. (Elens et al., 2013).

A frequency of 5-7% in the Caucasian population has been associated with *CYP3A4*22* and decreased *CYP3A4* activity. (Sanchez Spitman et al., 2017). Other studies, however, reported no association between *CYP3A4* variation and plasma concentrations of endoxifen or tamoxifen, but with some metabolic ratios (endoxifen/4-OH-Tam)(Sidibe et al., 2024).

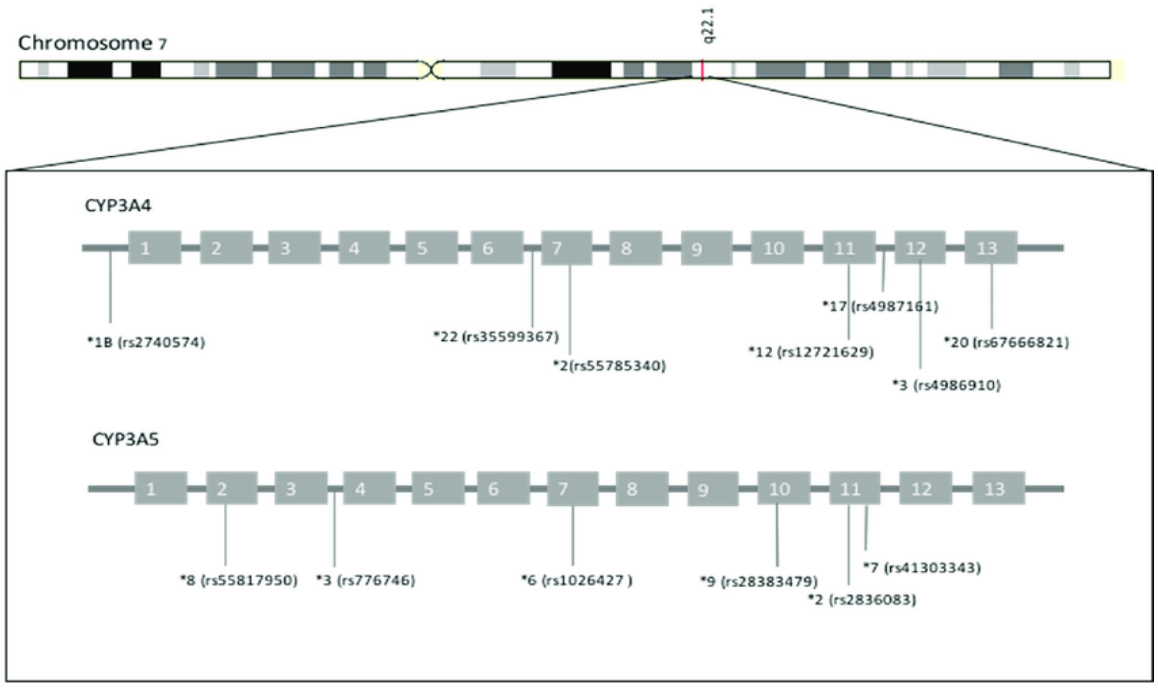

Figure (1-3): Single nucleotide polymorphisms of the *CYP3A4* gene on chromosome 7 (Saiz-Rodríguez et al., 2020)**.**

1.11.7.2. The impact of *ABCB1* gene variation on tamoxifen

One of the broad superfamilies of major active transporters found across all known forms of life is the ATP-binding cassette (ABC) subfamily B, which includes *ABCB1*. Multidrug resistance gene 1 (MDR1) is another name for this gene. P-gp, encoded by the *ABCB1* gene, is involved in the energy (ATP)-dependent excretion of drugs and is located on chromosome 7 (Zinzi et al., 2014).

A large number of cancer patients acquire resistance to chemotherapeutic medications, and *ABCB1* is the gene that is primarily responsible for this mechanism. (Leslie et al., 2005). A shorter disease free survival period (DFS) and a higher risk of disease recurrence were shown to be associated with this protein's overexpression in breast cancer tumors. (Tsukamoto et al., 1997). A wide range of illness susceptibilities and therapy outcomes are associated with this very polymorphic gene (Cizmarikova et al., 2010).

Because of their binding to P-gp and their status as P-gp substrates, tamoxifen, and its active metabolites 4-hydroxytamoxifen, and endoxifen may not be delivered to important target tissues, hence decreasing the efficacy of tamoxifen treatment. (Teft et al., 2011).

Less information known about the effect of ABCB1 transporters in endoxifen fate and response. However, several studies have studied the relationship between ABCB1 variations and breast cancer patients' chances of relapse and metastasis when taking tamoxifen. To date, thousands of SNPs have been identified in the ABCB1 gene. One of the most important ABCB1 gene polymorphism is 3435C > T (rs1045642) in exon 26, a synonymous polymorphism which alters gene expression, protein activity and substrate specificity (İçduygu et al., 2020).

1.8. Aims of the Study

1. Study the distribution of genotypes of *CYP3A4* and *ABCB1* genes among breast cancer Iraqi women receiving tamoxifen.

2. Detect the *CYP3A4*22* G>A (rs35599367) and *ABCB1* C3435T (rs1045642) genetic polymorphisms among breast cancer Iraqi women included in this study.

3. Evaluate whether there are any relationships between *CYP3A4* (rs35599367) and *ABCB1* (rs1045642) gene polymorphisms and the plasma levels of CA15.3, estradiol level, calcium, and lipid profile in tamoxifen treated patients

4. Investigate the influence of *CYP3A4* and *ABCB1* gene polymorphisms (rs35599367 and rs1045642) on tamoxifen efficacy and occurrence of hot flash in breast cancer patients.

Chapter two:

Patients, Materials and Methods

2.1. Patients

This cross-sectional observation study was carried out between November 2022 to April 2023 at Imam AL-Hassan AL-Mujtaba Teaching Hospital and Imam Al-Hussein Medical City in holy Kerbala. This study included one hundred females with breast cancer. Patients who were recently diagnosed with breast cancer came to the oncology center for subsequent checkups. The study's methodology was approved by the College of Pharmacy's Scientific and Ethical Committee at the University of Kerbala. Following an explanation of the study's purpose and design, each patient agreed to a permission form.

2.1.1. Criteria for patients' inclusion

Aged 45 and older, female, diagnosed with breast cancer, taking 20 mg of tamoxifen daily for a minimum of 4 months, and positive for ER and/or PR, as standard adjuvant therapy. All Patients had previously completed all primary surgery, radiation, and adjuvant chemotherapy prior to tamoxifen treatment.

The study patients were divided into two groups: responders and non-responders, based on their plasma levels of estradiol and tumor marker CA15.3. The classification was determined through careful analyses of individual patient data, taking into account specific cutoff values for estradiol and CA15.3 values referred to the normal kit values used in the measurements. Patients with levels above a certain threshold were considered non-responders, while those below were responders. This grouping method allowed us to study how treatment outcomes varied based on hormone and tumor marker profiles.

2.1.2. Criteria for patients' exclusion

Exclusion criteria included if tamoxifen was taken in conjunction with adjuvant chemotherapy or radiation therapy, and if they were taking other adjuvant endocrine therapies. Concurrent medications that induce or inhibit *CYP2D6*, *CYP3A4* metabolizing enzymes and *ABCB1* efflux transporter, and individuals who had a medical history of gastrointestinal problems or surgeries that could have affected tamoxifen absorption were also not included in the research. Fluoxetine and clarithromycin are strong inhibitors of *CYP2D6* and *CYP3A4* respectively. While phenytoin and rifampin are strong inducers of CYP34 enzymes.

2.2. Healthy Control Group

Healthy controls were female who show the same characteristics of the patient's group, except were free from breast cancer. This group consists of one hundred healthy women provides a critical comparison group for biochemical analysis (Estradiol level, Ca 15.3, Calcium and lipid profiles).

2.3. Materials

2.3.1. Chemicals and kits

Table (2-1) represents' various kits with chemicals and their manufactures' origin.

2.3.2. Instruments and equipment

Table (2-2) represent the instruments, equipment and the manufacturing companies utilized in genetic and biochemical analysis.

Table (2-1): Chemicals and kits used with their manufacture and origin.

Chemicals and kits	Manufacturing company	Origin country
Absolute Ethanol	Honeywell	Germany
AddPrep Genomic DNA Extraction Kit	ADDBIO	Korea
Agarose powder	CONDA	Spain
Ca15.3 detection Kit	DIRUI	China
Calcium Kit	Mindray	China
DNA Ladder Marker(100bp)	BIONEER	Korea
Estradiol Detection Kit	DIRUI	China
Ethidium Bromide	Sigma	USA
Lipid Profile Kit (triglyceride, cholesterol, HDL and LDL)	Mindray	China
Nuclease Free Water	BIONEER	Korea
PCR Smart mix 1	SolGent	Korea
Primers	Macrogen	Korea
Tamoxifen	AstraZeneca	Swedish
Tris- Borate- EDTA (TBE) Buffer	Marliju	Korea

Table (2-2): Instruments, equipment and the manufacturing companies

Instruments, equipment	Manufacturing company	Origin country
Digital Camera	Canon	England
DIRUI CM – 180 Chemiluminescence Immunoassay Analyzer	DIRUI	China
Gel electrophoresis apparatus	Cleaver scientific	UK
HERAEUS FRESCO 21 Centrifuge	ThermoFisher	Germany
Hot plate stirrer	LabTEch	Korea
Incubator	BBINDER	Germany
Micropipettes	SLAMMED	Japan
Mindray BS -series (BS-430 pro) analyzer	Mindray	China
Nanodrop	Thermo scientific	Germany
PCR machine (Thermocycler)	ThermoFisher	Germany
Refrigerator	Hitachi	Jaban
Sensitive balance	AND	Taiwan
Smart spin mini centrifuge	JoanLab	China
UV- Trans illuminator	Major Science	Taiwan
Vortex mixer	Vortex stirrer	China

2.4. Methods

2.4.1. Clinical data collection

Patients' self -information were collected from them while they were undergoing treatment. These include patients' weight, age, family history, marital status, and number of births, type of delivery (normal or caesarean section), use of contraception, date of first menarche, breast-feeding, presence of other diseases and presence of hot flashes, joint pain or other side effects.

Date of breast cancer diagnosis, cancer stage, tumor site: right, left or bilateral, immunohistochemistry status, use of chemotherapy and radiation, surgery type, time on tamoxifen medication, and duration were additional details collected from the medical file of women who gave their informed consent.

2.4.2. Blood sample collection

All the women who took part in study, about 5 ml of venous blood was taken and around 2 ml of it was put into an EDTA tube (Ethylenediaminetetraacetic acid) for molecular analysis.

Serum was separated from the remaining 3 ml of blood and placed in gel tube utilized to evaluate biochemical parameters such calcium, estradiol, tumor marker level, and lipid profile.

2.4.3. Biochemical analysis

2.4.3.1. Estimation of serum cancer antigen tumor marker CA15.3

For the quantitative determination of cancer antigen 15.3 in human serum in vitro by the chemiluminescence immunoassay (G. Wang et al., 2014). This reagent is detected by the double antibody sandwich method based on the chemiluminescence immunoassay.

Reference Range: <32.4 U/mL

2.4.3.2. Estimation of serum estradiol

This is for quantitative determination of E2 in human serum or plasma *in vitro* by the chemiluminescence immunoassay (Xin et al., 2010).

Estradiol level kit is tested by the competitive method based on chemiluminescence immunoassay.

Reference Rang: Postmenopausal female < 32.2 pg/ml

2.4.3.3. Estimation plasma calcium level

The *in vitro* quantitative measurement of calcium level in serum by using Mindray BS series (BS- 430 Pro) analyzer (Bauer, 1981).The calcium assay is obtained by a photometric Arsenazo III method.

2.4.3.4. Estimation of serum lipid profile

2.4.3.4. A. Estimation of total cholesterol

Total cholesterol (CHO) in serum was determined *in vitro* using the enzymatic colorimetric technique. Cholesterol esters are broken to free cholesterol and fatty acids by the action of the cholesterol esterase enzyme (ChE). The enzyme cholesterol oxidase (CHOD) then oxidizes cholesterol to cholest -4-en-3-one and hydrogen peroxide.

Due to the action of the peroxidase enzyme (POD), the hydrogen peroxide affects the oxidative coupling of phenol and 4-aminoantipyrin (4-AAP) to produce red quinone-imine dye.

Cholesterol esters + H_2O $\xrightarrow{ChE}$ Cholesterol + Free fatty acid

Cholesterol + O_2 $\xrightarrow{CHOD}$ Cholest-4-en-3-one + H_2O_2

2 H_2O_2 + 4-AAP + Para hydroxy benzoic acid $\xrightarrow{POD}$ Quinone-imine dye + 4 H_2O

The content of cholesterol is exactly proportional to the color intensity of the dye produced, as assessed by an increase in absorbance at 512 nm (Allain et al., 1974)

2.4.3.4. B. Estimation of serum triglyceride

In vitro triglyceride (TG) estimation was performed using the enzymatic quantitative colorimetric method. Triglycerides are catalyzed to yield H2O2 through a series of enzymatic catalysis steps by lipase, glycerol kinase, and Glycerol-3- Phosphate Oxidase, which oxidizes 4-aminoantipyrinel to yield a colored dye of quinoneimine. The increase in absorbency is proportional to the concentration of triglycerides (TG, 1997).

Triglycerides + 3 H_2O $\xrightarrow{LPL}$ Glycerol + 3 Free fatty acid

Glycerol + ATP $\xrightarrow{GK + Mg2+}$ Glycerol-3-phosphate + ADP

Glycerol-3-phosphate + O_2 $\xrightarrow{GPO}$ Dihydroxyacetone phosphate + H_2O_2

H_2O_2 + 4-AAP + Aniline color of original material $\xrightarrow{POD}$ Quinone imine pigment + H_2O

2.4.3.4. C. Estimation of serum high-density lipoprotein

High-density lipoprotein (HDL) levels are determined directly in serum. The method's fundamental principle is as follows. Under assay conditions, the apo-B containing lipoproteins in the specimen are reacted with a blocking reagent, rendering them non-reactive with the enzymatic cholesterol reagent.

As a result, apo-B containing lipoproteins are totally excluded from the assay, and only HDL cholesterol is identified under the conditions of the assay. For the HDL-cholesterol measurement, sulfated alpha-cyclodextrin in the presence of Mg+2 forms complexes with apo-B containing lipoproteins, and polyethylene glycol-coupled cholesteryl esterase and cholesterol oxidase (Hafiane & Genest, 2015). The amount of HDL cholesterol present is proportionally correlated with the amount of blue quinoneimine dye produced.

2.4.3.4. D. Estimation of serum low density lipoprotein

The concentration of LDL (Low-Density Lipoprotein) was estimated indirectly using Fried Ewald and colleagues' equation, which has become the more prevalent method in clinical laboratories (de Cordova & de Cordova, 2013). LDL-C mmol/L = [Total-cholesterol] - [HDL-C] - [TG/5]

2.4.4. Genetic analysis

2.4.4.1. Extraction of genomic DNA from blood sample

The genetic and extraction of DNA were performed at Laboratory of molecular biology in College of Pharmacy – Kerbala University. **AddPrep Genomic DNA Extraction Kit** offer simple, rapid and cost-effective method for isolating genomic DNA from blood and various biological samples to yield pure DNA suitable for storage and immediate application.

At first, 22.5 and 48 ml of ethanol added to Washing 1 and Washing 2 Solution respectively before use.

The following steps for DNA extraction from whole blood:

1. About 20 µl of Proteinase K solution was added to a 1.5 ml micro-centrifuge tube.
2. From blood sample, 200 µl was transferred to the 1.5 ml micro-centrifuge tube with Proteinase K solution.
3. About 200 µl of Binding Solution was added to the sample tube, and mixed well by pulse-vortexing for 15 sec.
4. Incubated at 56℃ for 10 min.
5. About 200 µl of absolute ethanol was added and mixed well by pulse-vortexing for 15 sec: After this step, briefly spun down to get the drops clinging under the lid.
6. Carefully, the lysate was transferred into the upper reservoir of the spin column with 2.0ml collection tube without wetting the rim.
7. Centrifuge at 13,000 rpm for 1 min done, the flow-through Poured off, and the spin column assembled with the 2.0 ml collection tube.
8. About 500 µl of Washing 1 Solution was added to the spin column with collection tube, centrifuged at 13,000 rpm for 1 min.

The flow-through poured off, and the spin column assembled with the 2.0 ml collection tube.

9. About 500 µl of Washing 2 Solution was added to the spin column with collection tube and centrifuged at 13,000 rpm for 1 min, the flow-through Poured off and the spin column with the 2.0 ml collection tube.
10. The spin column was dried by additional centrifugation at 13,000 rpm for 1 min to remove the residual ethanol in spin column.
11. The spin column was transferred to the new 1.5 ml micro-centrifuge tube.
12. About 100 ~ 200 µl of Elution Solution added to the spin column with micro-centrifuge tube, and let stand for at least 1 min.
13. The genomic DNA eluted by centrifugation at 13,000 rpm for 1 min.

2.4.4.2. Quantitation of DNA by spectrophotometric method

Nanodrop is a nanospectrophotometric technique for measuring the concentration and purity of isolated DNA. The equipment recorded the concentration of DNA after cleaning the micro detector from the blank and applying 1μL of samples to the nanodrop micro detector.

2.4.4.3. Polymerase chain reaction

In the current study, amplification refractory mutation system polymerase chain reaction (ARMS-PCR) and PCR amplification of specific alleles (AS-PCR) techniques were used for detecting rs35599367 and rs1045642 SNPs of *CYP3A4* and *ABCB1* genes.

2.4.4.4. Primers Design

The primers created using Primer-BLAST software using https://WWW.ncbi.nlm.nih.gov/websites and bought as a lyophilized package from Macrogen, Korea.

The primer sequences that utilized for amplification analysis of *CYP3A4* and *ABCB1* genes for SNPs identification are shown in Tables (2-3) and (2-4) respectively.

Table (2-3): Primers sequences of *CYP3A4*22* (G>A) (rs35599367).

(Abed et al ., 2022)

Primers	Primer sequence (5 ’->3 ’)	Primer size (bp)	Product size (bp)
I-F allele G	GATGCAGCTGGCCCTACG	18	215
I-R allele A	AGTGTCTCCATCACACCCAGT	21	297
O-F	AGGGGTCTTGTGGATTGTTGA	21	474
O-R	CACCTGTCTTGAGCCCCTTAG	21	474
I-F: Inner Forward, I-R: Inner Reverse, O-F: Outer Forward, O-R: Outer Revers			

Table (2-4): Primers sequences of *ABCB1* C3435T (rs1045642)

Primers	Primer sequence (5 '->3 ')	Primer Size (bp)	Product Size (bp)
Reverse Allele G	GGGTGGTGTCACAGGAAGAGATC	23	400
Reverse Allele A	GGGTGGTGTCACAGGAAGAGATT	23	
Forward	TAAGGCTGACAAAGGTGGAGCC	22	

Lyophilized primers first dissolved in a particular amount of nuclease-free water to create a stock solution with a concentration of 100 pmol/µl. Next, a diluted work solution was created by adding 90µl of nuclease-free water to 10µl of each stock solution r to get (10 pmol/ µL) as a final concentration (working solution). This work solution was stored at -20°C until use.

Table (2-5): The volumes of nuclease free water added to each primer to obtain 100 pmol/µl concentration according to manufacture instruction

Primers		Volume of nuclease free water added (µl)
G>A (rs35599367	C3435T (rs1045642)	
O-F	Reverse Allele A	250
O-F	Reverse Allele G	250
I-F	Forward	250
I-R		250

2.4.4.5. Polymerase chain reaction optimization conditions

Following a number of trials, the PCR reaction optimized to determine the ideal annealing temperature and the ideal number of amplification cycles.

2.4.4.6. Running the polymerase chain reaction

In a microcentrifuge tube, the PCR mixture was created by adding 25 µl of smart mix (SolGent/Korea), 2 µl of each primer, 5 µl of DNA and the volume was brought to 50µl with nuclease free water. Amplified segments separated by gel electrophoresis apparatus using agarose gel 1.5% and ethidium bromide stain and observed under ultraviolet (UV) trans-illuminator. DNA bands were photographed using a UV trans illuminator, and the molecular weights of the bands were determined using a DNA ladder (100–1500 bp).

Mixing the PCR component with DNA and employing the optimum PCR program are shown below in table (2-6), (2-7) and (2-8).

Table (2-6): PCR mixtures for genotyping of *CYP3A4*22* (G>A) (rs35599367) genetic polymorphism

Component	Volume (µl)
Inner Forward Primer	2
Inner Reverse Primer	2
Outer Forward Primer	2
Outer Forward Primer	2
DNA Templet	5
Nuclease Free Water	12
Smart mix	25
Total	50

Table (2-7): PCR mixtures for genotyping of *ABCB1* C3435T (rs1045642) genetic polymorphism

Component	Volume (µl)
Forward Primer	2
Reverse allele G	2
Reverse allele A	2
DNA Templet	5
Nuclease Free Water	14
Smart mix	25
Total	50

Table (2-8): PCR conditions for genotyping of *CYP3A4*22* (G>A) (rs35599367) and *ABCB1* C3435T (rs1045642) genetic polymorphisms

Step	Temperature ºC	Minutes : Seconds	cycles
Initial Denaturation	95	03:00	1
Denaturation	95	00.30	32
Annealing	61	00:30	
Extension	72	0.1:00	
Final Extension	72	05:00	1

2.4.4.7. Agarose gel electrophoresis

1. To make Agarose gel, 1.5g of agarose powder was dissolved in 100ml of 1x TBE buffer (Tris-Borate-EDTA), which is prepared by taken 10ml of 10x TBE buffer and 90ml of distilled water.
2. On a hot plate, the solution was boiled for a few minutes and avoid bubbles until the gel solution appeared clear and pure.
3. Once the solution had cooled, three microliters of ethidium bromide were added to the gel
4. To make wells for the loading of PCR products, the comb was attached to the end of the tray.
5. Carefully remove the comb from the agarose gel once it had hardened, which took 20 minutes after cautious pouring it into the tray.
6. To install the gel, a gel electrophoresis tank is utilized. The tank was filled with 1X TBE-electrolysis buffer until it was three to five millimeters above the surface of the gel.
7. Five microliters of DNA ladder were added to one well, and five microliters of each PCR product was added to the other wells.

8. Tray fixed inside an electrophoresis chamber.
9. For the distance between the cathode and anode, the voltage of the electrophoresis apparatus was adjusted to produce an electrical field of 5 v * cm-.
10. Bands were identified at the end of the run using a UV trans-illuminator adjusted to 320-336 nm.
11. Gel photographs were taken using a digital camera gel (Lee et al., 2012).

2.4.5. Hot flash surveys

The hot flash severity survey was adapted from Sloan et al. (Sloan et al., 2001). In-person surveys were conducted during clinic visits where blood samples were obtained. Data gathered from surveys included the average number of mild, moderate, severe, and very severe hot flashes experienced per day. The average number of mild, moderate, severe, and very severe hot flashes experienced each day were multiplied by a severity factor (mild = 1, moderate = 2, severe = 3, and very severe = 4) and values were cumulated to determine the hot flash severity score (HFSS).

2.4.6. Body mass index calculation

The Body mass index (BMI) for each patient was calculated by dividing a patient's weight in kilograms by their height in meters squared. (Kannan et al., 2017). $BMI = kg/m^2$

2.5. Statistical Analysis

The presented data were collected in an Excel sheet and analyzed using the Statistical Package for the Social Sciences (SPSS version 26). The data were presented as frequencies and percentages or mean and standard deviation in appropriate tables and graphs. Independent t-test, one-way ANOVA, and two-way ANOVA were used where appropriate to assess the association between related variables. Post hoc analysis, including Tukey's multiple comparison test, was employed to further explore significant differences between group means. Least significant differences (LSD) tests were also applied when needed.

For the analysis of genetic polymorphisms, Hardy-Weinberg equilibrium was assessed using the Hardy-Weinberg equation $p^2+2pq+q^2=1$, where p represents the frequency of the dominant allele and q represents the frequency of the recessive allele. This calculation evaluated whether the observed genotype frequencies were consistent with expected proportions. The chi-square and Fisher exact test statistics were used to compare the observed and expected values, determining whether any differences between them were statistically significant.

In addition, a Pearson correlation test was used to examine the relationship between the parameters under study. Statistical significance was considered when the p-value was equal to or less than 0.05 or 0.01 ($P \leq 0.05$ or 0.01).

Chapter three

Results

3.1. Demographic characteristics of the study population:

Table (3-1) and figure (3-1): display demographic characteristics of the study population. The mean age of the participants is 49.17 years (±4.77 standard deviation). The mean body mass index is 31.19 kg/m² (±4.935), signifying an average BMI with moderate variability. However, the duration of tamoxifen use varies widely, with a mean of 27.57 months (±25.38). Similarly, the mean duration of the disease is 3.540 years (±3.067), showing substantial variability.

Table (3.1): Description of demographic characteristics of the studied patients (n=100)

Variables	Statistical values
Age groups m±SD, (n)	
Age (Years) m±SD	49.17±4.77, (100)
45-49	46.11±1.355, (61)
50-54	51.56±1.121, (27)
55-59	56.17±0.9832, (6)
60-65	62.5±2.074, (6)
BMI (Kg/m^2) m±SD	
BMI (Kg/m^2) m±SD	31.19±4.935
Normal weight	8%
Overweight	35%
Obese	57%
Duration of tamoxifen (months) m±SD	27.57±25.38
Duration of disease (Years) m±SD	3.540±3.067
Results are presented as mean (m) ± standard deviation (SD). BMI stands for body mass index. N= number of patients	

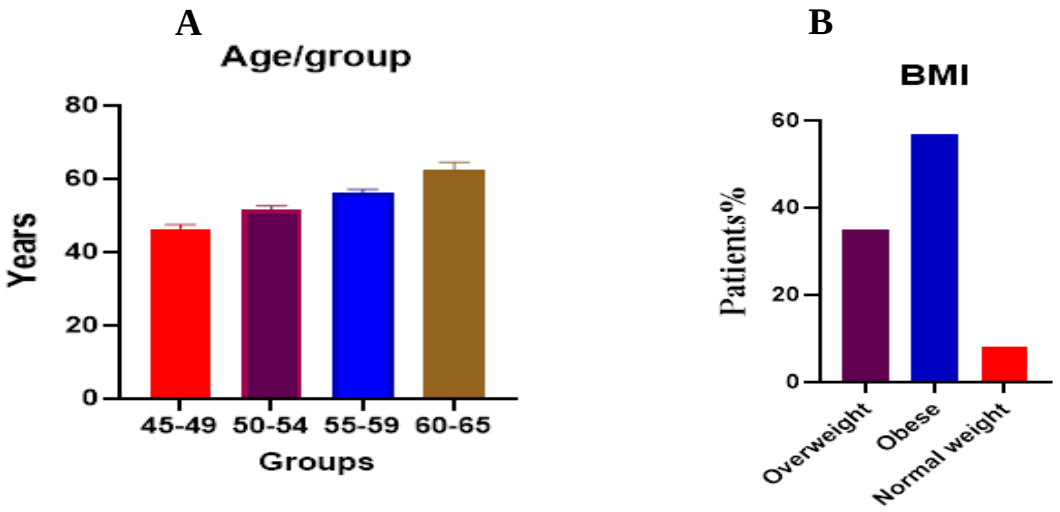

Figure (3-1): A) Shows age categories/years of the study patients. B) Refers to the categories of body mass index BMI of the study participants

3.2. Profile of demographic and clinical characteristics of breast cancer patients:

Table (3-2) provides a comprehensive snapshot of the study population's demographic and clinical characteristics. Notably, 47% of participants have a family history of breast cancer, while 42% use contraceptives. Most participants have had 1-5 children (73%) and tumours located on the left (51%) or right (46%) side. Furthermore, 54% breastfeed, and 53% have undergone mastectomy, with 94% and 80% receiving chemotherapy and radiotherapy, respectively. Immunohistochemical testing reveals 30% HER2-positive cases and varying ER/PR statuses. Most participants are at stage II (67%) of breast cancer, with hot flashes (82%) being a common side effect, and recurrence observed in 5% of cases.

Table (3-2): Description of demographic and disease characteristics of the studied patients (n=100)

Characteristics	Value		Percentage %
Family history of breast cancer	No		53
	Yes		47
Contraceptive use	Yes		42
	No		58
Number of births	Nulliparity		14
	1-5 child		73
	6-8 children		13
Site of breast tumor	Left		51
	Right		46
	Bilateral		3
Breast feeding	Mix		22
	No		10
	Yes		54
	Nulliparity		14
Type of surgery	Breast conserving		39
	Mastectomy		53
	Mastectomy and breast conserving		2
	No surgical interruption		6
Use of chemotherapy	Yes		94
	No		6
Use of radiotherapy	Yes		80
	No		20
Immunohistochemical test	HER2	Negative	70
		Positive	30
	Positive for both ER/PR		92
	ER positive /PR negative		6
	ER negative/PR positive		2
Cancer stage	I		14
	II		67
	III		19
Side effects	No signs		30
	Hot Flashes		82
	joint pain		45
	Mood change		15
	Fatigue		3
	Ocular toxicity		2
	Headache. Anorexia.		2
Recurrence of breast cancer	Yes		5
	No		95

Results are presented as percentages. HER2 stands for Human epidermal growth factor receptor 2.

3.3. Comparative analysis of biomarker profiles in healthy individuals and breast cancer patients:

Table (3-3) conducts a detailed comparative analysis of biomarker profiles between a group of healthy individuals and breast cancer patients. Estradiol levels exhibit a significant reduction in patients compared to healthy individuals (P = 0.0418), implying its potential relevance as a diagnostic marker for breast cancer. Additionally, CA15.3 and calcium levels are notably higher in patients (P = 0.0081) and (P = 0.0005) respectively.

Conversely, lipid profiles encompassing cholesterol, triglycerides, HDL (High-Density Lipoprotein), and LDL (Low-Density Lipoprotein) do not manifest significant differences between the two groups.

Table (3-3): Description of mean levels of laboratory biomarkers of the studied patients (n=100)

Biomarker	Healthy, Mean±SD N=100	Patients, Mean±SD N=100	P value	P value summary
Estradiol	24.05 ± 6.552	19.29 ± 15.64	**0.0418**	*
CA15.3	17.92 ± 6.218	24.16 ± 15.80	**0.0081**	**
Calcium	8.700±0.6643	9.355 ± 1.190	**0.0005**	***
Cholesterol	178.1 ± 37.66	181.0 ± 42.43	0.6778	ns
Triglycerides	206.6 ± 135.3	177.9 ± 155.8	0.2681	ns
HDL	42.66 ± 12.60	44.37 ± 12.38	0.4292	ns
LDL	136.8 ± 39.56	145.5 ± 36.43	0.1823	ns

Results shown as mean ± standard deviation. P value > 0.05 is considered non-significant (ns). * mean significant. T.test was used in this table.

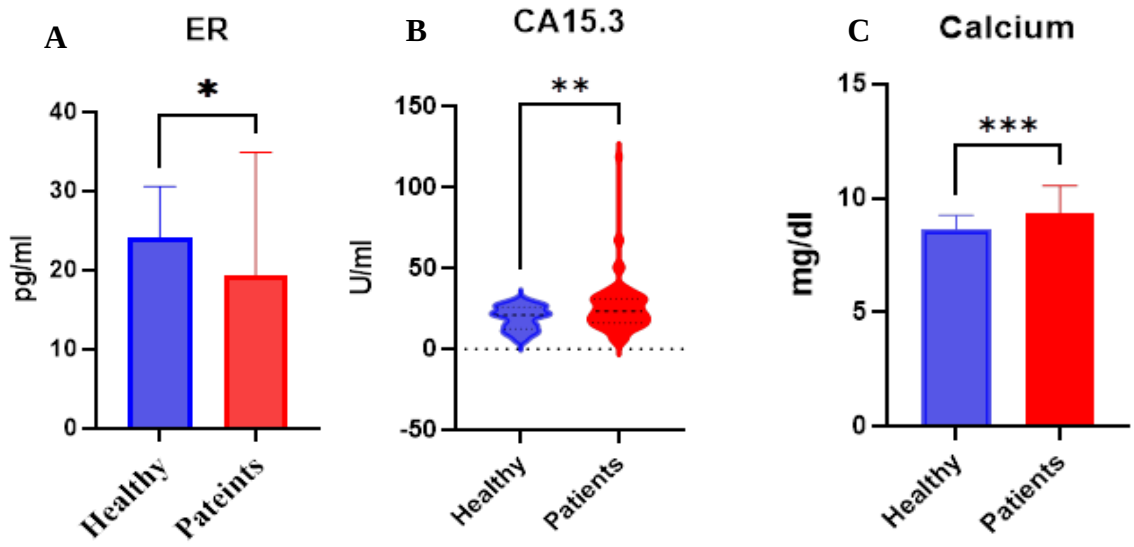

Figure (3-2): Describe the significant differences the mean levels of the laboratory biomarkers between a group of healthy individuals and breast cancer patients. A) plasma estradiol. B) plasma CA15.3, C) plasma calcium. The data was presented as mean±SD * P<0.05.

3.4. Age-related variations in biomarker levels: Insights from a comparative analysis:

Table (3-4) provides an extensive examination of various biomarkers across distinct age categories, ranging from 45-49, 50-54, 55-59, to 60-65 years, within the study population. The data reveals that none of the analyzed biomarkers, including estradiol, CA15.3, calcium, cholesterol, triglycerides, HDL, and LDL, exhibit statistically significant differences across the specified age groups, as all P values surpass the conventional threshold of 0.05.

Table (3-4): Comparison of laboratory biomarkers with age categories of studied patients

Biomarkers	Age Categories				
	45-49 n=61	50-54 n=27	55-59 n=6	60-65 n=6	P- value
Estradiol	15.84±11.27	17.29±16.21	13.42±7.992	9.132±7.623	0.595
CA15.3	22.99±14.4	23.89±21.93	21.65±12	24.45±3.978	0.9857
Calcium	9.411±1.449	9.26±0.6102	9.55±0.295	9.017±0.7574	0.8166
Cholesterol	182.5±46.77	180.4±36.49	178±32.18	171.7±35.98	0.9408
Triglycerides	178.5±189.4	175.2±85.76	218±80.97	142.8±47.05	0.8744
HDL	45.57±14.29	42.81±9.068	37.83±6.21	45.67±5.68	0.4385
LDL	146.8±39.12	145.3±35.04	134.4±21.94	143.1±29.58	0.8848
Results shown as mean ± standard deviation, P value > 0.05 considered non-significant. ANOVA test was used in this table					

3.5. Association of biomarker profiles with BMI categories: Insights into lipid metabolism and weight classification:

In the presented table (3-5) and figure (3-3): showcasing biomarker data across different BMI categories (Normal weight, Overweight, and Obese).

Firstly, it is noteworthy that estradiol, CA15.3, and calcium levels do not exhibit statistically significant variations among the BMI categories, as evidenced by their non-significant p-values ($p > 0.05$). However, the picture changes when we examine lipid-related biomarkers. HDL levels display a significant difference between the BMI categories ($p = 0.00432$).

Specifically, individuals in the normal weight category have notably higher HDL levels compared to those in the overweight and obese categories. Similarly, cholesterol levels also demonstrate significant variations across BMI categories ($p = 0.028$).

Here, individuals classified as obese exhibit higher cholesterol levels compared to their normal weight and overweight counterparts. Likewise, triglyceride levels significantly vary with BMI (p = 0.0478). Obese individuals tend to have substantially higher triglyceride levels than those in the normal weight and overweight groups. Conversely, LDL levels do not display any significant differences across BMI categories (p = 0.7779).

Table (3-5): Comparison of laboratory biomarkers with BMI categories of studied patients

Biomarker	BMI category				value summary
	Normal weight n=8	Overweight n=35	Obese n=57	P value	
Estradiol	26.49±20.06	17.28±12.83	18.49±15.9	0.3487	ns
CA15.3	33±35.9	22.58±15.06	22.27±12.11	0.2041	ns
Calcium	9.363±0.3249	9.12±0.6356	9.498±1.48	0.3385	ns
HDL	51.13±17.91	44.67±11.35	41.36±9.105	**0.0432**	*
Cholesterol	164.1±21.57	171±32.42	192.5±46.72	**0.028**	*
Triglycerides	107.9±37.12	139.9±71.15	212.3±195.2	**0.0478**	*
LDL	138.2±31.73	143.1±30.21	147±41.01	0.7779	ns
Results shown as mean ± standard deviation, P value > 0.05 are considered non-significant. * mean significant. ANOVA test was used in this table					

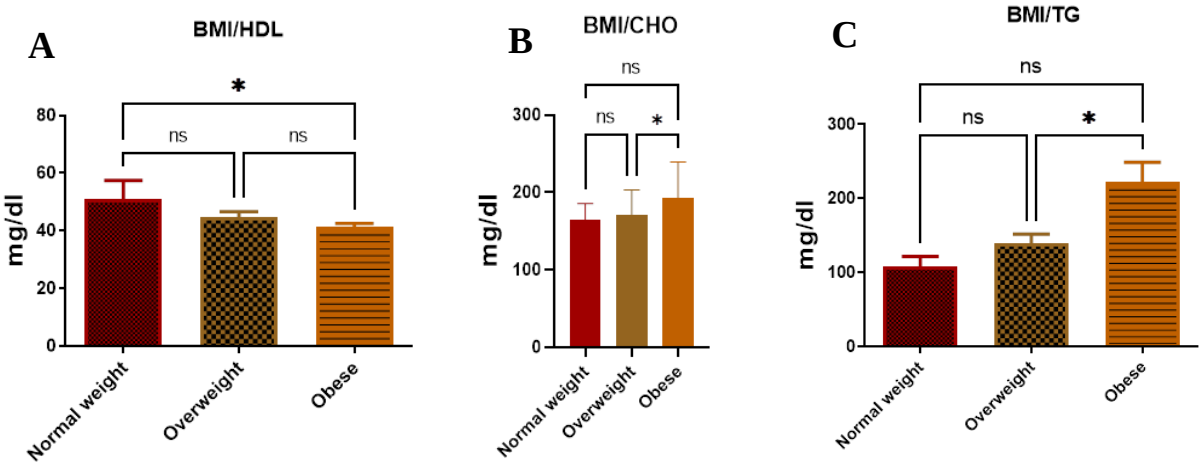

Figure (3-3): Describes the laboratory biomarkers comparisons based on BMI categories. A) HDL refers to plasma HDL levels, B) CHO refers to plasma cholesterol levels. C) TG refers to triglyceride levels. The data was shown as mean±SD. * P<0.05.

3.6. Impact of duration of tamoxifen treatment on biomarker profiles in breast cancer patients:

Table (3-6) presents data on various biomarkers categorized by the time since diagnosis (≤5 Years and >5 Years) within a study population.

The biomarkers examined include estradiol, CA15.3, calcium, HDL, cholesterol and triglycerides. Estradiol, there is a significant difference in estradiol levels between the two groups (P value = 0.0453), with those diagnosed within the last 5 years having higher levels compared to those diagnosed more than 5 years ago. CA15.3 levels do not significantly differ between the two time since diagnosis groups (P value = 0.4102). Calcium levels also do not show a significant difference between the two groups (P value = 0.3512).

HDL levels do not significantly differ between the two groups (P value = 0.3767). Cholesterol levels remain consistent between the two groups (P value = 0.97). Triglyceride levels also show no significant variation between the two groups (P value = 0.7605).

In summary, the data suggests that the time since diagnosis has a significant impact on estradiol levels, with those diagnosed more recently having higher levels. However, CA15.3, calcium, HDL, cholesterol, and triglycerides do not significantly differ between the two groups of time since diagnosis.

Table (3-6): Comparison of laboratory biomarkers with duration of tamoxifen treatment groups

Biomarkers	≤5 Years n= 86	>5 Years n= 14	P value	P value summary
Estradiol	21.25±16.04	11.94±9.338	0.0453	*
CA15-3	25.19±12.19	22.13±9.576	0.4102	ns
Calcium	9.4±1.239	9.079±0.8097	0.3512	ns
HDL	44.81±12.99	41.64±7.334	0.3767	ns
Cholesterol	181±42.5	181.4±43.61	0.97	ns
Triglycerides	179.8±166	166±68.53	0.7605	ns
Results shown as mean ± standard deviation, P value > 0.05 considered non-significant and * mean significant				

3.7. Genetic Analysis

3.7.1. Genotyping of *CYP3A4*22* (G>A) (rs35599367)

In Figure (3-4), the results of ARMS-PCR analysis were presented for the genetic polymorphism of *CYP3A4**22 (G>A) (rs35599367) within our study cohort. Lane M signifies a DNA ladder. Lanes 7, 9, 10, 11, 12, 14, 16, 17, 18, and 19 exhibit GG genotype (wild type) homozygous individuals, displaying a clear band with a molecular size of 215 base pairs (bp). Lanes 1, 2, 4, 6, 13, 15, and 20 represent AA genotype (mutant) homozygous individuals, characterized by a 297 bp band. Lanes 3, 5, 8, and 21 denote GA genotype (heterozygous) individuals, showcasing both 215 bp and 297 bp bands. The determination of amplicon size was accomplished by comparing it with a DNA ladder ranging from 100 to 1500 bp. Electrophoresis was conducted at 45 volts.

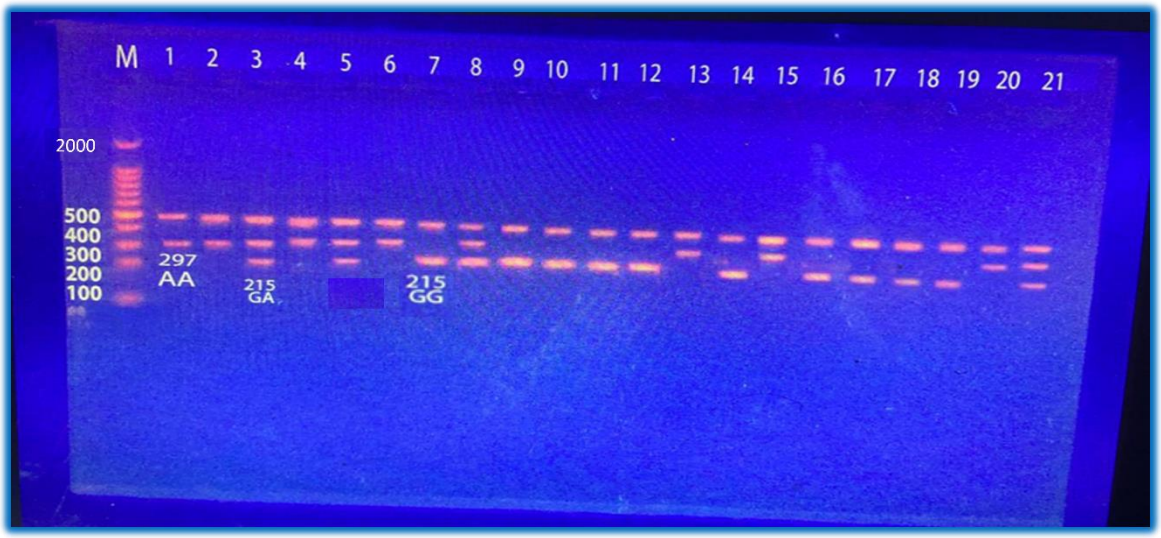

Figure (3-4): Genotyping of *CYP3A4*22* (rs35599367) by using ARMS-PCR

3.7.1.1. Genotype and allele frequency analysis of *CYP3A4*22* (G>A) (rs35599367) in Iraqi women with breast cancer

The provided data in the table (3-7) presents findings of the genotype and allele frequencies of the *CYP3A4*22* (G>A) (rs35599367) from 100 individuals. Analysis of genotypic distribution reveals 39% GG, 35% AA and 26% GA genotypes. The major allele frequencies indicate, 52% for allele G and 48% for allele A. Significant testing with a P<0.0001 shed the light of a highly significant association between the SNP and the trait under investigation.

Table (3-7): Genetic variation analysis of *CYP3A4*22* (G>A) (rs35599367) in breast cancer patients (n=100)

Gene	Genotype	Group n=100	Frequency %	Allele		P value
				G	A	
*CYP3A4*22*	GG	39	0.39	0.52	0.48	<0.0001
	GA	26	0.26			
	AA	35	0.35			
P value <0.05 considered significant, data shows as percentage.						

3.7.1.2. Hardy-Weinberg equation analysis of *CYP3A4*22* (G>A) (rs35599367) in Iraqi women with breast cancer

In the context of the genetic analysis previously discussed, the table (3-8) displays detailed of the provided results for a specific genotype within a sample of 100 individuals calculated according to the Hardy-Weinberg equilibrium and utilizes the Fischer exact test to assess the observed genotype frequencies against their expected counterparts.

The observed genotype percentages show allele G in 52% and allele A in 48% of the population, which deviated from Hardy-Weinberg equilibrium expected calculations, with allele G at 0.2704 and allele A at 0.2304. Fischer exact test yield significant p-values of 0.001 for comparisons as GA/AG genotype and observed vs expected frequencies for GG, GA and AA genotypes.

Table (3-8): Hardy–Weinberg equilibrium for (rs35599367) genotype in studied patients

Genotype/observed % n=100		Hardy–Weinberg equilibrium/ expected %			Fischer exact test	P-value
G	A	G	A	GA/AG	GG/observed vs GG/expected	**0.001**
0.52	0.48	0.2704	0.2304	0.4992	GA/observed vs GA/expected	**0.001**
					AA/observed vs AA/expected	**0.001**
P <0.05 considered significant, data shows as percentage.						

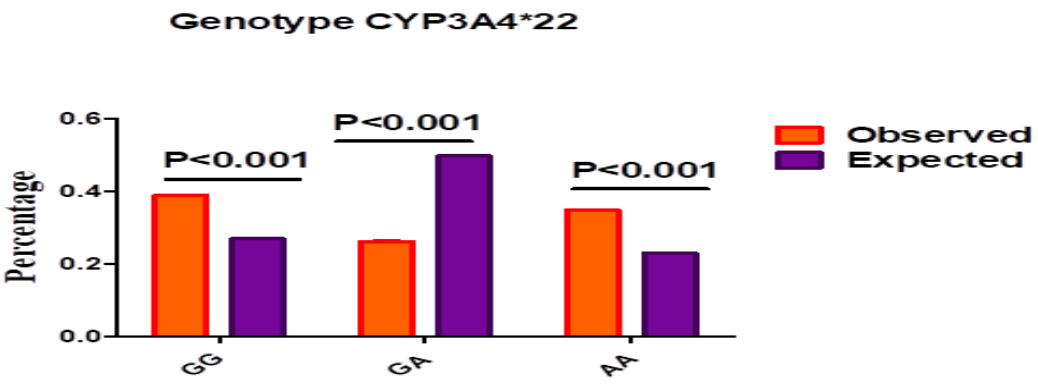

Figure (3-5): genotype distribution (%) among study participants (observed) and Hardy-Weinberg analyses (expected). The data was analyzed using Fischer exact test P-value. All genotypes show significant deviations from the expected distribution (p = 0.001, *). Significance threshold: P < 0.05**

3.7.2. Genotyping of *ABCB1* Gene 3435C>T (rs1045642)

In Figure (3-6), the AS-PCR yielded a clear 400 base pair (bp) amplicon, which was sized by comparison with a 100-1500 bp DNA ladder. Lane M indicating the DNA ladder for size reference. Lanes 1 and 6 represented individuals with the TT genotype (mutant type), lanes 4, 7, 8 and 9 represented individuals with the CC genotype (wild type), and lane 2, 3, 5 and 10 represented individuals with the CT genotype (heterozygous). The gel electrophoresis was conducted at 45 volts.

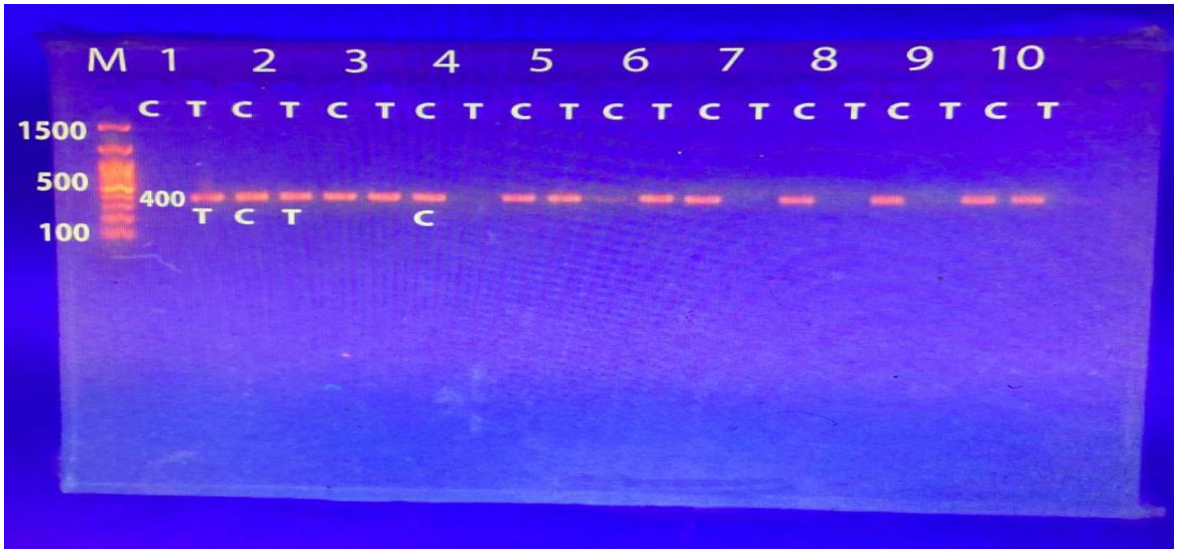

Figure (3-6): Detection of *ABCB1* 3435C>T (rs1045642) genetic polymorphism by using AS- PCR.

3.7.2.1. Genotype and allele frequency analysis of *ABCB1 3435C>T (rs1045642*) in breast cancer patients

Continuing with the genetic analysis from previous findings, the below table provides an in-depth examination of the *ABCB1* gene SNP (3435C>T, rs1045642) polymorphism within the same sample of 100 individuals. The genotype distribution reveals 28%CC, 29% CT, 43% TT genotypes alongside allele frequencies of 42.5% for allele C and 57.5% for allele T. of the alleles. Clear significant association is presented by a P-value of <0.0001, indicating that *ABCB1* SNP is strongly linked to the trait under investigation.

Table (3-9): Distribution of *ABCB1 3435 C>T gene (rs1045642*) in breast cancer patients

Gene SNP	Genotype	Group n=100	Frequency %	Allele		P value
				C	T	
ABCB1	CC	28	0.28	0.425	0.575	< 0.0001
	CT	29	0.29			
	TT	43	0.43			
P value <0.05 considered significant, data shows as percentage.						

3.7.2.2. Hardy-Weinberg equation analysis of *ABCB1 gene 3435C>T* in Iraqi women with breast cancer

Table (3-10) presents findings of the *ABCB1* 3435C>T genetic analyses, indicating genotype frequencies, Hardy-Weinberg equilibrium, Fischer exact test results and corresponding P-values. Fischer exact test has employed to compare expected to observed genotype frequencies, which reveals highly significant differences for CC, CT and TT genotypes alongside with P<0.001.

Table (3-10): Hardy–Weinberg equilibrium for (rs35599367) genotype in studied patients

Genotype/observed % n=100		Hardy–Weinberg equilibrium/ expected %			Fischer exact test	P-value
C	T	C	T	CT/TC	CC/observed vs CC/expected	0.001
0.425	0.575	0.1806	0.3306	0.4887	CT/observed vs CT/expected	0.001
					TT/observed vs TT/expected	0.001
P value <0.05 considered significant. Data shows as percentage						

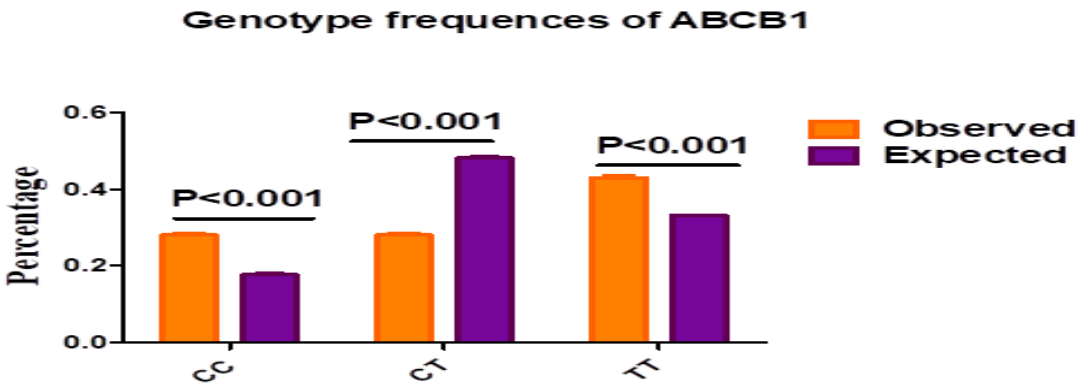

Figure (3-7): genotype distribution (%) among study participants (observed) and Hardy-Weinberg analyses (expected). The data was analyzed using Fischer exact test. All genotypes show significant deviations from the expected distribution (p = 0.001, *). Significance threshold: P < 0.05.**

3.8. Relationship between demographic characteristics and *CYP3A4 and ABCB1 genes*

3.8.1. Genotypic variations of *CYP3A4*22* rs35599367 G>A, across demographic and clinical parameters in breast cancer patients

Table (3-11) provides information of rs35599367 SNP genotype distribution alongside various demographics and clinical parameters in breast cancer women.

The section illustrates significant association between genotype and demographic factors such as age, BMI, duration of the disease, history of the disease, number of the children, chemotherapy, radiotherapy, markers of immunohistochemistry markers (ER, PR, HER2), cancer stage, side effects, clinical symptoms and chronic diseases. In addition, the results are presented both as the number of patients (n) and the percentage within each genotype category for each parameter.

Table (3-11): Difference among demographic characteristic in rs35599367 SNP.

Demographic parameters		**Genotype, n=100**			**P value**	
		GG % 39	**GA %** 26	**AA %** 35		
Age (Years)	45-49	21	17	23	Colum factor	0.1942
	50-54	13	8	6		
	55-59	3	0	3	Row factor	**0.0002**
	60-65	2	1	3		
BMI	<18.5	1	0	0	Colum factor	0.3227
	18.5–24.9	2	3	8		
	25–29.9	9	6	12	Row factor	**0.002**
	>30	26	15	18		
Duration of disease(years)	≤5 Years	26	23	28	Colum factor	0.3000
	>5 Years	13	3	7	Row factor	**0.0190**
Duration of tamoxifen(years)	≤5 Years	33	24	3	Colum factor	0.4540
	>5 Years	6	3	4	Row factor	0.2070
Family history	No	17	13	23	Colum factor	0.7133
	Yes	22	13	12	Row factor	0.6018
Contraceptive use	Yes	17	11	14	Colum factor	0.0500
	No	22	15	21	Row factor	**0.0234**
Number of births	Nulliparity	5	4	5	Colum factor	0.0984
	(1-4) children	27	2	21		
	(5-8) children	7	2	9	Row factor	**0.0384**

Site of breast tumor	Left	17	13	21	Colum factor	0.5071
	Right	22	11	13		
	Bilateral	0	2	1	Row factor	**0.0185**
Breast feeding	Mix	1	5	7	Colum factor	0.8653
	No	4	3	3		
	Yes	2	14	2	Row factor	0.8653
	Nulliparity	5	4	5		
Type of surgery	Breast conserving	14	11	14	Colum factor	0.5741
	Mastectomy	22	1	21		
	Mastectomy and breast conserving		2		Row factor	0.5741
	No surgical interruption	3	3			
Use of chemotherapy	Yes	36	25	33	Colum factor	0.3350
	No	3	1	2	Row factor	**0.0085**
Use of radiotherapy	Yes	32	21	27	Colum factor	0.3214
	No	7	5	8	Row factor	0.0171
Immunohistochemical section	Positive for both ER/PR	0.36	0.23	0.33	Colum factor	0.4761
	ER positive /PR negative	0.03	0.02	0.01		
	ER negative/PR positive	0	0.01	0.01	Row factor	**0.0014**
	HER2					
	Positive	0.09	0.12	0.11	Colum factor	0.1612
	Negative	0.3	0.14	0.24	Row factor	0.6724
Cancer stage	I	5	5	5	Colum factor	0.4637
	II	33	18	15		
	III	6	7	6	Row factor	**0.0367**
Recurrence	Yes	3		2	Colum factor	0.1371
	No	36	26	33	Row factor	**0.0199**
Side effects/ Hot flash degree	No sign	9	2	7	Colum factor	0.4002
	Mild	8	6	4		
	Moderate	10	2	7		
	Severe	8	9	7	Row	0.9009

	Very severe	4	7	10	factor	
Other symptoms	No signs	13	10	12	Colum factor	0.3807
	Elevated liver enzyme		1			
	Fatigue	1		2		
	Headache. Anorexia.	0		2		
	joint pain	16	10	14		
	Ocular toxicity	2			Row factor	**0.0004**
	Mood change	4	6	5		
	Nausea	1	1			
	Vitiligo	1				
Chronic diseases	No chronic disease	34	20	25	Colum factor	0.3292
	Diabetes mellitus	3	2	5		
	Hypertension	4	4	8	Row factor	**0.0017**
	Hypothyroidism	1		1		
Results are shown as n= number of patients and percentage, two-way ANOVA was used to calculate factor and column effect, P <0.05 was considered significant						

3.8.2. Genotypic variations of *ABCB1 3435 C>T (1045642)*, across demographic and clinical parameters in breast cancer patients:

To analyze the relationship between demographic characteristics and the *ABCB1* 3435 C>T (1045642), we start by looking at the distribution of genotypes (CC, CT, and TT) across different demographic parameters. This helps us identify any potential associations or patterns. Across a variety of demographic factors including: age, BMI, duration of the disease, duration of tamoxifen use, contraceptive, breastfeeding, history of the disease, number of the children, type of surgery, chemotherapy, radiotherapy, markers of immunohistochemistry markers (ER, PR, HER2), cancer stage, side effects, clinical symptoms and chronic diseases, significant association with genotype variations was found, as indicated by low P-values.

Table (3-12): Difference among demographic characteristic in rs1045642 SNP

Demographic parameters		Genotype, n=100			P value	
		CC % 28	CT % 29	TT % 43		
Age (Years)	45-49	15	16	30	Colum factor	0.4559
	50-54	9	9	9		
	55-59	4	1	1	Row factor	**0.0067**
	60-65	0	3	3		
BMI	<18.5	0	0	1	Colum factor	0.0490
	18.5–24.9	0	7	8		
	25–29.9	5	9	10	Row factor	**<0.0001**
	>30	19	20	21		
Duration of disease(years)	≤5 Years	20	23	34	Colum factor	0.3794
	>5 Years	8	6	9	Row factor	**0.0415**
Duration of tamoxifen(years)	≤5 Years	21	25	35	Colum factor	0.1914
	>5 Years	4	6	9	Row factor	**0.0170**
Family history	No	13	17	23	Colum factor	0.1560
	Yes	15	12	20	Row factor	0.4380
Contraceptive use	Yes	15	12	15	Colum factor	0.3435
	No	13	17	28	Row factor	0.4447
Number of births	Nulliparity	1	2	5	Colum factor	0.7038
	(1-4) children	24	12	36		
	(5-8) children	4	13	3	Row factor	0.0783
Site of breast tumor	Left	13	11	27	Colum factor	0.4910
	Right	14	17	15		
	Bilateral	1	1	1	Row factor	**0.0366**
Breast feeding	Mix	10	1	11	Colum factor	0.4138
	No	2	5	3		
	Yes	16	15	23	Row factor	**0.0173**
	Nulliparity	0	8	6		
Type of surgery	Breast conserving	10	12	17	Colum factor	0.3679
	Mastectomy	14	16	23		
	Mastectomy and breast		1		Row factor	**0.0194**

	conserving					
	No surgical interruption	5	1	1		
Use of chemotherapy	Yes	27	28	39	Colum factor	0.2570
	No	1	1	4	Row factor	**0.0093**
Use of radiotherapy	Yes	22	22	36	Colum factor	0.4645
	No	6	7	7	Row factor	**0.0473**
Immunohistochemical section	Positive for both ER/PR	25	27	4	Colum factor	0.2545
	ER positive /PR negative	3	2	1		
	ER negative/PR positive			2	Row factor	0.4357
	HER2					
	Positive	12	1	8	Colum factor	0.4550
	Negative	17	18	35	Row factor	0.1240
Cancer stage	I	2	4	8	Colum factor	0.3301
	II	16	21	29		
	III	9	5	6	Row factor	**0.0116**
Recurrence	Yes	4	1		Colum factor	0.2444
	No	24	28	43	Row factor	0.0941
Side effects/ Hot flash degree	No sign	2	8	8	Colum factor	**0.0486**
	Mild	5	5	8		
	Moderate	4	4	11		
	Severe	9	7	8	Row factor	0.7575
	Very severe	6	5	10		
Other symptoms	No signs	7	11	17	Colum factor	0.4585
	Elevated liver enzyme	0	1	0		
	Fatigue	1	2	0		
	Headache. Anorexia.	2	0	0		
	joint pain	11	14	15		
	Ocular toxicity	0	1	1	Row factor	<**0.0001**
	Mood change	6	1	7		
	Nausea	0	2	0		
	Vitiligo	1	0	0		

Chronic diseases	No chronic disease	20	23	36	Colum factor	0.4569
	Diabetes mellitus	6	1	3		
	Hypertension	5	5	6	Row factor	**0.0047**
	Hypothyroidism	1		1		
Results are shown as n= number of patients and percentage, two-way ANOVA was used to calculate factor and column effect, P <0.05 was considered significant						

3.9. The impact of tamoxifen treatment on laboratory profiles in respect to *CYP3A4* and *ABCB1* gene variations in the study participants:

3.9.1. Effect of *CYP3A4*22 G>A* SNP on laboratory parameters in breast cancer women undergoing tamoxifen treatment

Table (3-13) and figure (3-8) represent that rs35599367 G>A SNP (*CYP3A4*22*) genotype plays an important impact on laboratory parameters in response to tamoxifen treatment. The data was presented as mean values and standard deviations of the three parameters (calcium, estradiol and CA15.3) based on different genotypes of *CYP3A4*22* (GG, GA, AA), along with Tukey's multiple comparison calculations indicating the significance of differences between the groups.

While, no significant differences were observed in calcium levels or estradiol levels across genotypes (P>0.05), there were clear significant differences in CA15.3 plasma levels, specifically between AA genotype carriers, and GG or GA carriers (P<0.05).

Table (3-13): Genotype-dependent effects of tamoxifen on laboratory parameters in breast cancer patients: *CYP3A4*22* SNP analysis

Parameter	*CYP3A4*22*, Mean±SD			Tukey's Multiple Comparison Test	P- value
	GG, n=39	GA, n=26	AA, n=35		
Calcium	9.328±0.5491	9.219±0.5455	9.486±1.881	GG vs GA	0.4349
				GG vs AA	0.6179
				GA vs AA	0.4865
Estradiol	15.72±11.21	18.49±15.97	18.55±14.35	GG vs GA	0.7320
				GG vs AA	0.6861
				GA vs AA	>0.9999
CA 15.3	21.41±10.14	19.75±10.36	30.16±21.14	GG vs GA	0.5313
				GG vs AA	**0.0132**
				GA vs AA	**0.0122**
Results are shown as mean±SD, P <0.05 was considered significant, n=number of patients.					

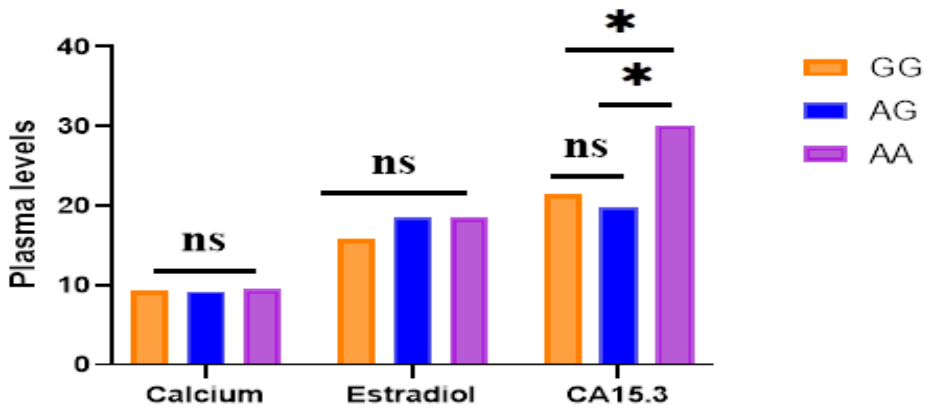

Figure (3-8): Describes genotype-dependent effects of tamoxifen on laboratory parameters in breast cancer patients: *CYP3A4*22* SNP analysis

3.9.2. Effect of rs35599367 SNP genotypes on lipid profile changes in breast cancer patients undergoing tamoxifen treatment:

Table (3-14) delves into the influence of genetic variant, rs3559967, within the *CYP3A4*22* gene, on lipid profile markers in the same study of breast cancer patients receiving tamoxifen therapy. The table presents the mean serum levels and standard deviations of lipid profile markers (Cholesterol, Triglycerides, HDL, LDL) in breast cancer patients based on their rs35599367 SNP genotypes (GG, GA, and AA).

Significant differences were found between GG and GA as well as GG and AA genotypes for cholesterol plasma levels (P=0.001 and P=0.01, respectively), and more likely, between GG and GA alleles for TG plasma levels P=0.01.

Table (3-14): Impact of *CYP3A4* rs3559967 genotype on lipid profile changes in breast cancer patients undergoing tamoxifen treatment

Parameter	*CYP3A4*22*, Mean±SD			Tukey's Multiple Comparison Test	P - value
	GG, n=39	GA, n=26	AA, n=35		
Cholesterol	161.3±21.41	198.5±51.32	184.1±39.89	GG vs GA	**0.001**
				GG vs AA	**0.01**
				GA vs AA	0.341
Triglyceride	140.1±59.76	257.9±275.3	172.5±67.51	GG vs GA	**0.01**
				GG vs AA	0.231
				GA vs AA	0.124
HDL	43±9.976	45.88±15.7	44.77±12.23	GG vs GA	0.245
				GG vs AA	0.312
				GA vs AA	0.162
LDL	139.5±33.55	146.8±40.74	148.8±36.35	GG vs GA	0.324
				GG vs AA	0.143
				GA vs AA	0.453
Data was presented as mean±SD, P<0.05 was considered significant, n=number of patients.					

3.9.3. Effect of *ABCB1 C>T* SNP on laboratory profiles in breast cancer women undergoing tamoxifen treatment

Table (3-15) and figure (3-9) demonstrate the effect of tamoxifen on laboratory variables based on the genotype variations of the *ABCB1* rs104542 C>T. This experiment helps to understand how this SNP affects different genotypes and changes in laboratory parameters in response to tamoxifen therapy in breast cancer patients**.** Exploring plasma calcium, the mean calcium levels for the CC, CT, and TT genotype groups are 9.582, 9.217, and 9.3, respectively. Tukey's test does not reveal any significant differences in calcium levels between any of the genotype groups.

Not surprisingly, no significant differences were observed in the estradiol plasma levels among all the genotypes (CC vs CT: p = 0.5971, CC vs TT: p = 0.6235, CT vs TT: p = 0.9905), which may suggest that estradiol level is not associated with ABCB1 genotype variations.

Unlike estradiol findings, CA15.3 showed clear differences between CC and CT (P=0.001) and between CC and TT (P=0.0001), while no noticeable change was noticed between CT and TT (P=0.1324).

Table (3-15): Genetic variations in *ABCB1* rs1045642 C>T SNP and their impact on biomarker levels in breast cancer patients undergoing tamoxifen treatment

Parameters	*ABCB1*, Mean±SD			Tukey's Multiple Comparison Test	Significant P < 0.05
	CC, n=28	CT, n=29	TT, n=43		
Calcium	9.582±2.067	9.217±0.522	9.3±0.601	CC vs CT	0.3610
				CC vs TT	0.4015
				CT vs TT	0.5473
Estradiol	21.78±18.04	20.21±15.16	18.12±14.19	CC vs CT	0.9258
				CC vs TT	0.6156
				CT vs TT	0.8529
CA 15.3	33.2±23.99	20.87±9.527	18.34±9.422	CC vs CT	**0.001**
				CC vs TT	**0.0001**
				CT vs TT	0.1324
Data was presented as mean±SD, P<0.05 was considered significant, n=number of patients.					

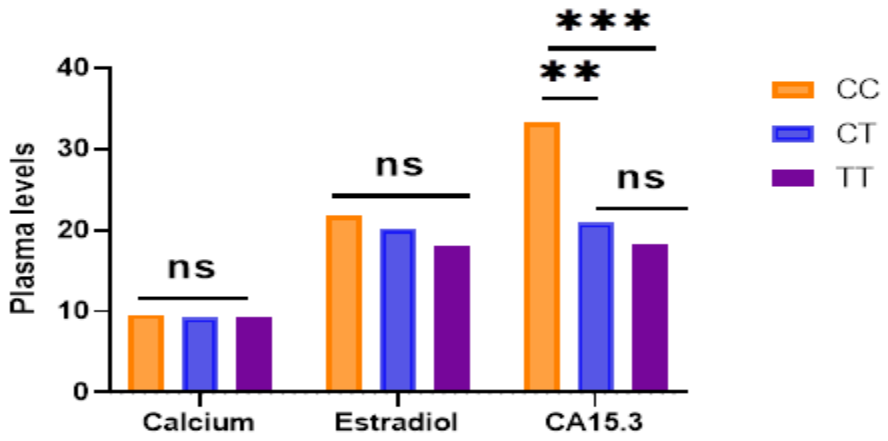

Figure (3-9): describes genetic variations in *ABCB1* rs1045642 C>T SNP and their impact on biomarker levels in breast cancer patients undergoing Tamoxifen treatment. The data was shown as mean±SD. P<0.05 considered significant.

3.9.4. Effect of *ABCB1* rs1045642 C>T SNP genotypes on lipid profile changes in breast cancer patients undergoing tamoxifen treatment:

In the context of breast cancer patients undergoing tamoxifen treatment, the effect of *ABCB1* rs1045642 C>T SNP genotypes on various laboratory parameters were explored previously. Building upon that analysis, the table (3-16) provides a deeper insight into the influence of these genetic variants (CC, CT, and TT) on specific lipid profile markers, including cholesterol, triglycerides, HDL, and LDL. The results suggest that no significant differences in CHO, TG, or LDL levels across the CC, CT and TT genotypes (all p-values > 0.05). However, notable findings emerged in HDL levels, with the TT genotype showing significantly higher levels compared to CC and CT genotypes (P<0.05) demonstrating the impact of this genetic variant on HDL concentrations during tamoxifen treatment.

Table (3-16): Differential influence of *ABCB1* rs1045642 C>T SNP on lipid profile levels in breast cancer patients receiving tamoxifen treatment

Parameter	*ABCB*1, Mean±SD			Tukey's Multiple Comparison Test	P -value
	CC, n=28	CT, n=29	TT, n=43		
Cholesterol	188.2±55.77	188±47.79	171.7±23.77	CC vs CT	0.453
				CC vs TT	0.143
				CT vs TT	0.674
Triglyceride	175.6±79.86	200.3±164.9	137.7±64.57	CC vs CT	0.875
				CC vs TT	0.967
				CT vs TT	0.365
HDL	41.25±9.702	41±11.59	48.67±13.34	CC vs CT	0.930
				CC vs TT	**0.05**
				CT vs TT	**0.05**
LDL	143.7±35.25	147.9±50.08	144.9±25.53	CC vs CT	0.453
				CC vs TT	0.675
				CT vs TT	0.875

Data was presented as mean±SD, P<0.05 was considered significant, n=number of patients.

3.10. Association of rs35599367 and rs1045642 SNPs genotypes with treatment response according to estradiol plasma levels:

Then, the potential genetic associations were investigated with treatment response and how these associations might be influenced by serum estradiol levels. Table (3-17) displays a cross-tabulation of genetic data for two SNPs, rs35599367 and rs1045642, segmented by responder and non-responder groups and further categorized based on plasma estradiol levels. For *CYP3A4* G>A rs35599367, the genotypes GG, GA, and AA are distributed among responders and non-responders. The genotype GG presented with highest proportion of responders 31% compared to GA and AA genotypes with significant observed P<0.001 across genotypes according to the Chi-square calculation.

Likewise, for *ABCB1* C>T rs1045642, the genotypes CC, CT, and TT are allocated to responders and non-responders, with respective responder percentages of (20, 21, 31) %, respectively. Aelle TT showed the highest proportion of responder individuals compared to the genotypes CT and CC with P value=0.001.

Table (3-17): Cross-tabulation of *CYP3A4* and *ABCB1* genotypes response according to plasma estradiol level

SNP	Genotype	Responders %	Non-responders %	Chi-square statistic	P value according to Chi-square test
***CYP3A4* G>A rs35599367**	GG	31	8	24.304	<0.001
	GA	17	9		
	AA	24	11		
Total	n=100	72	28		
***ABCB1* C>T rs1045642**	CC	20	8	16.420	<0.001
	CT	21	8		
	TT	31	12		
Total	n=100	72	28		
Data was presented as number and percentage					

3.11. Comprehensive analysis of genetic variations, treatment response, and serum biomarker (CA15.3) levels in two SNPs (rs35599367 and rs1045642):

Table (3-18) displays a cross-tabulation of genetic data for two SNPs, rs35599367 and rs1045642, segmented by responder and non-responder groups and further categorized based on plasma CA15.3 levels. For *CYP3A4* G>A rs35599367, the genotypes GG, GA, and AA are distributed among responders and non-responders.

According to Chi-square calculations, the genotype GG presented with highest proportion of responders 30% compared to GA and AA genotypes with observed P<0.065 which was marginally significant across genotypes. Likewise, for *ABCB1* C>T rs1045642, the genotypes CC, CT, and TT are allocated to responders and non-responders, with respective responder percentages of (12, 23, 38) %, respectively. Aelle TT showed the highest proportion of responder individuals compared to the genotypes CT and CC with P value=0.001.

Table (3-18): Cross-tabulation of *CYP3A4* and *ABCB1* genotypes response according to serum CA15.3 level

SNPs	Genotype	Responders %	Non responders %	Chi-square statistic	P value according to Chi-square test
***CYP3A4* G>A rs35599367**	GG	30	9	5.479	**0.065**
	GA	19	7		
	AA	24	11		
Total	n=100	73	27		
***ABCB1* C>T rs1045642**	CC	12	16	25.088	**<0.001**
	CT	23	6		
	TT	38	5		
Total	n=100	73	27		
Data was presented as percentage, n=number of patients.					

3.12. Correlation between *ABCB1* and *CYP3A4* gene variations and hot flashes in women undergoing tamoxifen treatment

Understanding the relationship between inflammation and genetics, particularly its role in genetic diversity, cancer progression and clinical outcome, can provide valuable insights. This calculation examines in detail the complex association between gene mutations and thermal radiation scores in cancer patients. It looks for correlations when categorizing burnout scores according to severity from ‘no symptoms’ to ‘very severe’.

The study involves patients with genetic mutations and no genetic mutations, giving a comprehensive view of how genetic factors may affect fluorescent experiences.

Then, ingenuity pathway analyses (IPA) bioinformatics tool was used to test the interaction between ABCB1 and CYP genes, which confirmed interactions across several pathways. This intrigued us to study the association and correlation of hot flashes as a side effect of tamoxifen response in patients with breast cancer and genetic variations within both genes without analyzing the gene genotypes separately for each gene as presented in figure (3-10).

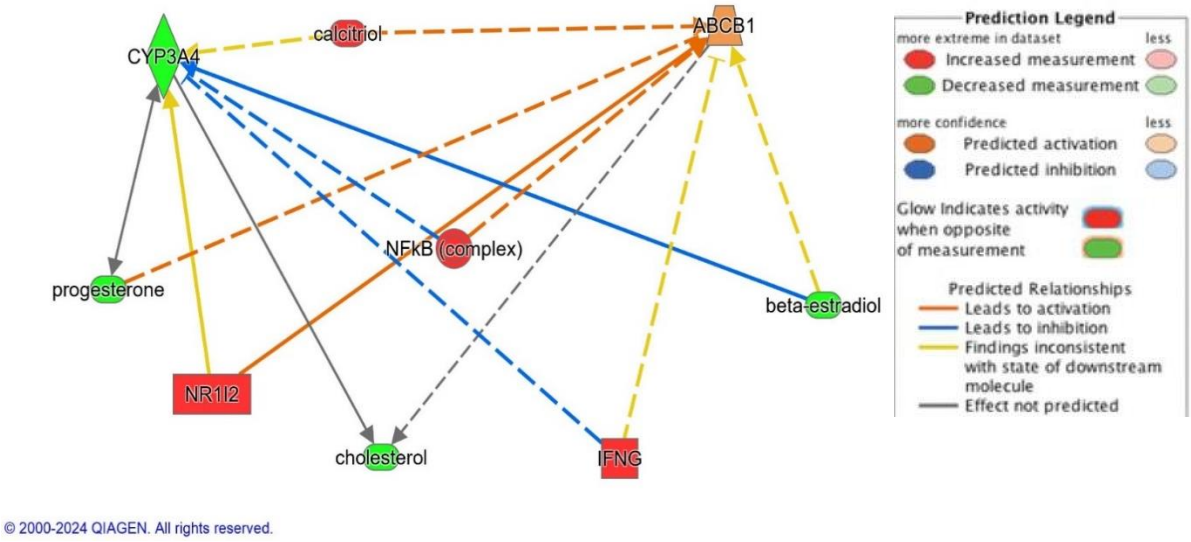

Figure (3-10): represents *CYP3A4* and *ABCB1* genes interaction through different pathways according to the IPA bioinformatic tool.

The co-relationship of *CYP3A4* and *ABCB1* was tested with hot-flash score, which is not acceptable for inverted table patients, and has not been investigated. For patients with mutant and no mutant gene variations, the analysis reveals no correlation (R Square = 0.2967) between gene variations and hot flash scores, with a p-value of 0.342. The confidence intervals provide a range for this correlation's strength. Subdividing hot flash scores by symptom severity, from "No Signs" to "Very Severe," alongside corresponding mean estradiol levels, allows for a comprehensive assessment of the gene variation's impact across different symptom severities.

Similarly, for patients with mutant gene variations, no significant correlation is observed (p-value = 0.6875), while patients with wild gene allele show a statistically significant correlation (p-value = 0.0499) with strong negative Pearson association (r=-0.8784).

Table (3.19): Correlation of CYP3A4 and ABCB1 gene variations interaction and hot flashes in breast cancer patients.

Parameter			Mutant and no mutant	R Square	Pearson r	P-value
Hot flashes scores	**Scores**	**# of patients**	**Mean of Estradiol**	0.2967	-0.544	0.342
No signs	0	18	52.204			
Mild	1	18	143.945			
moderate	2	19	20.794			
severe	3	24	23.228			
very severe	4	21	21.472			
Total		100				
Parameter			**Mutant**	**R Square**	**Pearson r**	**P-value**
No signs	0	9	19.8311	0.06151	0.2480	0.6875
Mild	1	10	17.141			
moderate	2	10	13.649			
severe	3	11	13.595			
very severe	4	8	25.498			
Total		48				
Parameters			**Wild**	**R Square**	**Pearson r**	**P-value**
No signs	0	10	79.46	0.7717	-0.8784	0.0499
Mild	1	9	29.708			
moderate	2	10	26.672			
severe	3	12	17.069			
very severe	4	11	9.560			
Total		52				

Continue with the previous step, an analysis of the association between *CYP3A4* genotype variations, in particular rs35599367, and hot flashes in breast cancer patients was done as presented in the table (3.20). Interestingly, the results revealed that a weak association (R square=0.1051) with hot flashes scores, indicated by a modest negative correlation (Pearson r=-0.3241) with a P value more than statistic threshold. Conversely, a strong association was found between wild allele and hot flashes score (R square= 0.8447) indicated with a robust negative correlation (Pearson r=-0.9191) that is statistically significant P value<0.0273.

Table (3.20): Correlation of *CYP3A4* gene variations and hot flashes in breast cancer patients.

Parameter			***CYP3A4* G>A rs35599367**	**Statistical calculations**		
Hot flashes scores	**Score**	**# of patients**	**Mutant alleles Mean of Estradiol**	**R Square**	**Pearson r**	**P-value**
No signs	0	11	21.870	0.1051	-0.3241	0.5946
Mild	1	14	171.215			
moderate	2	12	17.015			
severe	3	17	14.46			
very severe	4	11	31.135			
Total		65				
Parameters			**Wild alleles Mean of Estradiol**	**R Square**	**Pearson r**	**P-value**
No signs	0	7	99.871	0.8447	-0.9191	0.0273
Mild	1	4	48.5			
moderate	2	7	27.2714			
severe	3	7	18.11			
very severe	4	10	10.844			
Total		35				

Furthermore, the correlation of *ABCB1* genotype variations in particular rs1045642 was calculated, and hot flashes in the study's participants. As described in the table (3.21), the findings revealed a strong correlation between ABCB1 mutant gene alleles and severity of hot flashes with R square=0.7824, with robust negative regression value (Pearson r=-0.8845) that is statistically significant as presented by P value<0.0463.

Conversely, a wild allele exhibited a weak correlation with the hot flashes severity as indicated by (R square=0.08557, Pearson r=-0.2925 and P value=0.6329).

Table (3.21): Correlation of *ABCB1* gene variations and hot flashes in breast cancer patients.

Parameter			***ABCB1 C>T rs1045642***	**Statistical calculations**		
Hot flashes scores	**Score**	**# of patients**	**Mutant alleles Mean of Estradiol**	**R Square**	**Pearson r**	**P-value**
No signs	0	15	56.278	0.7824	-0.8845	0.0463
Mild	1	13	29.875			
moderate	2	15	17.992			
severe	3	15	10.507			
very severe	4	14	14.028			
Total		62				
Parameters			**Wild alleles Mean of Estradiol**	**R Square**	**Pearson r**	**P-value**
No signs	0	3	31.833	0.08557	-0.2925	0.6329
Mild	1	5	14.676			
moderate	2	4	25.625			
severe	3	9	16.375			
very severe	4	7	24.452			
Total		38				

Chapter Four: Discussion

4. Discussion

Tamoxifen is a highly effective drug for treating hormonal-positive breast cancer patients. However, a significant proportion of these patients, approximately 20% - 30%, may experience relapse (Ali *et al.*, 2016). It has been found that through previous studies that variations in the cytochrome P450-3A4 enzyme can greatly influence the response to tamoxifen by altering its metabolism into its active form, endoxifen (Sanchez Spitman et al., 2017). While *CYP3A4* polymorphisms have been extensively studied in Western countries, the findings on its impact on tamoxifen concentrations and clinical outcomes for treated patients are inconsistent and remain unclear (de Vries Schultink *et al.*, 2015).

The extent of *ABCB1* expression in breast cancer has been described by many authors over the last thirty years. However, the extent of expression differs among the studies, and there is no consensus regarding its potential role in carcinogenesis or in the tumor response to antineoplastic drugs. (Sensorn *et al.*, 2013; Skinner, Palkar and Hong, 2023).

Therefore, we look to explore the possible prognostic and predictive role(s) of *CYP3A4* and *ABCB1* polymorphisms in metastatic and/or locally recurrent inoperable breast cancer patients from Iraq who were treated with tamoxifen. These could help us to identify patients who will not respond to tamoxifen treatment and accordingly they should shift to other therapeutic options that help to improve clinical outcomes in those non-responding patients.

4.1. Impact of demographic characteristics on biomarkers profiles and treatment outcomes:

In this cross-sectional study, we included comprised breast cancer patients, and their demographic characteristics that provide valuable context for understanding the potential impact of genetic polymorphisms on tamoxifen resistance. The study reveals that the average age of the participants was 49.17 (45-65) years, with a standard deviation of 4.77, reflecting the age distribution within the group. Their mean BMI was 31.19 (17.55-42.73) kg/m², with a standard deviation of 4.935, and BMI categories indicates 8% within normal weight, 35% are overweight, and 57% are obese, which may indicate variability in weight status. On average, patients had been on tamoxifen therapy for 27.57 months, with a wide standard deviation of 25.38, suggesting considerable variation in treatment duration. Furthermore, the mean duration of breast cancer diagnosis was 3.540 years, with a standard deviation of 3.067, underscoring the chronic nature of the disease.

A comprehensive profile of demographic and clinical characteristics among breast cancer patients shed light on important factors that can be considered a risk factors for breast cancer or can influence treatment response and overall outcomes.

The obtained results found that family history and contraceptive use are not strongly associated with breast cancer, as the percentages are close to 50% for both variables, while in other studies found that the risk of breast cancer was 67% higher in women with a first-degree relative diagnosed with the same disease and twice as high in those with multiple affected relatives. The fact that both patients had a heritable genetic predisposition (the BRCA mutation) could explain this connection (Bravi, Decarli and Russo, 2018).

The obtained findings also suggest that most of the patients had 1-5 children and breastfed them, which are factors that may reduce the risk of breast cancer as it is believed that breastfeeding lowers the number of menstrual cycles a woman has in her lifetime, which in turn reduces her cumulative exposure to endogenous hormones and increases the differentiation of her breast ductal cells, making her less vulnerable to carcinogens (Kwan *et al.*, 2015). However, these factors did not prevent them from developing the disease, suggesting that other factors may be more influential. Furthermore, our findings indicate that most of the patients had tumors on the left breast, which agree with many previous studies that suggested a higher incidence of left breast cancers, which may be due to hormonal variations, environmental factors or diagnostic biases (Al Saad *et al.*, 2022; Zheng *et al.*, 2024).

The majority of the patients in our study underwent mastectomy and received intensive chemotherapy or radiotherapy due to the aggressive nature of their tumors. However, our data indicates that there are some encouraging signs: most of the patients had positive ER/PR and negative HER2 status, which are both associated with a decreased risk of recurrence and a greater chance of successful treatment (Wang *et al.*, 2011).

Additionally, it is worth noting that most of the patients were diagnosed at stage II, indicating locally advanced tumors that have not spread to other organs. With proper care, this stage has a good chance of recovery, but it also requires careful monitoring and follow-up (Trayes and Cokenakes, 2021). The patients in the current study showed symptoms of hot flashes and joint pain, which are common clinical signs of hormonal chemotherapy (Condorelli and Vaz-Luis, 2018). Managing these effects is essential when it comes to ensuring that patients continue taking medications and the quality of people’s lives.

Additionally, there were a few patients who developed cancer again, which is an encouraging result indicating that their treatment was successful and their tumors had good prognosis. Nevertheless, recurrence can still take place after numerous years; therefore, life-long surveillance and screening become mandatory.

4.2. Effects of tamoxifen treatment on laboratory parameters

The comparative analyses of the biomarkers profile of patients of this study reveals that breast cancer patients showed dramatically lower levels of estradiol ($p=0.0418$) and higher levels of CA15.3 ($p=0.0081$) compared to healthy individuals, aligning with known breast cancer characteristics. Calcium levels are elevated in patients ($p=0.0005$), though the clinical relevance of this difference may warrant further investigation. In contrast, lipid biomarkers, including cholesterol, triglycerides, HDL, and LDL, do not significantly differ between the two groups ($p>0.05$, ns).

Not surprisingly, our findings agree with previous studies, which highlight the potential utility of estradiol and CA15.3 antigens as valuable markers of serum tumors in breast cancer patients (Li *et al.*, 2020; Atakpa *et al.*, 2021; Tarighati, Keivan and Mahani, 2023; Masoudi, 2023).

Continuing to the next step, we examined the changes in biomarkers levels that associated with age. This is because fluctuating in biomarkers including proteins, lipid and hormones is linked to the aging physiology (Green and Hillersdal, 2021).

Clearly, our data revealed that among the 45-65 age range, there were no significant differences in the biomarkers assessed, including estradiol, CA15.3, calcium, cholesterol, triglycerides, HDL, and LDL. This answer into the question the belief that age alone drives changes in these biomarkers within this particular group.

Instead, other important factors such as health status, genetics background and lifestyle should be counted. These results highlight the value of a comprehensive assessment when examining biomarker profiles and their possible bearing on the course and results of breast cancer treatment.

Exploring how BMI and clinical laboratory biomarkers are correlated in breast cancer patients is essentially to fully understand the disease, its advancements and how body weight could potentially impact the overall health. With this knowledge we can precisely assess progress of the individualized treatment plans, and enhance care for those with breast cancer (Meyer *et al.*, 2022). Interestingly, our findings reveal that a strong connection in some laboratory biomarkers based on BMI status. Normal weight patients showed more than two folds' increase in the HDL plasma levels comparing to the obese and overweight patients who exhibited elevated levels of cholesterol and triglycerides.

It is worth to notice that there is no discernible difference in the levels of calcium, LDL, CA15.3, and estradiol between the various BMI categories. These findings agree with previous research that highlighting the impact of weight on specific biomarkers in patients with breast cancer. Thus, it is critical to take their BMI into account when evaluating their general health and any potential risk factors (Liu *et al.*, 2018; Hu *et al.*, 2020; Chen *et al.*, 2023) (Nardelli et al., 2024), which suggesting that weight status critically impact specific biomarkers in patients with breast cancer. Intriguingly, the data reveals that normal weight patients had higher HDL

Tamoxifen is a widely used adjuvant therapy for ER+ breast cancer; however, drug resistance in about 30% of cases can lead to recurrence and metastasis, the primary causes of death (Amiruddin *et al.*, 2022). In depth examine the association of tamoxifen with several laboratory biomarkers may lead to understand the cellular processes underlying tamoxifen resistance and indicator of poor overall survival (Dal Berto *et al.*, 2021).

In this current study we compared laboratory biomarkers in two groups of breast cancer patients based on the duration of tamoxifen treatment. The first group received tamoxifen treatment for less than or equal to 5 years, while the other received treatment for more than 5 years. We found a significant difference in estradiol levels between the two groups, with longer treatment duration associated with lower estradiol levels. However, there were no significant differences in other biomarkers, including CA15.3, calcium, HDL, cholesterol, and TG, based on treatment duration. These findings have been suggested by other groups in which they found that long term tamoxifen treatment significantly reduced estrogen levels, which may suggest a promising mechanism that can be used to predict resistance to tamoxifen (Woo *et al.*, 2015; Dal Berto *et al.*, 2021).

Hence, these findings underscore the potential impact of tamoxifen treatment duration on estradiol regulation, which is relevant in breast cancer management. In respect, the duration of treatment does not significantly influence the other tested biomarkers.

4.3.1. Frequency of *CYP3A4*22 (rs35599367) G>A* gene polymorphism in Iraqi breast cancer patients:

*CYP3A4*22* G>A variant is located in intron 6 of the *CYP3A4* gene. It was first identified by Dai et al. and later found by Fukushima-Uesaka et al. in a Japanese population with a frequency of 0.189 and with lowers liver *CYP3A4* mRNA expression (Fukushima-Uesaka et al., 2004) (Holmberg *et al.*, 2019).

In the current study we specifically examined the association of *CYP3A4*22* gene polymorphism with breast cancer. Among the three genotypes (GG, GA, and AA) analyzed, the GG genotype is the most prevalent at 39%, followed by AA at 35% and GA at 26%, which is consistent with previous study in Iraqi population (Abed, 2022), and also agree with previous study in United States in which carrying high percentage of mutant allele (Hertz *et al.*, 2017), while mutant genotype (AA) detected in others studies with very low frequency for example in Jordanian patients (2%) (Al-Eitan *et al.*, 2019) and (5%-7%) in Caucasian (Hertz *et al.*, 2017) while Asian and African population below (1%) (Holmberg *et al.*, 2019).

The Hardy-Weinberg principle is a fundamental concept in population genetics that states that allele and genotype frequencies in a population will remain constant from generation to generation in the absence of other evolutionary influences.

The Hardy-Weinberg principle can be used to study gene polymorphism by comparing the observed genotype frequencies in a population with the expected genotype frequencies under the Hardy-Weinberg equilibrium.

If the observed genotype frequencies differ significantly from the expected genotype frequencies, it suggests that some evolutionary force is acting on the population (Chen, 2010; McMurran, 2010). Surprisingly, variation analysis of *CYP3A4*22* (rs35599367) G>A in a cohort of 100 breast cancer patients revealed that among the three genotypes (GG, GA, and AA) analyzed in this current study revealed that individuals with allele G had a frequency of 52% and those with allele A had a frequency of 48%. Deviations from the Hardy-Weinberg equilibrium, as indicated by the Fischer exact test, reveal significant departures for all genotypes (GG, GA, and AA) from the expected values.

The observed GG genotype significantly deviates from the expected GG genotype, while the observed GA genotype significantly differs from the expected GA genotype (mean difference -0.2366, $p < 0.001$). The AA genotype also deviates from the expected AA genotype (mean difference 0.1194, $p < 0.001$). These significant deviations from Hardy-Weinberg equilibrium suggest that there are specific factors influencing the genetic distribution of the *CYP3A4*22* (rs35599367) G>A gene SNP in this breast cancer patient population.

Many previous studies agreed with results obtained in this study, in which they found that the *CYP3A4*22* genotypic distribution did not adhere to Hardy-Weinberg population laws (Olagunju et al., 2014)(Oetting et al., 2018)(Abed, 2022). There could be multiple reasons for this phenomenon, including potential issues with genotyping; however, the exact underlying cause remains unclear and was not discussed in the original articles (Zeigler-Johnson *et al.*, 2002).

Thus, these findings highlight the potential impact of genetic variants in these genes on breast cancer and suggest the need for further studies to understand the underlying genetic mechanisms causing the occurrence and potential implications of these barriers under the pathogenesis of disease and treatment outcome.

4.3.2. Frequency of *ABCB1 3435C>T (rs1045642)* gene polymorphism in Iraqi breast cancer patients:

The results of the present study revealed three genotypes: CC, CT, and TT are in the line with earlier studies in Chinese and Iraqi population, our data reveals that the most common genotype was the homozygous mutant TT (43%), followed by heterozygous CT (29%) and homozygous CC (28%) (Wu *et al.*, 2012) (Mahdi *et al.*, 2021) (Hasan *et al.*, 2023). Previously, a study conducted by Abouhalima et al. on women in Jordan discovered that those with the T allele were twice as unlikely to get breast cancer, while those with the CC genotype were more common in breast cancer patients (Abuhaliema et al., 2016).Another findings revealed a higher prevalence of the T allele compared to the C allele among Jordanians (Salem et al., 2014).

In Hardy-Weinberg equilibrium, the genotype frequencies for CC (42.5%), CT (33.1%), and TT (24.4%) exhibit noteworthy deviations, signifying non-randomness. The genotypes CC, CT and TT exhibit significant deviations from the anticipated frequencies, as indicated by p-values of 0.0001 and mean differences of -0.201, 0.180625 and 0.099, respectively. The discrepancies observed in each genotype from the Hardy-Weinberg equilibrium suggest certain factors that may have influenced the genetic distribution of *ABCB1* in a sample of breast cancer patients such as ethnic and environmental background of the participants, methods of the analysis and sample size.

4.4.1. Genotype-dependent effects of tamoxifen on laboratory parameters in breast cancer patients: *CYP3A4*22 G>A* SNP analysis

Numerous studies involving breast cancer patients with variety of breast cancer treatment including tamoxifen, docetaxel and exemestane have demonstrated patients with breast carcinoma who carried the 6 intronic of *CYP3A4*22* rs35599367 variant have low enzymatic activity (Thummel and Lin, 2014) (Bins *et al.*, 2019), classified as poor metabolizer along with elevated steady state drug concentrations (Hertz *et al.*, 2017) (Sanchez Spitman *et al.*, 2017).

In light of this, breast cancer patients who had the rs35599367 SNP had an elevated level of tamoxifen and its metabolites (Teft *et al.*, 2013). The current study explored the impact of *CYP3A4*22* SNP on laboratory parameters, including calcium, estradiol and CA15.3 levels. While there are no significant differences in calcium levels among GG, GA, and AA genotypes. The GA and GG genotypes were significantly associated with lower CA15.3 levels, indicating a potential effect of gene variations on tumor marker levels. Estradiol levels were marginally lower in the GG genotype, suggesting a crucial role on hormone levels in a subset of patients. These findings suggest that the GG genotype may be significantly related to specific laboratory parameter variations and could be a key factor in individual responses to treatment and prognosis in breast cancer patients (Baxter et al., 2014), (Sanchez Spitman et al., 2017), (Abed et al., 2022). These findings underscore the importance of examining gene variations in breast cancer patients for tailoring personalized treatment plans. Understanding the impact of genetic variations on laboratory parameters can provide valuable insights into disease aggressiveness, hormone receptor status, treatment decisions, and survival rates (Demurtas et al., 2021).

4.4.2 *CYP3A4*22* SNP influence on lipid profiles in tamoxifen-treated breast cancer patients:

Here in this study, we examined the impact of *CYP3A4*22* rs35599367 SNP genotypes on lipid profile in breast cancer patients undergoing tamoxifen treatment. Obviously, the data indicate that GG allele is dramatically associated with lower cholesterol levels compared to GA and AA genotypes (means difference -37.24, P< 0.001, -22.77, P<0.01) respectively. Furthermore, GG genotype exhibits significantly lower triglyceride levels compared to GA genotype, with a mean difference of -117.8 (p = 0.01) but no significant difference in HDL and LDL levels between genotypes. The use of tamoxifen has been associated with reduced levels of LDL and TC, and a higher proportion of HDL. This is thought to be due to tamoxifen's structural resemblance to estrogen, which helps in safeguarding blood lipids during endocrine therapy (Song *et al.*, 2021).

Furthermore, there was not a strong correlation between HDL and LDL plasma levels and genotype variation. Instead, our findings suggest that gene polymorphism may impact plasma cholesterol, triglyceride levels in the patients with breast cancer underwent tamoxifen treatment. Hence gene variations may alter lipid metabolism, which emphasize the needs for genotype-based monitoring and care during tamoxifen therapy (He *et al.*, 2020, 2022; Song *et al.*, 2021; Pakiet *et al.*, 2023).

4.5.1. Genotype-dependent effects of tamoxifen on laboratory parameters in breast cancer patients: *ABCB1* rs1045642 C>T SNP analysis:

The present study demonstrated significant differences between genotypes regarding the levels of the CA 15.3 biomarker that may determine the modes of treatment. But no significant differences are observed in case of calcium and estradiol levels among genotypes.

The recent studies show that the CT/TT SNP rs1045642 (variant allele) of the gene *ABCB1* does not increase the probability of relapse, but predicts a favorable outcome following therapy. However, individuals with the CC genotype of the *ABCB1* C3435T develop metastases and recurrences faster (Rafiee et al., 2018).

Some research findings pertaining the relationship of *ABCB1* polymorphisms and therapy success in tamoxifen-dependent breast cancer sufferers. In 95 patients with breast cancer receiving tamoxifen treatment, the study evaluated the clinical relationship between *ABCB1* polymorphisms and metastasis and recurrence risk. The findings showed that patients with homozygous CC genotypes of *ABCB1* C3435T had reduced time to tumor recurrence (Teh et al., 2012). Comparable outcomes were found in a different study involving thirty Thai patients who had breast cancer (Sensorn et al., 2013). In patients receiving tamoxifen adjuvant therapy, patients with heterozygous *ABCB1* 3435 CT genotype showed significantly shorter disease free survival than those with homozygous 3435 CC genotype (Sensorn et al., 2013).

In fact, subjects carrying the TT genotype showed lower expression of intestinal P-gp than did carriers of the CC genotype. This suggests that because the capacity of *ABCB1* transporters may be decreased, patients with TT may respond better to treatment than those with wild genotypes, which is consistent with our findings (Nurmatova et al., 2022). It implies that *ABCB1* 3435C.T may have an effect on recurrence risk.

4.5.2. *ABCB1* rs1045642 C>T SNP influence on lipid profiles in tamoxifen-treated breast cancer patients:

Clearly, there were no significant differences in cholesterol and LDL levels between genotypes variations in spite patients with TT allele exhibited lower triglycerides level compared to CT allele.

Additionally, there was an interesting trend with TT genotype patients having higher levels of HDL compared to those with CC and CT genotypes. These results suggest that this genetic variation may have an impact on certain aspects of the lipid profile in breast cancer patients, potentially influencing treatment outcomes in a clinical setting.

Many studies explored the relationship between tamoxifen treatment and blood lipid profiles in patients with breast cancer. Through their research, Li and his team endeavored to examine the impact of *ABCB1* rs1045642 C>T SNP on the lipid profile levels of breast cancer patients undergoing chemotherapy. Their findings indicated that the presence of the minor-T-allele (CT/TT) of rs1045642 was linked to a more positive outcome in patients treated with Gemtuzumab ozogamicin (X. Li *et al.*, 2018).

Furthermore, studies examined the levels of lipid lipoprotein in females with breast cancer at the time of diagnoses and during chemotherapy administration as markers of treatment response. The studies showed significantly lower levels of dyslipidemia in the breast cancer group compared to the normal group. However, cholesterol may be significantly worsened following chemotherapy. Therefore, lipid monitoring, prevention and treatment of dyslipidemia should be performed for patients with breast cancer at initial diagnosis and during the development of chemotherapy (Yao *et al.*, 2020).

4.6. Explore genotype variations according to tamoxifen response:

Understanding the correlation between tamoxifen treatment response and plasma estradiol levels further solidifies the significance and efficacy treatment of patients with breast cancer (L. Li *et al.*, 2018).

Therefore, we examined tamoxifen response based on the genotype variations of two SNPs *CYP3A4**22 (rs35599367) and *ABCB1* (rs1045642), with a specific focus on plasma estradiol levels. A Chi-square statistic with 2 degree of freedom reveals that patients with GG allele at rs35599367 and TT allele at rs1045642 have a higher chance with a P value <0.001 of response to tamoxifen treatment comparing to the other genotypes. Interacting between plasma E2 levels and genetic variations and tamoxifen response is crucial for breast cancer treatment. Therefore, understanding this area further enhances patients care and personal medicine outcomes (Ximenez *et al.*, 2019; Faltinová *et al.*, 2021; Malathi, Balakrishnan and B. S, 2021)

Then tamoxifen response was examined based on the genotype variations of two SNPs *CYP3A4*22* (rs35599367) and *ABCB1* (rs1045642), with a focus on serum CA15.3 levels. Interestingly, our results reveal that patients who carry GG allele at rs3559967 showed a marginally significant tendency toward higher response (P<0.065) comparing to the other genotypes. On the other hand, *ABCB1* C>T rs1045642, allele TT showed the highest proportion of responder individuals compared to the genotypes CT and CC with P value=0.001.

Previously, scientists proposed that the genotype variations impact preoperative levels of CA15.3 in Chinese women with breast cancer. More studies examined the effectiveness of chemotherapy treatment according to the *ABCB1* gene variations in the patients with breast cancer. The findings showed that for patients treated with Gemtuzumab ozogamicin, the TT genotype of ABCB1 rs1045642 was linked to a better outcome (Perrier *et al.*, 2020).

Additional research examining the impact of the *ABCB1* SNP on calicheamicin-mediated cytotoxicity discovered that patients with the variant allele (CT/TT) had a markedly better prognosis than those with the genotype CC (Al-Azawi *et al.*, 2006; Sychev *et al.*, 2022). All things considered, gene variations are critical to the response to tamoxifen treatment in patients with breast cancer, and these discoveries may help design effective therapeutic approaches that take into account the genetic makeup of individual patients.

4.7. Correlation of gene variation and hot flashes in cancer patient

Finally, the effect of *CYP3A4*22* and *ABCB1* gene variations on hot flashes occurrence were examined as it is crucial to advance personalized medicine, predict an individual's risk of experiencing hot flashes, optimize treatment choices and dosages (Al-Ali et al., 2022).

For patients with both mutant and no mutant gene variations, with hot flashes scores, the Pearson correlation coefficient (r) was -0.544 with a p-value of 0.342, suggesting a moderate negative relationship between estradiol levels and hot flashes scores, in spite of its not statistically significant. However, the wide confident interval (-0.9638-0.6499) suggests a wide range of possible correlation values. The calculations were breakdown by mutant status and no mutant status. Interestingly the results showed varying degrees of correlation between the severity of hot flashes and estradiol plasma levels. For mutant group, there was no statistically significant correlation between estradiol plasma levels and hot flashes scores comparing to the no mutant or wild group that showed a strong negative correlation between estradiol levels and hot flashes scores with a $P<0.0499$ ($r=-0.8784$).

The correlation between the *CYP3A4**22 gene (specifically the G>A polymorphism at rs35599367) and hot flashes in breast cancer patients was further calculated. For mutant alleles, the R square value was 0.1051 for patients with no signs of hot flashes suggesting a weak correlation between estradiol plasma levels and hot flashes symptoms. In addition, Pearson value -0.3241, indicating a weak negative statistically not significant correlation. Conversely, for the wild alleles, the R square was 0.8447 for patients with no symptoms of hot flashes which may indicate a strong negative correlation between estradiol levels and hot flashes signs as indicating with a P value less than 0.0273. Similar interpretations can be found for the other severity of hot flashes in the wild alleles group. Certainly, the data recommend that the wild alleles of the *CYP3A4* gene may play a crucial role in modulating hot flashes severity in women with breast cancer disease comparing to those who carry *CYP3A4* rs35599367 C>T mutant alleles.

A correlation analyses between variations in the *ABCB1* gene C>T of rs1045642 and hot flashes in breast cancer patients, divided by mutant and wild alleles was calculated. A strong relationship was found between estradiol levels and hot flashes signs in the patients with mutant alleles as indicated by Pearson correlation coefficient value=-0.8845 with a P value 0.0463. Conversely, for patients with wild or no mutant alleles, the analyses revealed that a weak relationship between E2 levels and hot flashes signs as indicated with a P value 0.6329 r value equal to -0.2925.

Therefore, the current study implies there is a significant correlation between *ABCB1* and *CYP3A4*22* gene mutations and the occurrence of hot flashes in Iraqi breast cancer women.

Hale et al explored genome wide analyses, and his findings suggested that there are 12 multi loci model that able to predict hot flashes in women under the effect of tamoxifen treatment, which emphasizing the need for confirmation through additional studies (Hale, Cuzick and Sestak, 2020). Therefore, employing predictive models for side effects like hot flushes could enhance our understanding of individual responses to tamoxifen and potentially shed light on the underlying biological pathways involved.

4.8. Study Conclusions

1. In a group of Iraqi breast cancer patients, the *CYP3A4*22 G>A (rs35599367*) and *ABCB1 C>T* (rs35599367) genes had a high level of genetic diversity and with variety of genotypes and frequencies.

2. There was a higher frequency of wild genotype GG compared to mutant types GA and AA for rs35599367, while a higher frequency of mutant type TT for rs1045542 SNP compared to wild genotypes CC.

3. There was no significant correlation found between *CYP3A4*22* and *ABCB1* genes variations and plasma levels of estradiol, calcium and some lipid profile testing, while there was a significance reduction in tumor marker CA15.3 level for both SNPs.

4. According to the lowest estradiol and tumor biomarker plasma levels, patients with the wild GG genotype of rs35599367 are good responder group with severe hot flashes, and for the mutant TT genotype of rs1045542 represented a more promising response to tamoxifen therapy with strong correlation for severity of hot flashes.

4.9. Recommendations

1. In order to have a better understanding of the effects of *CYP3A4*22* and *ABCB1* variants, more large-scale and multi center studies are needed for Iraqi breast cancer women receiving tamoxifen.

2. The inter-individual variability in tamoxifen response may be influenced by genetic polymorphisms in other drug transporters and enzymes implicated in tamoxifen metabolism and may warrant further investigation.

3. Our recommendation for the clinical setting is the development of genetic testing that can predict an individual's reaction to tamoxifen treatment, as well as the creation of tailored medications that are more effective and safer.

4. Determination the plasma level of tamoxifen metabolites is necessary to demonstrate the impact of gene polymorphisms of the metabolizing enzyme on the bioavailability of tamoxifen.

5. Establishing special center to record the results of genetic studies in specific data base for Iraqi population.

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Appendices

Patient name **Age** **Weight** **Height**

Educated state **Menarche age**

Social activity of women: **Smoking**

hormonal treatment

breast cancer history

Number of births: **Normal delivery or CS**

Breast feeding: **Bottle** **Mixed**

Tumor site: Left **Right** **Bilateral**

Type of surgery: **Mastectomy** **Breast conserving**

Use of adjuvant: **Radiotherapy** **Chemotherapy**

Receptor states: **ER, PR and HER2**

Stage and grading:

Other disease:

Side effects:

Hot flash degree:

Other drugs used:

Time of tamoxifen use:

Date of breast cancer diagnosis:

الخلاصة

خلفية الدراسة: يعتبر سرطان الثدي من أكثر الأمراض التي يتم تشخيصها لدى النساء على مستوى العالم. تاموكسيفين هو مُعدِّل انتقائي لمستقبلات هرمون الاستروجين، ويعتبر الاساس في علاج سرطان الثدي الإيجابي للمستقبلات الهرمونية. تاموكسيفين هو دواء أولي ويتم تحويله إلى النواتج الايضية الأكثر حيوية بواسطة العديد من إنزيمات التمثيل الغذائي. علم الوراثة الدوائي له تأثير بالغ الاهمية على التباين بين الأفراد الذي يؤثر على الاستجابة العلاجية، ومن المهم دراسة الجينات متعددة الأشكال المتضمنة في حركية عقار التاموكسفين. لذلك، فإن تعدد الأشكال الجيني لانزيم التمثيل الغذائي المهم لعقار التاموكسفي*CYP3A4* والناقل الى الخارج لعقار التاموسكفين والنواتج الايضية له*ABCB1* قد يكون له تأثير محتمل على النتائج العلاجية للتاموكسيفين.

تهدف الدراسة إلى: الكشف عن الأشكال الجينية ل *CYP3A4*22 G>A (rs35599367)* و *ABCB1 C3435T (rs1045642)* بين النساء العراقيات المصابات ب سرطان الثدي المشمولة في هذه الدراسة والتحقيق في تأثير هذه المتغيرات الجينية على فعالية عقار تاموكسيفين.

المرضى وطريقة العمل: هذه دراسة مقطعية رصدية أجريت في مدينة الإمام الحسين الطبية ومستشفى الإمام الحسن المجتبى التعليمي / مركز الأورام في كربلاء / إلى جانب مختبرات كلية الصيدلة / جامعة كربلاء التي أجريت بين نوفمبر 2022 وأبريل 2023 . تم اختيار مائة مريضة مصابة بسرطان الثدي الذي كان مستقبل هرمون الاستروجين و / أو مستقبل هرمون البروجسترون إيجابيا. تم تسجيل المرضى الذين تتراوح أعمارهم بين 45 عامًا وما فوق، والذين يتناولون جرعة يومية من عقار تاموكسيفين 20 ملغ لمدة أربعة أشهر على الأقل، في هذه الدراسة. شملت الدراسة أيضًا مائة امرأة سليمة توفر مجموعة مقارنة مهمة للتحليل الكيميائي الحيوي (مستوى الاستراديول، علامة الورمCA15.3 ، الكالسيوم وملف الدهون).

تم سحب خمسة ملليلتر من الدم الوريدي من كل مريض بعد التوقيع على استمارة الموافقة المكتوبة مسبقا لقياس المعايير البيو كيميائية مثل استراديول، CA15.3، الكالسيوم، وملف الدهون بالإضافة إلى التحليل الجيني. تم استخدام نظام طفرة التضخيم الحراري وتفاعلات سلسلة البلمرة الخاصة بالأليل للكشف عن *CYP3A4*22 G>A* (rs35599367) و *ABCB1 C3435T* (rs1045642) على التوالي.

النتائج: أظهرت النتائج التي تم الحصول عليها أن هناك متغيرات وراثية مختلفة لـ *CYP3A4*22* (rs35599367) G>A و *ABCB1 C3435T* (rs1045642) بالنسبة إلى (rs3559967) G>A فإن النمط الوراثي المتماثل الزيجة السائد (GG) هو النمط الجيني الأكثر شيوعًا متبوعًا بالأنماط الجينية المتماثل الزيجة الطافر (AA) والأنماط الجينية المتغاير الزيجة الطافر (GA) تكون أقل شيوعًا بمعدل تكرار 39% و35% و26% على التوالي. لكن بالنسبة لـ (rs1045642 *C3435T*)، فإن النمط الوراثي المتماثل الزيجة الطافر (TT)هو النمط الجيني الأكثر شيوعًا، في حين أن الأنماط الجينية المتغايرة الزيجة الطافرة ((CT)والمتماثلة السائدة (CC)أقل بمعدل تكرار 43% و29% و28% على التوالي. أظهرت مستويات الاستراديول والكالسيوم وLDL في المصل ارتباطا غير معنوي، في حين تبين ان مستوى دلالة الورم في المصل CA15.3 ارتباطًا معنويًا بين تعدد أشكال النوكليوتيدات المفردة الاثنان المدروسة لجينات *CYP3A4* و*ABCB1* بقيمة p أقل من 0.05. علاوة على ذلك، وجدت الدراسة الحالية أن من التأثيرات الجانبية الهبات الساخنة تحدث بنسبة عالية تبلغ حوالي 82% مع ظهور جديد للمرض بنسبة قليلة حوالي 5%.

الاستنتاج: خلصت الدراسة الحالية إلى وجود علاقة ارتباط بين التباين الوراثي في جينات *CYP3A4*22* و*ABCB1* والتباين في الاستجابة لعقار التاموكسفين بين النساء العراقيات المصابات بسرطان الثدي حيث أن النمط الجيني المتماثل الزيجة السائد GG من *CYP3A4 * 22 rs35599367* والنمط الجيني المتماثل الزيجة الطافر TT من *ABCB1 rs1045542* هي مجموعات ذات استجابة جيدة مع حدوث الهبات الساخنة الشديدة.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء

كلية الصيدلة

تأثير تعدد الأشكال الجيني لـ *CYP3A4* و *ABCB1* على فعالية عقار تاموكسيفين لدى النساء العراقيات المصابات بسرطان الثدي

رسالة

مقدمة الى مجلس كلية الصيدلة – جامعة كربلاء

كجزء من متطلبات نيل شهادة الماجستير في علم الادوية والسموم

من قبل

أرجوان فؤاد حسين

بكالوريوس صيدلة (جامعة كربلاء 2015)

بإشراف

الأستاذ الدكتور

احمد صالح صاحب

الأستاذ مساعد الدكتور

جمال علي عاشور

2024 ميلادي

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