Thesis for doctoral degree (Ph.D.)
2019

Pharmacokinetics and Pharmacogenetic Studies of Cyclophosphamide based Chemotherapy and Tamoxifen in Ethiopian Breast Cancer Patients

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Pharmacokinetic and Pharmacogenetic Studies of Cyclophosphamide based Chemotherapy and Tamoxifen in Ethiopian Breast Cancer Patients

A Thesis Submitted to the School of Graduate Studies of Addis Ababa University, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy (Ph.D.) in Clinical Pharmacology.

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January, 2019

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DECLARAION

I, the undersigned, declare that this Ph.D. thesis is my original research work and has not been presented for a degree in any other university.

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Place and date of submission Addis Ababa, Ethiopia, October, 2019

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Acknowledgment

I would like to take this opportunity to express my heartfelt and sincere appreciations to all those who have made a huge contribution to this work troughout my studies.

The past six years were years with lots of challenges and ups and downs. However, the wise and careful guidance as well as full support of **Professor Eyasu Makonnen** was there to push me forward. I testify that that you were, for me, not only a supervisor. The fatherly treatment you afforded me has served as an inspiration and energizer for the completion of this study. You were always there when I needed advice. I always enjoyed the rich discussions we had as well as your swift and critical comments to my manuscripts and thesis. I would like to express my uttermost appreciations for your wisdom, support and guidance throughout the study period.

I would also like to convey my biggest gratitude and appreciations to **Professor Eleni Aklillu**. I feel very proud to be invited and work in your research group under your supervision. You have taught me the essence of research and science. I have learned a lot from your amazing strength, intelligence, and diligence. I am always amazed by your pin point eye to meticolosly review my manuscripts. I will never forget all your help and all the great tips for living in Sweden, as well as arranging to Ugandan visit. Dear **Professor Eleni**, from the bottom of my heart, thank you very much! For everything you did for me, from the very warm welcoming me to your research group to ultimately making this five-year journey a success. I hope to stay in contact with you.

I would also to thank my advisors **Dr Daniel Seifu, Dr Getnet Yimer** and **Dr Matheos Assefa** for their constructive advice and moral support. My gratitude also goes to **Dr Alan Fotoohi**, for linking me to ECM research group to analyse of cyclophosphamide. I am very grateful to **Professor Moustapha Hassan** for welcoming me to his lab to analyze cycolphospahmide. I would also like to thank all my co-authors especially **Dr Jackson Mukonzo** for welcoming me to Makarare University to do modeling work, **Ronald Kutessa** for teaching me NONMEMs, a PK/PD modeling software and R, a software for statistical computing and graphics. Thank you all for all your collaboration and help. My special thanks also goes to **Najjemba Letisha, Susan**

Karungi and **Aida Kawuma** from Makarere University for sharing their experiences during NONMEM modeling work.

I would like to thank **Jimma University** for providing me the study leave. I would also like to thank **Addis Ababa University** for making this study possible. I am deeply grateful to **Armauer Hansen Research Institute (AHRI)**, for the financial support required for this study. I would also like to extend my special thanks to **Dr Abraham Aseffa** and **Dr Rawleigh Howe**, for all your guidance and positive support during my studies. I would also like to extend my sincere appreciations to all the staff members of AHRI for their kindness and support, particularly, Dr Martha Zewdie, Dr Kididst Bobosha, and Mr Hailemichael Getachew. I am also very grateful to have wonderful lab members Abby, Matias, Rajabu at Karolinska Institutet (Sweden), and Beti from AAU core lab for their kindness and very supportive conversations.

I also owe a great deal of gratitude to Seada Muktar (my wife), and my kids for their love, patience and moral support during the study period. I would also like to thank my study participants for their willingness to participate in the study.

Above all, with genuine humility, I acknowledge Your Aid and Grace. O' my **God (Allah)**, Without Your Guidance, Cause, Innumerable Blessings, and Support, this work would not have been possible.

Abstract

Introduction: *Breast cancer is the most frequently diagnosed cancer among women in both developed and developing countries. Its treatment involves a multi-modality approach to eradicate residual cancer and prevent recurrent disease. Large inter-individual variations in the phamacokinetics and treatment outcome of cyclophosphamide and tamoxifen have been reported. In addition to other patient factors such as age, sex and etc, pharmacogenetic variations have been implicated to potentially influence the pharmacokinetics of anticancer drugs resulting in suboptimal effects and/or unpredictable toxicity. This thesis contributes to better increase our understanding of the implication of pharmacogentic variations in the treatment of breast cancer.*

Objective: *The aim of this thesis was to investigate the effect of genetic polymorphism of drug metabolizing enzymes and transporter proteins on the pharmacokinetics and pharmacodynamics of cyclophosphamide and pharmacokinetics of tamoxifen metabolites among Ethiopian breast cancer patients.*

Methods*: The study was conducted in breast cancer patients who were on CPA based chemotherapy (chemotherapy group) and on tamoxifen (tamxifen group). Drug and metabolite quantification was performed using HPLC (cyclophosphamide) or LC/MS/MS (Tam and its metabolites). Genotyping of candidate genes encoding drug metabolizing enzymes and transporter proteins, relevant to cyclophosphamide and tamoxifen metabolism and transport including CYP2B6, CYP2D6, CYP2C9, CYP2C19, CYP2J2, CYP3A5, POR, and ABCB1 and UGT2B15, was done using TaqMan™ allele specific PCR. CYP2D6 gene copy number was also determined by TaqMan™ Copy Number Assay. Among chemotherapy group, hematological toxicity (neutropenia, anemia, and thrombocytopenia) were also monitored throughout chemotherapy cycle. A population PK-PD modeling of cyclophosphamide was performed using non linear mixed effect modeling (NONMEM) software.*

Result*: **In study I**, the incidence of chemotherapy induced grade 3 or 4 hematological toxicity was 51.0% (95% confidence interval (CI) = 44.54 – 57.46%). Most of the hematologic toxicities were neutropenic toxicity (50.2%). CYP2J2*7 variant allele and low baseline white blood cells count (grade 1 leukocytopenia), or low neutrophils count (grade 1 or 2 neutropenia), were associated with increased risk of grade 3 or 4 hematologic toxicity in the subsequent cycles. Patients carrying CYP2C9 *2 or *3 alleles were associated with lower incidence of hematologic toxicity. **In study II**, the overall actual average RDI was 81.9%. The proportion of patients who received reduced RDI < 85% of the standard/planned dose intensity was 56.6%. The independent risk predictors of reduced RDI were CYP2J2*7 allele, BMI < = 18.4 kg/m^2 (underweight), low baseline leukocyte count, and low baseline neutrophils count. On the other hand, the odds of receiving reduced RDI was lower in patients with CYP2B6 *6/*6 genotype. **In study 3**, the PK analysis showed that cyclophosphamide was described by one compartment pharmacokinetic model with population clearance, and apparent volume of distribution of 5.41 L/hr and 46.5 L, respectively. The inter-individual variability (IIV) in clearance was* 51% (38.9% and 49.3% for 500 and 600 mg/m^2 CPA regimen group, respectively). *CYP3A5 and CYP2C9 genotypes, body surface area and cyclophosphamide dosing regimen were identified as significant predictors of PK parameters. Plasma cyclophosphamide exposure moderately and*

positively predicts neutropenic toxicity. ***In study IV,*** *the proportion of patients with low endoxifen concentration (below 5.9 ng/mL) was 35.8%. Large inter-individual variability in endoxifen concentration and* $MR_{E/NDM}$ *was observed (coefficient of variation 74.6% and 59%, respectively). An increase in CYP2D6 activity score (AS) was associated with a corresponding increment in endoxifen concentration and metabolic ratios (*$MR_{E/NDM}$*,* $MR_{E/4\text{-}HT}$ *and* $MR_{4HT/Tam}$*). ABCB1 was also significantly associated with* $MR_{E/NDM}$ *(*$p = 0.042$*) and* $MR_{E/4\text{-}HT}$ *(*$p = 0.015$*). Carriers of G allele for ABCB1 had a lower metabolic ratios for* $MR_{E/NDM}$ *(0.022 vs 0.041;* $p = 0.042$*) and* $MR_{E/4\text{-}HT}$ *(2.52 vs 3.02,* $p = 0.016$*) compared to the wild type allele (ABCB1, allele A). Moreover, CYP2D6 diplotype explained 29% of the variability in endoxifen concentration and 46% of the variability in* $MR_{E/NDM.}$ *Similarly, CYP2D6 phenotype explained 26.3% of the variability in endoxifen concentration and 40.9% of the variability in* $MR_{E/NDM}$*.*

In conclusion*, we report high rates of chemotherapy-induced hematological toxicities causing larger proportion of patients to receive inadequate RDI. Patients carrying CYP2J2 *7 allele and with low baseline WBC or ANC are at a higher risk for chemotherapy induced hematologic toxicities and receiving reduced RDI. Such patients require prior support and close follow up during chemotherapy. CYP3A5, and CYP2C9 genotypes, CPA dosage regimen, BMI, and BSA, influence the PK of CPA. Plasma CPA exposure moderately and positively predicts neutropenic toxicity. On the other hand, CYP2D6, POR and ABCB1 explain the interindividual variations of endoxifen. Tamoxifen therapy guided by CYP2D6 genotyping in clinical practice may assist treatment decision-making for optimal patient benefit.*

Jemal Hussien Ahmed, 2019

List of Scientific Papers

1. ***Jemal Hussien Ahmed***, Eyasu Makonnen, Getnet Yimer, Daniel Seifu, Abebe Bekele, Mathewos Assefa, Abraham Aseffa, Rawleigh Howe, Alan Footohi, Moustapha Hassan, Eleni Aklillu. ***CYP2J2*7 Genotype Predicts Risk of Chemotherapy-Induced Hematologic Toxicity and Reduced Relative Dose Intensity in Ethiopian Breast Cancer Patients***. *Frontiers in Pharmacology | Pharmacogenetics and Pharmacogenomics, 10|481 (2019).*
https://doi.org/10.3389/fphar.2019.00481

2. *Jemal Hussien Ahmed,* Ronald Kutessa Bisaso, Eyasu Makonnen, Jackson Kijumba Mukonzo, Alan Fotoohi, Daniel Seifu, Abraham Aseffa, Rawleigh Howe, Moustapha Hasen, and Eleni Aklillu. ***Population Pharmacokinetic, Pharmacogenetic and Pharmacodynamic analysis of Cyclophosphamide in Breast Cancer Patients* (*Submitted*).**

3. ***Jemal Hussien Ahmed***, Eyasu Makonnen, Abraham Aseffa, Rawleigh Howe, Alan Footohi, Eleni Aklillu. ***CYP2D6 Genotype Predicts Plasma Concentrations of Tamoxifen Metabolites in Ethiopian Breast Cancer Patients***. *Cancers* 2019, *11*(9), 1353; *doi:10.3390/cancers11091353*

4. Jemal Hussien Ahmed, Eyasu Makonnen,Alan Fotoohi,Getnet Yimer,Daniel Seifu,Mathewos Assefa,Wondmagegnehu Tigeneh,Abraham Aseffa,Rawleigh Howe and Eleni Aklillu. ***Vitamin D Status and Association of VDR Genetic Polymorphism to Risk of Breast Cancer in Ethiopia*.** *Nutrients* 2019, *11*(2), 289; *https://doi.org/10.3390/nu11020289*

Table of contents

List of acronyms and abbreviations

AAU	Addis Ababa University
AAPBCR	Addis Ababa population based cancer registry
ABCB1	Adenosine triphosphate (ATP)-binding cassette, subfamily B member 1
AC	Adriamycin/doxorubicin and cyclophosphamide
ACN	Acetonitrile
AC-T	Sequencial Adriamycin and cyclophoshamide followed by pacliTaxel
ADR	Adverse drug reactions
AFCRN	African cancer registry network
ALDH	Aldehyde dehydrogenase
ALT	Alanine aminotransferase
ANC	Absolute neutrophil count
ANOVA	Analysis of variance
ARV	Antiretroviral drugs
AS	Activity score
AST	Aspartate aminotransferase,
AUC	Area under the curve
BC	Breast cancer
BMI	Body mass index
BRCA1	Breast cancer susceptibility gene (Human tumor suppressor gene) type 1
BRCA2	Breast cancer susceptibility gene (Human tumor suppressor gene) type 2
BSA	Body surface area
CA	Cancer
CAR	Constitutive androstane receptor
CI	Confidence interval
CH_3CN	Acetonitrile
CL	Clearance
CMF	Cyclophosphamide, methotrexate, and fluorouracil
CNV	Copy Number Variation
COV	Covariance
CPA	Cyclophosphamide
C_t	Plasma trough concentration
CV	Coefficient of variation
CWRES	Conditional weighted residuals
CYP	Cytochrome P450 enzyme systems
CTCAE	Common Terminology Criteria for Adverse Events
dATP	Adenine deoxyribotriphosphate
dCTP	Cytosine deoxyribotriphosphate
DFS	Disease Free Survival
dGTP	Guanine deoxyribotriphosphate
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dTTP	Thymidine deoxyribotriphosphate
DV	Dependent varaiable
EBC	Early stage breast cancer
EBCTG	Early breast cancer trialist's collaborative group
EDTA	Ethylenediamine tetra acetic acid
EPS	Epsilon
ER	Estrogen receptor
GCP	Good clinical practice
GLP	Good laboratory practice
GOF	Goodness of fit plot

GST	Glutathione S-transferase
FAC	Fluorouracil, doxorubicin, and cyclophosphamide
FEC	Fluorouracil, epirubicin, and cyclophosphamide
5-FU	5-flourouracil
GST	Glutathione S-transferase
G-CSF	Granulocyte colomny stimulating factor
HIV	Human immuno deficiency virus
HPLC	High performance liquid chromatography
4-HT	4-Hydroxy tamoxifen
IPRED	Individual prediction
IRB	Institutional Ethics Review Board
IQR	Interquartile range
IV	Intravenous
KCl	Potassium chloride
Kel	Elimination rate constant
NDT	N-desmethyl tamoxifen
NRERC	National research ethics review committee
NONMEM	Nonlinear mixed effect modeling
OFV	Objective function value
4OH-CPA	4-hydroxycyclophosphamide
OS	Overall survival
PCR	Polymerase chain reaction
PD	Pharmacodynamics
PGx	Pharmacogenetics
Pg	P-glycoprotien
PK	Pharmacokinetics
PRED	Prediction
PXR	Pregnane X receptor
POR	*Cytochrome P450 oxidoreductase*
QA	Quality assurance
QoL	Quality of life
RDI	Relative dose intensity
RP-HPLC	Reversed phase high performance liquid chromatography
SEM	Standard error of the mean
SOP	Standard operating procedure
SPSS	Statistical package for social sciences
Tam	Tamoxifen
$T_{1/2}$	Half -life
TDM	Therapeutic drug monitoring
TY	Typical value
UV/VIS	Ultraviolet/visible light
VAL	Typical Value
V_D	Volume of distribution
VPC	Visual predictive check
WBC	White blood cells counts
WHO	World health organization

List of figures

1 Introduction

1.1 Breast cancer: epidemiological perspectives

Cancer has become the leading cause of death and the single most important barrier to increasing life expectancy in every country of the world in the 21st century (WHO, 2018). An estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012. Eight million (57%) of these new cancer cases and 5.3 million (65%) of the cancer related deaths were in low- and middle-income countries (Ferlay et al., 2015). A new report suggests that the global cancer burden has risen in 2018 to 18.1 million cases (5.8% in Africa) and 9.6 million cancer deaths (7.3% in Africa) (Figure 1) (Bray et al., 2018).

The term "breast cancer" refers to a malignant proliferation of epithelial cells lining lobules, or ducts of the breast (Lippman, 2008). Breast cancer is by far the most frequently diagnosed cancer among females, both in developed and developing regions of the world (Figure 1). The incidence and mortality of the disease are also rapidly growing worldwide. An estimated 1.7 million women were diagnosed with breast cancer in 2012. Since the 2008 estimates, breast cancer incidence has increased by more than 20%, while mortality has increased by 14% in 2012 (Ferlay et al., 2015). The new global cancer report also suggests an increase in the global incidence of breast cancer cases. Approximately 2.1 million new breast cancer cases have been estimated in 2018. The report has also revealed that, among females, breast cancer ranks as the leading cause of cancer death in the vast majority of countries (Bray et al., 2018).

Worldwide trends show a rising burden of cancers in developing countries going through rapid societal and economic changes (Ferlay et al., 2015). This could be associated with reproductive, dietary, and hormonal risk factors attributed to the shift in lifestyles towards typical of industrialized countries (Morhason-Bello et al., 2013). Africa and Asia are reported to have a higher proportion of cancer deaths (7.3% and 57.3%, respectively) compared with their incidence (5.8% and 48.4%, respectively). The higher mortality trend is likely due to the higher frequency of cancer types associated with poorer prognosis, along with limited access to timely diagnosis and treatment (Bray et al., 2018). It is projected that the number of new cancer cases per year will increase by 70% in Africa between 2012 and 2030 due to demographic changes alone – faster than in any other region of the world (Ferlay et al., 2015).

Despite the growing burden, cancer control programs and the provision of early detection and treatment services are limited in most African countries (Jemal et al., 2012). This could be attributed to the limited resources and other pressing public health problems, including communicable diseases such as acquired immune deficiency syndrome (AIDS)/human immunodeficiency virus (HIV) infection, malaria, and tuberculosis (Morhason-Bello et al., 2013). It may also be in part due to lack of awareness about the magnitude of the current and future cancer burden among policy makers, general public, and private or public health agencies (Aniebue and Aniebue, 2010; Morhason-Bello et al., 2013).

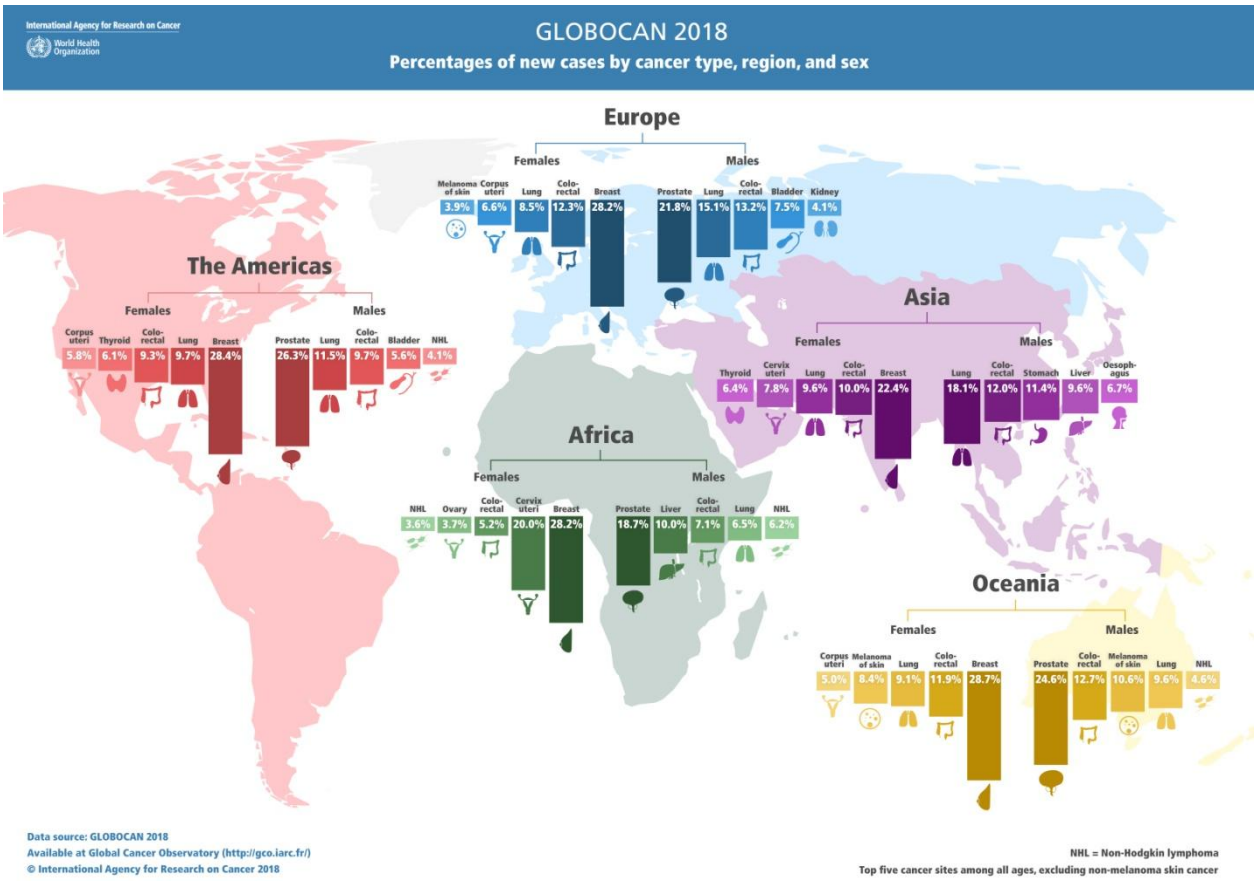


Figure 1: Global cancer incidence rates (per 100,000) incidence rates by type, region and sex in 2018 (Source: ("GLOBOCAN, Global cancer statistics," 2018); *http://www.iarc.fr/en/media-centre/iarcnews/2018/gco_globocan2018.php*)

The burden of breast cancer is also rising in women of sub-Saharan Africa including Ethiopia (Jemal et al., 2012). Though national figure regarding the prevalence of cancer in Ethiopia are non-existent, according to the Addis Ababa cancer registry (AACR), breast cancer has been reported to account for 33% of cancer cases followed by cancer of the cervix (17%) (AACR, 2014). This report demonstrates a shift from previous reports in which cervical cancer was the most commonly diagnosed cancer in Ethiopia (Getahun et al., 2013). This indicates that breast cancer has become a major threat to the lives and productivity of women in the country. Moreover, a large population of breast cancer patients in Ethiopia has been reported to present for medical care too late when the disease has reached at a more advanced stages which decreases the rate of cure (Ersumo, 2006). Lack of access to and use of timely mammographic screening in the country could contribute to the late diagnosis of the cancer. Breast tumors in Sub Saharan Africa population are described to be more aggressive compared to those in western countries (Morris and Mitchell, 2008) with large proportions of young patients affected by the disease (Ersumo, 2006; Burson et al., 2010).

1.1.1 Breast cancer risk factors

Several risk factors for breast cancer have been well documented. However, for the majority of women presenting with breast cancer, it is not possible to identify specific or major risk factors indicating that the search for the etiology of this disease is largely incomplete (Lacey et al., 2009). A familial history of breast cancer increases the risk by a factor of two or three (Michaud *et al.*, 2008). Some mutations, particularly in *BRCA1*, *BRCA2*, *p53* and *HER* genes result in a very high risk for breast cancer (Lu et al., 2010; Abbad et al., 2018). Reproductive factors associated with prolonged exposure to endogenous estrogens, such as early menarche, late menopause (age 55 years or later), late age at first child birth (≥ 30 years) are among the most important risk factors for breast cancer. Exogenous hormones also exert a higher risk for breast cancer. Oral contraceptive and hormone replacement therapy users are also at higher risk than non-users (Lacey et al., 2009).

1.2 Modalities of breast cancer treatment

There are five types of standard treatment modalities for patients with breast cancer: Surgery, radiation therapy, chemotherapy, hormonal therapy and molecularly targeted therapy (Edward and Longo, 2008). The modalities are often used in combination. Surgery and radiation therapy are considered local treatments and provide the best chance of cure for patients with localized cancers. Chemotherapy, hormonal therapy and biologic therapy (including immunotherapy and gene therapy) are systemic treatment methods and are used for disseminated cancers (Medina and Fausel, 2008).

Surgery represents the oldest cancer treatment modality and plays a major role in the diagnosis and treatment of cancer. It remains the treatment of choice for solid tumors, diagnosed in the early stages. Most patients with breast cancer have undergone surgery to remove the cancer. Unfortunately, only about 40% of cancer patients are cured by surgery. A large fraction of patients with solid tumors (about 60%) have metastatic disease that is not accessible for removal (Edward and Longo, 2008). Even when the disease is not curable by surgery alone, the removal of tumor can obtain important benefits, including local control of tumor, preservation of organ function, debulking that permits subsequent therapy to work better, and staging information on extent of involvement (Michaud et al., 2008).

Radiation therapy involves exposure of cells to ionizing radiation to treat cancer. It can be used alone or together with chemotherapy to produce cure of localized tumors and control of the primary site of disease in tumors that have disseminated. X-rays and gamma rays are the forms of radiation most commonly used to treat cancer. Radiation causes breaks in DNA and generates free radicals from cancer cells that may damage cell membranes, proteins and organelles (Edward and Longo, 2008).

1.2.1 Principles of chemotherapy

Chemotherapy is a cancer treatment that uses drugs to stop the growth of cancer cells, either by killing the rapidly proliferating cells or by stopping them from dividing (Chu and Sartorelli, 2012). The *era* of modern cancer chemotherapy was born in 1942, when Goodman and Gilman

first administered nitrogen mustard to patients with lymphoma. Since then, numerous anti-neoplastic agents have been developed, and a variety of chemotherapy regimens have been investigated in various type of cancer (Michaud et al., 2008).

Cytotoxic chemotherapy plays a pivotal role in the medical management of cancer. It may be indicated as a primary, neoadjuvant, adjuvant or palliative treatment modality. Treatment with cytotoxic drugs is the primary curative modality for a few diseases, including leukemias, lymphomas, choriocarcinomas, and testicular cancer. Most solid tumors are not curable with chemotherapy alone, either because of the biology of the tumor or because of advanced disease at presentation. Chemotherapy in this setting is often initiated for palliative purposes (Medina and Fausel, 2008).

Currently, the use of preoperative systemic therapy is gaining favor in both early stage and locally advanced breast cancers. This approach of therapy, referred to as neo-adjuvant or primary systemic therapy, usually consists of chemotherapy, but in special circumstances may also include hormonal therapy (*e.g.*, in inoperable patients with significant co-morbidities). Advantages of preoperative systemic therapy include a decrease in the size of the tumor to minimize surgery; and determining the response to chemotherapy/hormone therapy *in vivo* (Edward and Longo, 2008; Michaud, *et al.*, 2008).

One of the most important roles for cancer chemotherapy is its use as an adjuvant to local treatment modalities such as surgery or radiation therapy, and this has been termed adjuvant chemotherapy (Schlotter et al., 2008). Adjuvant therapy is systemic therapy that is administered to treat any existing micro-metastases remaining after surgical excision of localized disease. It is well established that adjuvant chemotherapy reduces the incidence of both local and systemic recurrence and improves the survival outcome of patients by effectively prolonging both disease-free survival (DFS) and overall survival (OS) (Lemos Duarte et al., 2012). It also plays a pivotal role in increasing the cure of early breast cancer. Chemotherapy regimens with clinical activity against advanced disease may also have the potential to provide long term survival advantage following surgical resection of the primary tumor, provided the appropriate dose and schedule are used (Michaud, *et al.*, 2008).

Postoperative use of systemic adjuvant chemotherapy with six cycles of cyclophosphamide, methotrexate, and fluorouracil (CMF protocol) or of fluorouracil, doxorubicin, and cyclophosphamide (FAC) has been shown to significantly reduce the relapse rate and prolong survival. Alternative regimens with equivalent clinical benefit include four cycles of doxorubicin and cyclophosphamide (AC) and six cycles of fluorouracil, epirubicin, and cyclophosphamide (FEC). Furthermore, advances in the treatment of early-stage breast cancer, in particular the development of taxane-based regimens, have led to increased use of adjuvant chemotherapy (Michaud et al., 2008; Chu and Sartorelli, 2012).

Despite the major survival gains of chemotherapy, breast cancer can recur and cause death in a large number of patients (Lemos Duarte et al., 2012). Traditionally, adjuvant chemotherapy has been used for patients with classic prognostic factors such as positive lymph nodes, younger age, no expression of hormone receptors, and presence of vascular invasion. However, recent data have shown that early stage breast cancer is formed by different sensitivities to chemotherapy (von Minckwitz et al., 2009). Attempts to maximize efficacy of cancer treatment have not been limited to the addition of new drugs; maintaining the dose and schedule are also of central concern in delivery of chemotherapy (Lemos Duarte et al., 2012). The consequences of dose delays and reductions are significant, potentially affecting short and long-term outcomes as well as patient's quality of life (QoL) (Wildiers & Reiser 2011). It has been observed that achieving maximal benefit of chemotherapy requires the maintenance of dose intensity (Bonadonna et al., 2005; Terada et al., 2009; Loibl et al., 2011).

Dose intensity (DI) is defined as the amount of drug delivered to a patient over the time course of treatment. Relative dose intensity (RDI) is the ratio of the actual dose intensity delivered to the reference standard/planned dose intensity for a chemotherapy regimen (Citron, 2004; Bonadonna et al., 2005; Vavra *et al*., 2013). Following 20 years' follow up of randomized studies of adjuvant CMF in operable breast cancer cohort study, Benadonna *et.al*, reported that patients who received RDI of 85% or more have a longer relapse free and overall survival. The authors showed that treatment with below this threshold of RDI is associated with poorer survival outcomes (Bonadonna et al., 1995, 2005). Other studies have also demonstrated a strong association between RDI and disease-free and overall survival, in both early stage and metastatic

cancers especially for lymphoma (Terada et al., 2009; Yamaguchi et al., 2011) and cancer of the breast (Uptigrove et al., 2010; Loibl et al., 2011; Sandy and Della-Fiorentina, 2013). In light of these considerations, chemotherapy regimen and the actual dose delivered to patients with breast cancer have important public health implications (Bonadonna *et al.* 2005; Wildiers & Reiser 2011).

Mathematical models of tumor behavior hypothesize that small tumors would harbor a higher proportion of dividing cells than larger ones which would make the former more sensitive to chemotherapy (Medina and Fausel, 2008). Accordingly, applying chemotherapy in shorter intervals would expose the small tumor, which would not have much time to re-grow-to another cycle of chemotherapy. This would increase tumor cell killing exponentially (Medina and Fausel, 2008; Lemos Duarte et al., 2012). This been also been substantiated with a study that compared dose-dense (14-day cycles with primary prophylactic GCSF support) and standard (21-day cycles) schedules in patients with node-positive breast cancer. Dose-dense schedules are associated with significantly improved disease-free and overall survival (Marc L. Citron et al., 2003). In the subsequent studies, higher RDI has been shown to have the greatest impact particularly for patients with early-stage disease in which curative intent with adjuvant chemotherapy is the goal (Havrilesky et al., 2015; Sandy and Della-Fiorentina, 2013; Vavra et al., 2013).

1.2.2 Pharmacokinetics of cyclophosphamide

Cyclophosphamide (CPA) is a bifunctional alkylating agent widely employed for various indications such as acute and chronic lymphatic leukemia, solid tumors like breast cancer, and for immune-suppression in preparative regimens during bone marrow transplantation (Timm et al., 2005). It has remained a stable component in many of the chemotherapy combinations used in breast cancer treatment (Stearns et al., 2004). CPA is rarely used as a single agent in breast cancer and thus it is difficult to ascertain whether the efficacy or toxicity seen in combination treatments is related to CPA or other agents in the combination (Stearns et al., 2004; Timm et al., 2005). The dose of CPA in the setting of neoplastic disease can vary greatly depending on the therapeutic regimen. This has ranged from doses of 2–6 mg/kg body weight (low dose) to >6000 mg/m^2 body surface area (high dose). CPA

can be administered orally in dosages of 100–200 mg/day when used as an immunosuppressant to prevent graft rejection and in the treatment of some chronic autoimmune disorders such as rheumatoid arthritis, autoimmune skin diseases, multiple sclerosis, systemic vasculitides and systemic lupus erythematosus (Jonge *et al.*, 2005; Timm et al., 2005).

The volume of distribution (V_D) of CPA ranges between 30–50L, while the elimination half-life ($t_{1/2}$) ranges between 5–9 hours over a large concentration range. The plasma half-life appears to be shorter for children and young adults compared with adults as a result of increased *CYP* activity (Jonge *et al.*, 2005). Total systemic clearance ranges from 4–5L/h, most of which is non renal clearance (Hasan *et al*, 1999). As it has low plasma protein binding capacity, its low renal clearance may not be associated to plasma protein binding rather to its extensive renal tubular reabsorption. Renal clearance is dependent on urine flow and thus, enhanced hydration may increase renal clearance of the drug (Jonge *et a*l., 2005).

CPA consists of a nitrogen mustard group (bischloroethylamine) attached to an oxazophosphorine ring (Figure 2). It is predominantly activated by hepatic cytochrome P450 (*CYP*) enzyme system (Griskevicius, *et al.*, 2003). Various CYP enzymes have been demonstrated to be involved in the 4-hydroxylation of CPA in humans, including *CYP2B6*, *CYP2C9*, *CYP2C19*, *CYP3A4* and *CYP3A5*. Among them, *CYP2B6* has been reported to have the highest intrinsic clearance for CPA 4-hydroxylation (Griskevicius, *et al*., 2003; Nakajima *et al.*, 2007). The current understanding of its activation is presented in Figure 2. After CPA administration, approximately 70-80% of the dose is converted into 4 hydroxycyclophosphamide (4OH-CPA), which exists in equilibrium with its ring-open tautomer aldophosphamide. Because 4-hydroxycyclophosphamide and aldophosphamide cannot be distinguished with common assays, the name 4-hydroxycyclophosphamide is commonly used to denote both 4-hydroxycyclophosphamide and aldophosphamide. Plasma concentrations of 4OH-CPA are a good marker of the alkylating activity of CPA (Xie et al., 2003; Nakajima et al., 2007).

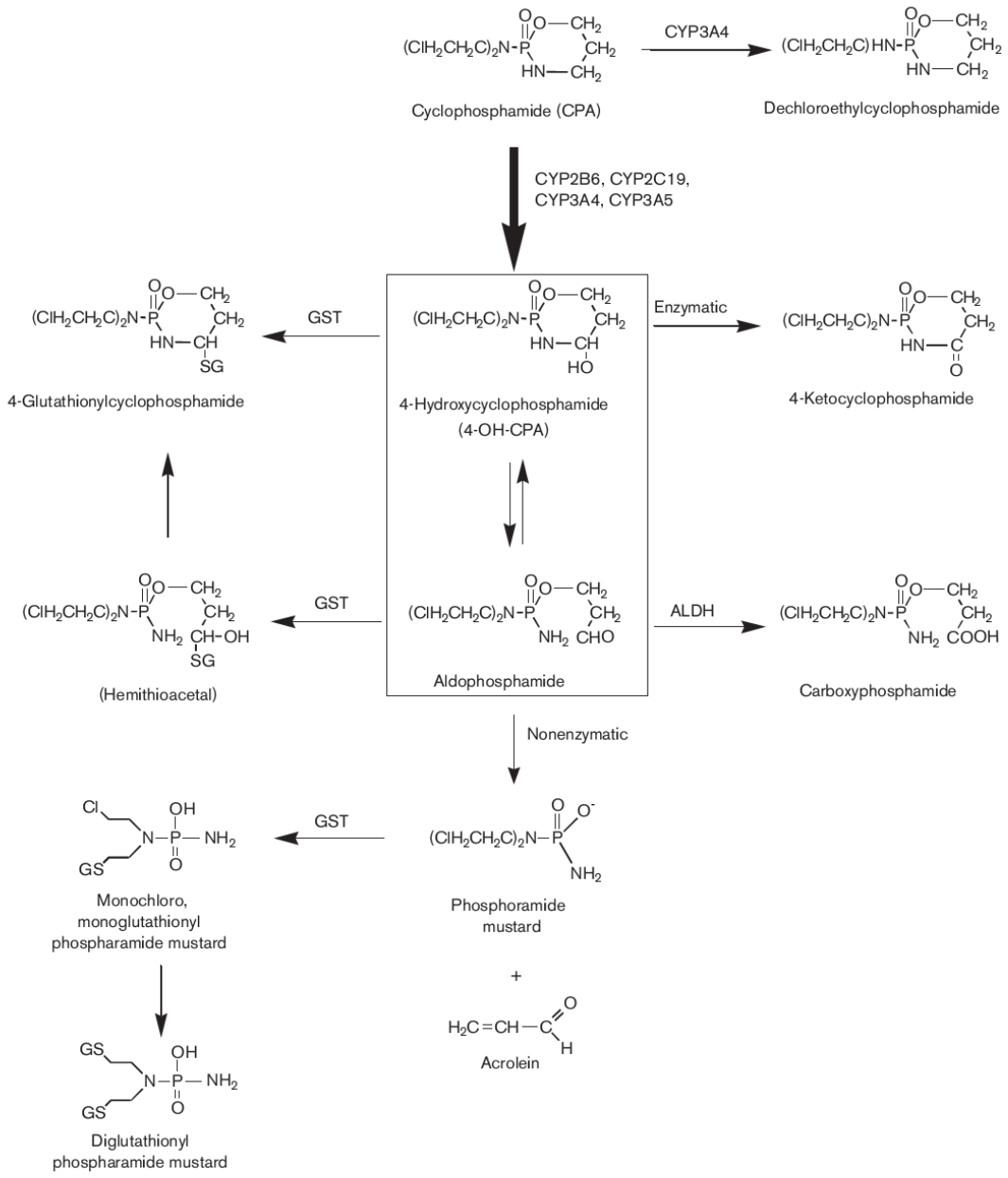


Figure 2: Activation and inactivation pathways of cyclophosphamide in humans (Nakajima *et al.*, 2007).

4-Hydroxycyclophosphamide readily diffuses into cells and is not cytotoxic. It is very unstable and spontaneously decomposes into phosphoramide mustard and acrolein. Phosphoramide mustard is a bi-functional DNA alkylating agent and is considered to be the ultimate metabolite responsible for the alkylating effect of CPA (Nakajima *et al.*, 2007; Ekhart, *et al.*, 2008).

However, circulating phosphoramide mustard may not contribute to cytotoxicity because it is largely ionized at physiological pH and may not enter cells. Therefore, only the intra-cellularly formed phosphoramide mustard fraction may be considered cytotoxic. Since 4-hydroxycyclophosphamide readily diffuses into cells, followed by spontaneous liberation of phosphoramide mustard inside the cell, it may function as a transport molecule delivering phosphoramide mustard into cells. The 4-hydroxycyclophosphamide systemic concentration may, therefore, reflect the intracellular activation state of CPA (Xie *et al*., 2003; Jonge *et al.*, 2005; Nakajima *et al*., 2007; Ekhart, *et al*., 2008).

After short intravenous infusions of CPA (30 minutes to 1 hours), peak levels of 4-hydroxycyclophosphamide, phosphoramide mustard, 2-dechloroethylcyclophosphamide and aldophosphamide reach within 0.5–3 hours, 3–6 hours, 8–15 hours and 4 hours after the start of infusion, respectively (Jonge *et al.*, 2005). The pattern of metabolism after oral administration is similar to that after intravenous administration. The exposure to 4-hydroxycyclophosphamide and phosphoramide mustard was similar after oral and intravenous administration of the same CPA dose. In cerebrospinal fluid, both phosphoramide mustard and CPA could be detected, while carboxyphosphamide and 2-dechloroethylcyclphosphamide were not found (Jonge *et al.*, 2005; Nakajima *et al.*, 2007; Ekhart, *et al.*, 2008).

The metabolites, 4OH-CPA and phosphoramide mustard are detoxified by *Glutathione-S-transferase* (*GST*) to form 4-glutathionylcyclophosphamide and diglutathionyl-phosphoramide mustard, respectively. The isoforms *GSTA1* and *GSTP1* are mainly involved in the metabolism of CPA. Aldophosphamide is metabolized by the aldehyde dehydrogenase (ALDH) enzymes, *ALDH1A1* and *ALDH3A1* to carboxyphosphamide. Direct detoxification of CPA also occurs *via* side chain oxidation resulting in the formation of 2-dechloroethylcyclophosphamide. This reaction is predominantly mediated by *CYP3A4* and *CYP3A5* and accounts for less than 5% of the total elimination of CPA (Jonge *et al.*, 2005; Nakajima *et al*., 2007). Thus, hepatic metabolism plays major role in CPA elimination. The liver appears to be protected through the enzymatic formation of the inactive metabolites 4-ketocyclophosphamide and carboxyphosphamide (Nakajima *et al.*, 2007). Acrolein, formed in the degradation of 4-hydroxycyclophosphamide to phosphoramide mustard, is a highly reactive aldehyde and may

enhance CPA induced cell damage, possibly by depletion of cellular glutathione by conjugation. It is excreted into the urine as 3-hydroxypropylmercapturic acid. Glutathione may function as a detoxifier of reactive electrophiles, thereby protecting cells against 4-hydroxycyclophosphamide by limiting its breakdown to phosphoramide mustard. By depletion of cellular glutathione, acrolein exacerbates the cytotoxicity of CPA (Jonge *et al*., 2005; Nakajima *et al.*, 2007).

CPA treatment may result in severe side effects such as anemia, leukocytopenia, thrombocytopenia, gonadal toxicity and development of secondary tumors (Timm *et al*., 2005). The acute and delayed type of cytostatic drug-induced nausea and vomiting are presumably the result of direct stimulation of the chemoreceptor trigger zone by various nitrogen mustard metabolites of CPA. Nausea and vomiting is seen in most patients who receive doses above 50 mg/kg body weight. Moreover, significant urotoxicity is attributed to the direct toxic effect of the CPA metabolite, acrolein (Stearns, *et al.*, 2004).

1.2.3 Endocrine therapy: Tamoxifen

About two third of breast cancer patients express estrogen receptors (ERs) and are dependent on estrogen for growth (Saladores, *et al*., 2013). This population of patients is considered candidate for anti-oestrogen endocrine therapies. Selective estrogen receptor modulators (SERMs) and aromatase inhibitors are effective therapies for hormone receptor-positive breast cancers as they deprive breast cancer cells of estrogens or estrogenic growth stimuli (de Vries Schultink *et al*., 2015).

Tamoxifen (Tam) is one of the most widely used drugs for women with estrogen receptor-positive breast cancer. The current recommended dose for Tam in the adjuvant, metastatic, and preventive settings is 20 mg/day. Women receiving adjuvant Tam therapy have reduced risk of recurrence and mortality as compared to women not receiving adjuvant Tam therapy (EBCTG, 2005). This observation, coupled with evidence of Tam's tolerability including beneficial estrogenic effects on the lipid profile and bone density, led to Tam being the hormonal agent of choice for both pre- and postmenopausal women (Saladores *et al* 2013). Five years of Tam is currently the standard of care for premenopausal women with early breast cancer. In

postmenopausal women Tam is used alone or in sequence with aromatase inhibitors (Saldores *et al.*, 2015). Tam for 5 years has been shown to be highly effective in the adjuvant treatment of ER-positive breast cancer, reducing breast cancer recurrence by nearly 40% and cancer-specific mortality by approximately a third during the first 15 years (EBCTG, 2005). Further reductions in breast cancer recurrence and mortality have been observed by continuing Tam therapy for 10 years with 25% mortality reduction relative to 5 years (Davies *et al.*, 2013; Gray *et al*., 2013).

Although Tam has proven to be an effective drug, not all women with estrogen receptor-positive tumors derive benefit from Tam therapy. Women who receive Tam at the same dose, as in current daily practice, can have different clinical outcomes (Saldores et al., 2015). Approximately ~30–50% of the patients relapse or eventually die from the disease (EBCTG, 2005). In addition, occurrence of side effects may also differ between individuals, some of whom experiencing treatment-limiting side effects, while others do not have any side effects (Saldores *et al*., 2013). Identifying predictive biomarkers of response to Tam, other than expression of ERs, and the use of these markers to individualize Tam therapy has become a subject of intensive research. Currently, much attention has been given to genetic polymorphisms in drug-metabolizing enzymes involved in the metabolic activation of Tam into endoxifen (de Vries Schultink *et al.*, 2015).

Tam undergoes extensive primary and secondary liver metabolism *via* two metabolic pathways: N-demethylation and 4-hydroxylation (Figure 3). The predominant metabolic pathway is the demethylation of Tam to N-desmthyl-tamoxifen (NDM) primarily mediated by *CYP3A4/5,* followed by *CYP2D6* mediated oxidation to form (Z)-4-hydroxy-*N*-desmethyl-tamoxifen (Endoxifen). This pathway is considered to contribute more than 90% of Tam metabolism to endoxifen. The second and minor metabolic pathway involves hydroxylation of Tam to (Z)-4-Hydroxy-tamoxifen (4-HT), which can then be further metabolized to Endoxifen. This reaction pathway is mediated by *CYP2C9, CYP2C19, CYP2D6, CYP3A4,* and *CYP3A5* (Klein et al., 2013).

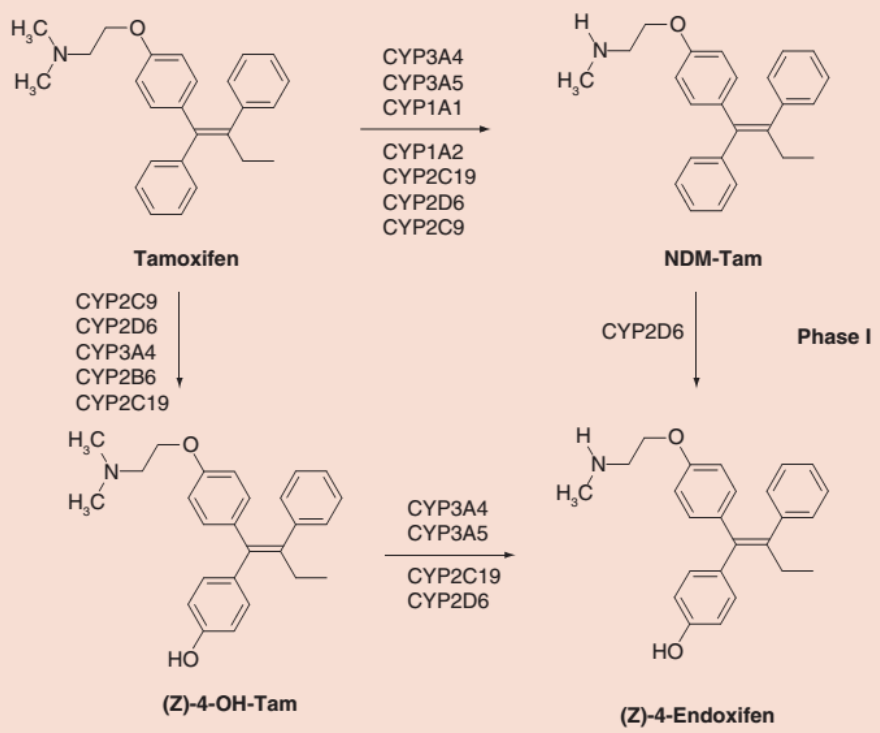


Figure 3: The Phase I metabolism of tamoxifen (Saldores *et al*., 2013)

Both 4-HT and Endoxifen are known to have significantly higher efficacy at the ER compared with the parent drug (Johnson *et al*., 2004; Lim *et al.*, 2005). Because *CYP2D6* is responsible for the hydroxylation of NDM to endoxifen (Klein et al., 2013), genetic variations that cause differences in the *CYP2D6* metabolizer status may play a role in individual therapeutic benefit. This has led to research into the possible association between *CYP2D6* genotype and maximum benefit from Tam therapy (Saldores *et al.*, 2013). There is a wide degree of inter-individual and interethnic differences in the steady-state plasma concentrations of Tam and its metabolites which have been shown to influence therapeutic outcome (Mürdter et al., 2011; Schroth et al., 2017). The deviations in plasma concentrations of Tam and its metabolites can be due to genetic variants present in the genes encoding various proteins involved in regulating its disposition or drug–drug interactions (Saldores *et al.*, 2013).

1.3 Role of genetic variations on responses to drugs

Despite drugs are the cornerstone of modern therapeutics, drug therapy faces many challenges such as non response to standard therapy and adverse drug reactions, which sometimes gets serious or even lethal (Fagerlund and Braaten, 2001). It has been known that the same doses of a medication are associated with considerable heterogeneity in efficacy and toxicity across human populations. This heterogeneity in response can lead to unpredictable life threatening or even fatal adverse effects in small groups of patients (Robertson et al., 2002). The inter-individual difference in drug response cannot be explained only by factors such as patients' age and weight, co-morbidity, lifestyle, co-medication, renal and liver function, unfavorable drug-drug interactions and poor compliance of patients (non genetic factors). Although these factors are always of great importance for the understanding of sub-optimal drug therapy, genetic factors has been accepted as one of the main factors in the inter-individual differences observed in drug responses and toxicities (Evans and Johnson, 2001). Genetic factors can account for 15%–30% of inter-individual differences in drug metabolism and response (Ingelman-Sundberg and Rodriguez-Antona, 2005). It has been reported that, out of drugs frequently cited in adverse drug reaction (ADR), 59% have been found to be metabolized by at least one enzyme with a variant allele associated with decreased drug metabolism (Phillips et al., 2001).

1.3.1 Molecular mechanisms of genetic variation

Genetic variation can be defined as nucleotide sequence differences in a gene. Sequence differences can be in coding regions, regulatory regions (*i.e.*, promoters, enhancers), introns, at 5' or 3' flanking regions, etc (Efferth and Volm, 2005). The molecular mechanisms of genetic variation include splice site mutations resulting in exon skipping (*e.g.*, *DPD*, *CYP2C19*), microsatellite nucleotide repeats (*e.g.*, *CYP2D6*), gene duplication (*e.g.*, *CYP2D6*), point mutations resulting in early stop codons (*e.g.*, *CYP2D6*), enhanced proteolysis (*e.g.*, *TPMT*), altered promoter functions (*e.g.*, *CYP2A5*, *UGTIA1*), critical amino acid substitutions (*e.g.*, *NAT2*, *CYP2D6*, *CYP2C19*, *CYP2C9*), or large gene deletions (*e.g.*, *GSTM1*, *CYP2D6*) (Evans and Johnson, 2001). In the coding sequences, genetic variation may occur at the level of genes that code for metabolizing enzymes, transporters, receptors, and/or ion channels and molecules of signal transduction cascades that may have a major impact on drug response. The effects can

thus be described as pharmacokinetic (affecting drug concentrations) and pharmacodynamic (affecting drug action) (Ingelman-Sundberg, et al 2007).

1.3.2 Pharmacogenetics of Cytochromes P450 system

The vast majority of phase I reactions are catalyzed by cytochrome P450 superfamily of hemoproteins with multiple isoforms. Cytochrome P450s are the most thoroughly studied pharmacogenetic enzymes with consequences in drug response and toxicity (Johansson and Ingelman-Sundberg, 2011). From *CYP* families present in humans, *CYP1*, *CYP2* and *CYP3* are responsible for > 90% of all phase I metabolism of drugs in clinical use in humans among which *CYP2C9*, *CYP2C19* and *CYP2D6* metabolize 40–45% of all drugs on the market. The CYP450 enzymes are also known to be highly polymorphic which creates problem in drug dosing (Ingelman-Sundberg, 2004). The mutations in the *CYP* genes can produce enzyme products with abolished, reduced, altered or increased enzyme activity influencing pharmacokinetic parameters such as drug bioavailability, distribution, metabolism and tissue binding (Ingelman-Sundberg 2004; Ingelman-Sundberg, *et al* 2007).

1.3.2.1 *CYP2B6*

CYP2B6 is a member of the cytochrome P450 family primarily expressed in the liver and makes up approximately 2–10% of the total hepatic *CYP* content (Zanger and Hofmann, 2008). At present, 38 variant have been reported (*https://www.pharmvar.org/gene/CYP2B6*, accessed 25/May/2019). There is high inter-individual variation in *CYP2B6* mRNA expression, ranging from 20 to 250-fold, which may be attributed to differential transcriptional regulation and inherited genetic variation (Wang and Tompkins, 2008). *CYP2B6* is responsible for the metabolism of clinically important drugs; including, cyclophosphamide, ifosfamide, bupropion, diazepam, phenobarbital, rifampicin, phenytoin, carbamazepine, effavirenz, and nevirapine. Many of the drugs that are metabolized by *CYP2B6* are also inducers of the gene (through PXR and CAR). Owing to the existence of extensive genetic polymorphism as well as strong inhibitors and inducers, its activity is highly variable in the population (Zanger and Klein, 2013).

The most common variant allele in all populations studied to date harbors two amino acid changes, Q172H and K262R, and is termed *CYP2B6*6* (Zanger and Klein, 2013). The allelic frequency of this allele in Caucasians is 26%, while in Japanese frequency was found to be 16% (Hiratsuka et al 2002). The allele frequencies of *CYP2B6*6* were reported to be 29.8% among Ethiopians (Habtewold et al., 2011), 41% in Zimbabweans (Maimbo et al., 2012). For some clinically used drugs including the antiretroviral agents efavirenz and nevirapine, *CYP2B6* single nucleotide polymorphisms have been shown to be useful predictors of pharmacokinetics and drug response. *CYP2B6*6* allele is associated with reduced enzymatic activity and higher incidence of antiretroviral induced liver toxicity in Ethiopian HIV patients (Yimer et al., 2012).

The widely used anticancer and immunosuppressant prodrug CPA depends on bioactivation to 4-hydroxycyclophosphamide for cytotoxic activity. Bioactivation is highly variable in cancer patients and has been attributed mainly to *CYP2B6* with contributions from *CYP2C19* and *CYP3A4* (Raccor et al., 2012). Cyclophosphamide 4-hydroxylation was initially reported to be enhanced in livers genotyped *CYP2B6*6/*6* (Xie et al., 2003), which was also observed in several later *in vivo* studies (Xie *et al.*, 2006; Nakajima *et al.*, 2007; Torimoto and Kohgo, 2008). However, other subsequent *in vivo* studies analyzing pharmacokinetics and clinical outcome presented contradictory or negative results (Singh *et al*., 2007; Ekhart *et al.*, 2008; Melanson *et al*., 2010;Yao *et al.*, 2010).

A recent finding by Tsuji *et al.* (2016) indicated that, being non-*CYP2B6*6* carrier was a significant predictor of grade 4 neutropenia in breast cancer patients treated with doxorubicin and CPA combination chemotherapy (Tsuji *et al*., 2016). On the contrary, few other studies concluded that *CYP2B6* genotype was not significantly associated with myelotoxicity (Haroun *et al.*, 2015; Yao *et al.*, 2010). Such inconclusive results could be explained by different study size, design or difference in the relative expression levels of *CYP2B6* which may help determine *in vivo* kinetics and toxicity of drugs metabolized by these enzymes.

1.3.2.2 *CYP2C9*

CYP2C9 is the most abundant among the *CYP2C* family and comprises approximately one-third of the total hepatic P450 content (Xie *et al.*, 2002). It is involved in the metabolism of more than 100 drugs, with Warfarin (coumarin anticoagulant), phenytoin, and tolbutamide (sulfonylurea) are examples of narrow therapeutic index drugs (Scordo et al., 2001; Hirota et al., 2013). The *CYP2C9* gene has more than 60 known variant alleles, many of which have been associated with reduced enzyme activity (*https://www.pharmvar.org/gene/CYP2C9*, accessed May-25-2019), with *CYP2C9*3*, and to a lesser extent **2*, having the most clinical relevance. At least one *CYP2C9*2* or **3* allele is carried by 20 and 12% of Caucasians, respectively. *CYP2C9*2* and **3* are rare in African-American and Asian populations with the wild-type comprising more than 95% of these populations. In the Ethiopian population, *CYP2C9*2* and *CYP2C9*3* alleles occur at a frequency of 4 and 2%, respectively (Scordo et al., 2001).

The *CYP2C9*2* and **3* alleles have single base substitutions in exons 3 and 7 resulting in amino acid changes at residue 144 (Arg to Cys) and 359 (Ile to Leu), respectively (Higashi *et al.*, 2002; Xie *et al* 2002). These two alleles **2* and **3* have ~40% and ~10% of normal enzyme activity, respectively (Higashi et al., 2002; Schwarz et al., 2008). In human liver microsomes, the intrinsic clearance of CPA by CYP2C9.2 and CYP2C9.3 samples was approximately threefold lower than that by CYP2C9.1. Because of the apparently low K_m (0.49 mM for *CYP2C9*1*, the contribution of *CYP2C9* to CPA 4-hydroxylation has been presumed to increase at low concentrations of CPA (Griskevicius et al., 2003).

1.3.2.3 *CYP2C19*

The *CYP2C19* gene has more than 35 known variant alleles (*https://www.pharmvar.org/gene/CYP2C19* accessed May-25-2019). The two most important null alleles are *CYP2C19*2*, which occurs almost exclusively in Caucasians (2 to 5%), and *CYP2C19*3*, which occurs primarily in Asians. The causal mutation of **2* is located in exon 5 and leads to aberrant splicing, whereas that of *3 in exon 4 creates a premature stop codon. A further clinically relevant variant that may produce an ultra-rapid phenotype is the promoter variant 806C>T (*CYP2C19*17*). *CYP2C19*2* and *CYP2C19*3* are together responsible for the majority of PM alleles, of which *CYP2C19*3* is mainly found in Asians (~25%) (Sistonen et al.,

2009). The *CYP2C19*17* allele associated with an ultra-rapid phenotype seems relatively common in Swedish and Ethiopians (allele frequencies of 18%) and Chinese (4%) (Sim *et al.*, 2006).

Many drugs, including proton pump inhibitors (omeprazole), (*S*)-mephenytoin (anticonvulsant), and diazepam (anxiolytic) are metabolized by the polymorphic cytochrome P450 (CYP) 2C19 enzyme. *CYP2C19* has been shown to contribute to the bioactivation of CPA in human liver microsomes (Griskevicius et al., 2003). The pharmacokinetics of CPA has also been shown to be influenced significantly by *CYP2C19* genotype at doses <=1000 mg/m^2 (Timm et al., 2005). Another study also showed that a combined role for both *CYP2C19* and *CYP2B6* in the bioactivation of CPA *in vitro* and in patients (Helsby et al., 2010). The presence of at least one loss of function (LoF) allele at either *CYP2C19* or *CYP2B6* resulted in a significant decrease in the bioactivation of CPA (Helsby et al., 2010).

1.3.2.4 *CYP2D6*

CYP2D6 is one of the most studied cytochrome P450 families responsible for metabolism of approximately 25% of all marketed drugs, including antidepressants, antiarrhythmics, antipsychotics adrenergic receptor blockers, and tamoxifen (Williams et al., 2004; Eichelbaum *et al.*, 2006; Zhou *et al.*, 2008). The frequency and genetic basis of major variants of *CYP2D6* are well documented. Owing to its highly polymorphic nature, currently, more than 113 distinct alleles are known on the CYP allele nomenclature website (*https://www.pharmvar.org/htdocs/archive/cyp2d6.htm;* accessed May 2019). The interest in studying the *CYP2D6* gene continues to grow because of its significant contribution to inter-individual variability in drug metabolism, resulting in higher incidence of adverse events or lack of therapeutic efficacy. Comprehensive genotype–phenotype correlation studies in Caucasians using debrisoquine or sparteine as probe-drug clearly indicate the impact of *CYP2D6* polymorphisms on *CYP2D6* function *in vivo* and result in the discrimination of the poor (PM), intermediate (IM), extensive (EM) and ultra-rapid (UM) metabolizer phenotypes.

The EM status is determined by the presence of two normal functioning alleles (*e.g.*, **1*, **2*, **35*). The PM status is defined by a lack of *CYP2D6* enzyme function and is determined by the

presence of two nonfunctional alleles (so-called null alleles, *e.g., *3, *4, *5, *6, *7,* etc). Subjects carrying one partially defective *CYP2D6* allele (*e.g., *9, *10,*17, *41*,) together with another partially defective or null allele are classified as IM (*e.g., *9/*41* or *4/*41*). Moreover, heterozygous carriers of one defective and one normal *CYP2D6* allele (*e.g., *1/*4)* tend to also have a lower median *CYP2D6* enzyme activity compared with wild-type carriers (*e.g., *1/*1*). As this group is not as clearly defined as EMs and PMs but represents a rather heterogeneous group, many of the current studies combine heterozygous carriers together with classically defined IMs into one decreased function phenotype. The UM phenotype can be inferred from *CYP2D6* gene duplications/amplifications of fully functional alleles (*e.g., *1xN/*2)* resulting in excessive enzymatic function (Saldores *et al.*, 2013).

There are significant interethnic differences of *CYP2D6* allele frequencies across geographic regions and populations leading to a shift of metabolizer phenotype prevalence with higher frequencies of IMs in Asians and UMs in Arabic/North African countries and Ethiopians as compared with populations of European descent (Aklillu *et al* 1996; Sistonen *et al.*, 2007). In the European population, approximately 5–10% are PMs, 10–15% are IMs and approximately 5–10% are UMs. *CYP2D6* PM alleles in individuals of African and Asian descent appears to be rare. *CYP2D6*17* is the most common reduced function allele in African populations while the IM **10* allele is more prevalent in Asian populations ranging from a frequency of 38 to 70% (Bradford *et al.*, 2002).

1.3.2.5 *CYP2J2*

Cytochrome *P450 2J2 (CYP2J2)* is an enzyme mainly found in human extrahepatic tissues, with predominant expression in the cardiovascular systems, intestine, kidney, and lower levels in the lung, pancreas, brain, liver (Xu et al., 2013). It is an epoxygenase enzyme that catalyzes the metabolism of structurally diverse therapeutic compounds, particularly in extra-hepatic tissues (Berlin et al., 2011). In the pharmacogenetic variation consortium website, 8 known variant alleles are described (*https://www.pharmvar.org/gene/CYP2J2*). *CYP2J2*7* is the most common known functional *CYP2J2* variant studied, occurring at frequencies of 2.1%-17%. The defining SNP for *CYP2J2*7* (rs890293), is located in the proximal promoter of *CYP2J2*, substituting a "T" for the "G" found in the wild-type gene (*https://www.pharmgkb.org/vip/PA166169433*).

Several clinical studies investigated the association of *CYP2J2*7* with different cardiovascular and cerebrovascular diseases (Berlin *et al.*, 2010). A study by El-Serafi *et al.*, (2015) demonstrated the role of *CYP2J2* in the bioactivation of CPA. Using enzyme kinetic studies, it was shown that *CYP2J2*7* is over expressed during CPA treatment, and the bioactivation of the drug was significantly correlated to *CYP2J2*7* expression (Ibrahim El-Serafi et al., 2015). In another study, *CYP2J2* is reported to be highly expressed in human and mouse hematological cell lines, as well as in peripheral blood and bone marrow cells of leukemia patients (Chen et al., 2011). The cytotoxic effect of CPA in hematological cell lines has also been associated with *CYP2J2* expression, despite absence of *CYP2B6* (Xie *et al*., 2002). As *CYP2J2*7* is associated with increased enzyme activity (El-Serafi *et al.*, 2015), patients carrying this variant allele could activate cytotoxic agents such as CPA to 4-hydroxycyclophosphamide and become at a higher risk for toxicity. The heart, intestine and urinary bladder express high levels of *CYP2J2* (Xu et al., 2013); consequently, local CPA bioactivation may explain CPA treatment-related toxicities in these organs.

1.3.2.6 *CYP3A4* and *CYP3A5*

The *CYP3A* isoforms *CYP3A4* and *CYP3A5* together account for approximately 30% of hepatic P450 content and are involved in over 50% of oxidative drug metabolism, including immunosuppresants, chemotherapeutics, macrolide antibiotics, antidepressants, anxiolytics, antipsychotics, opiates, calcium channel blockers, and statins (Pinto and Dolan, 2011). *CYP3A4* and *CYP3A5* are also involved in the activation of CPA as well as in side-chain oxidation (N-dechlroethylation), leading to the formation of the inactive metabolite 2-dechloroethylcyclophosphamide (Huang et al., 2000). These CYP enzymes also contribute to Tam metabolism, particularly during the conversion of the parent drug to both NDM and 4HT (Saladores et al., 2013). *CYP3A4* and *CYP3A5* are expressed in liver and intestine, with *CYP3A5* being the predominant form expressed in extra-hepatic tissues. *CYP3A4* shows remarkable inter-individual variability attributed to environmental factors including medication and food, but its variability is also influenced by gender and age (Pinto and Dolan, 2011).

CYP3A4

Several polymorphisms within the gene have been extensively studied to alter the catalytic activity of the enzyme (*https://www.pharmvar.org/gene/CYP3A4*). However, these variations do not appear to result in absence of functional protein and have not impacted clinical care to date. *CYP3A4 *1B* allele, a promoter variant, has been associated with altered enzyme activity (Mutagonda et al., 2017). A rare allele of Brazilian origin, *CYP3A4*20*, has been shown to contain a premature stop codon which does result in a truncated protein and complete loss of function (Westlind-Johnsson et al., 2006). Subjects of this genotype might be susceptible to side effects during drug therapy with substrates or inhibitors of *CYP3A4*. In addition, intron 6 polymorphism *CYP3A4* (*CYP3A4*22*) has been shown to markedly affect expression of *CYP3A4* and could influence lipid-lowering response to statin drugs (Wang et al., 2011). However, in Greek patients with primary hypercholesterolemia, the impact of this SNP was not observed on lipid-lowering response to statins (Ragia et al., 2015).

CYP3A5

CYP3A5 expression is highly polymorphic with 25 allelic variants of *CYP3A5* reported by various investigators and listed on the Pharmacogene Variation Consortium web (*https://www.pharmvar.org/gene/CYP3A5*). *CYP3A5*3* (rs776746) is the most frequent and well-studied variant allele of *CYP3A5* (splice site mutation) resulting in a non-functional protein. *CYP3A5*3* is the most frequent and well-studied variant allele of *CYP3A5*. The frequency is higher in populations of European descent (73–93%) compared with those of Asian (74–77%) and African descent (27–50%) (Roy et al., 2005; Kurose et al., 2012). A slight increase in endoxifen levels in individuals carrying at least one copy of the normal function allele has been reported (Jin et al., 2005), but subsequent studies did not confirm this (Lim et al., 2011). It is likely, however, that the *CYP3A5*3* deficiency allele contributes to variation in the levels of the primary metabolite NDM. The other two most studied *CYP3A5* alleles are **6* (rs10264272) and **7*.

1.3.2.7 *CYP450 Oxidoreductase (POR)*

All microsomal P450 enzymes require cofactors for their oxidative catalytic activities. *Cytochrome P450 oxidoreductase (POR*), a membrane-bound enzyme in the endoplasmic reticulum, is the only cofactor that donates electrons to microsomal P450 enzymes (Flück et al., 2007). *POR* is a flavoprotein that contains both flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) as cofactors and uses NADPH as the source of electrons. The two flavin cofactors are tightly bound to different domains and these flavins serve as intermediates for the supply of electrons from nicotinamide adenine dinucleotide phosphate (NADPH), which gives up a pair of electrons. Electrons pass from NADPH through FAD to FMN in the *POR* protein. Following a conformational change, the FMN-binding domain of the *POR* interacts with the oxidation/reduction-partner binding site of the P450 enzymes so that electrons reach the P450 heme iron (Fe^{3+}) to achieve catalysis of drug oxidation (Hart et al., 2008).

It is reasonable to assume that disruption of electron flow in the *POR* protein would have destructive effects on oxidation of drugs by all microsomal P450 enzymes. The gene encoding human *POR* is highly polymorphic which is of considerable clinical significance to impact the metabolism and curative effects of clinically used drugs (Hart *et al.*, 2008; Hu et al., 2012). There are about 48 variant alleles (*https://www.pharmvar.org/gene/POR*). Genetic polymorphisms in the cofactor genes have been found to play an important role in *CYP* dependent metabolism of drugs and xenobiotics *in vitro* (Sandee et al., 2010; Subramanian et al., 2012). *POR*28* has been reported to be the variant known to increase *CYP3A5* activity but decrease the activity of *CYP2D6* (Zhang et al., 2013). The recent results of El Serafi, *et al* (2015), showed that *POR* gene expression was significantly ($p < 0.001$) up-regulated after the CPA infusion, of which approximately 50% of them were carriers for *POR*28* (El-Serafi *et al*., 2015).

1.3.3 The pharmacogenetics of *ABCB1*

ABC genes encode transporter and channel proteins possessing multiple membrane-spanning domains that form a pore, and intracellular nucleotide-binding domains for ATP-dependent translocation of substrates or ions across the cell membrane (Jones and George, 2004).

ABCB1 (also known as Multi-Drug Resistance 1 (*MDR1*)) is one of many ubiquitous adenosine triphosphate (ATP)-binding cassette (ABC) genes present in all kingdoms of life. It is responsible for cellular homeostasis, nutrient uptake, resistance to xenotoxins, antigen processing, cell division, bacterial immunity, pathogenesis and cholesterol and lipid trafficking. All eukaryotic ABC proteins are efflux pumps (Wolking et al., 2015).

A great number of studies has been carried out to establish the role of *ABCB1* genetics in various phenotypes such as P-glycoprotien (Pg) expression, function, drug response, and disease susceptibility with little consensus. Most genotype-phenotype associations are not substantiated by study replication, meaningful sample size, and appropriate multi-testing correction. Despite much work to ascertain the genetic contribution of *ABCB1* on drug disposition and disease susceptibility, the accumulation of studies to date are unclear. Until data are amassed to form a consensus about the role of genetics in P-gp-related phenotypes, the primary clinical focus on Pg relates to its role in (1) multi-drug resistance, and (2) drug-drug interactions (Kimchi-Sarfaty et al., 2007; Wolking et al., 2015).

There is growing evidence that altered *ABCB1* activity may affect tamoxifen pharmacokinetics and possibly tamoxifen efficacy in breast cancer patients. The primary active tamoxifen metabolites, endoxifen and 4-hydroxytamoxifen, are substrates for *ABCB1* (Iusuf et al., 2011). The three common SNPs in the protein coding region of *ABCB1* gene (rs1128503 (1236T>C), rs2032582 (2677T>G/A), and rs1045642 (3435T>C) have been the focus of many pharmacokinetic and disease association studies (Wang et al., 2005) with inconsistent and controversial results (Leschziner et al., 2007). Other studies have also investigated the relationship between the C3435T polymorphism of *ABCB1* gene and risk of breast cancer; whose results are also conflicting (Tazzite et al., 2016; Amjadi et al., 2018). The number and frequency of SNPs observed varies by ethnicity. In black African population, *ABCB1* rs1045642 (3435T>C and rs3842 have been studied and reported to occur with allele frequencies of 20-25%. The relevance of *ABCB1*c.4036A>G (rs3842A>G) has been consistently demonstrated in African population in relation to the PK of antiretroviral drugs. Several studies have showed that *ABCB1*c.4036A>G (rs3842A>G) is a significant predictor of plasma and intracellular efavirenz

concentration in Uganda (Mukonzo et al., 2009, 2013), South Africa (Swart et al., 2012), Ethiopia and Tanzania (Ngaimisi et al., 2013) as well as in other populations (Elens et al., 2010).

1.4 Rationale of the study

Despite drugs are the cornerstone of modern therapeutics, drug therapy faces many challenges such as non response to standard therapy and adverse drug reactions, which sometimes gets serious or even lethal. It has been established that the same doses of a medication are associated with considerable heterogeneity in efficacy and toxicity across human populations. The inter-individual difference in drug response cannot be explained only by factors such as patients' age and weight, co-morbidity, lifestyle, co-medication, renal and liver function, unfavorable drug-drug interactions and poor compliance of patients (non genetic factors). Although these factors are always of great importance for the understanding of sub-optimal drug therapy, genetic factors are currently accepted as one of the main factors in the inter-individual differences observed in drug responses and toxicities.

Large inter-individual variability in therapeutic response and/or adverse reactions have been reported to CPA based treatment of breast cancer. High pharmacokinetic variability coupled with heterogeneity of the disease, which demonstrates differential response to chemotherapeutic agents, can make it difficult to predict how a patient will respond to cancer chemotherapy. Unable to predict predisposes to increased risk of both toxicity and sub-therapeutic dosing in the individual patient. Consequently, breast cancer can recur and cause death in a large number of patients. In Ethiopia, the pharmacokinetics of CPA among Ethiopian populations has not been investigated before and the inter-patient PK variations and the sources of variability are unknown.

It has been indicated that pharmacogenetic differences (polymorphisms in key genes) can account for 15%–30% of inter-individual differences in drug metabolism and response. Various polymorphic cytochrome P450 (CYP) enzymes have been shown to catalyze the metabolism of CPA including *CYP2B6, CYP2C19, CYP2C9* and CYP3A4/5, cytochrome P450 oxidoreductase (*POR*) and cytochrome P450 epoxygenases (*CYP2J2*). Genetic variations in these genes may

explain the inter-individual differences in the PK and PD of CPA. However, the clinical relevance CPA pharmacogenetics has not been demonstrated in SubSaharan Africans in general and Ethiopia in particular. This country is a population with uniquely different in terms of socio-economic and environmental factors, SNP frequency, types of co-morbidities and associated co-treatments from white populations and even from other Africans.

Anticancer chemotherapeutic regimens usually have a narrow therapeutic index and are known to suppress the hematopoitic system. Consequently, patients receiving chemotherapy are at risk of chemotherapy induced myelosuppresion specifically neutropenia, thrombocytopenia and anemia which may predispose patients at risk of life threatening complications and infections. In clinical practice, myelosuppressive effects resulting from the previous cycle of chemotherapy, usually necessitate chemotherapy dose-delays, dose-reductions, and/or medication discontinuations, interfering with the delivery of optimal treatment. Neutropenia is the major dose-limiting toxicity and the primary driver of chemotherapy dose delays and reductions. The consequences of such dosing modifications are huge in patients with curable breast cancer which may potentially compromise the efficacy of chemotherapy, disease control, survival outcome as well as patient's quality of life (QoL). In Ethiopia, studies depicting the clinical progress of cancer management are very little. One retrospective study demonstrated that the 5-year cumulative probabilities of distant metastasis-free survival (MFS) for breast cancer was 72% (for stages 1 and 2) and 33% (for stage 3) (Kantelhardt *et al*., 2014). Yet, chemotherapy induced hematological toxicities, reduced chemotherapy dose intensities and associated risks among Ethiopian breast cancer patients are lacking.

Although Tam has proven to provide a significant clinical benefit in endocrine therapy of breast cancer, not all women with estrogen receptor-positive tumors derive benefit from Tam therapy. Women who all receive Tam at the same dose, as in current daily practice, can have different clinical outcomes (Saldores *et al.,* 2015) and the disease recurs in approximately ~30–50% of the patients and/or eventually die from the disease **(**EBCTG, 2005**)**. In addition, occurrence of side effects may also differ between individuals, some of them experiencing treatment-limiting side effects, while others do not have any side effects (Saldores et al., 2013). Identifying predictive biomarkers of response to Tam, other than expression of ERs, and the use of these markers to

individualize Tam therapy has become a subject of intensive research. Currently, much attention has been given to genetic polymorphisms in drug-metabolizing enzymes involved in the metabolic activation of Tam into Endoxifen especially *CYP2D6* (de Vries Schultink *et al.*, 2015).

Pharmacokinetic studies stressing the link between patients' *CYP2D6* genotype and plasma levels of active tamoxifen metabolites in individuals undergoing Tam therapy has been investigated in European and Asian descent (Lim, *et al.*, 2011; Mürdter *et al.*, 2011; Madlensky, *et al.*, 2011). It has been shown that 29% of Ethiopians are *CYP2D6* gene duplicators (categorized as ultrarapid metabolizers) (Aklillu *et al.*, 1993). Given this unique *CYP2D6* genotype, there is no study showing how *CYP2D6* genetics influence plasma endoxifen levels, in SubSaharan African population in general and Ethiopian women in particular.

The present thesis describes the PK of CPA in Ethiopian breast cancer patients and explains the inter-subject PK variation using a population PK-PD modeling approach. The study also explores the impact of CPA based anticancer regimen in terms of hematologic toxicities and potential risk factors of toxicities in our patient populations. In addition, the adequacy of chemotherapy dose intensity received by the individual breast cancer patients has been evaluated. The prevalence and predictors of reduced relative dose intensity has also been determined. Moreover, we also investigated the influence *CYP2D6*, *CYP2C9, CYP2C19*, *CYP3A5, POR* and *ABCB1* polymorphisms on the plasma level of the active Tam metabolites in Ethiopian women receiving adjuvant Tam therapy.

From these perspectives, oncologists and other health care workers in our setup can better understand the pharmacokinetics of CPA and Tam and response behavior of CPA, in Ethiopian patients for better treatment approaches. It would also provide pertinent information to consider when designing a treatment approach for a specific individual patient. Thus, the result may help implement the appropriate use of CPA and Tam in breast cancer therapy according to patient and clinical characteristics. Consequently, the study result may help improve the quality of oncology services such that patients will get better treatment. The finding would also be a baseline data for possible further dose optimization studies.

2. Objectives of the study

2.1 General objective

The aim of this study was to investigate the effect of genetic polymorphism and other patient demographic and clinical factors on the pharmacokinetics (PK) and pharmacodynamics (PD) of CPA and PK of Tamoxifen in Ethiopian females with breast cancer receiving CPA-based chemotherapy and Tamoxifen.

2.2 Specific objectives

- To assess the incidence and risk factors of chemotherapy induced hematologic toxicities of CPA based chemotherapy.
- To determine the prevalence and predictors of reduced relative dose intensity (RDI) of CPA based chemotherapy.
- To develop a population pharmacokinetic/pharmacodynamics (PK/PD) model of CPA in Ethiopian breast cancer patients.
- To investigate the effects of *CYP2D6, CYP2C9, CYP2C19, CYP3A5, POR, ABCB1* and *UGT2B1*5 polymorphisms on plasma level of tamoxifen and its metabolites.

3 Participants and methods

3.1 Study area/setting and period

The study was conducted at the oncology clinic, Tikur Anbessa Specialized Hospital (TASH), Addis Ababa University, Addis Ababa. Genotyping was performed at the Division of Clinical Pharmacology, Department of Laboratory of Medicine, Karolinska Institutet Huddinge, Stockholm, Sweden. CPA quantification was conducted at the Experimental Cancer Medicine (ECM), Clinical Research Centre (NOVUM), Department of Laboratory Medicine, Karolinska Institutet, Huddinge, Stockholm, Sweden. While, plasma Tam and its metabolites were quantified at the therapeutic drug monitoring (TDM) laboratory, department of clinical pharmacology, Karolinska Institutet Hospital, Huddinge, Stockholm, Sweden.

3.2 Description of the study area and setup

Addis Ababa is the capital city of Ethiopia, the second populous country in Africa. Based on the United Nations report for the year 2016, the population of Ethiopia has been estimated to be above 100 million. According to the office of the Central Statistics Agency (CSA, 2012) of Ethiopia, the population of Addis Ababa city by the year 2012 was 3.049 million. TASH is a public teaching specialized referral hospital with 800-beds under Addis Ababa University and provides both teaching and clinical care service in different medical disciplines. The Addis Ababa population based cancer registry is the first population based cancer registry in the country established in September 2011 at the Radiotherapy Center, TASH, Addis Ababa University. At present, the Radiotherapy Center of TASH is the major referral center that provides oncologic care service in the country. The population based cancer registry of Addis Ababa city has reported that breast cancer is the leading cancer case accounting for about 33% of cancer cases followed by cancer of the cervix (17%) (AACR, 2014).

The common first line combination chemotherapy regimens for breast cancer in TASH comprised of (1) six cycles **FAC** (5-**F**lourouracil 500 mg/m^2, Doxorubicin [or **A**driamycin] 50 mg/m^2, and **C**yclophosphamide 500 mg/m^2), **(2)** four cycles **AC** (**D**oxorubicin 60 mg/m^2 and **C**yclophosphamide 600 mg/m^2), **(3)** sequential **AC - T** (four cycles of **D**oxorubicin 60 mg/m^2 and **C**yclophosphamide 600 mg/m^2 followed by another 4 cycles of Paclitaxel 175 mg/m^2) and

(4) six cycles **CMF** (**C**yclophosphamide 600 mg/m^2, **M**ethotrexate 40 mg/m^2 and **F**lourouracil 600 mg/m^2). Figure 4 lists the first line chemotherapy regimens employed for breast cancer treatment and the administration procedures at the radiotherapy center of TASH.

1. FAC
Fluorouracil 500mg/m^2, (IV bolus), day 1
Doxorubicin 50mg/m^2, IV, day 1,
(Continuous infusion over 2 hours)
Cyclophosphamide 500mg/m^2, IV, day 1
(short IV infusion (½-1 hours duration)

Repeat cycles every 21 days for 6 cycles

2. AC
Doxorubicin 60mg/m^2, IV infusion, day 1
Cycophosphamide 600 mg/m^2, IV, day 1

Repeat cycles every 21 days for 4 cycles

3. AC-T (AC → Paclitaxel)
Doxorubicin 60mg/m^2, IV infusion, day 1
Cycophosphamide 600 mg/m^2, IV, day 1
Repeat cycles every 21 days for 4 cycles

Followed by:
Paclitaxel 175mg/m^2 IV infusion over 3hours

Repeat cycles every 21 days for 4 cycles

4. CMF
Cycophosphamide 600 mg/m^2, IV, day 1
Methotrexate 40mg/m^2, IV, day 1
Fluorouracil 600mg/m^2, IV, day 1

Repeat cycles every 21 days for 6 cycles

Figure 4: Common first line chemotherapy regimens for breast cancer in the outpatient day care of Tikur Anbessa Specialized Hospital.

3.3 Study design

A prospective cohort study design was employed to assess hematologic toxicities and relative dose intensity. A cross sectional study design was employed to investigate the population PK-PD modeling of CPA as well as PK of tamoxifen. A schematic representation of the study group is presented in Figure 5.

3.4 Population

3.4.1 Source population

All patients diagnosed (both clinical and laboratory diagnosis) with breast cancer at the radiotherapy center of TASH, Addis Ababa University, Addis Ababa.

3.4.2 Study population

All patients with breast cancer, receiving CPA containing chemotherapy regimen (neo-adjuvant, adjuvant or palliative) or Tam at the radiotherapy center of TASH, during the study period and who fulfill the following inclusion criteria below.

3.4.2.1 Inclusion criteria

For CPA-based chemotherapy group

- Patients who were capable and willing to provide signed voluntary informed consent to participate and agreed to give blood sample for the purpose of the study.
- age above 18 years and above
- Women with early stage, locally advanced or metastatic breast cancer
- Women with a Karnofsky's performance score (Annex III) of at least 70% (at diagnosis)
- A woman with baseline white blood cell (WBC) count ≥ 3,000/mm3; absolute neutrophil count ANC) ≥ 1500/ mm3; and a platelet count ≥ 100,000/mm^3 (during sampling for PK study).
- Baseline Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), and Serum Creatinine (SCr) ≤ 2.5 times the upper limits of normal; total bilirubin ≤ 2 times the upper limits of normal.
- Conventional dosage of cyclophosphamide (doses of 500-600 mg/m^2 I.V of CPA)

For tamoxifen group

- Patients who are capable and willing to provide signed voluntary informed consent to participate and agreed to give blood sample for the purpose of the study.
- Women with a Karnofsky's performance score of at least 70% (during visit)
- Patients taking tamoxfien 20 mg/day for at least three months.

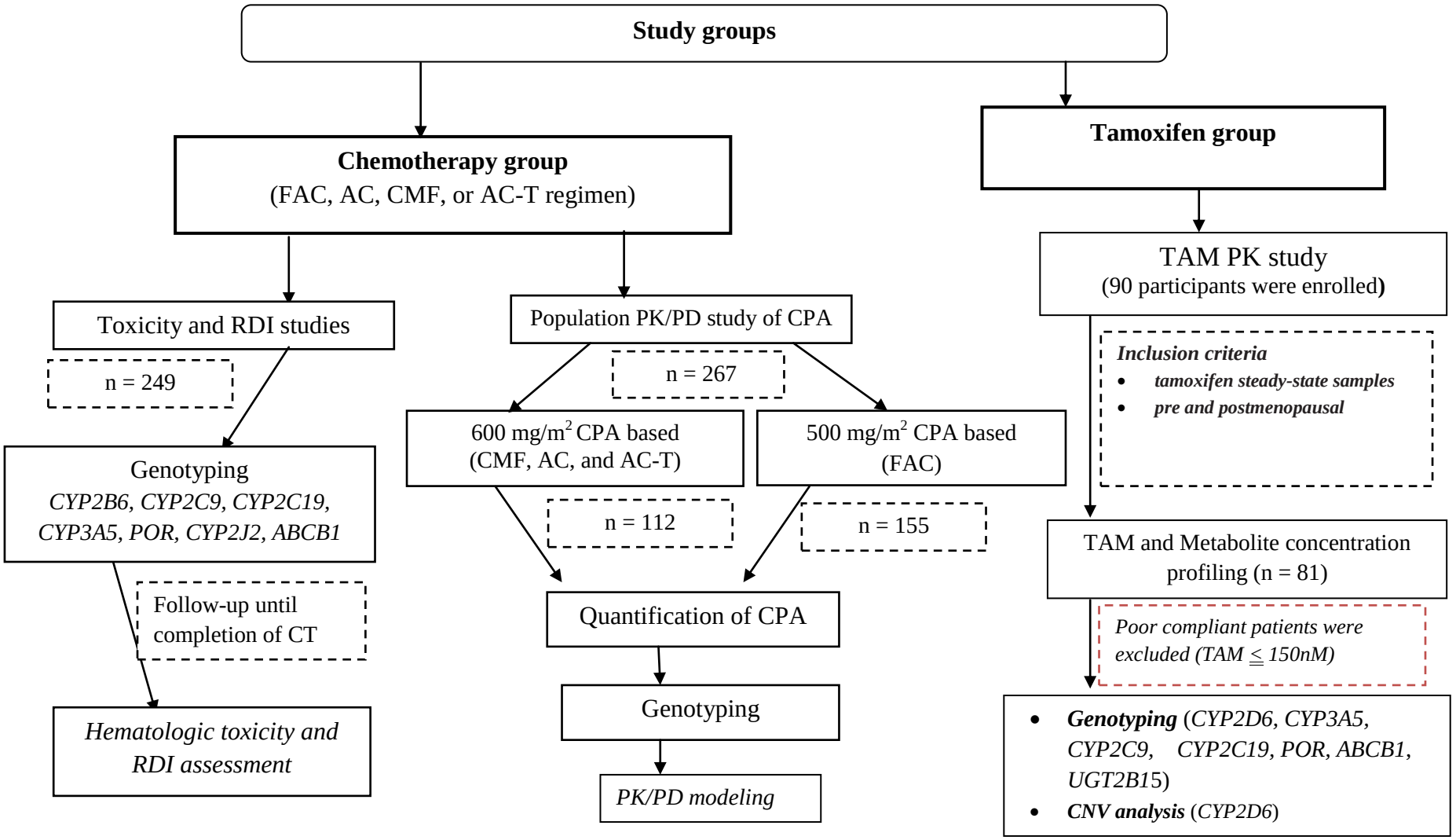


Figure 5: Study flow diagram depicting study groups and participant recruitment

3.4.2.2 Exclusion criteria

CPA-based Chemotherapy group

- ✓ Patients with multiple primary tumor types.
- ✓ patients on concurrent radiotherapy
- ✓ Pregnant women
- ✓ Breast cancer patients with male gender
- ✓ Patients with prior history of anemia
- ✓ Patients who took their first cycle of chemotherapy at private clinic and come for subsequent follow-up to the study hospital.

Tamoxifen group

- ✓ Patients taking known *CYP2D6* inhibitor (*e.g.*, Fluoxetine, paroxetine, bupropion, duloxetine, Chlorpheniramine, Chlorpromazine and amiodarone)/inducer (*e.g.*, Dexamethasone and Rifampin) as co-medication within 28 days.
- ✓ Patients with unavailable data (information) regarding co-medication history
- ✓ History of GI surgery (which might decrease the absorption of oral Tam).
- ✓ Non adherent patients to take regular Tam
    - o At the level of enrollment, medication adherence was assessed using self-report in the past 28 days.
    - o At the level of data analysis (patients were identified as non-compliant (plasma TAM concentrations < 150 nM and were excluded) and compliant (Tam $\geq$ 150 nM) (Saladores et al., 2015).

3.5 Variables

3.5.1 Outcome measures

- o Chemotherapy induced hematologic toxicity
- o Reduced relative dose intensity (reduced RDI)
- o PK variability to CPA
- o PK variability to Tam

3.5.2 Explanatory variables

- Socio-demographics (age, weight, body mass index (BMI), body surface area (BSA)
- Genotype of drug metabolizing enzymes' and transporter genes
- Co-morbidities (HIV, cardiovascular diseases, diabetes, etc)
- Stage of cancer
- Lymph node status
- Tumor size at diagnosis
- Renal function estimates (SCr, BUN)
- Liver function estimates (AST, ALT, ALP)

3.6 Chemicals and reagents

The following chemicals, reagents and kits were used: QIAamp^{R} genomic DNA extraction Kit (QIAGEN^{R}, Sweden), Cyclophosphamide (Sigma Aldrich Co, Germany), Ifosfamide (Vnr079111, Denmark), acetonitrile (ACN) (Merck KGaA, Germany), methanol (Merck KGaA, Germany), potassium dihydrogen phosphate (Merck KGaA, Germany), and potassium monohydrogen phosphate (Merck KGaA, Germany), *ortho*-phosphoric acid (GR grade, Merck KGaA, Germany), Zinc sulphate pentahydrate (Sigma Aldrich, Germany), Formic acid, Ammonium phormate, Water (Millipore Milli Q) Taqman^{R} fast advanced master mix and Taqman SNP genotyping master mix (Applied biosystems, Sweden).

3.7 Methods

3.7.1 Assessment of hematologic toxicity and dose intensity

At baseline, patients' medical records, biopsy reports, radiologic imaging results (X-RAY, ultrasound, CT scan (where available)) and laboratory investigations (blood counts and organ function estimates (Alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), serum creatinine (SCr) and blood urea nitrogen (BUN)) levels were recorded. In addition, other pretreatment demographic, clinical and tumor characteristics include age at diagnosis, menopausal status, performance status, weight, height, body, site of tumor, histologic type of the tumor, degree of differentiation, tumor size, lymph node involvement, tumor receptor status, co-morbidity (assessed and classified based on the Charlson Co-morbidity

Index) (Breccia et al., 2011), chemotherapy panel, *i.e*., treatment intent (neo-adjuvant, adjuvant or metastatic), chemotherapy regimen and primary or secondary G-CSF use. At the first cycle, the planned dose and schedule of each chemotherapy regimen were noted. In the subsequent cycles, follow-up clinical information including actual chemotherapy dose received and date of administration, results of blood laboratory investigations (differential blood counts, and blood chemistry), dose intensity reducing events (*i.e*., dose reduction and/or delays) occurred across the cycles were also recorded.

Differential white blood cell, hemoglobin and platelet counts were noted at every cycle, in the course of chemotherapy. Toxicity events were graded according to the United States National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE), version 4.0 (CTCAE, 2010,). Accordingly, grade 3 and 4 anemia was defined as hemoglobin value of 6.5 – 8.0 g/dL and < 6.5g/dL, respectively. Platelet counts between 25,000 – 50,000/ mm^3 and < 25,000/ mm^3 were classified as grade 3 and grade 4 thrombocytopenia, respectively. Neutropenia was defined as grade 3 (neutrophil count =500 – < 1,000/ mm^3) and grade 4 (neutrophil count < 500/ mm^3). Grade 2 toxicities were also considered to be grade 2 anemia (hemoglobin level 8.0 – 10.0 g/dL), grade 2 thrombocytopenia (platelets count between 50,000 – 75,000/ mm^3), grade 2 neutropenia (neutrophil count 1000 – < 1,500/mm^3).

Cycles were 3 weeks long (planned every 21 days) in the study setup. A dose reduction was described as ratio of the actual dose given compared with the standard or planned dose. Dose delay was described as the actual cycle length compared with standard or planned cycle length. A dose reduction was defined as a ≥15 % reduction relative to standard/planned dose. Dose/treatment delay was defined as ≥ 15% delay in days relative to the standard cycle length for chemotherapy (Wildiers and Reiser, 2011).

The dose intensity (DI) of each agent was calculated by dividing the total received dose of the agent to the total number of weeks of treatment. The relative dose intensity (RDI) of each agent was expressed as the total delivered dose of the agent per unit time (*i.e*., weeks) as a percentage of the standard/planned dose intensity. The RDI for the regimen represents the average RDI for each chemotherapeutic agent in a given regimen (Hryniuk et al., 1998). According to Bonadonna

et al (2005) the bench mark RDI was defined as 85%. The proportion of patients receiving less than 85% of the reference dose was determined. Both planned and unplanned reductions in RDI (< 85%) were calculated for each chemotherapy panels (neo-adjuvant, adjuvant and metastatic). The reference dose intensity for each drug was considered to be the established dose in clinical trials in mg/m^2 per unit time or the planned dose at by the oncologist in the setup (in weeks) (Mamounas *et al.*, 2005; Anderson *et al.*, 2006).

3.7.2 Blood sampling design and processing

In 17 patients, blood samples (3 mL) were collected at five sampling points (30 minutes before CPA infusion starts (0hrs), and 4, 8, 12 and 22 hrs after the start of infusion. These patients were admitted to phase I clinical trial center of College of Health sciences, AAU, to receive their first cycle chemotherapy and monitored at this center overnight. For all other patients, blood samples were collected in the day care ward at, 0 hr (30 minutes before CPA infusion starts), 1/2, and 3/4 or 5/6 hr after CPA infusion starts. Blood samples were collected in prechilled heparin tubes and the heparinized blood was immediately centrifuged on bed side at 3500g for 3 min at 4^oC to separate the plasma (Nakajima et al., 2007). Additional 2mL of blood sample was also drawn at 0 hr using EDTA tube for DNA extraction. The plasma samples and EDTA bloods were then stored at $–80^oC$ until analysis.

3.7.3 Genotyping

Genomic DNA was isolated from peripheral leukocytes in whole blood samples using QIAamp DNA Midi Kit (Qiagen GmbH, Hilden, Germany). Genotypes of common functional variant alleles of drug metabolizing enzymes' genes relevant for CPA or Tam bioactivation or disposition including, *CYP2B6*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP3A5*, *CYP2J2*, *POR*, *ABCB1* and *UGT2B15* were carried out using Taqman allele specific PCR (Applied Biosystems Genotyping Assays) as described previously (Hatta and Aklillu, 2015). In brief, genotyping was performed using TaqMan® drug metabolism genotyping assay reagents for allelic discrimination (Applied Biosystems Genotyping Assays) with the following ID numbers for each SNP: C__7817765_60 for *CYP2B6*6* (c.516G4T, rs3745274), C__26201809_30 for *CYP3A5*3* (c.6986A4G, rs776746), C__25625805_10 for *CYP2C9*2* (rs1799853), C__27104892_10 for

*CYP2C9*3* (rs1057910), C__25986767_70 for *CYP2C19*2* (rs4244285), C__27861809_10 for *CYP2C19*3* (rs4986893), C_9581699_80 for *CYP2J2*7* (rs890293), C_8890131_30 for *POR*28* (rs1057868), C__27102425_10 for *CYP2D6*2* (rs16947), C__27102431_D0 for *CYP2D6*4* (rs3892097), C__11484460_40 for *CYP2D6*10* (rs1065852), C___2222771_A0 for *CYP2D6*17* (rs28371706), C__11711730_20 for *ABCB1* (rs3842), C_7586657_20 for *ABCB1c*.3435C>T (rs1045642), C_27028164_10 for *UGT2B15*2* (rs1902023), and C_9440184_20 for *UGT2B15*4* (rs4148269). Genotyping was carried out on QuantStudio 12 K Flex Real-Time PCR system (Life Technologies Holding, Singapore). The final volume for each reaction was 10 µl, consisting of TaqMan® fast advanced master mix (Applied Biosystems, Waltham, MA, USA), TaqMan 20X/40X drug metabolism genotyping assays mix (Applied Biosystems) and genomic DNA. The PCR profile consisted of an initial step at 60 °C for 30 s, hold stage at 95 °C for 10 min and PCR stage for 40 cycles step 1 with 95 °C for 15 and step 2 with 60 °C for 1 min and after read stage with 60 °C for 30 s. Genotypes were assigned using the manual calling option in the allelic discrimination application, using QuantStudio software (Applied Biosystems, Life Technologies Stockholm, Sweden). The characterized SNPs were selected on the basis of their potential or influence on the functionality of enzymes and transporters proteins obtained from public databases.

3.7.4 Copy number variation (CNV)

The *CYP2D6* gene copy number for the patient DNA samples was determined using TaqMan Copy Number Assay (Life Technologies, CA) and according to the manufacturer's recommendations. The genomic DNA samples were amplified in 96-well plates on QuantStudio™ 12K Flex Real-Time PCR system (Applied Biosystems Life Technologies Holding, Singapore) using comparative $\Delta\Delta C_T$ method. *CYP2D6* exon 9 (TaqMan Copy Number Assay ID: Hs00010001_cn), and intron 6 (TaqMan Copy Number Assay ID: Hs04502391_cn) were used as primers. RNase P (ID: 431683) was used as the internal control for copy number analysis (Life Technologies, CA). All the samples were run in triplicates. The final volume for each reaction was 10 µL, consisting of TaqMan® Genotyping Master Mix (Applied Biosystems, Waltham, MA, USA), TaqMan® Copy Number Assay mix (20x, Applied Biosystems, USA), TaqMan® Copy Number Reference Assay mix (20x, Applied Biosystems, USA). The PCR

conditions consisted of an initial step at 60 °C for 30 s, hold stage at 95 °C for 10 min and PCR stage for 40 cycles step 1 with 95 °C for 15 and step 2 with 60 °C for 1 min and after read stage with 60 °C for 30 s. Relative quantification of *CYP2D6* gene copy number was performed by using CopyCaller Software (Life Technologies, CA). A DNA samples with known *CYP2D6* gene copy number carrying 1 gene copy, and 2, 3 and 4 *CYP2D6* gene copies) were obtained from previous study (Aklillu et al., 1996) and used as positive controls.

3.7.5 Determination of plasma CPA concentrations

The method described by El Serafi *et al* (2015) was used to quantify the plasma concentrations of CPA with some modifications. To each 250µL of thawed plasma samples, 25µL of internal standard (Ifosfamide, 20mM) and 1.25 ml ethyl acetate was added. The mixture was vortexed for 15 seconds and then centrifuged at 5000 rpm, 4^{o}C for 10 minutes. The organic fraction was transferred to a clean tube and evaporated to dryness under vaccum centrifuge (Maxi dry Lyo). The residue was then dissolved in 100µL of mobile phase, and the resulting solution was put in ultrasonic bath for 15 seconds. 50µL of the resulting solution was injected into the HPLC system. The HPLC system consisted of an LKB-2150 pump, Gilson-234 auto-injector with a 100-µl sample loop, ZORBAX Extend-C18 column (150 × 4.6 mm, 3.5 µm, Agilent, Santa Clara, CA, USA) with a C18 guard column (Agilent, Santa Clara, CA, USA) and a UV/VIS detector, (Milton Roy Spectro Monitor 310). The mobile phase consisted of ACN/0.05 M KH_2PO_4 buffer, pH 2.8 (24:76 v/v), and the flow rate was 1 ml/min. Detection was .monitored at 195nm using a Milton Roy UV Spectro-Monitor 310 detector (Pont-Saint-Pierre, France). Under these conditions, the retention times for ifosfamide and CPA were 5.5 and 6 min, respectively. Standard curve was constructed from CPA free plasma to which increasing concentrations of CPA (5–2000mmol/L) and fixed concentration of internal standard were added. The quantification limit of CPA was 5 µM. The standard curve was linear in the range of 5–1000mmol/L (regression coefficient, r = 0.9987). Quality control samples (three samples, at a concentration of 25, 100, and 1000 µM) were included routinely at each run.

3.7.6 Determination of plasma tamoxifen and its metabolites

Tamoxifen (Tam) and its major metabolites N-desmethyltamoxifen (NDM), (Z)-4-hydroxytamoxifen (4HT), and (Z)-Endoxifen, (E) were quantified by reversed phase liquid chromatography followed by electrospray tandem mass spectrometry (LC-MS/MS). Briefly, to each 200 µL of thawed plasma samples, 400 µL internal standard solution (containing 2 ng/mL of Z/E-Endoxifen-d_5, and 4-OH-tamoxifen-d_5, and 20 ng/mL of N-desmethyltamoxifen-d_5, and tamoxifen-d_5) was added and mixed for 10 minutes in a micro plate shaker (Troemner Vx-2400 Multitube mixer). The mixture was centrifuged at 3400 rpm at 4^{o}C for 10 min. The supernatant was then transferred to a new glass vials and evaporated to dryness under vacuum centrifuge. The residue was dissolved with 50 µL methanol over micro plate shaker for 10 min and the resulting solution was then centrifuged at 3400 rpm for 10 min at 4^{o}C. Ten micro liter (10 µL) of the solution was injected into the LC system (Dionex Ultimate 3000, UHPLC focused, Thermo scientific, Germany) coupled with a mass spectrometer (TSQ Quantiva, Thermo scientific). Separation occurred on Accucore C-18 column (150x2.1 mm, Thermo scientific). The mobile phase for gradient elution consisted of solution A (10 mmol/L ammonium formate with 0.005% formic acid in water) and solution B (10 mmol/L ammonium formate with 0.005% formic acid in 99% methanol). The LC conditions were: column oven temperature 40^{o}C, flow rate 0.500 mL/min, auto sampler temperature 10^{o}C. Detection was in positive ion mode using electrospray ionization and monitored using Trace Finder software (Thermo scientific). Under this conditions, the retention times were 3.25, 3.26, 3.38, 4.03, and 4.11 min for E-Endoxifen, Z-Endoxifen, 4-OH-tamoxifen, and N-desmethyl-tamoxifen, and tamoxifen, respectively. Calibration curves were constructed by plotting the ratio of the area of the compound and the internal standard against the standard analyte concentration. Three quality control samples were also included at each run.

3.7.7 Population pharmacokinetic modeling

The plasma concentration data were prepared in excel spread sheet and saved as coma separated (csv) file. The csv file was imported into Rstudio (version 3.5.1) on 64 bit windows platforms. The structure of the data was then explored by plotting scatter plots and *ggplots* of concentration or log concentration *versus* time for the data set. The data set was then formatted to NONMEM

data file and a population PK model for CPA was built using nonlinear mixed effect modeling (NONMEM) program (version 7.3). Additional software tools were also used as a workbench to facilitate the use of NONMEM. PsN (version 3.4.2), was used for performing advanced modeling and simulation with NONMEM and it allows the implementation of bootstrap and visual predictive checks (VPCs). Xpose (version 4.5.0), a module for R software (version 3.1.0), was used as a tool for plotting and analyzing NONMEM output. Pirana (version 2.9.6), was used as a graphical user interface for NONMEM, PsN and Xpose/R for model management, model execution, output generation and interpretation of results.

To build the base model, different compartmental models (one, two or three compartments) were fitted to the dataset, starting with a one compartment. The compartments models were specified in NONMEM using ADVAN6 subroutine. First order conditional estimation with Interaction (FOCE-I) was used to estimate parameters, theta, omega and sigma. The parameters were estimated in dose normalized conditions (normalized based on BSA). The structural model was parameterized with clearance (CL) and volume of distribution (V). Inter-individual variability (IIV) was specified by exponential error function (VALUE (Y) = THETA * EXP (ETA)). Additive (Y = Typical Value (TY) + EPS), proportional (Y = TY * (1 + EPS)), combined proportional plus additive (Y = TY * (1 + EPS (1)) + EPS (2)) and exponential error models was attempted to describe residual error (to account within subject variability, experimental errors and model misspecification. Model discrimination for competing models was based on a change (drop) in the objective function value (OFV) ($\alpha = 0.05$, χ^2 distribution) and goodness of fit (GOF) plots. Prediction based diagnostic plots (Observations (DV) *vs* Individual predictions (IPRED) or DV *vs* population predictions (PRED)), and residual based diagnostic plots (conditional weighted residuals (CWRES) *vs* IPRED or *vs* TIME) were plotted. Parameter standard errors (%SEM) and coefficient of variations (%CV) were obtained using a covariance option ($COV) in the NONMEM control file.

3.7.7.1 Covariate analysis

Prior to testing for covariates in the model, plots (box plots or scatter plots) of each of the covariates (socio-demographic, clinical, and genotype) were performed against the parameters

using R. Because shrinkage was higher than 30% in all the parameter estimates, we developed a visual predictive check (VPC) and chose to include all pertinent covariates in the covariate model building process. The covariates explored were demographics characteristics (weight, BSA, BMI, age), CPA dosage regimen (500 *vs* 600 mg/m^2 based CPA), baseline renal and liver function estimates (SCr, BUN, ALT, AST, ALP), pharmacogenetic variables (*CYP2B*6*, *CYP2C9*2*, *CYP2C9*3*, *CYP2C19*2*, *CYP2C19*3*, *CYP3A5*3*, *CYP3A5*6*, *CYP2J2*7*, *POR*28,* and *ABCB1*).

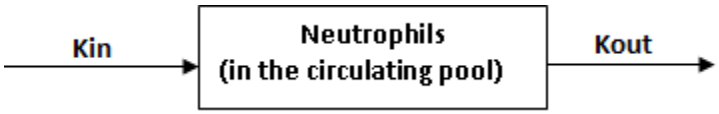
Based on the built PK model, covariate analysis was performed using stepwise covariate modeling (SCM) procedure after creating a configuration file. This involves both forward (α = 0.05) and backward elimination steps (α = 0.01). In the first phase (forward elimination) of the SCM, covariates are added to the base model in stepwise manner based on statistical significance (decrease in OFV). The effect of each potential covariate on each parameter is independently tested with a 5% significance level corresponding to a change in OFV > 3.84 for one degree of freedom. The covariate with the large drop in OFV are added to the model ($p < 0.05$). The procedure is repeated for all covariates until no more significant covariates are identified. In the backward elimination step, the level of significance is set to 1% threshold corresponding to a change in OFV > 6.63 for one degree of freedom and the covariates selected in the forward step are removed from the model one at a time. The covariates which cause an increase in greater than 6.63 ($p > 0.01$) upon removal are retained in the model.

When the SCM is completed, the final covariate model with included covariate relations was subjected to simulation-based model diagnostic. Visual predictive checks (VPC) was created simulating 1000 replicates based on the final model parameters using bootstrap to assess how well the model predicts the observed data on which the model is conditioned, with 5th and 95th percentile confidence interval (CI).

3.7.8 Pharmacodynamic modeling

Absolute neutrophil count (ANC) at baseline and at day 20 post of CPA administration of the first cycle CPA based chemotherapy were used to develop the neutropenic toxicity model. A semi mechanistic (indirect response) and empiric (direct drug effect) PD models were applied

(Felmlee et al., 2012) to describe CPA-related neutropenic toxicity. Initially, the indirect response model was explored. It was assumed that cells from myeloblasts to myelocytes are chemotherapy sensitive and bone marrow leukocyte inhibition depends on sensitive cell exposure (unlike the stem cells which divide but are less sensitive to chemotherapy than mitotic hematopoeitc precursors). Thus, we chose the inhibition of cell production model (Woo et al., 2008) as described by the schematic representation (Figure 6 and Equation 1), where k_{in} is the zero order production rate constant, *i.e.*, the rate of entry of neutrophils into the circulating pool, k_{out} (K_{in}/ANC_o) is the first order elimination rate constant, *i.e.*, the rate of disappearance of neutrophils from the circulating pool (Figure 1). E_{max} is defined as the maximum fractional factor of inhibition or a factor that inhibits neutrophil synthesis as an anticancer chemotherapy effect ($0 < E_{max} \leq 1$). ANC is the drug effect (neutrophil count at any time t) and ANC_o is the baseline neutrophil count. Since area under the concentration time curve (AUC) represents the systemic exposure, we incorporated the AUC, from time zero to day 20 after the first dose of CPA in the PD model to describe the relationship between AUC and drug effect. AUC_{50} represents the area under the curve associated with 50% of the maximum effect (E_{max}).



$$\frac{d(ANC)}{dt} = K_{in}\left(1 - \frac{E_{max} \times AUC}{AUC_{50} + AUC}\right) - K_{out} \times ANC \quad \ldots\ldots\ldots\ldots (1)$$

Figure 6: Schematic representation of indirect response model

For AUC significantly less than AUC_{50} (*i.e.*, $AUC_{50} >> AUC$), AUC in the denominator of equation 1 can be considered negligible and the relationship can be reduced to Equation 2, with $m = E_{max}/AUC_{50}$ is the slope of the relationship. Subsequently, K_{in}, slope (m) of the relationship and ANC_o were estimated using NONMEM.

$$\frac{dA\,(ANC)}{dt} = K_{in}\,(1 - m \times AUC) - K_{out} \times ANC \quad \ldots\ldots\ldots\ldots\ldots (2)$$

On the other hand, empiric model (a linear direct drug effect model) was also separately adapted to the AUC data to correlate neutrophil count according to Equation 3, from which ANC_o and slope (*m*) were estimated using NONMEM.

$$Y\ (ANC) = ANC_o - \text{slope}\ (m) \times AUC \quad \ldots\ldots\ldots\ldots\ (3)$$

3.8 Data management

The patient data and blood samples were collected by trained clinical nurses working in the radiotherapy center of TASH. The accuracy of information on dosing and timing of sample collections were monitored by the PI. Standard operating procedures (SOPs) were followed for data/sample collection, blood sample pretreatment, genotyping and HPLC assays.

3.9 Statistical analysis

Descriptive statistics were computed to explore the demographic characteristics, clinical profiles, genotype frequencies of participants. *Chi*-square test was used to compare the observed and expected genotype frequency according to Hardy–Weinberg equilibrium. Univariate followed by multivariate Cox proportional hazard and logistic regression were used to estimate risk predictors of toxicity and reduced RDI, respectively.

Population PK/PD modelling were performed using NONMEM. Interpatient variability in plasma CPA and tamoxifen was described with %CV. ANOVA were run for comparison of genotype and chemotherapy regimens, BSA, and BMI groups. Linear regression analysis was used to see the association between CYP polymorphisms, socio-demographic and clinical factors and plasma levels of tamoxufen and its metabolites. All the tests were considered significant at *p* values of 0.05. The data were analyzed using SPSS for Windows (version 21.0, IBM Corporation, New York, USA). Graphs were prepared using *ggplot2* package in R (version 3.5.1)

3.10 Ethical considerations

The study protocol was approved by Institutional Review Board (IRB) of the college of health sciences, Addis Ababa University, Armauer Hansen Research Institute Ethical Review Committee (AHRI/AAERC) and National Research Ethics Review Committee (NRERC) of the

Federal democratic republic of Ethiopia. A written information sheet, prepared both in Amharic and English, was provided to study participants. A signed informed consent was obtained from each patient before enrolment in the study. During the investigation, the right of the patient not to participate or refuse to give blood sample for the purpose of the study or to withdraw at any time from the study was respected. Each patient was designated only by unique code (BC-000) and all patient records were kept confidential (except for those involved in the study) such that the filled forms were kept in a secured cabinet in the principal investigator's office.

4 Results

4.1 Chemotherapy induced grade 3 or 4 hematological toxicity (*Paper I*)

A total of 249 breast cancer patients (median age = 39 years) were included. Invasive ductal carcinoma was the most common (85.9%) type of cancer and larger proportion (73.1%) of patients had node positive tumor. Majority (65.9%) of the patients in all chemotherapy panels (neo-adjuvant, adjuvant or metastatic) received 5-Flourouracil, Doxorubicin, and Cyclophosphamide (FAC) combination regimen. The mean doses of these drugs administered per cycle were 797.5 mg ± 77.37 (5-Flourouracil) and 822.08 mg ± 124.3 (CPA), while the median Doxorubicin dose administered was 82.5 mg (IQR 10.0).

4.1.1 Incidence of chemotherapy induced grade 3 or 4 hematological toxicity

The incidence of chemotherapy induced grade 3 or 4 hematological toxicity was 51.0% (95% confidence interval (CI) = 44.54 – 57.46%). Most of the hematologic toxicity events were neutropenic toxicities, 50.2% (95% CI = 43.83 – 56.56%). The incidence of grade 3 or 4 anemia and thrombocytopenia across the cycles was 2% and 1.2%, respectively. Paired sample *t*-test showed there was significant decrease in the mean absolute neutrophil counts in each cycles compared to the baseline ($p < 0.05$ for the trend across the cycles).

4.1.2 Risk predictors of chemotherapy induced grade 3 or 4 hematological toxicity

Multivariate Cox proportional hazard regression analysis indicated that, patients who took chemotherapy with low baseline white blood cells count (grade 1 leukocytopenia, hazard ratio (HR) = 2.748; 95% CI, 1.466 - 5.149, $p < 0.001$), or low neutrophils count (grade 1 or 2 neutropenia) (HR = 2.746; 95% CI, 1.732 – 4.353, $p < 0.001$), were at increased risk of experiencing grade 3 or 4 hematologic toxicity in the subsequent cycles. In addition, the risk of toxicity was also higher in patients carrying *CYP2J2*7* variant allele (HR = 1.819; 95% CI, 1.141 – 2.899, $p = 0.012$). Patients carrying *CYP2C9* *2 or *3 alleles were also associated with lower incidence of hematologic toxicity (3.1% *vs*, 11.4%, $p = 0.024$, Chi square test). Controlling for *POR* genotype, it was found that toxicity was significantly reduced in patients with *CYP2C9* *2 or *3 alleles who also carried *POR**28 ($p = 0.003$). No such association was observed among *POR*1/*1* carriers ($p = 0.124$). Although *Chi* square test showed association of *CYP2C9* variant

alleles to toxicity, these alleles did not reach to statistical significant level in the final multivariate regression analysis. There was also no association between *CYP2B6, CYP2C19*, *POR* or *ABCB1* genotype with risk for hematologic toxicity.

4.2 Reduced relative dose intensity (*Paper I*)

4.2.1 Prevalence of reduced relative dose intensity

The overall actual average RDI was 81.9% (95% CI = 80.52% - 83.28%). The proportion of patients who received reduced RDI < 85% of the standard/planned dose intensity was 56.6% (95% CI = 50.32% - 62.88%). Our finding indicates that a higher proportion of breast cancer patients in Ethiopia are receiving reduced RDI due to subsequent hematologic toxicities associated dose/treatment delays.

4.2.1 Predictors of reduced relative dose intensity

Results of multivariate logistic regression analysis showed that the independent risk predictors of reduced RDI were BMI < = 18.4 kg/m^2 (underweight) (Adjusted odds ratio (AOR) 5.975; 95% CI = 1.361 – 26.227), low baseline leukocyte count (AOR 6.092; 95% CI = 1.238 – 29.98, p = 0.026), low baseline neutrophils count (AOR 3.37; 95% CI = 1.412 – 8.045, p = 0.006) and *CYP2J2*7* allele (AOR 2.892; 95% CI = 1.238 - 6.647, p = 0.012). On the other hand, the odds of receiving reduced RDI was lower in patients with *CYP2B6 *6/*6* genotype (AOR 0.179; 95% CI = 0.049 – 0.656, p = 0.009). Analysis of variance showed statistically significant differences in average RDI between the different chemotherapy regimens, (p-value 0.005), baseline WBC toxicity grades (p-value 0.002) and baseline neutropnic toxicity grades (p-value 0.000). Patients receiving sequential AC-T had the highest average RDI value, 86.7%

4.3 Population pharmacokinetic and pharmacodynamic modeling (*paper 2*)

4.3.1 Population pharmacokinetic modeling

CPA concentration data were best described by one compartment pharmacokinetic model. Additive error model of residual variability performed better than proportional or exponential or combined error models. In the final model, the typical values for clearance and apparent volume of distribution of CPA were 5.41 L/hr and 46.5 L, respectively. The estimated inter-individual

variability (IIV) in clearance was 51% (38.9% and 49.3% for 500 and 600 mg/m^2 group, respectively). The mean clearance values of CPA were 3.97 and 5.81 L/h for patients who received 500 and 600 mg/m^2 based CPA regimen, respectively.

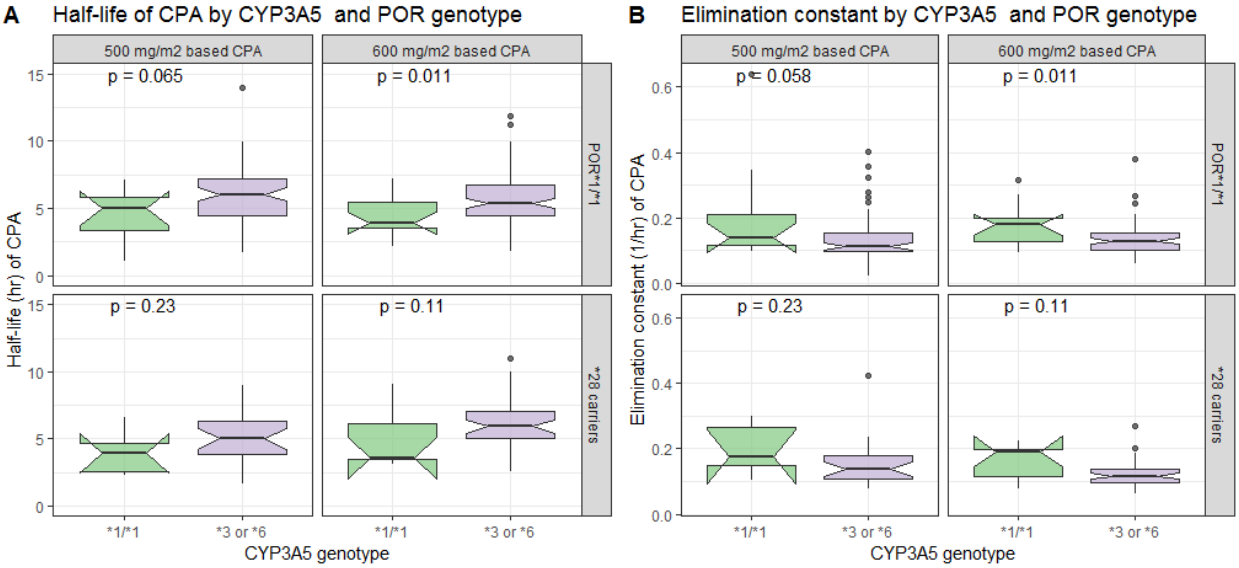


Figure 5: Half-life and elimination constant of cyclophosphamide by *CYP3A5* and *POR* genotype.

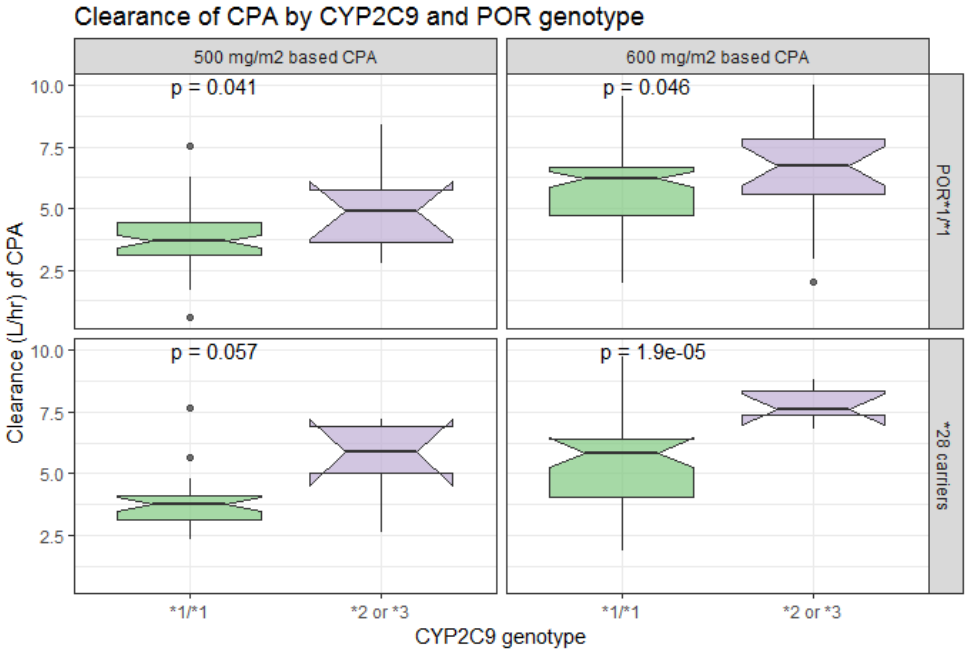


Figure 6: Clearance of cyclophosphamide by *CYP2C9* and *POR* genotype.

Covariate testing in NONMEM identified CPA dosage regimen (CPA 500 mg/m^2 or 600 mg/m^2 based CPA regimen), as significant predictor of clearance and V_D of CPA. BSA was also significantly associated with V_D of CPA. Accordingly, 500 mg/m^2 based CPA regimen was associated with a 32.3% lower than average clearance and 37.1% lower than average V_D of 600 mg/m^2. The mean clearance values of CPA were 3.97 and 5.81 L/h for patients who received 500 and for 600 mg/m^2 based CPA regimen, respectively. Similarly, a 0.1 m^2 unit increase in BSA was associated with a 5.6% increment in V_D of CPA. The mean V_D (33.5 L) in underweight group was also significantly lower compared to those of overweight (48.1 L) ($p < 0.001$) or obese patients (51.9 L) ($p < 0.001$). In patients who received 600 mg/m^2, but not in 500 mg/m^2, K_{el} and half-life of CPA was significantly different between BSA ($p < 0.001$, *ANOVA*). *Post hoc* analysis showed that patients with BSA > 1.75 m^2 had a longer half-life (6.71 hr) compared to those with BSA < 1.5 m^2 (4.78 hr) ($p < 0.001$) or BSA between 1.5-1.74 m^2 (5.42 hr) ($p = 0.004$).

In comparison to those with *CYP3A5*1/*1* genotype, patients carrying *CYP3A5*3* or **6* alleles had a lower elimination rate constant (0.124 *vs.* 0.16 hr^{-1}) ($p = 0.016$, *t*-test) and longer half life (5.58 *vs*. 4.34 hrs) in the 600 mg/m^2 group ($p = 0.022$, *t*-test). Furthermore, controlling for effect of *POR* genotype, the difference in mean K_{el} and $t_{1/2}$ between *CYP3A5* genotypes was evident among those with *POR*1/*1* ($p = 0.022$, *t*-test) but not in *POR*28* carriers. *CYP2C9 *2* or **3* carriers were also associated with increased clearance of CPA and reduced half life ($p < 0.05$). On the other hand, in both CPA regimen group, with *POR*1/*1* genotype, Clearance of CPA was moderately associated with *CYP2C9* genotype. In patients carryoing **28* for *POR* increased clearance was significantly associated in carriers of *CYP2C9 *2* or **3* alleles (Figure 5). On the other hand, in both CPA regimen group, with *POR*1/*1* genotype, Clearance of CPA was moderately associated with *CYP2C9* genotype. In patients carrying **28* for *POR* increased clearance was significantly associated in carriers of *CYP2C9 *2* or **3* alleles (Figure 6).

4.3.2 Population pharmacodynamic modeling

The linear direct response (empiric) model was successfully fitted to the neutrophil count with combined proportional and additive error model performed well compared to the additive or proportional error models. The parameter estimates for the slope, and ANC_o were -1.42 and 3450, respectively. Accordingly, a 1 unit increase in the AUC of CPA was associated with a decrease in neutrophil count by 1.42.

4.4 Factors influencing the concentrations of tamoxifen and its metabolites (*paper 3*)

This section of our study focused on the impact pharmacogenetic and other factors on the pharmacokinetics (PK) of tamoxifen and its metabolites. A total of 20 (25.9%) patients had *CYP2D6* copy number of 3 or more. The allele frequencies of *CYP2D6 *5* (gene deletion), and **1* or *2 gene duplication/multiplication were 4.3 and 14.8%, respectively. Based on the CPIC *CYP2D6* phenotype assignment, 61.7 and 22.2% of the patients are predicted to be NMs and UMs, respectively. The prevalence of PMs and IMs was low, accounting for 1.2 and 3.7%, respectively.

The median concentration of endoxifen (7.94 ng/mL), was 2.6 times higher than 4-HT (3.04 ng/mL). The proportion of patients with low endoxifen concentration (< 5.9 ng/mL) was 35.8%. Pronounced interindividual variability in endoxifen concentration (coefficient of variation 74.7%) and in metabolic ratio of endoxifen to NDM ($MR_{E/NDM}$) (coefficient of variation 59%) was observed. Pearson correlation analysis between tamoxifen and its metabolites revealed a strong positive association between tamoxifen and formation of NDM (Pearson correlation coefficient (R) = 0.89), as well as tamoxifen and formation of 4-HT (R = 0.66). Thus, an increase in the concentration of tamoxifen was associated with a corresponding increase in NDM and 4HT levels. Formation of 4-HT is also accompanied by a corresponding formation of endoxifen (R = 0.86). However, the association between NDM and formation of endoxifen (R = 0.38) as well as tamoxifen and formation of endoxifen (R = 0.48) was moderate.

Analysis of variance showed that the median endoxifen level, $MR_{E/NDM}$ and $MR_{4HT/Tam}$ were significantly different among *CYP2D6* diplotype ($p < 0.001$) or genotype predicted phenotype

groups ($p < 0.001$) (Figure 2). Patients with UM/EM, UM/IM and UM/PM diplotypes were associated with higher median endoxifen concentrations or metabolic ratios ($MR_{E/NDM}$ and $MR_{4\text{-}HT/Tam}$). Similarly, an increase in the activity score of *CYP2D6* phenotype was associated with a corresponding increase in absolute endoxifen concentrations and metabolic ratio ($MR_{E/NDM}$) ($p < 0.001$).

In patients categorized into *CYP2D6* NM/IM diplotype group, significant difference in $MR_{E/NDM}$ ($p = 0.036$) and $MR_{E/4\text{-}HT}$ ($p = 0.022$) was observed between genotypes **1/*10*, **1/*17* and **2/*17*. Among patients classified under NM/NM diplotypes, significant differences were also found between **1/*1, *1/*2,* and **2/*2* genotypes in tamoxifen ($p < 0.001$), NDM ($p < 0.001$), and 4-HT concentrations ($p < 0.001$) as well as corresponding metabolic ratios; $MR_{E/NDM}$ ($p < 0.001$), $MR_{E/4\text{-}HT}$ ($p = 0.025$) and $MR_{4\text{-}HT/Tam}$ ($p = 0.007$).

In addition to *CYP2D6* genotypes, a moderate association *POR* genotype with endoxifen level was observed ($p = 0.035$). On the other hand, *ABCB1* genotype showed a trend of association with $MR_{E/NDM}$ ($p = 0.054$) and moderate association with $MR_{E/4\text{-}HT}$ ($p = 0.039$). Carriers of the *G allele* for *ABCB1* had a lower $MR_{E/NDM}$ (0.022 *vs* 0.041; $p = 0.054$) and $MR_{E/4\text{-}HT}$ (2.52 *vs* 3.02, $p = 0.039$) compared to the wild type genotype (*ABCB1 c.4036AA*). There was no significant effect of *CYP2C9*, *CYP2C19* or *CYP3A5* genotype on plasma endoxifen concentration or $MR_{E/NDM}$ ratio. No difference was also detected in the median plasma tamoxifen concentrations and its metabolites ($p > 0.05$), between pre and postmenopausal patients, and different BMI groups as well as with age.

Patients with at least one variant allele for *UGT2B15* (c.1568A > C, rs4148269) showed lower median plasma concentrations of tamoxifen (125.9 *vs*. 173.8 ng/mL, $p = 0.045$). The median NDM/Tam (1.86 *vs*. 1.54, $p = 0.004$) and E/4-HT (2.96 *vs*. 2.06, $p = 0.029$) ratios were also higher in carriers of *C* allele for *UGT2B15* (c.1568A>C, rs4148269) compared to the wild type genotype. However, there was no significant difference in median plasma tamoxifen metabolite concentrations or metabolic ratios between *CYP2C9, CYP2C19 CYP3A5, UGT2B15*2 (c.253G>T)* or *ABCB1 c.3435C>T* genotypes. No difference was also detected in the median

plasma tamoxifen concentrations and its metabolites ($p > 0.05$), between pre- and postmenopausal patients, and different BMI groups as well as with age.

The impact of *CYP2D6* diplotype or phenotype on the interpatient variability of endoxifen or $MR_{E/NDM}$ was modeled using linear regression. The results indicated that *CYP2D6* diplotype explained 28.2% of the variability in endoxifen concentration (coefficient of determination (R^2) = 0.282, $p < 0.001$). Similarly, *CYP2D6* phenotype explained 26.3% of the variability in endoxifen concentration ($R^2 = 0.263$, $p < 0.001$). *POR* contributed 3.4% of the variability in absolute endoxifen concentration ($R^2 = 0.034$, $p = 0.054$). *CYP2D6* diplotype explained 44% of variability in $MR_{E/NDM}$ and combined with *ABCB1c.4036A/*G genotype, it explained 46.7% ($R^2 = 0.467$, $p < 0.001$) of the variability in $MR_{E/NDM}$. Phenotype and *ABCB1c.4036A>G* explained 40.9% of the variability in $MR_{E/NDM}$ ($R^2 = 0.409$, $p < 0.001$). *ABCB1c.4036A>G* contributed 4% the variability in $MR_{E/NDM}$ ($R^2 = 0.040$, $p = 0.042$).

The impact of *CYP2D6* on endoxifen variability and $MR_{E/NDM}$ (a marker for *CYP2D6* metabolic activity) was assessed stratifying patients by *ABCB1* genotype groups. Interestingly, *CYP2D6* diplotype explained 43% of the variability in endoxifen concentration among *ABCB1c.4036G* carriers ($R^2 = 0.43$, $p = 0.004$) (explanatory power increased from 28.2 to 43%), compared to *AA* genotype ($R^2 = 0.231$, $p < 0.001$). Similarly, *CYP2D6* diplotype explained 65.2% of the variability in $MR_{E/NDM}$ (improved from 44 to 65.2%) among *ABCB1c.4036G* carriers ($R^2 = 0.652$, $p < 0.001$), compared to *AA* genotype ($R^2 = 0.356$, $p < 0.001$).

4. Discussion

In the present study, we first prospectively investigated the incidence of chemotherapy induced hematologic toxicity, the prevalence of reduced RDI, and associated risk factors including pharmacogenetic variations in drug mobilizing enzymes' genes relevant for the bio-activation or disposition of chemotherapeutic agents (***Paper I***). The study further examined the pharmacokinetic profile of CPA and developed a population PK-PD model for CPA. to show the link between pharmacokinetic parameter and neutrophil count after first cycle CPA treatment (***Paper II***). Furthermore, we also investigated the factors that influence the active Tam metabolite concentrations with special emphasis on *CYP2D6* in patients on tamoxifen (***Paper III***).

Our main finding include i) Higher incidence of grade 3 or 4 hematologic toxicities (51%), which was largely manifested as neutropenic toxicity (50.2%). Consequently, significant proportion of the patients (56.6%) received reduced RDI; ii) We have demonstrated significant association of *CYP2J2*7* and *CYP2C9* genotype with chemotherapy induced hematologic toxicity, and *CYP2B6* genotype with reduced RDI; iii) Low baseline blood count was also strong predictor of both chemotherapy induced hematologic toxicity and reduced RDI; iv) Plasma CPA concentration-time data were described by one compartment pharmacokinetic model. The inter-patient variability in clearance was 51%. Body surface area (BSA) and CPA dosage regimen (500 mg/m^2 *vs* 600 mg/m^2 based CPA regimen), *CYP3A5 and CYP2C9* genotypes were found to associate with PK parameters of CPA; v) AUC - neutropenic toxicity relationship was described by empiric direct response model and was positively associated; vi) In Tamoxifen treated patients, *CYP2D6* activity score (AS) was associated with endoxifen concentration and $MR_{E/NDM}$. A moderate effect of *POR*28* on plasma endoxifen concentration and *ABCB1c.4036A>G* genotype on $MR_{E/NDM}$ was also observed

Chemotherapy predisposes cancer patients to infections both by suppressing the production of neutrophils and by cytotoxic effects on the cells that line the alimentary tract impairing host protective mechanisms. Particularly, neutropenia remains a central concern in the delivery of chemotherapy in cancer patients. It is associated with the risk of life-threatening infections as

well as chemotherapy dose reductions and delays that may compromise treatment outcomes. (Crawford, 2006). In the present study, we observed a high incidence of grade 3 or 4 hematologic toxicities (51%), which was largely manifested as neutropenic toxicity (50.2%). Similar incidence of neutropenea has been reported in patients treated with chemotherapy for various types of malignancies in Italy (45.1%) (Repetto, 2009), USA (43%) (Laskey et al., 2012), Japan (50.5%) (Hashiguchi et al., 2015), Egypt (28%) and Nepal (31.2%) (Sah et al., 2018).

Currently, research activities have also focused to the identification of pharmacogenetic markers predictive of drug activity and tolerability for personalized care and treatment. In the present study, *CYP2J2*7* was identified as new pharmacogenetic risk factors of chemotherapy induced hematologic toxicity in Ethiopian breast cancer patients. Consequently, patients carrying this variant allele could possibly activate cytotoxic agents such as CPA to 4-hydroxycyclophosphamide at a faster rate and hence increase the risk for toxicity. Although *Chi*-square test showed association of *CYP2C9* variant alleles to toxicity, these alleles did not reach to statistical significant level in the final multivariate regression analysis which could be attributed to the lower sample size.

Compiling all the potential factors of hematologic toxicity into a comprehensive risk model would make it possible for clinicians to better estimate the likelihood of neutropenic complications in individual patients and thus, use appropriate prophylactic measures. Studies have shown that prophylactic use of primary G-CSF has been found to be a statistically significant predictor of reduced neutropenic events and thus can reduce the risk of myelosuppresive complications during chemotherapy and help facilitate the delivery of adequate RDI particularly in high risk patients (M. L. Citron et al., 2003; Citron, 2004; Lyman et al., 2013; Weycker et al., 2014).

Hematologic toxicities are the main reasons for chemotherapy dose reductions or delays interfering in the optimal delivery of dose intensity. The major RDI reducing event in our study was treatment delay associated with hematologic toxicity. The present study result estimates that,

the prevalence of reduced RDI among Ethiopian patients could be as low as 50.3% and as high as 62.9%. Several studies have also shown that patients in various clinical settings are treated with a lower dose intensity of chemotherapy including in Canada (4.4%) (Raza et al., 2009), United States (30%) (Shayne et al., 2007, 2006) and Australia (12% in adjuvant and 36% metastatic setups) (Bae et al., 2014). However, a higher proportion of patients are receiving reduced RDI in our setup unacceptably, compared to the reports mentioned above.

Risk factors for reduced RDI have previously been reported including older age, lower pretreatment blood cell counts, BSA > 2 m^2, and/or nonuse of G-CSF (Lyman et al., 2003). The present study has revealed, underweight (BMI ≤ 18.4 kg/m^2), baseline grade 1 leukocyte toxicity and grade 1or 2 neutropenia as independent predictors of reduced RDI. Consequently, such patients are more likely to receive reduced RDI. On the other hand, *CYP2B6*6/*6* genotype was associated with a lower risk of receiving reduced RDI. In line with our finding, greater incidence of dose delay, indicative of reduced RDI, was observed during AC treatment in variant carriers of *CYP2B6*2* and *CYP2B6*5* (Bray et al., 2010). *CYP2B6* is the main enzyme that substantially metabolizes CPA into its active metabolite (4-hydroxy-cyclopsphmaide), which is known to induce hematologic toxicities (Nakajima *et al* 2007). Although *CYP2B6*6* genotype was not identified as a risk factor for hematologic toxicity, we found significant protective effect against reduced RDI. Previous study showed that *CYP2B6*6* allele is associated with reduced enzymatic activity and higher incidence of antiretroviral induced liver toxicity in Ethiopian HIV patients (Yimer et al., 2012). A recent research finding indicated that, being non-*CYP2B6*6* carrier was a significant predictor of grade 4 neutropenia in breast cancer patients treated with doxorubicin and CPA combination chemotherapy (Tsuji et al., 2016). By contrast, few other studies concluded that *CYP2B6* genotype was not significantly associated with myelotoxicity (Yao et al., 2010; Haroun et al., 2015). Such inconsistencies in the results could be attributed to the difference in the sample size and study population. Moreover, the relative expression levels of *CYP2J2* and *CYP2B6* may determine *in vivo* kinetics and toxicity of drugs metabolized by these enzymes.

As shown in ***paper II***, the plasma CPA concentration-time data were described by one compartment pharmacokinetic model, with typical values for population clearance and apparent volume of distribution 5.41 L/hr and 46.5 L, respectively. Our result indicates that one compartment model better described CPA concentration data, which is in agreement with previous reports (Timm et al., 2005; Joerger et al., 2007). In contrast, other studies described PK parameters using two compartment model (Nakajima et al., 2007; Ekhart et al., 2008). These findings may suggest that, the simple one or two compartment models may be sufficient to describe PK parameters in conventional doses of CPA administered by short IV infusion. The volume of distribution estimated in this study (46.5 L) is in concordance with previous report (de Jonge et al., 2005). Given that one compartment PK model described our data, together with V_D which approximates to the total body water, it is plausible that CPA achieves instantaneous distribution throughout the body. The population clearance of 5.41 L/hr estimated in the present study is also in agreement with those reported in breast cancer patients from France (Joerger et al., 2007) but significantly higher compared to the finding from Swedish and Japanase breast cancer patients (Hassan et al., 1999; Nakajima et al., 2007; Ekhart et al., 2008). Indeed, higher CYP enzyme activities including *CYP3A* in Ethiopian population compared to others have been reported previously (Gebeyehu et al., 2011; Aklillu et al., 2014), which may explain the higher CPA clearance rate in Ethiopians than Swedish or Japanese breast cancer patients.

On the other hand, earlier studies demonstrated that when CPA is administered in a high dose regimen (*e.g.*, 4000 mg/m^2) and by short infusion, a concentration-dependent PK model of CPA was developed with convex downward elimination curves (Chen et al., 1995, 1997). A time-dependent PK model was also described in patients receiving high dose CPA (6000 mg/m^2) in a prolonged infusion (96 hours) (Chen et al., 1995). Moreover, other studies developed a PK model for CPA incorporating the effect of auto-induction (Hassan et al., 1999) and the phenomena of both auto-induction and drug–drug interaction between CPA and thioTEPA (Huitema et al., 2001). These findings show that, in addition to other factors, PK of CPA could be influenced by the dosing history, and the duration of IV infusion.

Substantial variations in PK and exposure to CPA and its metabolites have been reported previously in both adults and children (de Jonge et al., 2005). These variations could be explained either by the differences in the level of expression of specific CYP enzymes or the level of activity of the individual enzymes (Zanger and Schwab, 2013) or other non-genetic factors (de Jonge et al., 2005). Results of previous studies regarding the roles of the individual CYP enzymes' genes to PK differences of CPA are inconsistent. In this study, the variant alleles in *CYP2B6*, *CYP2C19*, *CYP2J2*, and *ABCB1* were not associated with PK of CPA. In agreement with our finding, previous studies demonstrated that polymorphisms in *CYP2B6* and *CYP2C19* did not have significant role in variations of CPA PK (Ekhart et al., 2008; Fernandes et al., 2011; Afsar et al., 2012; Raccor et al., 2012). The lack of association of *ABCB1* genotype was also supported by previous study (Kim et al., 2013).

On the other hand, the findings of Xie et al demonstrated that patients with *CYP2B6 G516T* variants had a two-fold higher CPA clearance compared to those carrying the wild type (Xie et al., 2003). In Japanese breast cancer patients, those homozygous for *CYP2B6*6* (Q172H and K262R) had higher clearance than heterozygous or homozygous for *CYP2B6*1 (Nakajima et al., 2007). In another study, *CYP2C19*2* variant allele was associated with lower elimination rate constant in individuals receiving < 1000 mg/m^2 of CPA (Timm et al., 2005), which was not evident at higher doses. Notably, a significantly increased elimination rate of CPA was observed at CPA doses > 1000 mg/m^2, possibly due to CYP induction at higher doses (Timm et al., 2005). In our study, a dose dependent clearance of CPA was also observed. Patients receiving 600 mg/m^2 had significantly higher clearance as compared to 500 mg/m^2 CPA dosing (5.81 *vs*. 3.97 L/h, $p < 0.001$). CPA induces *CYP2C* and *CYP3A4* in human hepatocytes by increasing the enzyme production rate and thereby increasing the amount of enzyme by which CPA is metabolized (Hassan et al., 1999).

The present study also revealed that carriers of *CYP3A5*3* or **6* had lower mean elimination rate constant and prolonged half-life compared to those with *CYP3A5*1/*1* genotype ($p < 0.001$). In contrast, *CYP2C9 *2 or *3* carriers were associated with increased clearance of CPA. The observed effect of *CYP2C9* on CPA clearance was unexpected, as *CYP2C9 *2* or **3* alleles are

defective variant alleles associated with reduced *CYP2C9* enzyme activity (Griskevicius et al., 2003) resulting in reduced clearance of the drug. However, our finding is supported by Balasubramanian et al that reported that *CYP2C9 *2* allele increased CPA clearance and decreased its AUC (Balasubramanian et al., 2009). These findings may suggest that the extent and pattern of CYP activity and induction or inhibition may be variable among various patient populations. For example, *POR*28* was demonstrated to significantly alters *CYP2C9* activity in Swedish, but not in Korean healthy subjects (Hatta and Aklillu, 2015). Moreover, multiple competing CYP pathways involved in the biotransformation and clearance of CPA might also play a prominent role in *CYP2C9* defective patients, compensating for the absence of *CYP2C9* activity. Furthermore, previous study has shown higher CYP enzyme activities including *CYP3A* in Ethiopian population (Gebeyehu et al., 2011; Aklillu et al., 2014), which may explain the higher CPA clearance rate in the present study population.

A previous study demonstrated that *CYP2J2* expression was significantly up-regulated during CPA treatment and the bio-activation of the drug was significantly correlated to *CYP2J2* expression (I. El-Serafi et al., 2015). *CYP2J2*, expressed in high levels particularly in extra-hepatic organs such as the heart, intestine and urinary bladder, may be responsible for the local CPA bio-activation and may explain CPA treatment-related toxicities (Hashizume et al., 2002; Matsumoto et al., 2002; Lee et al., 2010). Recently, in a cohort study investigating the association of pharmacogenetic variations to hematologic toxicity in Ethiopian breast cancer patients, we reported that patients carrying *CYP2J2*7* variant alleles were associated with increased incidence of hematological toxicity (Ahmed et al., 2019). However, the present study could not identify association of *CYP2J2* genetic polymorphism and PK of first cycle CPA. The discrepancy could be explained by the increased *CYP2J2* expression as well as CYP induction in the subsequent chemotherapy cycles, which could facilitate formation of active metabolite and contributing for the increased incidence of hematologic toxicity.

Cytochrome P450 oxidoreductase (*POR*), a flavoprotein that supplies electrons to all CYP enzymes for their catalytic activity (Hart et al., 2008), was implicated to influence CYP catalyzed bio-activation of xenobiotics (Hu et al., 2012). In this study, *POR* was found to alter *CYP3A5* activity. Among patients with *POR*1/*1*, the mean K_{el} or $t_{1/2}$ between *CYP3A5*

genotypes was significantly different. However, the effect was not evident in *POR*28* carriers. Although the expression level of *POR* was not investigated in the present study, previous study demonstrated strong correlation between CPA clearance and *POR/CYP* ratio (Ibrahim El-Serafi et al., 2015), which might signify the importance of the level of expression of the gene in CPA metabolism and clearance.

The major toxic effect of CPA at conventional doses and the primary reason for change in the schedule of CPA based chemotherapy delivery to breast cancer patients is treatment associated neutropenia (Kozma et al., 2012). Consequently, one aspect of optimizing cancer chemotherapy involves describing neutropenic toxicity as a function of drug exposure. The present study demonstrated a positive relationship between AUC of CPA and neutropenic toxicity. A previous study also showed that breast cancer patients with complete response had significantly higher CPA AUC than patients with progressive disease (Joerger et al., 2007). The greater part of total systemic clearance of CPA is non renal clearance (de Jonge et al., 2005), and hence, these findings may suggest that exposure to CPA may be predictive of the amount of activated metabolite formed. High exposure to bio activated CPA was also reported to relate to the occurrence of veno-occlusive disease of the liver following high-dose CPA (de Jonge et al., 2006). In contrast, an inverse association was also reported between AUC of CPA and its treatment-related cardio-toxicity and event-free survival in women with breast cancer (Ayash et al., 1992; Petros et al., 2002). Patient with significantly lower AUC developed congestive heart failure in a high-dose CPA regimen (Ayash et al., 1992). Treatment effectiveness and occurrence of adverse effects like heart failure were also inversely related to CPA AUC in high-dose therapy (Petros et al., 2002). On the other hand, other studies could not establish association between CPA exposure and toxicity or relapse free survival in cancer patients treated with high-dose chemotherapy (Nieto et al., 1999; Veal et al., 2016). The discrepancies in the results of the various studies could be attributed to the differences in the sample size, patient population and richness of the PK and PD data in the individual studies.

The present study describes the population PK and PD of first cycle CPA in a resource limited setting. However, important limitations are also to be noted. First, the metabolite concentration

was not determined and consequently, it was not possible to incorporate it in the PK-PD model. In addition, as CPA is commonly used in combination with other chemotherapeutic agents in breast cancer treatment, their contribution in the observed neutropenic toxicity would be expected, however not addressed in this study. Moreover, concentration and response were collected at different time points. Owing to time-dependent signal transduction in hematopoiesis, simple empiric model adapted in this study may not account for this time lag between drug concentration and observed effects of CPA and the results also may not reflect the underlying physiological process. The PD observations (data points) in our study were also few (taken at baseline and day 20 post CPA administration) which might have hampered the successful development of the semi-mechanistic model.

In ***paper III,*** it was shown that the median concentration of endoxifen was 7.94 ng/mL (IQR 4.68 – 13.75 ng/mL), which was 2.6 times higher than 4-HT (3.04 ng/mL). The proportion of patients with low endoxifen concentration (below 5.9 ng/mL) was 35.8%. Large inter-individual variability in endoxifen concentration and $MR_{E/NDM}$ was observed (coefficient of variation 74.6% and 59%, respectively). Moreover, endoxifen concentration and $MR_{E/NDM}$ were found to increase with an increase in *CYP2D6* activity score, indicating a gene-dose effect of endoxifen formation. Several previous studies have also consistently demonstrated a significant gene-dose effect of *CYP2D6* polymorphism for tamoxifen metabolism (Jin et al., 2005; Saladores et al., 2015; Schroth et al., 2017; Puszkiel et al., 2019). Although the impact of *CYP2D6* on plasma endoxifen is significant, it was observed that *CYP2D6* diplotype categorizations were able to explain 29% of the IIV in plasma level of endoxifen, and thus larger part of the variability is explained by factors other than *CYP2D6*.

In the present study, *CYP2D6* diplotype and *ABCB1c.4036A/G* genotype explained 46.7% the variability in $MR_{E/NDM}$. With slightly different genotype interpretation applied for *CYP2D6* AS, previous study demonstrated that the contribution of *CYP2D6* to the IIV of endoxifen formation *via* NDM, as revealed from $MR_{E/NDM,}$ was 46% (Asians), 55% (Libansese) and 55% (Caucasians) (Saladores et al., 2015). Our result is comparable to Asians but lower than Lebanese or Caucasians. The explained variability of absolute endoxifen concentration by

CYP2D6 diplotype in our study (28.2%) was also lower than that reported in Asians and Caucasians (39-58%) (Schroth et al., 2017), suggesting that other factors are also contribute for the bioavailability of endoxifen in our study population.

Previous studies reported that variations in the concentration of endoxifen can be related to variations in tamoxifen concentrations itself (Balkenende et al., 2013; Fotoohi et al., 2016). A high between-patient variations in plasma tamoxifen (CV = 56.2%) and NDM concentrations (53.1%) was also observed in our study. The Pearson's correlation coefficients between endoxifen and tamoxifen (R = 0.48) as well as endoxifen and NDM (R = 0.38) also showed that, endoxifen level would not adequately be predicted by plasma levels of tamoxifen and NDM. This could mean that the extent of absorption of tamoxifen from the gut or the quality of the formulation or food-drug interaction could also be potential factors influencing the bioavailability of endoxifen.

Our study also showed significant differences in $MR_{E/NDM}$ between patients with genotype *CYP2D6 *1/*10*, **1/*17* and **2/*17*, which belong to EM/IM dipotype. Similarly, differences were also observed in endoxifen level between **1/*1*, **1/*2*, and **2/*2* genotypes which are grouped under same NM/NM diplotype groups (Figure 2). In agreement with our finding, recent study demonstrated that the concentrations of tamoxifen, endoxifen 4-HT, and NDM were significantly different between genotypes classed under same NMs (Puszkiel et al., 2019). The range of activity between *CYP2D6*1/*1*, **1/*2*, and **2/*2* genotypes could be explained by the differences in the level of expression of mRNA, associated with the presence or absence of enhancer SNP in the enhancer regions distant to the *CYP2D6* gene (Wang et al., 2014).

Previous studies showed that tamoxifen efficacy is dependent on attaining a certain threshold of endoxifen level. According to Madlensky et al. (2011), women in the upper quintiles of endoxifen concentration, but not of tamoxifen and other metabolite levels, had a lower recurrence rate compared to those in the lowest quintile. Moreover, endoxifen concentration >5.97 ng/mL was associated with a 30% lower risk of additional breast cancer events and proposed as the therapeutic threshold for endoxifen (Madlensky et al., 2011). Subsequent study

also demonstrated that, low endoxifen concentration (< 5.97 nM) was associated with distant relapse free survival (Saladores et al., 2015), indicating the significance of this therapeutic threshold. The results of our study showed that significant proportion of Ethiopian patients (35.8%) were unable to attain this proposed therapeutic threshold for endoxifen concentrations.

In our study, the low level of endoxifen was detected in patients classified to PM/PM, IM/PM, IM/IM, NM/PM, NM/IM, as well as NM/NM diplotypes. Poor/intermediate metabolizer genotype was identified as a predictor of low endoxifen concentration in earlier study (Madlensky et al., 2011). Moreover, it was also reported that 99% of PMs can be predicted by *CYP2D6* null alleles in homozygous variant or heterozygous genotype (Zanger and Schwab, 2013). Our result is in agreement with this notion, given that all the null or lower activity diplotypes (PM/PM, IM/PM, and IM/IM) were associated with low endoxifen level. In such patients, the clinical outcome of the cancer could be less favorable, though its association was not investigated in the present study. Thus, in line with the recent CPIC therapeutic recommendations, *CYP2D6* PMs in our study are recommended alternate hormonal therapy such as aromatase inhibitors (AIs) for post-menopausal women or AIs along with ovarian function suppression in premenopausal women. Escalation of tamoxifen dose from 20-40 mg/day can also be considered for PMs if there is contraindications to AIs (Goetz et al., 2018).

In contrast, other studies demonstrated that not all PMs have endoxifen concentrations below the proposed threshold concentration (5.9 ng/mL) (Madlensky et al., 2011; Teft et al., 2013). For example, it was observed that 24 % of the PMs were still able to generate therapeutic concentrations of endoxifen despite the lack of metabolic activity of *CYP2D6* (Teft et al., 2013). This could partly be attributed to environmental factors. A previous study demonstrated that black Africans were associated with lower rates of *CYP2D6*-dependent drug metabolism compared to Caucasians of the same apparent genotype (Aklillu et al., 2002). Moreover, among individuals of the same *CYP2D6* genotype, it was revealed that Ethiopians in living in Sweden had higher *CYP2D6* metabolic activity than Ethiopians in Ethiopia (Aklillu et al., 2002), implying the profound impact of environmental factors in *CYP2D6* catalyzed drug metabolism.

In the present study, patients with NM/IM (41.7%) and NM/NM diplotypes (31.4%), all belonging to NM phenotypes had lower endoxifen concentrations (< 5.9 ng/mL). Recently, it was reported that the activity of *CYP2D6* is impacted not only by genetic variations within the *CYP2D6* gene, but also by regulatory mechanisms including genetic polymorphisms in the enhancer regions or differential expression of transcription factors, which can modulate the rate of translation of mRNA into protein (Gaedigk et al., 2018). Genotype panels typically interrogate a limited number of SNPs identifying more commonly observed *CYP2D6* alleles and provide limited information on gene copy number and structural variants (Gaedigk et al., 2018). It is therefore possible that a patient carries allele(s) that was not tested for and that may contribute to the variability seen in these groups. In addition, the impact environmental factors on *CYP2D6* catalyzed drug metabolism cannot also be underestimated (Aklillu et al., 2002). Moreover, other individual-specific and biologic factors may also modulate *CYP2D6* expression levels or enzyme activity and contribute to the variability *CYP2D6* enzymatic activity. Emerging evidence showed the contribution of the microbiome on overall health and its role in drug metabolism and response (Koppel et al., 2017). A recent study also provided evidence that nutritional status, specifically fasting, alters P450-mediated drug metabolism in animal studies (de Vries et al., 2016). However, the extent a person's microbiome, nutritional and fasting status influences CYP activity and *CYP2D6* metabolizer status, intra- and interindividual variability, in particular, remains to be investigated in future studies. Notably, the low level of endoxifen detected in large proportion of patients with predicted NM phenotypes in our study may suggest the need for therapeutic drug monitoring (TDM) of endoxifen in addition to genotyping for the common functional *CYP2D6* variant alleles.

In addition to *CYP2D6*, genetic variants in genes such as *CYP2C9*, *CYP2C19*, *CYP3A5*, *POR*, and *ABCB1*, could also impact the plasma levels of tamoxifen metabolites. Cytochrome P450 oxidoreductase (*POR*) is the only electron donor for all microsomal cytochrome P450 monooxygenases (CYP) responsible for oxidation of more than 80% of drugs (Hart and Zhong, 2008). In our study, we observed that *POR*28* carriers were associated with low plasma endoxifen level, although the effect is moderate (p = 0.035). Previous reports indicated that genetic variants of *POR* (*e.g.*, *POR*28* (A503V)) were implicated in the impaired activity of

CYP2D6 and *CYP3A4* (Hart et al., 2008; Sandee et al., 2010), possibly contributing to genetic variability in drug metabolism.

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Apart from tamoxifen activating enzymes, genetic variants in drug transporters have been reported to influence pharmacokinetics and play a role to a variable degree in the clinical outcome of tamoxifen treatment and to confer treatment resistance for many anticancer drugs (Tulsyan et al., 2016). Results of our study showed that carriers of G allele for *ABCB1c.4036A>G* had a marginally lower $MR_{E/NDM}$ (0.025 *vs* 0.036; $p = 0.054$) compared to the wild type allele (*ABCB1*, *allele A*) indicating that polymorphism of *ABCB1* could influence exposure to endoxifen. Interestingly, among *ABCB1 G* carriers, improved predictive effect of the variability in endoxifen concentration (43%) and endoxifen formation (65.2%) from NDM was observed by *CYP2D6* diplotype. A previous study demonstrated that *ABCB1* influences the systemic exposure of tamoxifen and its active metabolites, 4-HT and endoxifen (Iusuf et al., 2011). Moreover, several other studies also substantiated the impact of *ABCB1* in relation to clinical outcome of tamoxifen. *ABCB1* polymorphism was associated with risk of recurrence or disease free survival (DFS), in breast cancer patients on tamoxifen treatment (Iusuf et al., 2011; Teh et al., 2012; Sensorn et al., 2016).

Variant alleles of *CYP2C9*, *CYP2C19* and *CYP3A5* could alter tamoxifen metabolism and exposure to its metabolites especially formation of 4-hydroxy-tamoxifen (Saladores et al., 2013). However, no evidence association was observed between *CYP2C9*, *CYP2C19* and *CYP3A5* genotypes and plasma concentrations of tamoxifen or its metabolites. Our finding is be

substantiated with previous studies (Jin et al., 2005; Lim et al., 2011; Teft et al., 2013). On the other hand, other studies have reported lower plasma concentrations of 4-HT and endoxifen among carriers of *CYP2C9*2* and/or **3* alleles (Mürdter et al., 2011; Saladores et al., 2015). The disparity in the results could be attributed to the difference in sample size or the variation in allele distributions.

Our study has some limitations. We present a significant impact of *CYP2D6* and a marginal effect of *POR* and *ABCB1* on the pharmacokinetics of tamoxifen and its metabolites. However, the sample size included in our study was not powered enough and might be a reason for lack of significant association of *POR* and *ABCB1* genotypes with tamoxifen and its metabolites PK. In addition, association of plasma concentration of the active tamoxifen metabolites to clinical outcome was not assessed. The treatment of cancer in patients with comorbidities can be challenging as these individuals are underrepresented in clinical studies (Lee et al., 2011). In addition to the *CYP2D6* genetics that much of the controversy around tamoxifen therapy has focused, drug-drug interactions (DDI) involving *CYP2D6* in polypharmacy for co-morbidities markedly increases the complexity of tamoxifen metabolism and efficacy. The concurrent use of *CYP2D6* inhibitors and tamoxifen would be expected to substantially reduce endoxifen concentrations, and there is clinical evidence to support this expectation (Borges et al., 2006; Jin et al., 2005; Stearns et al., 2003). For example, NMs who were also taking *CYP2D6* inhibitors had endoxifen plasma concentrations that were 58% lower than NMs not on *CYP2D6* inhibitors. Similarly, IMs on *CYP2D6* inhibitors had endoxifen concentrations that were 38% lower than IMs not on *CYP2D6* inhibitors (Jin et al., 2005). As tamoxifen has extraordinarily complex pharmacokinetics, with more than a dozen drug-metabolizing enzymes and transporters involved in its disposition, enzyme inducers may increase the activity of several of these pathways, including phase II enzymes, ABC transporters, and various CYP enzymes other than *CYP2D6* (Powers et al., 2016). There is a growing evidence that enzyme inducers can substantially alter the disposition of endoxifen, reducing tamoxifen efficacy (Hansten, 2018).

6. Conclusion and recommendations

The incidence of chemotherapy-induced hematological toxicities was high causing larger proportion of patients to receive reduced RDI in Ethiopian breast cancer patients. Patients carrying *CYP2J2 *7* allele and those with low baseline WBC or ANC are at a higher risk chemotherapy induced hematologic toxicities and to reduced RDI, and thus such patients need prior support such as prophylactic use of primary G-CSF before initiation of chemotherapy, close monitoring and follow up during chemotherapy. The potential impact of the observed reduced RDI on the survival outcome in this study population needs further investigation.

The PK profile CPA is better described by one compartment model in Ethiopian breast cancer patients. CPA also displays wide between patient variability in clearance, which is partly explained by CPA dosage regimen, BSA, BMI, *CYP3A5* and *CYP2C9* genotypes. The CPA plasma exposure moderately predicts neutropenic toxicity. Further studies, are recommended to explore the PK of the metabolite (4-Hydroxycyclophosphamide), incorporating auto-induction by CPA and drug interaction and correlate its impact in neutropenic toxicity. Future investigations aiming at the impact of the level expression of *CYP* enzymes relevant to CPA PK, including, *POR*, *CYP2J2*, *CYP2B6*, *CYP2C9*, and *CYP3A*, could also provide more information to describe the variability of and clinical impact of CPA based treatment. Moreover, PK-PD modeling involving the contribution of other anticancer drugs in CPA containing regimen needs to be explored.

A high rate of low plasma endoxifen level and wide between-patient variability in plasma concentration endoxifen, the main therapeutically active metabolites of tamoxifen, was also observed. *CYP2D6* genotype is a significant predictor of plasma endoxifen exposure. Tamoxifen therapy guided by *CYP2D6* genotyping could help patients who plan tamoxifen-based therapy. Therapeutic drug monitoring is recommended for those with low endoxofen level in normal metabolizer phenotypes. The marginal effect of *POR* and *ABCB1* on tamoxifen and its metabolites PK found in this study needs further investigation in a larger sample size. Despite the simplified AS system recommended by CPIC for genotype-phenotype translation, our study signals further refinement of the approach, probably considering other sources of variability such

as *POR* and *ABCB1*. Future studies are also recommended to investigate the impact of the *CYP2D6* genetic variations on the clinical outcome of breast cancer in Ethiopian patients.

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Annex: Publications

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 14 May 2019
doi: 10.3389/fphar.2019.00481

CYP2J2*7 Genotype Predicts Risk of Chemotherapy-Induced Hematologic Toxicity and Reduced Relative Dose Intensity in Ethiopian Breast Cancer Patients

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OPEN ACCESS

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Specialty section:
This article was submitted to Pharmacogenetics and Pharmacogenomics, a section of the journal Frontiers in Pharmacology

Received: *10 July 2018*
Accepted: *16 April 2019*
Published: *14 May 2019*

Citation:
*Ahmed JH, Makonnen E, Yimer G, Seifu D, Bekele A, Assefa M, Aseffa A, Howe R, Fotoohi A, Hassan M and Aklillu E (2019) CYP2J2*7 Genotype Predicts Risk of Chemotherapy-Induced Hematologic Toxicity and Reduced Relative Dose Intensity in Ethiopian Breast Cancer Patients. Front. Pharmacol. 10:481. doi: 10.3389/fphar.2019.00481*

Chemotherapy-induced hematologic toxicity is the primary reasons of dose reductions and/or delays, low relative dose intensity (RDI), and predicts anticancer response. We investigated the incidence and predictors of chemotherapy-induced hematologic toxicities and reduced RDI in Ethiopian breast cancer patients, and implication of pharmacogenetics variations. Breast cancer patients (n = 249) were enrolled prospectively to receive cyclophosphamide based chemotherapy. Hematological toxicity (neutropenia, anemia, and thrombocytopenia) were monitored throughout chemotherapy cycle. The primary and secondary outcomes were incidence of grade 3 or 4 toxicity and reduced RDI, respectively. *CYP2B6*6*, *CYP3A5*3*, *CYP2C9 (*2,*3)*, *CYP2C19 (*2,*3), CYP2J2*7, POR*28,* and *ABCB1 (rs3842)* genotyping were done. Cox proportional hazard and logistic regression were used to estimate risk predictors of toxicity and reduced RDI, respectively. Majority (73.5%) of the patients were < 45 years of age. The incidence of grade 3 or 4 hematological toxicity was 51.0% (95% CI = 44.54–57.46%). Multivariate Cox proportional hazard regression indicated *CYP2J2*7* genotype [Hazard ratio (HR) = 1.82; 95% CI = 1.14–2.90], pretreatment grade 1 leukopenia (HR = 2.75; 95% CI = 1.47–5.15) or grade 1 or 2 neutropenia (HR = 2.75; 95% CI = 1.73–4.35) as significant predictors of hematologic toxicities. The odds of having hematologic toxicities was lower in *CYP2C9*2 or *3* carriers (p = 0.024). The prevalence of reduced RDI was 56.6% (95% CI = 50.3–62.9%). Higher risk of reduced RDI was associated with *CYP2J2*7* allele [Adjusted odds ratio (AOR) = 2.79; 95% CI = 1.21–6.46], BMI ≤ 18.4 kg/m^2 (AOR = 5.98; 95% CI = 1.36–26.23), baseline grade 1 leukopenia (AOR = 6.09; 95% CI = 1.24–29.98), and baseline neutropenia

(AOR = 3.37; 95% CI = 1.41–8.05). The odds of receiving reduced RDI was lower in patients with *CYP2B6 *6/*6* genotype (AOR = 0.19; 95% CI = 0.06–0.77). We report high incidence of chemotherapy-induced hematological toxicities causing larger proportion of patients to receive reduced RDI in Ethiopian breast cancer patients. Patients carrying *CYP2J2*7* allele and low baseline blood counts are at a higher risk for chemotherapy-induced hematologic toxicities and receiving reduced RDI, and may require prior support and close follow up during chemotherapy.

Keywords: *CYP2J2*, *CYP2C9*, chemotherapy, hematologic toxicity, reduced relative dose intensity, breast cancer, Ethiopia

INTRODUCTION

Breast cancer has become the most commonly diagnosed cancer in women of Sub-Saharan African countries (Jemal et al., 2012). Though national figure regarding the prevalence of cancer in Ethiopia are non-existent, according to the Addis Ababa cancer registry (AACR), breast cancer accounts for 33% of cancer cases followed by cervical cancer (17%) (AACR, 2014). Chemotherapy plays a crucial role in increasing the cure rate of early breast cancer, provided that the appropriate dose is administered at recommended treatment schedule (Havrilesky et al., 2015). However, cancer chemotherapeutic regimens are known to suppress the hematopoietic system (Kozma et al., 2012), and hematologic toxicities remain the most common reasons for delaying treatment schedule and reducing relative dose intensity (RDI). Chemotherapy induced hematological toxicities are one of the key factors that affect delivery of chemotherapy. The prevalence of chemotherapy induced hematologic toxicities and complications vary between patients depending on type of treatment regimen received (Lyman et al., 2005; Crawford, 2006) and use of supportive care therapies such as recombinant granulocyte colony stimulating factor (G-CSF) which minimize the risk and maintain planned dose of chemotherapy on time (Crawford, 2006).

Various studies show that achieving maximal benefit of anticancer chemotherapy requires the maintenance of dose intensity (Bonadonna et al., 2005; Terada et al., 2009). Dose intensity (DI) is defined as the total amount of drug delivered to a patient per unit time; while RDI is the ratio of the actual dose intensity delivered to the standard dose intensity for a chemotherapy regimen (Citron, 2004; Vavra et al., 2013). Patients who received RDI of 85% or more of the standard dose have a longer relapse free and overall survival, while treatment with below this threshold of RDI is associated with poor survival outcomes (Bonadonna et al., 1995). There is a strong association between RDI and disease-free and overall survival, in both early stage and metastatic cancers especially for lymphoma (Terada et al., 2009) and breast cancer (Sandy and Della-Fiorentina, 2013; Vavra et al., 2013). Thus, RDI can be considered to be a clinical quality indicator and partly a surrogate marker for survival (Wildiers and Reiser, 2011).

Anthracycline-based or taxane-plus-anthracycline-based chemotherapy containing cyclophosphamide regimens are commonly used to treat early stage, locally advanced or metastatic breast cancer in resource limited settings (Anderson et al., 2006). Metabolic pathways of these drugs involve genetically polymorphic drug metabolizing enzymes and transporter proteins. Cyclophosphamide is a prodrug requiring bioactivation by genetically polymorphic cytochrome P450 enzymes that affect its disposition, including *CYP2B6* (Xie et al., 2003), *CYP2C9, CYP2C19* and *CYP3A4*/5 (Roy et al., 1999), and *CYP2J2* (El-Serafi et al., 2015). Several anticancer drugs including cyclophosphamide and doxorubicin are substrates of p-glycoprotein encoded by the polymorphic *ABCB1* gene (Leith et al., 1999). *ABCB1* variant alleles are associated with reduced clearance and significantly increased exposure level of doxorubicin (Lal et al., 2008). Some of the variant alleles are specific or occur at a higher frequencies in black Africans population than whites and Asians (Gebeyehu et al., 2011; Aklillu et al., 2016), and their impact on toxicities associated with treatment of common infectious diseases in sub-Saharan Africa is well characterized (Mugusi et al., 2012, 2018; Mukonzo et al., 2013; Yimer et al., 2014). However their impact on breast cancer associated toxicity remain to be investigated.

Characteristics of breast cancer and its treatment outcomes vary widely across patients and ethnically diverse populations (O'Donnell and Dollan, 2009), partly due to genetic variation (Westbrook, 2013). Breast cancer in black African women is commonly characterized by younger age of onset, as clinically aggressive, with high prevalence of triple negative tumor and higher mortality rates than age-matched Caucasian women (Morris and Mitchell, 2008; Lund et al., 2009). Treatment outcomes and pharmacogenetics of breast cancer chemotherapy are not well investigated in population of Sub-Saharan Africa. In Ethiopia, one retrospective study reported that the 5 years cumulative probabilities of distant metastasis-free survival (MFS) for breast cancer as being 72% (for stages 1 and 2) and 33% (for stage 3) (Kantelhardt et al., 2014). Yet, data on chemotherapy induced hematological toxicities, reduced chemotherapy dose intensities and associated risks from black

Abbreviations: ALP, Alkaline phosphatase; ALT, alanine aminotransferase; ANC, Absolute neutrophil count; AOR, adjusted odds ratio; AST; aspartate aminotransferase; BMI, body mass index; BSA, body-surface area; BUN; blood urea nitrogen; CI, confidence intervals; COR, Crude odds ratio; DI, dose intensity; DFS, disease free survival; G-CSF, granulocyte colony stimulating factor; HWE, Hardy-Weinberg equilibrium; HIV, human immunodeficiency virus; HR, hazard ratio; IRB, Institutional Review Board; MFS, Metastasis free survival; OS, overall survival; QOL, quality of life; RDI, relative dose intensity; SCr; serum creatinine; WBC, white blood cells.

Africans breast cancer patients including Ethiopians is lacking. Assessment of the incidence and identification of risk factors including pharmacogenetic markers for severe toxicities and reduced dose intensity is essential for personalized care and treatment. Thus, the objective of this study was to assess the incidence and prognostic markers including pharmacogenetics of chemotherapy induced hematologic toxicities and reduced RDI in female breast cancer patients from Ethiopia.

MATERIALS AND METHODS

Study Population and Design

This prospective cohort study was conducted at the radiotherapy center of Tikur Anbessa specialized hospital, Addis Ababa University, Addis Ababa, Ethiopia which is the only public oncologic care institution in the country currently. Newly diagnosed breast cancer patients who came to the center for breast cancer care during mid-June 2014 to mid-June 2015 were enrolled and monitored for hematologic toxicity and RDI until completion of their chemotherapy cycles. Study participants were followed up for 3–6 months depending on the chemotherapy regimen received and the number of cycles recommended by their respective clinician. All consecutive and volunteer adult female patients, pathologically and clinically diagnosed with breast cancer (stages I–IV) who received neo-adjuvant, adjuvant or palliative chemotherapy regimen in the outpatient day care ward and those with no prior history of anemia were included. These patients were those who took the first cycle of chemotherapy in this radiotherapy center and had been on follow-up of their chemotherapy. Patients who were less than 18 years of age, pregnant, with multiple primary tumor types, patients on concurrent radiotherapy, or patients who started chemotherapy at private clinic were excluded.

The main aim of this study was to determine the incidence and predictors of chemotherapy induced hematologic toxicities and reduced RDI. The sample size required to detect a risk ratio of 2.0 for grade 3 or 4 hematologic toxicity with 95% confidence and 80% power, and assuming 8% loss to follow up is calculated to be 269 (Dean et al., 2013). Ethical clearance was obtained from Institutional Review Board (IRB) of the college of health sciences, Addis Ababa University, Armauer Hansen Research Institute Ethical Review Committee (AAERC), and National Research Ethics Review Committee (NRERC) of the federal democratic republic of Ethiopia. Signed informed consent was obtained from individual patient prior to participation in the study.

Treatment, Follow Up, and Laboratory Analysis

The common chemotherapy regimens in the outpatient care at Tikur Anbessa Hospital comprised of six cycles of FAC (5-Flourouracil 500 mg/m^2, Adriamycin [Doxorubicin] 50 mg/m^2, and Cyclophosphamide 500 mg/m^2), four cycles of AC (Adriamycin 50 mg/m^2 and Cyclophosphamide 600 mg/m^2), six cycles CMF (Cyclophosphamide 500 mg/m^2, Methotrexate 40 mg/m^2 and Fluorouracil 500 mg/m^2, and sequential AC – T (four cycles of Adriamycin 60 mg/m^2 and Cyclophosphamide 600 mg/m^2 followed by another 4 cycles of Taxol 175 mg/m^2).

Cycles were 3 weeks long (planned every 21 days) in the study setup. A dose reduction was described as ratio of the actual dose given compared with the standard or planned dose. Dose delay was described as the actual cycle length compared with standard or planned cycle length. A dose reduction was defined as a $\geq$15% reduction relative to standard and dose delay was defined as $\geq$15% delay in days relative to the standard cycle length for chemotherapy (Wildiers and Reiser, 2011).

At baseline, patients' medical records, biopsy reports, radiologic imaging results [X-RAY, ultrasound, CT scan (where available)] and laboratory investigations (blood counts and organ function estimates [Alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), serum creatinine (SCr), and blood urea nitrogen (BUN)] levels were recorded. Pretreatment demographic, clinical and tumor characteristics collected include age at diagnosis, menopausal status, performance status, weight, height, body mass index (BMI, classified based on World Health Organization criteria), body-surface area (BSA), site of tumor, histologic type of the tumor, degree of differentiation, tumor size, lymph node involvement, tumor stage at diagnosis, tumor receptor status, co-morbidity (assessed and classified based on the Charlson Co-morbidity Index) (Breccia et al., 2011), chemotherapy panel, i.e., treatment intent (neo-adjuvant, adjuvant, or metastatic), chemotherapy regimen (planned dose and schedule), and primary or secondary G-CSF use. In the subsequent cycles, follow-up clinical information including actual chemotherapy dose received and date of administration, blood laboratory investigations, dose intensity reducing events (i.e., dose reduction and/or delays) occurred across the cycles were also recorded.

CYP2B6, *CYP2C9*, *CYP2C19*, *CYP3A5*, *CYP2J2*, *ABCB1,* and *POR* Genotyping

Genomic DNA was isolated from peripheral leukocytes in whole blood samples using QIAamp DNA Midi Kit (Qiagen GmbH, Hilden, Germany). Genotypes of common functional variant alleles of drug metabolizing enzymes' genes relevant for cyclophosphamide disposition or bioactivation including, *CYP2B*6*, *CYP2C9*2*, *CYP2C9*3*, *CYP2C19*2*, *CYP2C19*3*, *CYP3A5*3*, *CYP2J2*7*, and *POR*28* were carried out using Taqman® allele specific PCR (Applied Biosystems Genotyping Assays) as described previously (Hatta and Aklillu, 2015). In brief genotyping was performed using TaqMan® drug metabolism genotyping assay reagents for allelic discrimination (Applied Biosystems Genotyping Assays) with the following ID numbers for each SNP: C__7817765_60 for *CYP2B6*6* (c.516G4T, rs3745274), C__26201809_30 for *CYP3A5*3* (c.6986A4G, rs776746), C__25625805_10 for *CYP2C9*2* (rs1799853), C__27104892_10 for *CYP2C9*3* (rs1057910), C__25986767_70 for *CYP2C19*2* (rs4244285), C__27861809_10 for *CYP2C19*3* (rs4986893), C_9581699_80 for *CYP2J2*7* (rs890293), C_8890131_30 for *POR*28* (rs1057868), and C__11711730_20 for *ABCB1* (rs3842). Genotyping was carried out using QuantStudio 12K Flex Real-Time PCR system (Life

Technologies Holding, Singapore, Singapore). The final volume for each reaction was 10 μL, consisting of TaqMan® fast advanced master mix (Applied Biosystems, Waltham, MA, United States), TaqMan 20X drug metabolism genotyping assays mix (Applied Biosystems) and genomic DNA. The PCR profile consisted of an initial step at 60°C for 30 s, hold stage at 95°C for 10 min and PCR stage for 40 cycles step 1 with 95°C for 15 min and step 2 with 60°C for 1 min and after read stage with 60°C for 30 s.

Study Outcomes

The primary outcome measure was incidence of grade 3 or 4 hematologic toxicity during the course of chemotherapy. Toxicity events were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE), version 4.0 (CTCAE, 2010). Accordingly, grades 3 and 4 anemia were defined, respectively, as hemoglobin value of 6.5–8.0 g/dL and <6.5 g/dL. Platelet counts between 25,000 and 50,000/ mm^3 and <25,000/mm^3 were classified as grade 3 and 4 thrombocytopenia, respectively. Neutropenia was defined as grade 3 (neutrophil count 500 – < 1,000/ mm^3) and grade 4 (neutrophil count < 500/mm^3). Grade 2 toxicities were also considered to be grade 2 anemia (hemoglobin level 8.0–10.0 g/dL), grade 2 thrombocytopenia (platelets count between 50,000 and 5,000/mm^3), grade 2 neutropenia (neutrophil count 1000 – < 1,500/mm^3).

The secondary outcome was the average received RDI, defined as the proportion of the reference standard dose-intensity for each regimen actually received. Then, the proportion of patients receiving less than 85% of the reference dose was determined. Both planned and unplanned reductions in RDI (<85%) were calculated for each chemotherapy panels (neo-adjuvant, adjuvant and metastatic). The dose intensity (DI) of each agent was calculated by dividing the total received dose of the agent to the total number of weeks of treatment. The RDI of each agent was expressed as the total delivered dose of the agent per unit time (i.e., weeks) as a percentage of the standard/planned dose. The RDI for each regimen represents the average RDI for each chemotherapeutic agent in a given regimen (Hryniuk et al., 1998). The standard reference dose intensity for each drug was considered to be the established dose in clinical trials in mg/m^2 per unit time (in weeks) (Mamounas et al., 2005).

Statistical Analysis

Descriptive statistics was used to explore the demographic characteristics and clinical profiles including, toxicities, RDI, dose delays, and dose reductions of participants. Categorical variables were reported as percentages. Confidence intervals (95%) were computed for incidence proportion of overall grade 3 or 4 hematologic toxicities. Continuous variables were diagnosed for normality of the distribution and presented as mean ± standard deviations (SD) or median with inter quartile range (IQR). Paired sample *t*-test was used to compare the mean values of absolute neutrophil counts across the cycles and to determine if there were significant changes in neutropenic toxicities at each cycle compared to baseline.

Chi Square (χ^2) test was used to evaluate the allele and genotype frequencies if the patient population is in Hardy-Weinberg equilibrium (HWE). Cox proportional hazard regression model was used to estimate hazard risks factors for grade 3 or 4 hematologic toxicities. Results were expressed as hazard ratios (HRs) and 95% confidence intervals. Logistic regression analysis was performed to identify predictors of reduced RDI and the results were expressed as Odds ratios (OR) and 95% confidence intervals (CI). RDI categorization was based on the threshold of RDI < 85% and RDI ≥ 85%. In all multivariate regression models, variables with $p \leq 0.20$ in univariate analysis were used. Hosmer–Lemeshow goodness of fit test was used to assess the model fit (Hosmer-Lemeshow statistic ≥ 0.05). Backward elimination (likelihood ratio) was used as the variable selection method. The data were analyzed using SPSS for windows, version 21.0. A $p < 0.05$ was considered statistically significant for each test and then *Bonferroni* correction (as the number of hypotheses is fairly small) was applied for multiple comparisons.

RESULTS

Socio-Demographic Characteristics

A total of 285 patients were enrolled and followed up in the study. Of these, 36 patients were excluded: six patients had taken previous chemotherapy for breast cancer or non-Hodgkins lymphoma (NHL), 5 patients received concurrent radiotherapy, 6 patients were pregnant, 4 patients started chemotherapy at a private clinic and were referred to the radiotherapy center of the hospital (complete information was unavailable), 11 patients were lost to follow up for the second and subsequent cycles, 4 patients had poor performance (Karnosfsky's performance scale < 60). Thus, data from a total of 249 breast cancer patients were followed up and included in the analysis. The socio-demographic profiles of the patients are given in **Table 1** below. Larger proportions (73.5%) of patients were 45 years of age or younger (median age 39 years). Six (2.4%) and 31.3% patients had BSA range ≥ 2 and BMI range ≥ 25 kg/m^2, respectively. Twenty four (9.6%) and 11 (4.4%) patients were co-morbid with cardiovascular diseases and HIV/AIDS, respectively. Forty nine (19.7%) and six (2.4%) patients initiated their chemotherapy at baseline absolute neutrophil count of 1,500–2,500/m^2 (grade 1 neutropenia) and 1,000–1,500 m^2 (grade 2 neutropenia), respectively.

In this cohort of patients, invasive ductal carcinoma was the most common (85.9%) type of cancer and larger proportion (73.1%) of patients had node positive tumor (**Table 2**). Fifty five (22%) patients had at least two secondary involved organs that included to the lung (37 patients) or liver (28 patients). Hormone receptor status was obtained from 24 patients and triple negative breast cancer accounted for 25%. Adjuvant chemotherapy was given to 71.5% of the patients. Majority (65.9%) of the patients in all chemotherapy panels (neo-adjuvant, adjuvant or metastatic) received 5-Flourouracil, Doxorubicin, and Cyclophosphamide (FAC). The mean doses of these drugs administered per cycle were 797.5 mg ± 77.37 (5-Flourouracil) and 822.08 mg ± 124.3 (Cyclophosphamide), while the median Doxorubicin dose administered was 82.5 mg

TABLE 1 | Socio-demographic characteristics of breast cancer patients at the radiotherapy center, Tikur Anbessa specialized hospital, Addis Ababa University, Ethiopia, 2015.

Parameters	Statistic
Age (years) (median + IQR)*	39 + 14
BSA (m^2) (mean ± SD)	1.6 ± 0.17
BMI (Kg/m^2) (mean ± SD)	23.9 ± 4.65
Presence of co-morbidities, n (%)	40 (16.1%)
Cardiovascular co-morbidities, n (%)	24 (9.6%)
Presence of HIV/AIDS, n (%)	11 (4.4%)
Presence of diabetes, n (%)	3 (1.2%)
Baseline laboratory results	
WBC ($10^3/mm^3$) (median + IQR)	6.60 + 2.6
ANC ($10^3/mm^3$) (median + IQR)	3.48 + 1.98
Hgb (gm/dL) (median + IQR)	13.8 + 1.67
HCT (%) (mean ± SD)	41.3 ± 3.62
PLT ($10^3/mm^3$) (median + IQR)	291 + 108
ALT (U/L) (median + IQR)	20 + 14
AST (U/L) (median + IQR)	25 + 12.25
ALP (U/L) (median + IQR)	241 + 163
SCr (mg/dL) (mean ± SD)	0.87 ± 0.18
BUN (mg/dL) (median + IQR)	18 + 12

**ALP, Alkaline phosphatase; ALT, alanine aminotransferase; ANC, absolute neutrophil count; AST, aspartate aminotransferase; BUN, Blood urea nitrogen; BSA, body surface area; Hgb, hemoglobin; HCT, hematocrit; IQR, inter quartile range; PLT, platelet count; SCr, Serum creatinine; SD, standard deviation; WBC, white blood cell count.*

TABLE 2 | Tumor characteristics of breast cancer patients, at the radiotherapy center, Tikur Anbessa specialized hospital, Addis Ababa University, Ethiopia, 2015.

Parameters		Frequency, n (%)
Histologic type of invasive cancer	Ductal	214 (85.9%)
	Lobular	18 (7.2%)
	Mixed type	7 (2.8%)
	Special type	9 (3.6%)
Histologic grade	Grade I	26 (17.1%)
	Grade II	79 (52%)
	Grade III	47 (30.9%)
Positive nodal involvement		182 (73.1%)
Hormone receptor and Her2 expression	ER +	15 (62.5%)
	PR +	12 (50%)
	HER-2/neu	6 (25%)
	Triple negative	6 (25%)
Distant metastatic site	No known metastasis	52 (21.3%)
	Bone, lymph node, or lung only	140 (56.2%)
	Liver, CNS, Lung + other organs)	55 (22.1%)
Chemotherapy setting	Neoadjuvant	16 (6.4%)
	Adjuvant	178 (71.5%)
	Palliative	55 (22.1%)
Chemotherapy regimen	♣FAC	164 (65.9%)
	AC	48 (19.3%)
	AC – T	34 (13.7%)
	CMF	3 (1.2%)

♣FAC (5-Flourouracil 500 mg/m^2, Adriamycin [Doxorubicin] 50 mg/m^2, and Cyclophosphamide 500 mg/m^2); AC (Adriamycin 50 mg/m^2 and Cyclophosphamide 600 mg/m^2); CMF (Cyclophosphamide 500 mg/m^2, Methotrexate 40 mg/m^2, and Fluorouracil 500 mg/m^2; AC – T (Adriamycin 60 mg/m^2 and Cyclophosphamide 600 mg/m^2 followed by Taxol 175 mg/m^2).

(IQR 10.0). None of the study participants received primary colony stimulating factor (G-CSF), however, 4.8% of the patients received it during the course of chemotherapy in the subsequent cycles.

Incidence and Predictors of Chemotherapy Induced Grade 3 or 4 Hematological Toxicity

The overall incidence of chemotherapy induced grade 3 or 4 hematological toxicity was 51.0% [95% confidence interval (CI) = 44.54–57.46%]. Most of the hematologic toxicity events were neutropenic toxicities, 50.2% (95% CI = 43.83–56.56%). The incidence of grade 3 or 4 anemia and thrombocytopenia across the cycles was 2 and 1.2%, respectively. Paired sample *t*-test showed there was significant decrease in the mean absolute neutrophil counts in each cycles compared to the baseline ($p < 0.05$ for the trend across the cycles).

The overall allele frequencies of *CYP2B6*6, CYP2C9*2, CYP2C9*3, CYP2C19*2, CYP2C19*3, CYP3A5*3, CYP2J2*7, POR*28,* and *ABCB1 rs3842G* variant alleles were 34.8, 5.8, 1.5, 15.9, 1.8, 62.5, 15.2, 15.5, and 13.2%, respectively. All genotype frequencies were in consistent with HWE ($p > 0.05$). Comparison of genotype and allele frequencies between patients who developed chemotherapy induced grade 3 or 4 hematological toxicity versus treatment tolerant is presented in **Table 3**. After multiple comparisons, patients carrying *CYP2C9* *2 or *3 alleles had significantly lower incidence of hematologic toxicity (3.1% versus 11.4%, $p = 0.024$) (**Table 3**). Controlling for *POR* genotype, grade 3 or 4 hematologic toxicity was significantly lower in patients with *CYP2C9* *2 or *3 alleles who also carried *POR**28 ($p = 0.003$). No such association was observed among *POR**1/*1 carriers ($p = 0.12$). No interaction was also detected between POR and other genotypes ($p > 0.05$). On the other hand, the cumulative Kaplan–Meier hazard curves (**Figure 1**) showed association for the development hematologic toxicities among *CYP2J2* and *CYP2C9* genotype. There was no significant association between *CYP2B6, CYP2C19*, or *POR*28* and *ABCB1* genotype with risk for hematologic toxicity.

Univariate followed by multivariate Cox proportional hazard regression analysis were done to model predictors of grade 3 or 4 hematologic toxicities (**Table 4**). To avoid the interaction between pretreatment white blood cells count (WBC) and absolute neutrophils count (ANC) in the regression model (as neutrophils account for about 45–70% of WBC), two multivariate regression models were developed. First, all variables selected from univariate analysis, excluding ANC, were entered into multivariate model. In model 2, all variables except

TABLE 3 | Genotype and allele frequencies of candidate drug metabolizing enzymes genes by hematologic toxicities, at the radiotherapy center, Tikur Anbessa specialized hospital, Addis Ababa University, Ethiopia.

	Gene	Genotype	Grade 3 or 4 hematologic toxicity		*p*-value
			No	Yes	
			n (%)	n (%)	
Genotype frequency	*CYP2B6*	*1/*1	34 (40.9)	35 (43.2)	0.51
		*1/*6	37 (44.6)	39 (48.1)	
		*6/*6	12 (14.5)	7 (8.6)	
	CYP2C9	*1/*1	66 (79.5)	76 (93.8)	0.021
		*1/*2 or *3	15 (18.1)	5 (6.2)	
		*2/*2	2 (2.4)	0 (0)	
	CYP2C19	*1/*1	57 (68.7)	55 (67.9)	0.68
		*1/*2 or *1/*3	24 (28.9)	22 (4.9)	
		*2/*2 or *3/*3	2 (2.4)	4 (4.9)	
	CYP3A5	*1/*1	17 (20.5)	6 (7.4)	0.052
		*1/*3	37 (44.6)	40 (49.4)	
		*3/*3	29 (34.9)	35 (43.2)	
	POR	*1/*1	56 (67.5)	60 (74.1)	0.09
		*1/*28	27 (32.5)	18 (22.2)	
		*28/*28	0 (0)	3 (3.7)	
	CYP2J2	*1/*1	67 (80.7)	54 (66.7)	0.12
		*1/*7	13 (15.7)	23 (28.4)	
		*7/*7	3 (3.6)	4 (4.94)	
		Allele			
Allele frequency	*CYP2B6*	*6	61 (36.7)	53 (32.7)	0.44
	CYP2C9	*2	15 (9.04)	4 (2.5)	0.011
		*3	4 (2.4)	1 (0.6)	0.19
		*2 or *3	19 (11.4)	5 (3.1)	0.004
	CYP2C19	*2	26 (15.7)	26 (16.1)	0.92
		*3	2 (1.2)	4 (2.5)	0.39
		*2 or *3	28 (16.9)	30 (18.5)	0.7
	CYP3A5	*3	95 (57.2)	110 (67.9)	0.046
	POR	*28	27 (16.3)	24 (14.8)	0.72
	CYP2J2	*7	19 (11.4)	31 (19.1)	0.041

WBC were modeled. The result indicated that *CYP2J2*7* allele (HR = 1.819; 95% CI, 1.141–2.899, $p = 0.012$) and low baseline WBC count (grade 1 leukocytopenia) (HR = 2.748; 95% CI, 1.466–5.149, $p < 0.001$) were independent predictors of toxicity (model 1, **Table 4**). In model 2, low baseline ANC count (grade 1 or 2 neutropenia) (HR = 2.746; 95% CI, 1.732–4.353, $p < 0.001$), and *CYP2J2*7* allele (HR = 1.735; 95% CI, 1.085–2.775, $p = 0.021$) (model 2) were strong independent risk factors of hematologic toxicity. Thus, patients who took chemotherapy with low baseline white blood cells count, or low neutrophils count were at increased risk of experiencing grade 3 or 4 hematologic toxicity in the subsequent cycles. In addition, the risk of toxicity was also higher in patients carrying *CYP2J2*7* variant allele. Although Chi square test showed association of *CYP2C9* variant alleles to toxicity (**Table 3**), these alleles did not reach to statistical significance in the final multivariate regression analysis (**Table 4**).

Incidence and Predictors of Relative Dose Intensity

The overall actual average RDI was 81.9% (95% CI = 80.52–83.28%). Dose delay > 15% of the planned days was observed in 61.4% patients, whereas dose reduction was observed in 11.2% patients. The proportion of patients who received reduced RDI < 85% of the standard/planned dose intensity was 56.6% (95% CI = 50.32–62.88%). Three point six percent of the reduced RDI was planned dose reduction from the start of therapy as decided by senior oncologists to reduce the dose in metastatic conditions or due to co-morbidities. The remaining was unplanned RDI reduction associated with subsequent dose/treatment delays due to toxicities.

Results of multivariate logistic regression analysis (**Table 5**) showed that the independent risk predictors of reduced RDI were BMI ≤ 18.4 kg/m^2 (underweight) (AOR 5.975; 95% CI = 1.361–26.227, $p = 0.018$), low baseline leukocyte count (AOR 6.092; 95% CI = 1.238–29.98, $p = 0.026$), low baseline neutrophils count (AOR 3.37; 95% CI = 1.412–8.045, $p = 0.006$) and *CYP2J2*7* allele (AOR 2.892; 95% CI = 1.238–6.647, $p = 0.012$). On the other hand, the odds of receiving RDI less than 85% of the dose intensity was significantly lower in patients with *CYP2B6 *6/*6* genotype (AOR 0.179; 95% CI = 0.049–0.656, $p = 0.009$) irrespective of other factors.

DISCUSSION

In the present study, we prospectively investigated the incidence of chemotherapy induced hematologic toxicity, the proportion of reduced RDI, and associated risk factors including pharmacogenetics in drug mobilizing enzymes relevant for the disposition or bio-activation of chemotherapeutic agents. Our main finding include (i) higher incidence of grade 3 or 4 hematologic toxicities (51%), which was largely manifested as neutropenic toxicity (50.2%), that caused treatment delay in large number of patients. Consequently, significant proportion of the patients (56.6%) received reduced RDI; (ii) Low baseline WBC and neutrophil count is a strong predictor of both chemotherapy induced hematologic toxicity and reduced RDI; (iii) significant association of *CYP2J2*7* and *CYP2C9* genotype with chemotherapy induced hematologic toxicity, and *CYP2B6* genotype with reduced RDI. To the best of our knowledge, this is the first study to investigate the incidence and predictors of anticancer chemotherapy induced hematological toxicities, reduced chemotherapy dose intensities and associated risk factors among Ethiopian breast cancer patients, and the first pharmacogenetics association study for chemotherapy induced hematologic toxicity and RDI in Sub-Saharan Africa population.

Cyclophosphamide, the cornerstone of breast cancer chemotherapy in Ethiopia, is mainly metabolized to 4-hydroxy-cyclophosphamide by genetically polymorphic *CYP3A*, *CYP2B6*, *CYP2C9*, *CYP2C19*, and *CYP2J2* enzymes (El-Serafi et al., 2015). We investigated the association of common functional genetic

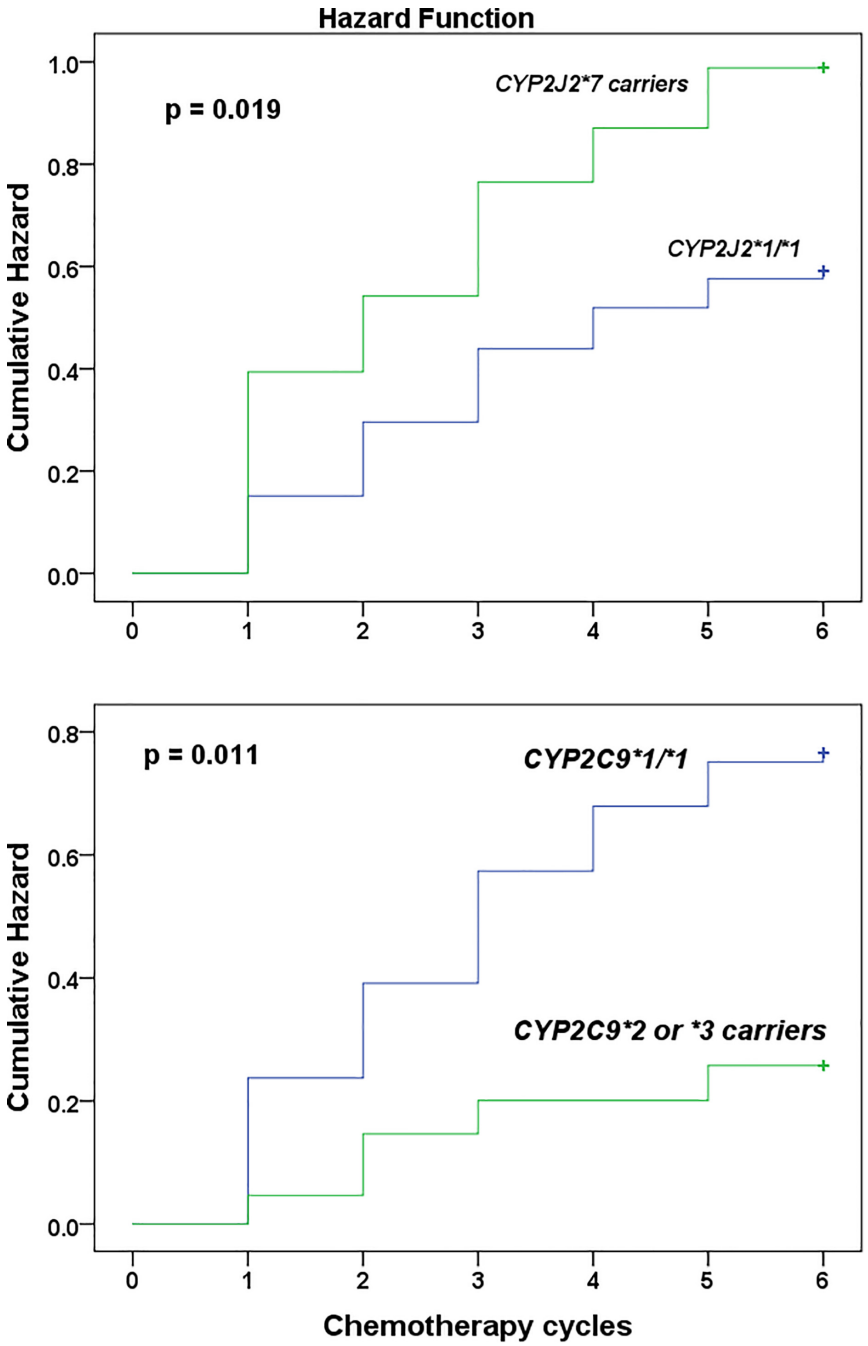

FIGURE 1 | Kaplan–Meier curves to estimate cumulative hazard for the development of chemotherapy induced grade 3 hematologic toxicity stratified by *CYP2J2**7 (top) and *CYP2C9* (below) genotype among Ethiopian female breast cancer patients at the radiotherapy center, Tikur Anbessa specialized hospital, Addis Ababa University, Ethiopia, 2015.

variant alleles of these enzymes with chemotherapy induced hematologic toxicity and RDI. We found that, patients with *CYP2J2*7* allele, baseline grade 1 leukocytopenia, and grade 1 or 2 neutropenia, were significantly associated with increased risk of grade 3 or 4 hematologic toxicity. Low baseline WBC count as an important predictor for chemotherapy induced hematologic toxicity was consistent with previous reports (Lyman et al., 2003; Jenkins et al., 2012; Tsuji et al., 2016). However, *CYP2J2*7* was identified as new pharmacogenetic risk factors of chemotherapy induced hematologic toxicity in Ethiopian breast cancer patients. *CYP2J2* is an epoxygenase enzyme that catalyzes the metabolism of structurally diverse therapeutic compounds, particularly in extra-hepatic tissues (Berlin et al., 2011). A recent study reported the role of *CYP2J2*

TABLE 4 | Cox proportional hazard regression results for incidence of grade 3 or 4 hematologic toxicity.

Parameters	Univariate analysis		Multivariate analysis			
	†OR (95% CI)	*p*-value	Model 1		Model 2	
			OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
Body surface area						
<1.5	1					
1.5–1.75	0.68 (0.45–1.04)	0.07				
>1.75	1.03 (0.62–1.72)	0.91				
Cardiovascular co-morbidity						
No	1					
Yes	0.56 (0.27–1.14)	0.11				
HIV/AIDS						
No	1					
Yes	0.62 (0.30–1.26)	0.19				
Baseline leukocyte toxicity grade						
Normal‡	1		1			
Grade 1£	3.20 (2.02–5.08)	< 0.001	2.75 (1.47–5.15)	< 0.001		
Baseline neutrophil toxicity grade						
Normald'	1				1	
Grade 1§ or Grade 2¥	2.32 (1.58–3.41)	< 0.001			2.75 (1.73–4.35)	< 0.001
CYP3A5						
*1/*1	1		1		1	
*3 carriers	2.34 (1.03–5.32)	0.042	2.04 (0.87–4.79)	0.05	2.12 (0.90–4.96)	0.08
CYP2C9						
*1/*1	1		1		1	
*2 or *3 carriers	0.35 (0.14–0.88)	0.024	0.40 (0.13–1.18)	0.10	0.49 (0.19–1.22)	0.12
CYP2C19						
*1/*1	1					
*2 or *3 carriers	1.08 (0.76–1.54)	0.66				
CYP2J2						
*1/*1	1		1		1	
*7 carriers	1.66 (1.05–2.64)	0.032	1.82 (1.14–2.9)	0.012	1.74 (1.09–2.78)	0.021
POR						
*1/*1	1					
*28 carriers	0.95 (0.58–1.56)	0.83				
ABCB1 rs 3842 A>G						
AA	1					
G carriers	0.75 (0.35–1.62)	0.46				

†*Odds ratio;* ‡*4,500–10,000/m*^{3}*;* £*3,000–4,500/mm*^{3}*;* d'*2,500–7,500/m*^{3}*;* §*1,500–2,500/m*^{3}*;* ¥ *1,000–1,500/m*^{3}*.*

in the bioactivation of cyclophosphamide (El-Serafi et al., 2015). Using enzyme kinetic studies, the authors revealed that *CYP2J2* is over expressed during cyclophosphamide treatment, and the bioactivation of the drug was significantly correlated to *CYP2J2* expression. In another study, *CYP2J2* is reported to be highly expressed in human and mouse hematological cell lines, as well as in peripheral blood and bone marrow cells of leukemia patients (Chen et al., 2011). The cytotoxic effect of cyclophosphamide in hematological cell lines has also been associated with *CYP2J2* expression, despite the lack of *CYP2B6* (Xie et al., 2002). Interestingly, we found increased risk for grade 3 or 4 chemotherapy induced hematologic toxicities in carriers of *CYP2J2*7*. As *CYP2J2*7* is associated with increased enzyme activity (El-Serafi et al., 2015), and patients carrying this variant allele could possibly activate cytotoxic agents such as cyclophosphamide to 4-hydroxycyclophosphamide at a faster rate and hence increased risk for toxicity.

The frequency distribution of *CYP2C9* genotype and defective variants alleles were significantly different between patients who developed grade 3 or 4 chemotherapy induced hematologic toxicities versus treatment tolerant (**Table 3**). *CYP2C9*2* or **3* allele frequency was significantly higher in treatment tolerant (11.4%) than chemotherapy induced hematologic toxicities cases (3.1%) or the general random Ethiopian population. The frequency of *CYP2C9*2* and *CYP2C9*3* alleles in healthy Ethiopians is 4 and 2%, respectively. Furthermore, univariate regression analysis indicated a significant association of *CYP2C9* with grade 3 or 4 chemotherapy induced hematologic

TABLE 5 | Logistic regression results for predictors of reduced RDI.

Parameters	Univariate analysis		Multivariate analysis			
	OR† (95% CI)	*p*-value	Model 1	*p*-value	Model 2	*p*-value
			OR (95% CI)		OR (95% CI)	
BSA (m^2)						
< 1.5	1.57 (0.82–3.02)	0.17	–		–	
1.5–1.75	1		–		–	
>1.75	0.81 (0.41–1.59)	0.53	–		–	
BMI (Kg/m^2)						
≤18.4	2.34 (0.88–6.24)	0.09	5.98 (1.36–26.2)	0.02	6.50 (1.45–29.05)	0.01
18.5–24.9	1		1		1	
25–29.9	0.92 (0.48–1.75)	0.79	0.75 (0.32–1.79)	0.52	0.79 (0.33–1.88)	0.59
≥30	0.68 (0.30–2.52)	0.35	0.65 (0.22–1.94)	0.44	0.57 (0.18–1.75)	0.32
Chemotherapy regimen						
FAC	1		–	–	–	–
CMF	0.41 (0.04–4.62)	0.47	–	–	–	–
AC	2.21 (1.09–4.49)	0.03	–	–	–	–
AC-T	0.65 (0.31–1.37)	0.26	–	–	–	–
Cardiovascular co-morbidity						
No	1		–		–	
Yes	0.42 (0.18–1.01)	0.05	–	–	–	–
Baseline leukocyte toxicity						
Normal‡	1		1		–	–
Grade 1£	6.52 (1.89–22.41)	0.003	6.09 (1.24–29.98)	0.026	–	–
Baseline neutropenic toxicity						
Normalđ	1		–		1	
Grade 1§ or 2¥	2.78 (1.42–5.43)	0.003	–		3.37 (1.41–8.05)	0.006
CYP2B6						
*1/*1	1		1		1	
*1/ *6	0.96 (0.52–1.79)	0.9	1.47 (0.73–2.99)	0.29	1.63 (0.79–3.36)	0.19
*6/*6			0.18 (0.05–0.66)	0.01	0.19 (0.06–0.77)	0.01
CYP2C9						
*1/*1	1		–		–	–
*2 or *3 carriers	0.87 (0.35–2.13)	0.17	–		–	–
CYP3A5						
*1/*1	1		–		–	–
*3 carriers	1.79 (0.075–4.26)	0.17	–		–	–
CYP2J2						
*1/*1	1		1		1	
*7 carriers	1.72 (0.84–03.50)	0.14	2.89 (1.26–6.65)	0.012	2.79 (1.21–6.46)	0.02
ABCB1						
AA	1	0.83				
Carriers of G	0.92 (0.43–1.97)					

†*Odds ratio;* ‡*4,500–10,000/m^3;* £*3,000–4,500/mm^3;* đ *2,500–7,500/m^3;* §*1,500–2,500/m^3;* ¥ *1,000–1,500/m^3.*

toxicities. Our result indicates that being carriers of defective variant alleles *CYP2C9*2* or *3 alleles and hence reduced *CYP2C9* enzyme activity is associated with a lower risk for chemotherapy induced hematologic toxicities. This is in line with a previous study reporting a threefold lower intrinsic clearance of cyclophosphamide CYP2C9.2 and CYP2C9.3 compared to CYP2C9.1 (Griskevicius et al., 2003). Thus, reduced cyclophosphamide bioactivation, in carriers of defective *CYP2C9* variant alleles, may be protective against cyclophosphamide induced hematologic toxicities. On the other hand, reduced cyclophosphamide bioactivation, in patients carrying *CYP2C9* defective alleles, may compromise the treatment success, increase risk of recurrence as well as worsen survival outcome. Consequently, future studies evaluating treatment successes based on *CYP2C9* genotype is needed to identify whether genotype-based cyclophosphamide dose modification is required or not. Although *CYP2C9* genotype was retained in the final multivariate regression model, the *p*-value did not reach significance, which might be due to a lower variant allele frequency and hence sample size.

Pharmacologically, the cytotoxic properties of chemotherapeutic agents form the basis for their anticancer as well as myelosuppressive effects (Kozma et al., 2012). In early stage breast cancer patients receiving CMF, greater myelosuppression during treatment had been shown to have better outcomes (Mayers et al., 2001). This indicates that chemotherapy induced hematologic toxicities could be predictive of anticancer response. In clinical practice, the primary response to hematologic toxicity during anticancer chemotherapy is dose reduction and/or treatment delays to allow cells to regenerate (Piccart et al., 2000). While toxicity could be avoided or reduced by varying schemes of dose attenuation and treatment delay, it is done at the expense of reduction in the dose intensity received.

Reductions of dose intensity from standard dose and dose-intensity of CMF may compromise desired survival advantages in breast cancer patients (Bonadonna et al., 1995; Piccart et al., 2000). Superior efficacy and survival benefits (both disease-free survival and overall survival) have been observed in those patients who received greater dose-intensity of CAF (Budman et al., 1998). The key role of dose density and intensity has also been substantiated with a study that compared dose-dense (14 days cycles with primary prophylactic GCSF support) and standard (21 days cycles) schedules in patients with node-positive breast cancer. Dose-dense schedules are associated with significantly improved disease-free and overall survival (Citron et al., 2003). In the subsequent studies, higher RDI has been shown to have the greatest impact particularly for patients with early-stage disease in which curative intent with adjuvant chemotherapy is the goal (Sandy and Della-Fiorentina, 2013; Vavra et al., 2013; Havrilesky et al., 2015).

RDI of less than 85% is widely considered as a benchmark for clinically important reduction in chemotherapy dose-intensity (Bonadonna et al., 2005; Wildiers and Reiser, 2011). Although achieving this RDI level is important to gain improved outcome, many patients in various clinical settings are treated with a lower dose intensity of chemotherapy (Piccart et al., 2000; Sandy and Della-Fiorentina, 2013). The present study result estimates that, the prevalence of reduced RDI received (56.6%) among Ethiopian patients could be as low as 50.3% and as high as 62.9%. This indicates that a higher proportion of patients are receiving reduced RDI in our setup unacceptably, compared to the finding reported in Canada (4.4%) (Raza et al., 2009), United States (30%) (Shayne et al., 2006, Shayne et al., 2007), and Australia (12% in adjuvant and 36% metastatic setups) (Bae et al., 2014). In line with the finding of our study, a nationwide survey involving breast cancer patients across the United States who received a similar chemotherapy regimens as our study, reported that 55% of women received less than 85% of the RDI (Lyman et al., 2003). The authors reported dose reductions in 40% of patients, and treatment delays up to 7 days in 24% of patients (Lyman et al., 2003). However, the major RDI reducing event in our study was treatment delay observed in 61.4% patients.

Several risk factors for reduced RDI have previously been reported in the literature including older age, and lower pretreatment blood cell counts, BSA > 2 m^2, and nonuse of G-CSF (Lyman et al., 2003). The present study has revealed that, underweight (BMI ≤ 18.4 kg/m^2), baseline grade 1 leukocyte toxicity and grade 1or 2 neutropenia were independent predictors of reduced RDI. Consequently, such patients are more likely to receive reduced RDI. On the other hand, *CYP2B6*6/*6* genotype was associated with a lower risk of receiving reduced RDI. In line with our finding, greater incidence of dose delay was observed during AC treatment in variant carriers of *CYP2B6*2* and *CYP2B6*5* (Bray et al., 2010). Other patient-related factors such as appointment cancelations, patient non-compliance and patient knowledge deficits may also contribute to clinically important reduction in chemotherapy dose-intensity (Piccart et al., 2000; Wildiers and Reiser, 2011; Kozma et al., 2012). *CYP2B6* is the main enzyme that substantially metabolizes cyclophosphamide into its active metabolite (4-hydroxy-cyclopsphmaide), which is known to induce hematologic toxicities. Although *CYP2B6*6* genotype was not identified as a risk factor for hematologic toxicity, we found significant protective effect against reduced RDI. *CYP2B6*6* allele is associated with reduced enzymatic activity and higher incidence of antiretroviral induced liver toxicity in Ethiopian HIV patients (Yimer et al., 2012). Reports regarding the impact of *CYP2B6*6* on chemotherapy induced hematologic toxicity are inconsistent. A recent finding indicated that, being non-*CYP2B6*6* carrier was a significant predictor of grade 4 neutropenia in breast cancer patients treated with doxorubicin and cyclophosphamide combination chemotherapy (Tsuji et al., 2016). By contrast, few other studies concluded that *CYP2B6* genotype was not significantly associated with myelotoxicity (Yao et al., 2010; Haroun et al., 2015). Such inconsistencies could be attributed to the difference in the sample size and study population. Moreover, the relative expression levels of *CYP2J2* and *CYP2B6* may determine *in vivo* kinetics and toxicity of drugs metabolized by these enzymes.

Although this study provides the first data on factors associated with chemotherapy associated hematologic toxicities and RDI, our study may not be powered enough to show significant association of other baseline clinical parameters including co-morbidity status, renal function (serum creatinine and blood urea nitrogen) and liver function estimates (Alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase) with either hematologic toxicity or reduced RDI. We did not incorporate information concerning potential influence of chemotherapy induced non-hematologic toxicities (such as peripheral neuropathy, fatigue, cardio-toxicity, mucositis, etc.), and patient preference (absenteeism on schedule) on reduced dose intensity. Some of the unplanned reductions in dose-intensity may relate to these factors. It is likely that, differences in PK parameters may further explain the incidence of hematologic toxicity in this cohort of participants. Future larger sample size studies are required to investigate the association of important clinical parameters with hematologic toxicity or reduced RDI in breast cancer.

Studies have shown that prophylactic use of primary G-CSF has been found to be a statistically significant predictor of reduced neutropenic events and thus can reduce the risk of myelosuppresive complications during chemotherapy and help facilitate the delivery of adequate RDI (Citron et al., 2003; Citron, 2004; Lyman et al., 2013; Weycker et al., 2014). However, none of the patients received primary colony stimulating factors in our study. Although the cost of CSF may impede the use of this agent in resource limited countries like Ethiopia, it could

be used more cost-effectively if treatment is targeted to patients with identifiable risk factors for subsequent neutropenic complications (Crawford, 2006).

CONCLUSION

In conclusion, we report high rates of chemotherapy-induced hematological toxicities causing inadequate RDI in large proportion of Ethiopian breast cancer patients. This study has identified a high activity *CYP2J2*7* and baseline grade 1 or 2 neutropenia is associated with higher risk of chemotherapy induced hematologic toxicity. On the other hand defective *CYP2C9* defective variant alleles and hence low CYP2C9 enzyme activity is protective against developing hematologic toxicity. *CYP2B6*6* genotype, BMI, baseline WBC and neutrophil counts, are predictors of reduced RDI. In general, results from this study provide relevant information to identify patients at greater risk of chemotherapy induced hematologic toxicity and to develop breast cancer treatment guideline in Ethiopia for proper management and patient care. Patients with *CYP2J2*7* alleles and low pretreatment WBC and ANC need prior support such as use the prophylactic use of primary G-CSF before initiation of chemotherapy, close monitoring and treatment follow up accordingly. The potential impact of the observed reduced RDI on the survival outcome in this study population needs further investigation.

ETHICS STATEMENT

Ethical clearance was obtained from Institutional Review Board (IRB) of the college of health sciences, Addis Ababa University, Armauer Hansen Research Institute ethical review committee, and National Research Ethics Review Committee (NRERC) of the federal democratic republic of Ethiopia. Signed informed consent was obtained from individual patient prior to participation in the study.

AUTHOR CONTRIBUTIONS

JA, EM, and EA designed the study. JA collected the data. JA and EA did genotyping, analyzed the data and wrote the manuscript. JA, EA, EM, GY, DS, AB, MA, AA, RH, AF, and MH involved in the discussion of results and critical review of the manuscript. All the authors have read and approved the final manuscript.

FUNDING

This work was supported by Addis Ababa University thematic research fund, and Armauer Hansen Research Institute (AHRI) through the BSPP Program, a grant obtained from Sida-Ethiopia Bilateral Program (Contribution No: 5108013506). The funding source did not have any role in collection, analysis, or interpretation of data, in writing the paper, or decision to submit it.

ACKNOWLEDGMENTS

We would like to extend our sincere appreciation to Armauer Hansen Research Institute (AHRI) and breast cancer thematic research group of the college of health sciences, Addis Ababa University for the financial assistance required for the study.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Population Pharmacokinetic, Pharmacogenetic and Pharmacodynamic analysis of Cyclophosphamide in Ethiopian Breast Cancer Patients

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Key words: Cyclophosphamide; Pharmacokinetics; *CYP3A5; CYP2C9;* BSA; Pharmacodynamics; Breast Cancer.

Abstract

Cyclophosphamide (CPA) containing chemotherapy regimen is the standard of care for breast cancer treatment in sub-Saharan Africa. Wide inter-individual variations in

pharmacokinetics (PK) of Cyclophosphamide (CPA) influence the efficacy and toxicity of CPA containing chemotherapy. Data on the pharmacokinetics (PK) profile of CPA and its covariates among black African patients is lacking. We investigated population pharmacokinetic/pharmacogenetic/pharmacodynamic (PK-PG-PD) of CPA in Ethiopian breast cancer patients.

During the first cycle of CPA-based chemotherapy, the population PK parameters for CPA were determined in 267 breast cancer patients. Absolute neutrophil count was recorded at baseline and day 20 post CPA administration. A population PK and covariate model analysis was performed using non-linear mixed effects modeling. Semi mechanistic and empiric drug response models were explored to describe the relationship between the area under concentration-time curve (AUC), and neutrophil toxicity.

One compartment model better described CPA PK with population clearance and apparent volume of distribution (V_D) of 5.41 L/hr and 46.5 L, respectively. Inter-patient variability in CPA clearance was 51%. Patients carrying *CYP3A5*3* or **6* alleles had lower elimination rate constant and longer half-life compared to wild type carriers. *CYP2C9 *2* or **3* carriers were associated with increased clearance of CPA. Patients who received 500 mg/m^2 based CPA regimen were associated with a 32.3% lower than average clearance and 37.1% lower than average V_D compared to patients who received 600 mg/m^2. A 0.1 m^2 unit increase in body surface area (BSA) was associated with a 5.6% increment in V_D. The mean V_D (33.5 L) in underweight group (BMI<18.5 kg/m^2) was significantly lower compared to those of overweight (48.1 L) or obese patients (51.9 L) ($p < 0.001$). AUC of CPA was positively correlated with neutropenic toxicity.

In conclusion, we report large between-patient variability in clearance of CPA. *CYP3A5* and *CYP2C9* genotypes, BSA, BMI and CPA dosage regimen influence PK of CPA. Plasma CPA exposure positively predicts chemotherapy-associated neutropenic toxicity.

Introduction

Cyclophosphamide (CPA) is a cornerstone of combination chemotherapy for the treatment of breast cancer (Stearns et al., 2004). Wide inter-individual variations in the pharmacokinetics (PK) of CPA and its treatment outcome including safety have been reported (de Jonge et al., 2005). CPA is a prodrug and requires bio-activation in the liver to 4-Hydroxy-cyclophosphamide (4-OH-CPA), which is subsequently converted to the ultimate alkylating metabolite, phosphoramide mustard, and acrolein, a urotoxic metabolite (Ekhart et al., 2008). Various cytochrome P450 (CYP) enzymes have been implicated to catalyze the 4-hydroxylation of CPA to 4-OH-CPA including *CYP2B6* (Xie et al., 2003), *CYP2C9* and *CYP3A4/5* (Roy et al., 1999), *CYP2C19* (Griskevicius et al., 2003; Timm et al., 2005), and *CYP2J2* (El-Serafi et al., 2015a). These enzymes are genetically polymorphic and may contribute to interindividual variation in CPA metabolic disposition and clinical response including chemotherapy-induced toxicities (Nakajima et al., 2007; Zanger et al., 2007; Ekhart et al., 2008). Apart from genetic factors, patient-specific characteristics such as disease status, body weight, age, body surface area, and hepatic and renal function status may influence CPA plasma exposure (de Jonge et al., 2005). A decrease in CPA clearance with increased body weight (Powis et al., 1987), impaired hepatic (de Jonge et al., 2005) or renal function (Haubitz et al., 2002) resulting in an increased systemic drug exposure is reported previously.

Identifying factors influencing PK parameters and exposure-toxicity relationship is critical for CPA dose optimization and personalized chemotherapy. The population pharmacokinetics of high dose CPA (4000-6000 mg/m^2), has previously been described by a time-dependent or concentration-dependent PK models (Chen et al., 1995, 1997). CPA auto-induction has been modeled by a mechanistic enzyme turnover model based on the data of both CPA and 4-hydroxycyclophosphamide (Hassan et al., 1999). On the other hand, Huitema et al reported a PK model for the bioactivation route of CPA incorporating both auto-induction and drug interaction between CPA and Thiotepa (Huitema et al., 2001).

CPA-based chemotherapy suppresses the hematopoietic system, impairing host protective mechanisms and limiting the doses of chemotherapy that can be tolerated. Neutropenia remains the major adverse event of CPA at conventional doses and the main concern in the delivery of CPA-based chemotherapy (Crawford et al., 2004). CPA-based chemotherapy-associated neutropenia is the primary reason for change in the schedule of chemotherapy delivery to cancer patients (Kozma et al., 2012). Particularly, patients manifesting febrile neutropenia, a serious complication of chemotherapy with risk of confusion, cardiac complications, hypotension, respiratory and renal failure, and even death, often require hospitalization and administration of antibiotics (Weycker et al., 2014). Chemotherapy-induced hematological adverse events cause a substantial economic burden on patients, caregivers and society at large (Liou et al., 2012). Consequently, one aspect of optimizing cancer chemotherapy involves describing neutropenic toxicity as a function of drug exposure or its pharmacokinetic parameters.

Ethnic differences in anticancer drug disposition is an important factor accounting for population variation in treatment response and tolerability (O'Donnell and Dolan, 2009). Hence PK, pharmacogenetics (PG) and exposure-toxicity relationship of CPA in various

populations require definition. CPA has remained a stable component in many of the chemotherapy combinations used in breast cancer CPA in sub-Saharan Africa including Ethiopia. To our knowledge, the PK profile of CPA and its covariates among black African breast cancer patients is yet to be investigated. Furthermore, the exposure-myelosuppressive toxicity relationship of CPA in black African population is not studied. Therefore, this study was aimed to investigate the population pharmacokinetics and pharmacodynamics (PD) of CPA and identify potential covariates including pharmacogenetic markers influencing PK of CPA in Ethiopian breast cancer patients.

Materials and Methods

Patients

A total of 267 female breast cancer patients were enrolled from the radiotherapy center of Tikur Anbessa specialized hospital, Addis Ababa, Ethiopia. The study protocol was approved by Institutional Review Board of the College of Health Sciences, Addis Ababa University (Ref No: 011/16/2016), Armauer Hansen Research Institute Ethical Review Committee (Ref No: PO26/16) and National Research Ethics Review Committee of the Federal Democratic Republic of Ethiopia (Ref No: 3.10/235/2017). Written informed consent was obtained from each patient before participation in the study. Breast cancer patients receiving CPA containing chemotherapy regimen with conventional dosage (500-600 mg/m^2) were included. The common CPA-based chemotherapy regimens in the outpatient care ward of the radiotherapy center were AC-T (Adriamycin 60 mg/m^2 and Cyclophosphamide 600 mg/m^2 followed by Taxol 175 mg/m^2), FAC (5-Flourouracil 500 mg/m^2, Adriamycin [Doxorubicin] 50 mg/m^2, and Cyclophosphamide 500 mg/m^2) and AC (Adriamycin 50 mg/m^2 and Cyclophosphamide 600 mg/m^2). Patients were stratified into 600 mg/m^2 CPA based regimen (AC and AC-T) and 500 mg/m^2 CPA based regimen (FAC). The individual dose of each drug was calculated based on body surface area (BSA) of the patient.

The criteria for inclusion were white blood cells count (WBC) $\geq$ 3,000/mm^3; absolute neutrophil count (ANC) $\geq$ 1500/mm^3; and platelet count (PLT) $\geq$ 100,000/mm^3; Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), and Serum Creatinine (SCr) $\leq$ 2.5 times the upper limits of normal; Karnofsky's performance score of at least 70% at presentation. For all included patients, socio-demographic data (age, weight, height, etc) and clinical profiles (stage of breast cancer, nodal status, tumor size, degree of differentiation, co-morbidity status, co-medication, pretreatment liver function (AST, ALT, ALP) and renal function estimates (SCr, BUN) were collected from patient medical charts. All patients were also given prophylactic pre-medications that comprised Cimetidine (400 mg i.v), Dexamethasone (8 mg i.v) and Ondansteron (8 mg i.v) prior to infusion of chemotherapy.

Blood Sampling

During the first cycle of CPA-containing chemotherapy, blood samples were drawn in EDTA tubes for genotyping and heparin tubes for CPA PK analysis. From all 267 patients, two blood samples were collected before CPA infusion starts (0 hour) for genotyping (2 mL) and CPA PK (2 mL). From 250 patients, additional blood samples (2 mL) were collected at

1(2), 3(4) and/or 5(6) hours after CPA infusion starts. From the remaining 17 patients, additional PK blood samples were also collected at 4, 8, 12 and 22 hours post CPA infusion initiation. These patients were admitted to receive their first cycle chemotherapy and monitored overnight. All PK blood samples were centrifuged within 30 minutes of collection at 3500xg and 4^{o}C for 3 minutes to separate the plasma (Nakajima et al., 2007). Both PK plasma and genotyping whole blood samples were then stored at –80^{o}C until analysis.

Determination of Plasma Cyclophosphamide Concentrations

The plasma concentrations of CPA was determined as described previously with some modifications (El-Serafi et al., 2015b). To each 250 µL of thawed plasma samples, 25 µL of internal standard (Ifosfamide, 20 mM, Vnr079111, Denmark) and 1.25 mL ethyl acetate were added. The mixture was briefly vortexed for 15 seconds and then centrifuged at 5000 rpm, 4^{o}C for 10 minutes. The organic fraction was transferred into a clean tube and evaporated to dryness under vacuum centrifuge. The residue was then dissolved in 100 µL of mobile phase, and the resulting solution was put in ultrasonic bath for 15 seconds. Fifty micro-liter of the solution was then injected into HPLC system. The HPLC system consisted of LKB-2150 pump, Gilson-234 auto-injector with a 100 µL sample loop, ZORBAX Extend-C18 column (150 × 4.6 mm, 3.5 µm, Agilent, Santa Clara, CA, USA) with a C18 guard column (Agilent, Santa Clara, CA, USA) and Milton Roy UV Spectro-Monitor 3100 detector (Pont-Saint-Pierre, France). The mobile phase consisted of acetonitrile/0.05 M KH_2PO_4 buffer (24:76 v/v, pH 2.8). The flow rate was 1 mL/min and detection was monitored at 195 nm. Data acquisition and processing were done using Clarity Chromatographic software (version 6.0, DataApex, Prague, Czech Republic). Under these conditions, the retention time was 5.5 and 6 min for Ifosfamide and CPA, respectively. The standard curve was constructed using CPA free plasma spiked with increasing concentrations (1–2000 µmol/L) of standard CPA (Sigma Aldrich Co, China). The quantification limit of CPA was 5 µmol/L. The standard curve was linear in the range of 5–1000 µmol/L (r = 0.998). Quality control samples (three samples, at a concentration of 25, 100, and 500 µmol/L) were included routinely at each run.

Genotyping

Genomic DNA was isolated from peripheral leukocytes in whole blood samples using QIAamp DNA Midi Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instruction. Genotyping was performed using TaqMan® drug metabolism genotyping assay reagents (Applied Biosystems Genotyping Assays) for allelic discrimination as described previously (Ahmed et al., 2019), with the following ID numbers for each SNP: C__7817765_60 for *CYP2B6*6* (rs3745274), C__26201809_30 for *CYP3A5*3* (rs776746), C__30203950_10 for *CYP3A5*6* (rs10264272), C__25625805_10 for *CYP2C9*2* (rs1799853), C__27104892_10 for *CYP2C9*3* (rs1057910), C__25986767_70 for *CYP2C19*2* (rs4244285), C__27861809_10 for *CYP2C19*3* (rs4986893), C_9581699_80 for *CYP2J2*7* (rs890293), C_8890131_30 for *POR*28* (rs1057868) and C__11711730_20 for *ABCB1* (rs3842). The genomic DNA samples were amplified in 96-well plates on QuantStudio^{TM} 12K Flex Real-Time PCR system (Applied Biosystems Life Technologies Holding, Singapore). The final volume for each reaction was 10 µL, consisting of TaqMan® fast advanced master mix (Applied Biosystems, Waltham, MA, USA), TaqMan 20X/40X

drug metabolism genotyping assays mix (Applied Biosystems, USA) and genomic DNA. The PCR conditions consisted of an initial step at 60 °C for 30 s, hold stage at 95 °C for 10 min and PCR stage for 40 cycles, step 1 with 95 °C for 15 and step 2 with 60 °C for 1 min and after read stage with 60 °C for 30 s. The characterized SNPs were selected on the basis of their potential to influence the functionality of enzymes to affect the disposition of CPA.

Population Pharmacokinetic Modeling

A population PK model of CPA was built using nonlinear mixed-effect modeling (NONMEM) program (version 7.30, ICON development solutions, Gaithersburg, Maryland). Additional software tools were also used as a workbench to facilitate the use of NONMEM including PsN (Lindbom et al., 2005) (version 3.4.2), Xpose (R Core Team, 2018) (version 4.5.0) and Pirana (Keizer et al., 2011) (version 2.9.6). A one, two and three-compartment models were fitted to the data set in that order. Differential equations were used to specify the compartment in NONMEM using ADVAN6 subroutine. First-order conditional estimation with interaction (FOCE-I) was used to estimate model parameters. The structural model was parameterized with clearance (CL), inter-compartmental clearance (Q) and compartmental volume of distribution (V_n), where n is the number of compartments. Inter-individual variability (IIV) in model parameters was assumed to be log-normally distributed and specified by exponential functions. Additive, proportional and combined proportional and additive residual error models were explored to account for within-subject variability, experimental errors, and model misspecification. The model with the lowest objective function value (OFV) was presumed to fit the data better. Residual-based and prediction-based goodness of fit plots (GOF) were also visually examined to determine the best fitting base model.

Based on the base model, covariate analysis was performed using stepwise covariate modeling (SCM) in NONMEM with the help of PsN SCM configuration file. The variables selected in covariate analysis were socio-demographics (BSA and BMI), CPA dosage regimen (500 *vs* 600 mg/m^2 based CPA), genotype (*CYP2BC6, CYP2C9, CYP2C19, CYP3A5, CYP2J2*, *POR* and *ABCB1*), comorbidity status (presence of cardiovascular diseases or HIV), and baseline organ function estimates (AST, ALT, ALP, SCr, BUN). The variables were selected based on clinical relevance or suggested impact on CPA disposition.

In the first phase of the SCM, covariates were added to the base model in a stepwise manner based on statistical significance (decrease in OFV). The effect of each potential covariate on each parameter was independently tested with a 5% significance level corresponding to a drop in OFV > 3.84 for one degree of freedom. The covariate with the largest change in OFV is added to the model. The procedure was repeated for all covariates until no more significant covariates are identified. In the backward elimination step, the level of significance was set at 1% threshold corresponding to a change in OFV > 6.63 for one degree of freedom or 9.210 for two degrees of freedom. The covariates selected in the forward step are removed from the model one at a time. The covariates which caused the largest statistically significant increase in OFV upon removal were retained in the model. Continuous covariates such as BSA and BMI were centered on their median values and the estimated shape parameter, THETA1, was coded as exponential factor as designated in equation 1:

$$\text{Clearance (CL)} = CL_{pop} \times (\text{BSA/median})^{\text{THETA1}}, \quad (1)$$

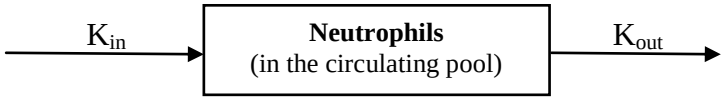
Where, CL_{pop} represents the population clearance value for patients with median BSA and THETA1 is the exponential factor for BSA, which describes the relationship with CL. Binary covariates were coded as 0 (wild type/no) and 1 (variant/yes) and modeled as designated in equation 2, where the shape parameter, THETA2, represents the proportional change in clearance compared to the reference category (*e.g.*, wild-type genotype).

$$CL = CL_{pop} \times (1 + \text{THETA2} * \text{genotype}), \quad (2)$$

Interpatient variability (IIV) in plasma CPA PK parameters were described by percentage coefficient of variation (CV) given by $\%CV = \text{Sqrt}(OMEGA^2)$. The predictive performance of the final covariate model was evaluated using prediction corrected visual predictive check (pcVPC). At least 200 simulated datasets were created using the final covariate model. Median, the 5^{th} and 95^{th} percentile of the observed data were compared to the 95% prediction intervals for the simulated data to ascertain the predictive performance of the final covariate model.

Modeling of Neutropenic Toxicity

Absolute neutrophil count (ANC) at baseline and at day 20 post CPA administration (in the first cycle) were used to develop the neutropenic toxicity model. A semi mechanistic (indirect response) and empiric (direct drug effect) PD models (Felmlee et al., 2012; Woo et al., 2017) were applied to describe CPA-related neutropenic toxicity. Initially, the indirect response model was explored. It was assumed that cells from myeloblasts to myelocytes are chemotherapy-sensitive and bone marrow leukocyte inhibition depends on sensitive cell exposure (unlike the stem cells which divide but are less sensitive to chemotherapy than mitotic hematopoeitc precursors). Thus, we chose the inhibition of cell production model (Woo et al., 2017) as described by the following schematic representation and equation (Equation 3), where k_{in} is the zero-order production rate constant, *i.e.*, the rate of entry of neutrophils into the circulating pool, k_{out} (K_{in}/ANC_o) is the first-order elimination rate constant, *i.e.*, the rate of disappearance of neutrophils from the circulating pool. E_{max} is defined as the maximum fractional factor of inhibition or a factor that inhibits neutrophil synthesis as an anticancer chemotherapy effect ($0 < E_{max} \leq 1$). ANC is the drug effect (neutrophil count at any time t) and ANC_o is the baseline neutrophil count. Since area under the concentration time curve (AUC) represents the systemic exposure, we incorporated the AUC, from time zero to day 20 after the first dose of CPA in the PD model to describe the relationship between AUC and drug effect (Equation 3). AUC_{50} represents the area under the curve associated with 50% of the maximum effect (E_{max}).



$$\frac{d(ANC)}{dt} = K_{in}\left(1 - \frac{E_{max} \times AUC}{AUC_{50} + AUC}\right) - K_{out} \times ANC, \quad (3)$$

For AUC significantly less than AUC_{50} (*i.e.*, AUC_{50} >> AUC), AUC in the denominator of equation 3 can be considered negligible and the relationship can be reduced to Equation 4, with $m = E_{max}/AUC_{50}$ is the slope of the relationship. Subsequently, K_{in}, slope (m) of the relationship and ANC_o were estimated using NONMEM.

$$\frac{d\,(ANC)}{dt} = K_{in}\,(1 - m \times AUC) - K_{out} \times ANC_o, \quad (4)$$

On the other hand, empiric model (linear direct drug response model) (Felmlee et al., 2012) was also separately adapted to the AUC data to correlate neutrophil count according to Equation 5, from which ANC_o and slope (m) were estimated using NONMEM.

$$Y = ANC_o - \text{slope } (m) \times AUC \quad (5)$$

Where, Y represents the drug effect (*i.e.*, absolute neutrophil count at any time t).

Statistical Analysis

χ^2-test was used to evaluate the genetic structure of the patient population (Hardy–Weinberg equilibrium). Descriptive statistics were computed to explore the demographic characteristics, clinical profiles, genotype frequencies of participants. Empirical Bayesian estimates of PK parameters were described as mean ± standard deviations (SD) or median with interquartile range (IQR). Inter-patient variability (IIV) in CPA PK parameters was described by coefficient of variation (% CV = Sqrt($OMEGA^2$). One-way ANOVA or independent sample t-test was also used in SPSS for Windows (version 21.0), to test for association between derived PK parameters (AUC, K_{el}, and $t_{1/2}$) and genotype or other patient-specific factors (*i.e.*, BMI, BSA, etc). Multiple comparisons were performed based on Tukey tests. Graphs were prepared using R software (version 3.5.1) (R Core Team, 2018).

Results

Socio-demographic and Clinical Profiles

CPA concentration was determined from a total of 532 plasma samples obtained from 267 female patients with breast cancer (average 2 plasma samples per patient). The socio-demographic and baseline laboratory results are summarized in Table 1. The median dose of CPA given to patients per cycle was 777.5 (Range 600-1000 mg) and 930 mg (Range 650-1150 mg), respectively for 500 mg/m^2 and 600 mg/m^2 based regimen group (the overall median CPA dose given per cycle was 870 mg (Range 600 – 1150 mg). The genotype and

allelic frequency distributions of candidate CPA metabolizing enzymes' and transporter genes are presented in Table 2. All the genotype frequencies were in line with Hardy–Weinberg equilibrium ($p > 0.05$).

Population Pharmacokinetic Modeling

CPA plasma concentration data were better described by one-compartment PK model. Additive error model of residual variability performed better than any of the other error models, *i.e*., proportional or exponential or combined error models. The parameter estimates of the population PK model of CPA are shown in Table 3. In the final model, the typical values for clearance and apparent volume of distribution of CPA were 5.41 L/hr and 46.5 L, respectively. The estimated inter-individual variability (IIV) in clearance was 51% (38.9% and 49.3% for 500 and 600 mg/m^2 group, respectively) (Table 3). The mean clearance values of CPA were 3.97 and 5.81 L/h for patients who received 500 and 600 mg/m^2 based CPA regimen, respectively.

Overall, goodness of fit plots shows that one compartment model describes the observed plasma concentrations adequately (Figure 1). The plot of observed concentration *vs*. population predicted concentration show uniformly distributed points on either side of lines of identity (Figure 1 top panel). The conditional weighted residuals (CWRES) are within acceptable range and are uniformly spread when CWRES are plotted against population predictions and also against time (Figure 1, bottom panel). Moreover, QQ plot and histogram of CWREs indicated normal distribution demonstrating good fit (Figure 2). Analysis of prediction corrected visual predictive check (pcVPC plot) showed that, for the base and final model, the observed concentrations are within the 5th and 95th % of the prediction intervals. However, concentrations are under-predicted at lower concentrations (Figure 3).

Covariate testing in NONMEM identified CPA dosage regimen (CPA 500 mg/m^2 or 600 mg/m^2 based), as significant predictor of clearance and V_D of CPA. BSA was also significantly associated with V_D of CPA (Table 4). Accordingly, 500 mg/m^2 based CPA regimen was associated with a 32.3% lower than average clearance and 37.1% lower than average V_D of 600 mg/m^2. Similarly, a 0.1 m^2 unit increase in BSA was associated with a 5.6% increment in V_D of CPA.

On the other hand, analysis of variance showed statistically significant difference in mean V_D between BMI groups ($p < 0.001$). The mean V_D (33.5 L) in underweight group (BMI < 18.5 kg/m^2) was significantly lower compared to overweight group (BMI 25.0-29.9 kg/m^2) (48.1 L) ($p < 0.001$) or obese patients (BMI $\geq$ 30 kg/m^2) (51.9 L) ($p < 0.001$). In patients who received 600 mg/m^2, but not in 500 mg/m^2, K_{el} and $t_{1/2}$ of CPA was significantly different between BSA ($p < 0.001$, *ANOVA*). *Post hoc* analysis showed that patients with BSA > 1.75 m^2 had a longer half-life (6.71 hr) compared to those with BSA < 1.5 m^2 (4.78 hr) ($p < 0.001$) or BSA between 1.5-1.74 m^2 (5.42 hr) ($p = 0.004$).

In comparison to those with *CYP3A5*1/*1* genotype, patients carrying *CYP3A5*3* or **6* alleles had a lower elimination rate constant (0.128 *vs* 0.179 hr^{-1} for 500 mg/m^2 and 0.124 *vs*. 0.16 hr^{-1} for 600 mg/m^2 group) and longer half-life (5.42 *vs*. 3.88 hrs for 500 mg/m^2 and 5.58

vs. 4.34 hrs for 600 mg/m^2 group) ($p < 0.001$, *t*-test) (Figure 4a and b). Further controlling for effect *POR* genotype, the difference in mean K_{el} or $t_{1/2}$ between *CYP3A5* genotypes was evident among those with *POR*1/*1*, but not in *POR*28* carriers, in both CPA regimen groups (Figure 5). *CYP2C9 *2* or **3* carriers were also associated with increased clearance of CPA and reduced half-life (Figure 4c and d). On the other hand, in both CPA regimen group, with *POR*1/*1* genotype, Clearance of CPA was moderately associated with *CYP2C9* genotype. In patients carrying *POR*28,* increased clearance was significantly associated in carriers of *CYP2C9 *2* or **3* alleles (Figure 6).

Pharmacodynamic Modeling

The linear direct response (empiric) model was successfully fitted to the neutrophil count. The goodness of fit plots is depicted in Figure 7. Combined proportional and additive error model performed well compared to the additive or proportional error models. The parameter estimates for the slope, and ANC_o were -1.42 and 3450, respectively. Accordingly, a one unit increase in the AUC of CPA was associated with a decrease in the neutrophil count by 1.42.

Discussion

In the present study, we developed population pharmacokinetics, pharmacogenetics and pharmacodynamics (PK-PG-PD) model for CPA in Ethiopian breast cancer patients at conventional CPA regimen. Our major findings include (i) PK of CPA was adequately described by one-compartment model, with estimated population clearance of 5.41 L/hr and V_D of 46.5 L, (ii) presence of large inter-individual variability in CPA clearance (51%), (iii) CPA dosage regimen, BSA, BMI, *CYP2C9* and *CYP3A5* genotypes significantly influence PK of CPA, and (iv) a positive correlation between CPA exposure (AUC) and neutropenic toxicity. To our knowledge, this is the first study to investigate the population PK-PD model for CPA in breast cancer patients from Sub-Saharan Africa.

Our result indicates that one-compartment model better described CPA concentration data, which is in agreement with previous reports (Timm et al., 2005; Joerger et al., 2007). In contrast, other studies described PK parameters using two-compartment model (Nakajima et al., 2007; Ekhart et al., 2008). These findings may suggest that the simple one or two-compartment models may be sufficient to describe PK parameters in conventional doses of CPA administered by short IV infusion. On the other hand, earlier studies demonstrated that when CPA is administered in a high dose regimen (*e.g*., 4000 mg/m^2) and by short infusion, a concentration-dependent PK model of CPA was developed with convex downward elimination curves (Chen et al., 1995, 1997). A time-dependent PK model was also described in patients receiving high dose CPA (6000 mg/m^2) in a prolonged infusion (96 hours) (Chen et al., 1995). Moreover, other studies developed a PK model for CPA incorporating the effect of auto-induction (Hassan et al., 1999) and the phenomena of both auto-induction and drug-drug interaction between CPA and thioTEPA (Huitema et al., 2001). These findings show that, in addition to other factors, PK of CPA could be influenced by the dosing history and the duration of IV infusion.

The volume of distribution estimated in this study (46.5 L) is also in agreement with a previous report (de Jonge et al., 2005). Given that one-compartment PK model described our data, together with V_D which approximates to the total body water; it is plausible that CPA achieves instantaneous distribution throughout the body. The population clearance of 5.41 L/hr estimated in the present study is also in agreement with those reported in breast cancer patients from France (Joerger et al., 2007) but higher compared to the finding from Swedish and Japanese breast cancer patients (Hassan et al., 1999; Nakajima et al., 2007; Ekhart et al., 2008). Indeed, higher CYP enzyme activities including *CYP3A* in Ethiopian population compared to others have been reported previously (Gebeyehu et al., 2011; Aklillu et al., 2014), which may explain the higher CPA clearance rate in Ethiopians than Swedish or Japanese breast cancer patients.

After covariate analysis in NONMEM, CPA regimen caused a reduction of coefficient of variation from 51 to 44.2% and thus accounting for 6.8% of the variability. Thus, larger part (44.2%) of the variability in CPA clearance remains unexplained. Similarly, substantial variations in PK and exposure to CPA and its metabolites have been reported previously in both adults and children (de Jonge et al., 2005). These variations could be explained either by the differences in the level of expression of specific CYP enzymes or the level of activity of the individual enzymes (Zanger and Schwab, 2013) or other non-genetic factors (de Jonge et al., 2005). Results of previous studies regarding the roles of the individual CYP enzymes' genes to PK differences of CPA are inconsistent. In this study, the variant alleles in *CYP2B6, CYP2C19, CYP2J2*, and *ABCB1* were not associated with PK of CPA. In agreement with our finding, previous studies reported that polymorphisms in *CYP2B6* and *CYP2C19* did not have significant role in variations of CPA PK (Ekhart et al., 2008; Fernandes et al., 2011; Afsar et al., 2012; Raccor et al., 2012). The lack of association of *ABCB1* genotype was reported by a previous study (Kim et al., 2013).

On the other hand, the findings of Xie et al. demonstrated, using human liver microsomes, that patients with *CYP2B6* G516T variants had a two-fold higher CPA clearance compared to those carrying the wild type (Xie et al., 2003). In Japanese breast cancer patients, those homozygous for *CYP2B6**6 (Q172H and K262R) had higher clearance than heterozygous or homozygous for *CYP2B6**1 (Nakajima et al., 2007). In another study, *CYP2C19*2* variant allele was associated with lower elimination rate constant in individuals receiving < 1000 mg/m2 of CPA (Timm et al., 2005), which was not evident at higher doses. Notably, a significantly increased elimination rate of CPA was observed at CPA doses > 1000 mg/m2, possibly due to CYP induction at higher doses (Timm et al., 2005). In our study, a dose-dependent clearance of CPA was also observed. Patients receiving 600 mg/m2 had significantly higher clearance as compared to 500 mg/m2 CPA dosing (5.81 vs. 3.97 L/h, $p < 0.001$). CPA induces *CYP2C* and *CYP3A4* in human hepatocytes by increasing the enzyme production rate and thereby increasing the amount of enzyme by which CPA is metabolized (Hassan et al., 1999).

The present study also revealed that carriers of *CYP3A5*3* or **6* had lower mean elimination rate constant and prolonged half-life compared to those with *CYP3A5*1/*1* genotype ($p < 0.001$). In contrast, *CYP2C9 *2 or *3* carriers were associated with increased clearance of CPA. The observed effect of *CYP2C9* on CPA clearance was unexpected, as *CYP2C9 *2* or **3* alleles are defective variant alleles associated with reduced *CYP2C9* enzyme activity (Griskevicius et al., 2003) resulting in reduced clearance of the drug.

However, our finding is supported by Balasubramanian *et al* that reported that *CYP2C9 *2* allele increased CPA clearance and decreased its AUC (Balasubramanian et al., 2009). These findings may suggest that the extent and pattern of CYP activity and induction or inhibition may be variable among various patient populations. For example, *POR*28* was demonstrated to significantly alters *CYP2C9* activity in Swedish, but not in Korean healthy subjects (Hatta and Aklillu, 2015b). In the present study, increased CPA clearance was significantly higher in carriers of *CYP2C9 *2* or *3* alleles who also carry *POR *28* allele. In both CPA regimen group and with *POR*1/*1* genotype, clearance of CPA was moderately associated with *CYP2C9* genotype. Moreover, multiple competing CYP pathways involved in the biotransformation and clearance of CPA might also play a prominent role in *CYP2C9* defective patients, compensating for the absence of *CYP2C9* activity. Furthermore, previous studies have shown higher CYP enzyme activities including *CYP3A* in Ethiopian population (Gebeyehu et al., 2011; Aklillu et al., 2014), which may explain the higher CPA clearance rate in the present study population.

A previous study demonstrated that *CYP2J2* expression was significantly up-regulated during CPA treatment and the bio-activation of the drug was significantly correlated to *CYP2J2* expression (El-Serafi et al., 2015a). *CYP2J2*, expressed in high levels particularly in extra-hepatic organs such as the heart, intestine and urinary bladder, may be responsible for the local CPA bio-activation and may explain CPA treatment-related toxicities (Hashizume et al., 2002; Matsumoto et al., 2002; Lee et al., 2010). In a recent cohort study investigating the association of pharmacogenetic variations to hematologic toxicity in Ethiopian breast cancer patients, we reported that patients carrying *CYP2J2*7* variant alleles were associated with higher incidence of hematological toxicity (Ahmed et al., 2019). However, the present study could not identify association of *CYP2J2* genetic polymorphism and PK of first cycle CPA. The discrepancy could be explained by the increased *CYP2J2* expression as well as CYP induction in the subsequent chemotherapy cycles, which could facilitate formation of active metabolite and contributing for the increased incidence of hematologic toxicity observed in the course of the chemotherapy cycles.

Cytochrome P450 oxidoreductase (*POR*), a flavoprotein that supplies electrons to all CYP enzymes for their catalytic activity (Hart et al., 2008), was implicated to influence CYP catalyzed bio-activation of xenobiotics (Hu et al., 2012). In this study, *POR* was found to alter *CYP3A5* activity. Among patients with *POR*1/*1*, the mean elimination constant (K_{el}) or half-life ($t_{1/2}$) between *CYP3A5* genotypes was significantly different. However, the effect was not evident in *POR*28* carriers. A previous study demonstrated a significant correlation between CPA clearance and *POR/CYP* ratio (El-Serafi et al., 2015b), which might signify the importance of the level of expression of the gene in CPA metabolism and clearance.

The major toxic effect of CPA at conventional doses and the primary reason for a change in the schedule of CPA based chemotherapy delivery to breast cancer patients are treatment associated neutropenia (Kozma et al., 2012). Consequently, one aspect of optimizing cancer chemotherapy involves describing neutropenic toxicity as a function of drug exposure. The present study demonstrated a positive relationship between AUC of CPA and neutropenic toxicity. A previous study also showed that breast cancer patients with complete response had significantly higher CPA AUC than patients with progressive disease (Joerger et al., 2007). The greater part of total systemic clearance of CPA is non-renal clearance (de Jonge et al., 2005), and hence, these findings may suggest that exposure to CPA may be predictive of

the amount of activated metabolite formed. High exposure to bioactivated CPA was also reported to relate to the occurrence of venous-occlusive disease of the liver following high-dose CPA (de Jonge et al., 2006). In contrast, an inverse association was also reported between the AUC of CPA and its treatment-related cardiotoxicity and event-free survival in women with breast cancer (Ayash et al., 1992; Petros et al., 2002). Patient with significantly lower AUC developed congestive heart failure in a high-dose CPA regimen (Ayash et al., 1992). Treatment effectiveness and occurrence of adverse effects like heart failure were also inversely related to CPA AUC in high-dose therapy (Petros et al., 2002). On the other hand, other studies could not establish association between CPA exposure and toxicity or relapse free survival in cancer patients treated with high-dose chemotherapy (Nieto et al., 1999; Veal et al., 2016). The discrepancies in the results of the various studies could be attributed to the differences in the sample size, patient population and richness of the PK and PD data in the individual studies.

The present study describes the population PK and PD of the first cycle CPA in a resource-limited setting. However, important limitations are also to be noted. First, the metabolite concentration was not determined and consequently, it was not possible to incorporate it in the PK-PD model. In addition, as CPA is commonly used in combination with other chemotherapeutic agents in breast cancer treatment, their contribution in the observed neutropenic toxicity would be expected. Moreover, concentration and response were collected at different time points. Owing to time-dependent signal transduction in hematopoiesis, the simple empiric model adopted in this study may not account for this time lag between drug concentration and observed effects of CPA and the results also may not reflect the underlying physiological process. The PD observations (data points) in our study were also few (taken at baseline and day 20 post CPA administration) which might have hampered the successful development of the semi-mechanistic model.

In conclusion, the PK profile of CPA is described by one-compartment model in Ethiopian breast cancer patients. CPA displays wide between patient variability in clearance, which is partly explained by variation in CPA dosage regimen, BSA, BMI, *CYP3A5,* and *CYP2C9* genotypes. CPA plasma exposure predicts chemotherapy-associated neutropenic toxicity. Further studies are recommended to explore the PK of the metabolite (4-Hydroxycyclophosphamide), incorporating auto-induction by CPA and drug interaction and correlate its impact in neutropenic toxicity. Moreover, PK-PD modeling involving the contribution of other anticancer drugs in CPA containing regimen needs to be explored.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article

Abbreviations

ALP: Alkaline phosphatase; **ALT**: alanine aminotransferase; **ANC**: Absolute neutrophil count; **ANOVA**: analysis of variance**; AST**; aspartate aminotransferase; **AUC**: area under the curve; **BMI**: body mass index; **BSA**: body-surface area; **BUN**; blood urea nitrogen; **CI**:

confidence intervals; **CL:** clearance**; CPA:** cyclophosphamide; **CV**: coefficient of variation; **CWRES**: conditional weighted residuals; **FOCE**: First-order conditional estimation; **GOF**: goodness of fit; **HWE**: Hardy-Weinberg equilibrium; **HIV**: human immunodeficiency virus; **IIV**: inter-individual variability; **IRB**: Institutional Review Board; K_{el}: eliminaton constant; NONMEM: non linear mixed effect modeling; **OFV**: objective function value; **pcVPC**: prediction corrected visual predictive check; **PD**: pharmacodynamics; **PG**: Pharmacogentics; **PK**: Pharmacokinetics; **SCM**: stepwise covariate modeling; **SCr**; serum creatinine; $\mathbf{t_{1/2}}$: half-life; $\mathbf{V_D}$: volume of distribution; **WBC**: white blood cells.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was supported by the Armauer Hansen Research Institute (AHRI) through the BSPP Program, a grant obtained from Sida-Ethiopia Bilateral Program (Contribution No: 5108013506). The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Acknowledgment

The authors would like to extend their sincere appreciation to Armauer Hansen Research Institute (AHRI) for the financial assistance required for the study.

Author's contribution

JHA, EM, and EA designed the study. JH collected the data. JH and EM did the genotyping. JH, RKB and EA analyzed the data and drafted the manuscript. JH, EA, EM, RKB, JKM, AA, RW, AF and MH involved in the discussion of results and critical review of the manuscript. All the authors have read and approved the final manuscript.

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722 **Table 1.** Baseline socio-demographic, clinical and laboratory values of participants

Variables*	**Values (mean/median)**	**Values N (%)**
Socio-demographic		
Age (years; median + IQR)	38 (33 – 48)	-
BSA (m^2; mean ± SD)	1.59 ± 0.20	-
BMI (kg/m^2; mean ± SD)	23.78 ± 4.8	-
Baseline differential blood parameters		
Neutrophils (10^3 cells/mm^3; median + IQR)	3645.5 (2645 - 4765)	-
Hemoglobin (g/dL; mean + SD)	13.91 ± 1.30	-
Platelet (10^3 cells/mm^3; median + IQR)	294 (249 – 346)	-
Baseline Liver function estimates		
ALT (U/L; median (IQR)	18 (13 - 27)	-
AST (U/L; median (IQR)	24 (19-31)	-
ALP (U/L; median (IQR)	208.5 (155 - 293)	-
Baseline Renal function estimates		
SCr (mg/dL; mean (± SD)	0.91 ± 0.2	-
BUN (mg/dL; median (IQR)	18.80 (14 – 24)	-
CRCL (mL/min; median (IQR)	76.40 (60.5 - 93.4)	-
CPA regimen		
500 mg/m^2 based	-	106 (39.7)
600 mg/m^2 based	-	161 (60.3)
Cardiovascular co morbidity		
Yes	-	23 (8.6)
No	-	244 (91.4)
Presence of HIV		

Yes	-	9 (3.4)
No	-	258 (96.6)

*ALP Alkaline phosphatase, ALT alanine aminotransferase, ANC absolute neutrophil count, AST aspartate aminotransferase, BUN, Blood urea nitrogen, BSA body surface area, CRCL Creatinine clearance, Hgb hemoglobin, HCT hematocrit, IQR inter quartile range, PLT platelet count, SCr Serum creatinine, SD standard deviation, WBC white blood cell count.

Table 2. Genotype and allele frequency distribution of candidate genes relevant to CPA activation or transport.

Gene	Genotype	Genotype frequency	Minor Allele frequency	
		N (%)	Allele	N (%)
CYP2B6	*1/*1	114 (42.7)	**6*	185 (34.6)
	*1/*6	121 (45.3)		
	*6/*6	32 (12)		
CYP2C9	*1/*1	229 (85.8)		
	*1/*2 or *1/*3	35 (13.1)	**2*	36 (6.74)
	*2/*2 or *3/*3	3 (1.1)	**3*	6 (1.12)
CYP2C19	*1/*1	188 (70.4)		
	*1/*2 or *1/*3	74 (27.7)	**2*	80 (15.0)
	*2/*2 or *3/*3	5 (1.9)	**3*	8 (1.5)
CYP3A5	*1/*1	32 (12)		
	*1/*3 or *1/*6	105 (39.3)	**3*	325 (60.9)
	*3/*3 or *6/*6	130 (48.7)	**6*	75 (14.0)
CYP2J2	*1/*1	197 (73.8)		
	*1/*7	62 (23.2)	**7*	78 (14.6)
	*7/*7	8 (3.0)		
POR	*1/*1	189 (70.8)		
	*1/*28	69 (25.8)	**28*	87 (16.3)
	*28/*28	9 (3.4)		
ABCB1 (rs3842, A>G)	*AA*	193 (79.1)		
	AG	44 (18)	*G*	58 (11.9)
	GG	7 (2.9)		

Table 3. Population pharmacokinetic parameter estimates

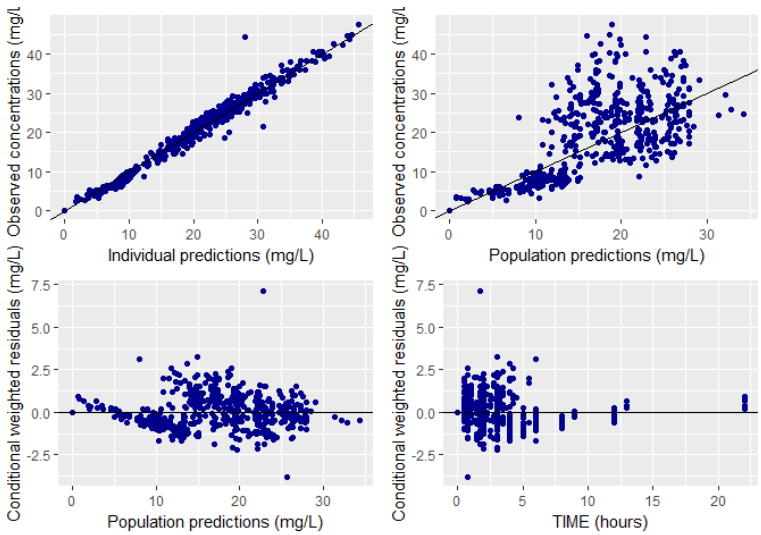
Description	OFV[δ]	Population estimate		Between subject variability (%SE)
		Parameter	%SE[γ]	
Base model	2372.44	CL	5.34 (4.4)	51% (8)
		V_D	30.4 (8.4)	44.7% (21)
Final model	2257.13	CL	5.41 (8.4)	44.2% (10)
		V_D	46.5 (13.2)	35.9% (11)
Residual variability				
Additive error 1		1.54 mg/L (24%)		
Additive error 2		0.0001 mg/mL		
Derived PK Parameters				
Elimination constant (K_{el}, hr^{-1}; median, IQR[†])		0.126 (0.1- 0.166)		
Half-life (hr, median, IQR)		5.48 (4.18 – 6.91)		
$AUC^{‡}_{0\text{-}day20}$ (µmol.h/L; median, IQR)		565.7 (444.9 - 828.1)		

[‡]AUC area under the curve; [†]IQR inter-quartile range; [δ]OFV objective function value; [γ]SE standard error.

Table 4. Covariate model analysis of cyclophosphamide

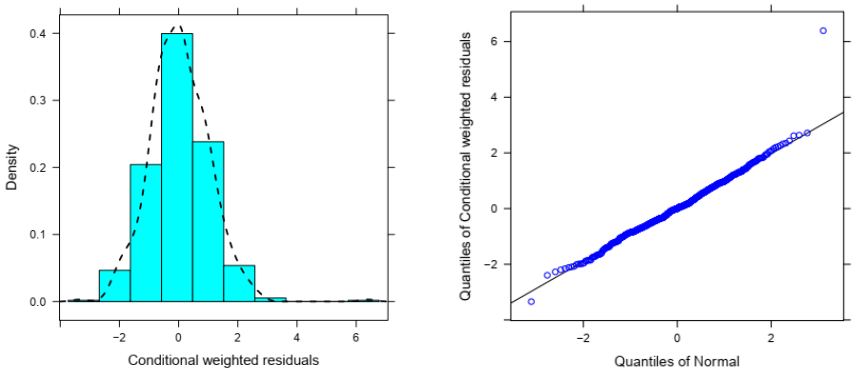
Parameters	Covariate	Covariate model	
		Covariate effect	THETA estimate
Clearance (CL)	CPA[♣] dose		
	500 mg/m^2	(1 + THETA1)	-0.323
	600 mg/m^2	1 (ref)	
Volume (VD)	BSA£	(BSA/1.58)**THETA2	0.861
	CPA dose		
	500 mg/m^2	(1 + THETA1)	-0.371
	600 mg/m^2	1 (ref)	

[£] BSA body surface area; [♣]*CPA* cyclophosphamide



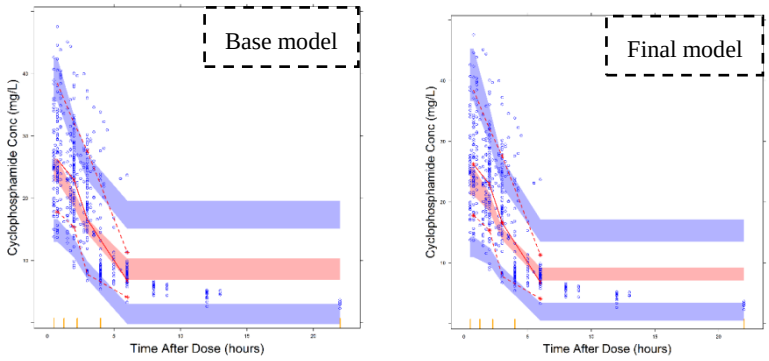
748

749 **Figure 1**. Goodness of fit plots for the final population PK model of CPA
750 (prediction-based (top panel) and residual based (bottom panel) goodness of fit
751 plots.



752

753 **Figure 2.** The distribution (histogram (left) and QQ plots (right) of CWRES



754

755 **Figure 3.** Prediction corrected visual predictive check plots for the base model and
756 final covariate models. The blue dots represent the distribution of observed plasma
757 CPA concentrations and the broken lines represent the 5th (bottom) and 95th
758 percentile (top line) of the median observed data. The shaded regions represent the
759 95% prediction interval for the observed concentrations.

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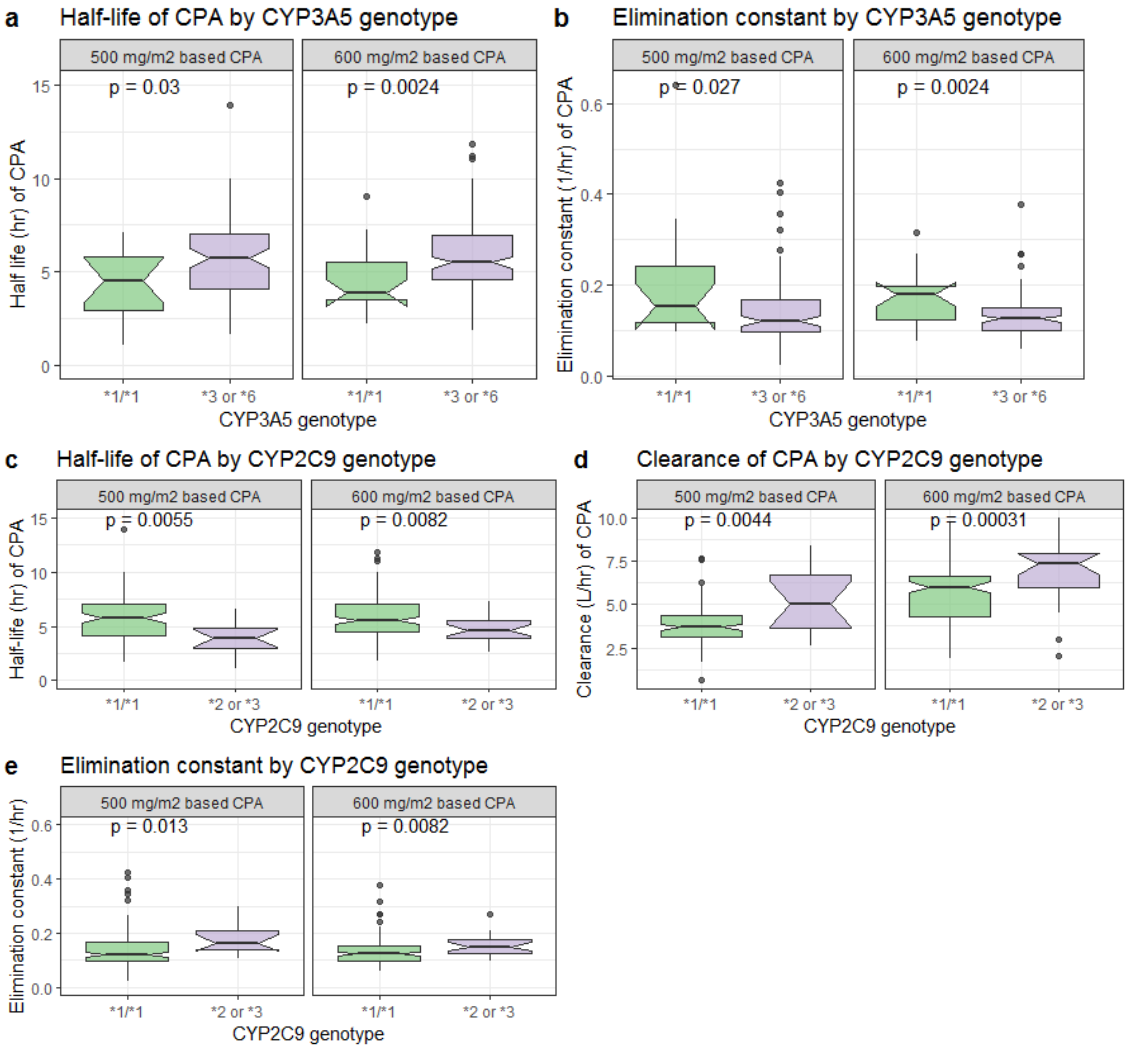
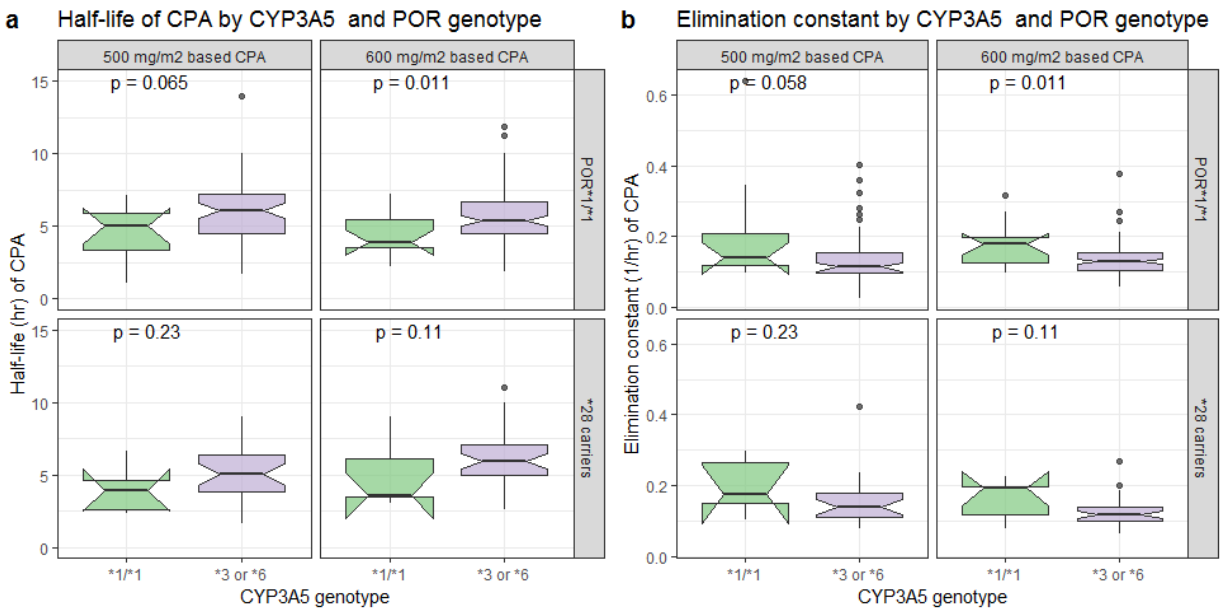


762

763 **Figure 4.** Clearance, elimination constant and half-life of cyclophosphamide by
764 *CYP3A5* (Figures a and b) and *CYP2C9* (Figures c, d and e) genotypes.

765

766



767

768 **Figure 5.** Comparison of half-life (a) and elimination constant (b) of
769 cyclophosphamide by *CYP3A5* and *POR* genotype.

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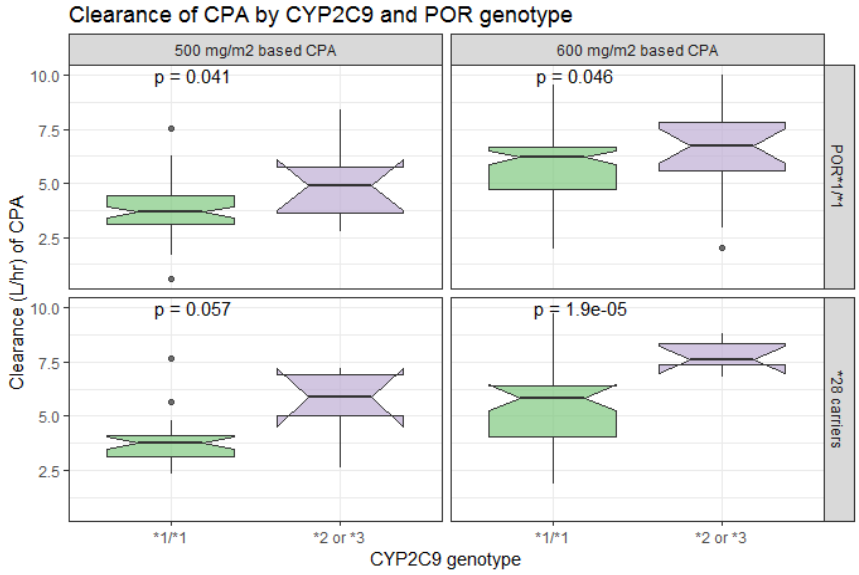
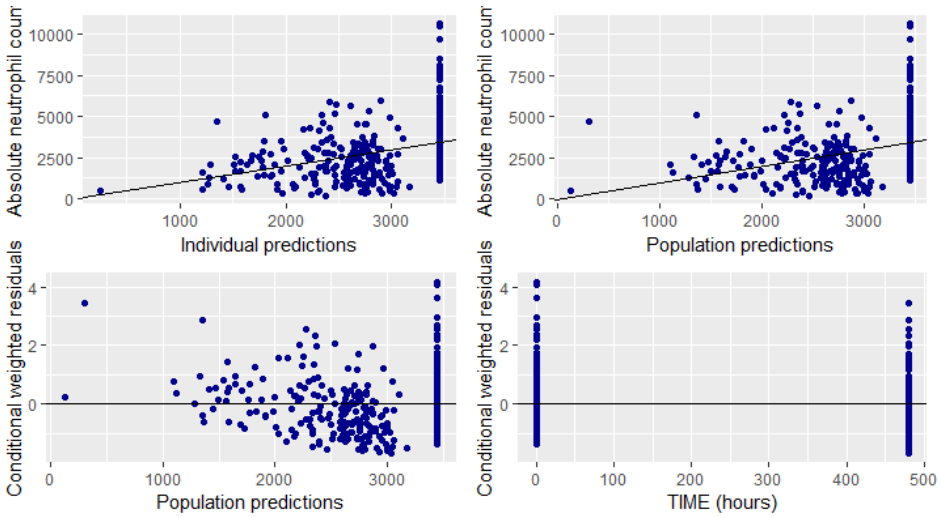


771

772 **Figure 6.** Clearance of cyclophosphamide by *CYP2C9* and *POR* genotype.



773

774 **Figure 7**. Goodness of fit plots for the final population PD model (absolute
775 neutrophil count, individual and population predictions were all in 10^3 cells/mm^3).

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Article

CYP2D6 Genotype Predicts Plasma Concentrations of Tamoxifen Metabolites in Ethiopian Breast Cancer Patients

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Received: 31 July 2019; Accepted: 6 September 2019; Published: 12 September 2019

Abstract: Tamoxifen displays wide inter-individual variability (IIV) in its pharmacokinetics and treatment outcome. Data on tamoxifen pharmacokinetics and pharmacogenetics from black African breast cancer patient populations is lacking. We investigated the pharmacokinetic and pharmacogenetic profile of tamoxifen and its major active metabolite, endoxifen, in Ethiopian breast cancer patients. A total of 81 female breast cancer patients on adjuvant tamoxifen therapy were enrolled. Tamoxifen (Tam) and its major metabolites, N-desmethyltamoxifen (NDM), 4-hydroxy-tamoxifen (4-HT), and (Z)-endoxifen (E) were quantified using LC-MS/MS. Genotyping for *CYP2D6*, *CYP2C9*, *CYP2C19*, *CYP3A5*, *POR*, and *ABCB1* and *UGT2B15* and copy number variation for *CYP2D6* were done. The proportion of patients with low endoxifen level (<5.9 ng/mL) was 35.8% (median concentration 7.94 ng/mL). The allele frequency of *CYP2D6* gene deletion (*5) and duplication (*1×N or *2×N) was 4.3% and 14.8%, respectively. Twenty-six percent of the patients carried duplicated or multiplicated *CYP2D6* gene. An increase in *CYP2D6* activity score was associated with increased endoxifen concentration and $MR_{E/NDM}$ ($p < 0.001$). The IIV in endoxifen concentration and $MR_{E/NDM}$ was 74.6% and 59%, respectively. *CYP2D6* diplotype explained 28.2% and 44% of the variability in absolute endoxifen concentration and $MR_{E/NDM}$, respectively. The explanatory power of *CYP2D6* diplotype was improved among *ABCB1c.4036G* carriers (43% and 65.2%, respectively for endoxifen concentration and $MR_{E/NDM}$) compared to *A/A* genotype. *CYP2C9*, *CYP2C19*, and *CYP3A5* genotypes had no significant influence on endoxifen concentration or $MR_{E/NDM.}$ In conclusion, we report a high rate of low endoxifen level as well as large IIV in tamoxifen and its metabolite concentrations. *CYP2D6* is significant predictor of plasma endoxifen level in a gene-dose dependent manner.

Keywords: tamoxifen; endoxifen; pharmacokinetics; pharmacogenetics; *CYP2D6*; *ABCB1*; breast cancer

1. Introduction

Tamoxifen is widely used as adjuvant therapy for women with estrogen receptor-positive breast cancer [1]. Its use for 5 years has been shown to be highly effective in reducing breast cancer recurrence and cancer-specific mortality [2]. Despite its proven clinical effectiveness, not all breast cancer patients

gain benefit from tamoxifen treatment; approximately 30–50% of patients relapse or eventually die from the disease [2]. Some individuals also experience treatment associated side effects [3].

The causes for breast cancer relapse or occurrence of side effects after tamoxifen treatment are attributed to a variety of factors, including pharmacogenetic variations in relevant drug-metabolizing enzymes involved in the metabolic activation of tamoxifen into endoxifen [1,4]. The bioactivation of tamoxifen involves several CYP450 drug metabolizing enzymes (DMEs) including, *CYP2D6*, *CYP3A4*, *CYP3A5*, *CYP2C9*, and *CYP2C19*, with key roles for *CYP2D6* and *CYP3A* [5]. The wide interindividual variability in the concentration of tamoxifen and its metabolites was mainly attributed to genetic polymorphism in *CYP2D6* [3,6–8]. Previous studies showed that patients with low *CYP2D6* enzyme activity or taking medication which inhibit *CYP2D6* activity were associated with lower endoxifen concentration [7,9]. Other studies also demonstrated that patients exhibiting endoxifen concentration below a proposed therapeutic threshold (5.9 ng/mL), were significantly associated with a higher risk of recurrence or death [3,10].

The link between patients' *CYP2D6* genotype and plasma levels of tamoxifen and its metabolites has been established in population of European and Asian descent [10–12], but not in sub-Saharan populations. Studies also showed that *CYP2D6* explains about 39–58% of the interpatient variability in endoxifen concentration [8] and residual variability is largely unexplained [7,12]. The extent to which *CYP2D6* determines the wide interpatient variability of endoxifen under standard tamoxifen treatment is not well characterized in black African populations. Although *CYP2D6* is the predominant enzyme in the metabolic conversion of tamoxifen into endoxifen, previous studies reported inconsistent findings about the association between *CYP2D6* polymorphism and clinical outcome of tamoxifen therapy.

The polymorphic *CYP2D6* gene exhibits pronounced interethnic differences in allele frequency distribution. Ethnic differences in tamoxifen metabolism and treatment outcome are also well recognized [13,14]. Reports on the association of *CYP2D6* genotype with clinical outcome in different population is not consistent, and hence lack of consensus to recommend genotype-based personalized tamoxifen treatment. About 29% of Ethiopians carry functionally active *CYP2D6* gene duplications and multiplications associated with increased enzyme activity [15]. Given this unique *CYP2D6* genotype constitution, the pharmacokinetic profile of tamoxifen in this population may have important implications for tamoxifen dosing recommendations to improve clinical effectiveness of the treatment. *CYP2C9*, *CYP2C19*, and *CYP3A5* are also involved in tamoxifen metabolism, but the impact of their genotype on the pharmacokinetics (PK) of tamoxifen are conflicting [4,6]. Furthermore, data on the impact of other genetic variations such as the role of *POR* and non-genetic determinants on endoxifen plasma concentration is limited. There is growing evidence that altered *ABCB1* activity may affect tamoxifen pharmacokinetics and possibly tamoxifen efficacy in breast cancer patients. The primary active tamoxifen metabolites, endoxifen and 4-hydroxytamoxifen, are substrates for *ABCB1* [16]. The three common SNPs in the protein coding region of *ABCB1* gene (rs1128503 (1236T>C), rs2032582 (2677T>G/A), and rs1045642 (3435T>C) have been the focus of many pharmacokinetic and disease association studies [17] with inconsistent and controversial results [18]. Other studies have also investigated the relationship between the C3435T polymorphism of *ABCB1* gene and risk of breast cancer; whose results are also conflicting [19,20]. On the other hand, the relevance of *ABCB1*c.4036A>G (rs3842A>G) has, however, been consistently demonstrated in African population in relation to the PK of antiretroviral drugs. Several studies have showed that *ABCB1*c.4036A>G (rs3842A>G) is a significant predictor of plasma and intracellular efavirenz concentration in Uganda [21,22], South Africa [23], Ethiopia and Tanzania [24] as well as in other populations [25]. We hypothesize that this SNP may also influence cellular transport of tamoxifen metabolites in our study population. In the present study, we investigated the pharmacokinetics profile and the influence of *CYP2D6*, *CYP2C9*, *CYP2C19*, *CYP3A5*, *POR*, *ABCB1* and *UGT2B15* polymorphisms on the plasma level of tamoxifen and its active metabolites in Ethiopian breast cancer patients receiving adjuvant tamoxifen.

2. Results

2.1. Sociodemographic Characterstics

A total of 89 patients (mean age = 39 ± 8.5) who were on tamoxifen treatment were enrolled. Of these, eight patients were excluded as the plasma level of tamoxifen and endoxifen were below the limit of quantification, possibly related to noncompliance to therapy. The remaining 81 patients, all with tamoxifen concentration >150 nM were included in the analysis. All the patients completed full course of chemotherapy after primary surgical treatment (modified radical mastectomy). The median duration of tamoxifen use was 11 months (IQR 6–18 months). The socio-demographic and tumor characteristics are displayed in Table 1.

Table 1. Socio-demographic and tumor characteristics of tamoxifen treated breast cancer patients at the radiotherapy center, Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia.

Parameters		Value
Socio-demographics		
Age (years, mean ± SD *)		39 ± 8.5
BMI (kg/m^2, mean ± SD)		24.6 (±3.86)
Chemotherapy regimen used		**N (%)**
FAC $^{\propto}$		37 (45.7)
AC		11 (11.1)
AC - T		35 (43.2)
Duration of tamoxifen use (months, median + IQR $^{\clubsuit}$)		11 (6–18)
Menopausal status		**N (%)**
Premenopausal		56 (69.1)
Postmenopausal		25 (30.9)
Tumor characteristics		**N (%)**
Histologic type of tumor	Ductal	79 (97.5)
	Lobular	2 (2.5)
Degree of differentiation	Well differentiated	21 (25.9)
	Moderately differentiated	44(54.3)
	Poorly differentiated	16 (19.8)
Lymph node involvement	Negative	15 (18.5)
	Positive	66 (81.5)
Distant metastatic site	No known distant metastasis	10 (12.3)
	Bone, lymph node, or lung only	63 (77.8)
	Liver, CNS, lung + other organs	8 (9.9)

$^{\clubsuit}$ IQR inter quartile range; * SD standard deviation; $^{\propto}$ FAC (5-Flourouracil 500 mg/m^2, Adriamycin [Doxorubicin] 50 mg/m^2, and Cyclophosphamide 500 mg/m^2), AC (Adriamycin 50 mg/m^2 and Cyclophosphamide 600 mg/m^2), and AC-T (four cycles of Adriamycin 60 mg/m^2 and Cyclophosphamide 600 mg/m^2 followed by another 4 cycles of Taxol 175 mg/m^2).

SNP genotyping results for *CYP2D6*, *CYP2C9*, *CYP2C19*, *CYP3A5*, *POR*, *ABCB1* and *UGT2B15* are presented in Table 2. All the genotype frequencies were in Hardy-Weinberg Equilibrium ($p > 0.05$). A total of 20 (25.9%) patients had *CYP2D6* copy number of 3 or more. The allele frequencies of *CYP2D6* *5 (gene deletion), and *1 or *2 gene duplication/multiplication were 4.3 and 14.8%, respectively. The frequencies of *CYP2D6* activity score (AS) and phenotype categories are presented in Table 3. Based on the CPIC *CYP2D6* phenotype assignment, 61.7 and 22.2% of the patients are predicted to be normal metabolizers (NMs) and ultra-rapid metabolizers (UMs), respectively. The prevalence of poor (PMs) and intermediate metabolizers (IMs) was low, accounting for 1.2 and 3.7%, respectively.

Table 2. The genotype and allele frequency distribution of tamoxifen metabolizing enzymes' and transporter genes.

Gene	Variant allele	Allele frequency (%)
CYP2D6	*2	33.3
	*4	4.9
	*5	4.3
	*10	1.9
	*17	10.5
	*1×N or *2×N †	14.8
CYP2C9	*2	4.3
	*3	7.4
CYP2C19	*2	11.7
	*3	1.2
CYP3A5	*3	67
POR	*28	12.4
ABCB1 c.4036A>G	G	14.8
ABCB1 c.3435C>T	T	16.9
*UGT2B15 *2*	T	20.2
*UGT2B15 *4*	C	40.3

† Number of *CYP2D6* gene copies.

Table 3. *CYP2D6* activity score and plasma concentrations of tamoxifen and its metabolite and respective metabolic ratios.

***CYP2D6* Activity Score**	**Phenotype Group †**	**N (%)**
>2	UM ¥	18 (22.2)
2	NM	37 (45.7)
1.5	NM	12 (14.8)
1	NM or IM	10 (12.4)
0.5	IM	3 (3.7)
0	PM	1 (1.2)
Plasma concentrations	**values**	**CV (%) ‡**
Tamoxifen (ng/mL, median + IQR #)	138 (105–200.5)	56.2
N-Desmethyl-tamoxifen (ng/mL, median + IQR)	257 (185.5–343)	53.1
4-Hydroxy-tamoxifen (ng/mL, median + IQR)	3.04 (2.15–4.0)	61
Z-Endoxifen (ng/mL, median + IQR)	7.94 (4.68–13.75)	74.7
Metabolic ratio	**values**	**CV (%) ‡**
$MR^{\varepsilon}_{E/NDM}$ (median + IQR)	0.038 (0.021–0.06)	59
$MR^{\gamma}_{NDM/Tam}$ (median + IQR)	1.81 (1.51–2.26)	28.6
$MR^{\lambda}_{4\text{-}HT/Tam}$ (median + IQR)	0.022 (0.016–0.029)	38.8
$MR^{\spadesuit}_{E/4\text{-}HT}$ (median + IQR)	2.85 (2.10–3.73)	43.9

† Phenotype translated based on Clinical Pharmacogenetics Implementation Consortium (CPIC) guideline for *CYP2D6* and tamoxifen therapy; ¥ UM ultrarapid metabolizer; NM normal metabolizer; IM intermediate metabolizer; PM poor metabolizer; ‡ coefficient of variation; # IQR interquartile range; $^{\varepsilon}$ metabolic ratio of endoxifen to N-desmethyl-tamoxifen; $^{\gamma}$ metabolic ratio of N-desmethyl-tamoxifen to tamoxifen; $^{\lambda}$ metabolic ratio of 4-Hydroxy-tamoxifen to tamoxifen; $^{\spadesuit}$ metabolic ratio of endoxifen to 4-hydroxy-tamoxifen.

2.2. Plasma Tamoxifen and Its Metabolite Concentration Profile

The plasma concentrations of tamoxifen and its metabolites are depicted in Table 3. The median concentration of endoxifen (7.94 ng/mL), was 2.6 times higher than 4-HT (3.04 ng/mL). The proportion of patients with low endoxifen concentration (<5.9 ng/mL) was 35.8%. This low level of endoxifen was detected in patients classified as PM/PM (1/1), IM/PM (3/3), IM/IM (1/1), NM/PM (6/9, 66.7%), NM/IM

(5/12, 41.7%), and NM/NM (11/35, 31.4%) diplotypes. There was pronounced interindividual variability in endoxifen concentration (coefficient of variation 74.7%) and in metabolic ratio of endoxifen to NDM (coefficient of variation 59%) (Table 3).

Pearson correlation analysis between tamoxifen and its metabolites revealed a strong positive association between tamoxifen and formation of NDM (Pearson correlation coefficient (R) = 0.89), and tamoxifen and formation of 4-HT (R = 0.66) (Figure 1). Thus, an increase in the concentration of tamoxifen was associated with a corresponding increase in NDM and 4HT levels. Formation of endoxifen is also strongly correlated with 4-HT level (R = 0.86). However, the correlation between NDM and formation of endoxifen (R = 0.38) as well as tamoxifen and formation of endoxifen (R = 0.48) was moderate.

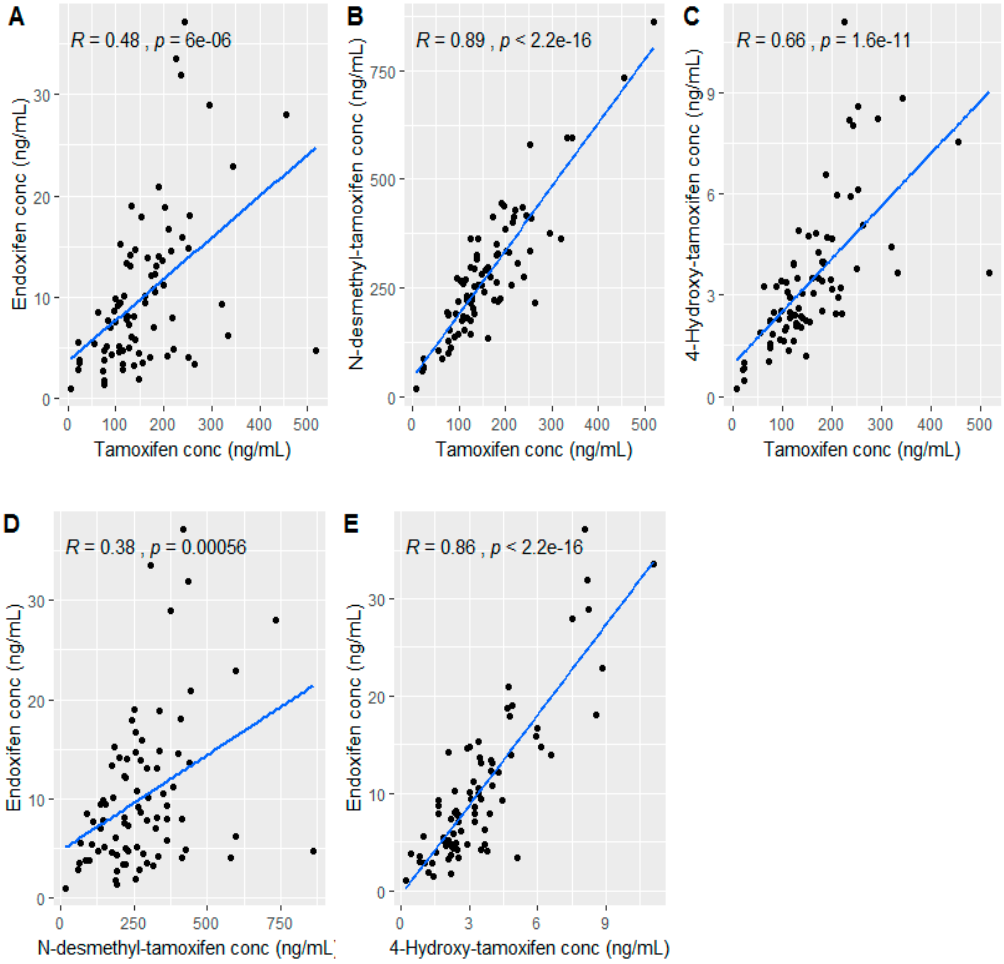

Figure 1. Scatter plot showing the Pearson's correlation between endoxifen versus tamoxifen (**A**), N-desmethyl-tamoxifen versus tamoxifen (**B**) and 4-hydroxy-tamoxifen versus tamoxifen (**C**), endoxifen versus N-desmethyl-tamoxifen (**D**) and endoxifen versus 4-hydroxy-tamoxifen (**E**).

2.3. Association of Pharmacogenetics and Pharmacokinetics of Tamoxifen Its Metabolites

Analysis of variance showed that the median endoxifen level, $MR_{E/NDM}$ and $MR_{4HT/Tam}$ were significantly different among *CYP2D6* diplotype ($p < 0.001$) or genotype-predicted phenotype groups ($p < 0.001$) (Figure 2). Patients with UM/EM, UM/IM and UM/PM diplotypes were associated with higher median endoxifen concentrations or metabolic ratios ($MR_{E/NDM}$ and $MR_{4\text{-}HT/Tam}$) (Figure 2A). Similarly, a corresponding increase in absolute endoxifen concentration and metabolic ratio ($MR_{E/NDM}$) was observed with an increase in the activity score of *CYP2D6* phenotype ($p < 0.001$) (Figure 2B).

In patients categorized into *CYP2D6* NM/IM diplotype group, significant difference in $MR_{E/NDM}$ ($p = 0.036$) and $MR_{E/4\text{-}HT}$ ($p = 0.022$) was observed between genotypes **1/*10*, **1/*17* and **2/*17*. Among patients classified under NM/NM diplotypes, significant differences were also found between **1/*1*, **1/*2*, and **2/*2* genotypes in tamoxifen ($p < 0.001$), NDM ($p < 0.001$), and 4-HT concentrations ($p < 0.001$) as well as corresponding metabolic ratios; $MR_{E/NDM}$ ($p < 0.001$), $MR_{E/4\text{-}HT}$ ($p = 0.025$) and $MR_{4\text{-}HT/Tam}$ ($p = 0.007$).

In addition to *CYP2D6* genotype, a moderate association *POR* genotype with endoxifen level was observed ($p = 0.035$) (Figure 3A). On the other hand, *ABCB1* (rs3842) genotype showed a trend of association with $MR_{E/NDM}$ ($p = 0.054$) and moderate association with $MR_{E/4\text{-}HT}$ ($p = 0.039$). Carriers of the *G allele* for *ABCB1* (rs3842) had a lower $MR_{E/NDM}$ (0.022 vs. 0.041; $p = 0.054$) and $MR_{E/4\text{-}HT}$ (2.52 vs. 3.02, $p = 0.039$) ratios compared to the wild type genotype (Figure 3B,C).

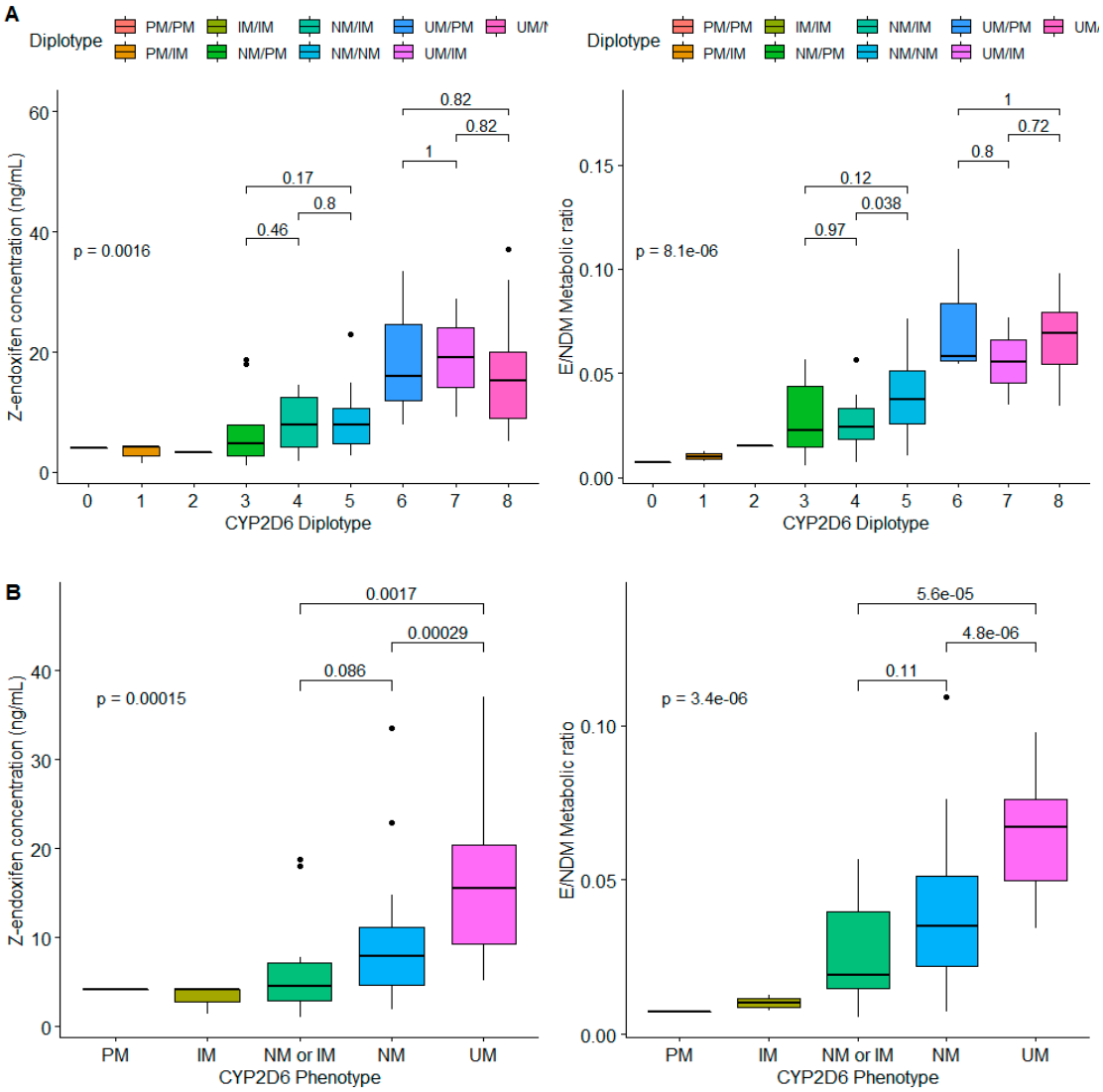

Figure 2. Plasma concentration of endoxifen and metabolic ratio ($MR_{E/NDM}$) by *CYP2D6* diplotype (**A**) and phenotype (**B**).

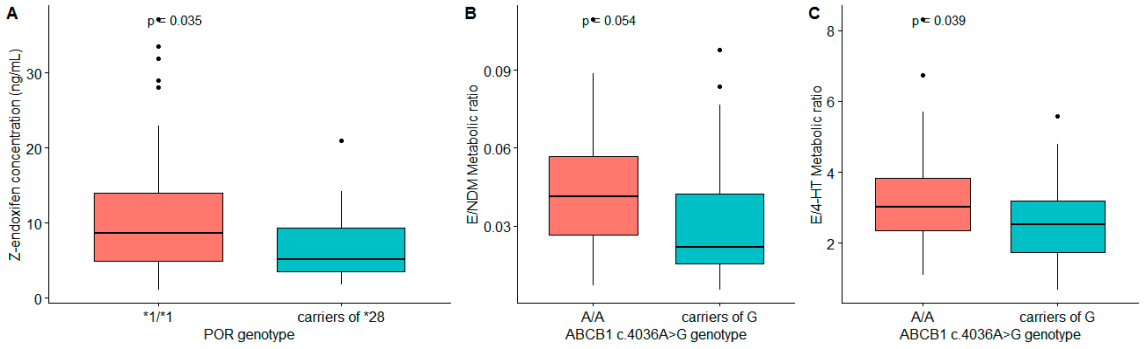

Figure 3. Plasma endoxifen concentration and metabolic ratios ($MR_{E/NDM}$ and $MR_{E/4\text{-}HT}$) plotted by *POR* (**A**) or *ABCB1 c.4036A>G* genotype (**B**,**C**).

Patients with at least one variant allele for *UGT2B15* (c.1568A>C, rs4148269) showed lower median plasma concentrations of tamoxifen (125.9 vs. 173.8 ng/mL, $p = 0.045$). The median NDM/Tam (1.86 vs. 1.54, $p = 0.004$) and E/4-HT (2.96 vs. 2.06, $p = 0.029$) ratios were also higher in carriers of *C* allele for *UGT2B15* (c.1568A>C, rs4148269) compared to the wild type genotype. However, there was no significant difference in median plasma tamoxifen metabolite concentrations or metabolic ratios between *CYP2C9*, *CYP2C19 CYP3A5, UGT2B15*2 (c.253G>T)* or *ABCB1 c.3435C>T* genotypes. No difference was also detected in the median plasma tamoxifen concentrations and its metabolites ($p > 0.05$), between pre- and postmenopausal patients, and different BMI groups as well as with age.

The impact of *CYP2D6* diplotype or phenotype on the interpatient variability of endoxifen or $MR_{E/NDM}$ was modeled using linear regression. The results indicated that *CYP2D6* diplotype explained 28.2% of the variability in endoxifen concentration (coefficient of determination (R^2) = 0.282, $p < 0.001$). Similarly, *CYP2D6* phenotype explained 26.3% of the variability in endoxifen concentration ($R^2 = 0.263$, $p < 0.001$). *POR* contributed 3.4% of the variability in absolute endoxifen concentration ($R^2 = 0.034$, $p = 0.054$). *CYP2D6* diplotype explained 44% of variability in $MR_{E/NDM}$ and combined with *ABCB1c.4036A*/G genotype, it explained 46.7% ($R^2 = 0.467$, $p < 0.001$) of the variability in $MR_{E/NDM}$. Phenotype and *ABCB1c.4036A>G* explained 40.9% of the variability in $MR_{E/NDM}$ ($R^2 = 0.409$, $p < 0.001$). *ABCB1c.4036A>G* contributed 4% the variability in $MR_{E/NDM}$ ($R^2 = 0.040$, $p = 0.042$).

The impact of *CYP2D6* on endoxifen variability and $MR_{E/NDM}$ (a marker for *CYP2D6* metabolic activity) was assessed stratifying patients by *ABCB1* genotype groups. Interestingly, *CYP2D6* diplotype explained 43% of the variability in endoxifen concentration among *ABCB1c.4036G* carriers ($R^2 = 0.43$, $p = 0.004$) (explanatory power increased from 28.2 to 43%), compared to *A/A* genotype ($R^2 = 0.231$, $p < 0.001$). Similarly, *CYP2D6* diplotype explained 65.2% of the variability in $MR_{E/NDM}$ (improved from 44 to 65.2%) among *ABCB1c.4036G* carriers ($R^2 = 0.652$, $p < 0.001$), compared to *A/A* genotype ($R^2 = 0.356$, $p < 0.001$).

3. Discussion

In the present study, we investigated the pharmacokinetic profile of tamoxifen and its active metabolites in breast cancer patients, and explored the impact of *CYP2D6*, *CYP2C9*, *CYP2C19*, *CYP3A5*, *POR*, *ABCB1* and *UGT2B15* genotypes on endoxifen and $MR_{E/NDM}$ in Ethiopians, a population with high frequency distribution of active *CYP2D6* gene duplication or multiplication. Our major findings include; (i) a wide between-patient variability in plasma concentrations of tamoxifen and its metabolites, (ii) significant proportion of patients (35.8%) with subtherapeutic plasma endoxifen concentrations (<5.9 ng/mL), (iii) *CYP2D6* diplotype is a significant predictor of both endoxifen concentration and $MR_{E/NDM}$, (iv) and a moderate effect of *POR*28* on plasma endoxifen concentration and *ABCB1c.4036A>G* genotype on $MR_{E/NDM}$. To our knowledge, this is the first study to investigate the pharmacokinetics and pharmacogenetics of tamoxifen in breast cancer patients from subSaharan

African population and to explore the impact of *POR* and *ABCB1c.4036A>G* pharmacogenetics on tamoxifen PK.

Although *CYP2D6* is involved in various tamoxifen metabolic pathways, it is the sole enzyme responsible for the formation of endoxifen from NDM [1]. Previous studies have consistently demonstrated a significant gene-dose effect of *CYP2D6* polymorphism for tamoxifen metabolism [3,6–8]. Our study also showed an increase in endoxifen concentration and $MR_{E/NDM}$ with increase in *CYP2D6* activity score, indicating a gene-dose effect of endoxifen formation. However, *CYP2D6* gene is highly variable among different ethnic groups, resulting in variations in concentrations of tamoxifen and its metabolites, mainly endoxifen [26,27].

In the present study, *CYP2D6* diplotype and *ABCB1c.4036A/G* genotype explained 46.7% the variability in $MR_{E/NDM}$. With slightly different genotype interpretation applied for *CYP2D6* AS, previous study demonstrated that the contribution of *CYP2D6* to the IIV of endoxifen formation via NDM, as revealed from $MR_{E/NDM}$, was 46% (Asians), 55% (Lebansese) and 55% (Caucasians) [3]. Our result is comparable to Asians but lower than Lebanese or Caucasians. The explained variability of absolute endoxifen concentration by *CYP2D6* diplotype in our study (28.2%) was also lower than that reported in Asians and Caucasians (39–58%) [7], suggesting that other factors could contribute for the bioavailability of endoxifen in our study population.

Previous studies reported that variations in the concentration of endoxifen can be related to variations in tamoxifen concentrations itself [28,29]. A high between-patient variations in plasma tamoxifen (CV = 56.2%) and NDM concentrations (CV = 53.1%) was also observed in our study. The Pearson's correlation coefficient between endoxifen and tamoxifen (R = 0.48) as well as endoxifen and NDM (R = 0.38) also showed that, endoxifen level would only moderately be predicted by plasma levels of tamoxifen and NDM. This could mean that the extent of absorption of tamoxifen from the gut or the quality of the formulation or food-drug interaction could also be potential factors influencing the bioavailability of tamoxifen thereby impacting the plasma level of endoxifen.

Our study also showed significant differences in $MR_{E/NDM}$ between patients with genotype *CYP2D6 *1/*10*, **1/*17* and **2/*17*, which belong to EM/IM dipotype. Similarly, differences were also observed in endoxifen level between **1/*1*, **1/*2*, and **2/*2* genotypes which are grouped under same NM/NM diplotype groups (Figure 2). In agreement with our finding, recent study demonstrated that the concentrations of tamoxifen, endoxifen 4-HT, and NDM were significantly different between genotypes classed under same NMs [8]. The range of activity between *CYP2D6*1/*1*, **1/*2*, and **2/*2* genotypes could be explained by the differences in the level of expression of mRNA, associated with the presence or absence of enhancer SNP in the enhancer regions distant to the *CYP2D6* gene [30].

Previous studies showed that tamoxifen efficacy is dependent on attaining a certain threshold of endoxifen level. According to Madlensky et al., women in the upper quintiles of endoxifen concentration, but not of tamoxifen and other metabolite levels, had a lower recurrence rate compared to those in the lowest quintile. Moreover, endoxifen concentration >5.97 ng/mL was associated with a 30% lower risk of additional breast cancer events and proposed as the therapeutic threshold for endoxifen [10]. A similar endoxifen level (13 nM) was also noted to occupy 90 % of the estrogen receptor (ER) in vitro and 93% of *CYP2D6* PMs fell below this concentration [12]. Subsequent study also demonstrated that, low endoxifen concentration (<5.97 ng/mL) was associated with distant relapse free survival [3], indicating the significance of this therapeutic threshold. The results of our study showed that significant proportion of Ethiopian patients (35.8%) were unable to attain this proposed therapeutic threshold for endoxifen concentrations. This is in agreement with previous study reported in Sweden (30%) [29], but higher compared to that reported in the U.S. (20%) [10].

In our study, the low level of endoxifen was detected in patients classified to PM/PM, IM/PM, IM/IM, NM/PM, NM/IM, as well as NM/NM diplotypes. Poor/intermediate metabolizer genotype was identified as a predictors of low endoxifen concentration in earlier study [10]. Moreover, it was also reported that 99% of PMs can be predicted by *CYP2D6* null alleles in homozygous variant or heterozygous genotype [31]. Our result is in agreement with this notion, given that all the null or lower

activity diplotypes (PM/PM, IM/PM, and IM/IM) were associated with low endoxifen level. In such patients, the clinical outcome of the cancer could be less favorable, though its association was not investigated in the present study. Thus, in line with the recent CPIC therapeutic recommendations, *CYP2D6* PMs in our study are recommended alternate hormonal therapy such as aromatase inhibitors (AIs) for post-menopausal women or AIs along with ovarian function suppression in premenopausal women. Escalation of tamoxifen dose from 20–40 mg/day can also be considered for PMs if there is contraindications to AIs [32].

In contrast, other studies demonstrated that not all PMs have endoxifen concentrations below the proposed threshold concentration (5.9 ng/mL) [10,33]. For example, it was observed that 24 % of the PMs were still able to generate therapeutic concentrations of endoxifen despite the lack of metabolic activity of *CYP2D6* [33]. This could partly be attributed to environmental factors. A previous study demonstrated that black Africans were associated with lower rates of *CYP2D6*-dependent drug metabolism compared to Caucasians of the same apparent genotype [34]. Moreover, among individuals of the same *CYP2D6* genotype, it was revealed that Ethiopians in living in Sweden had higher *CYP2D6* metabolic activity than Ethiopians in Ethiopia [34], implying the profound impact of environmental factors in *CYP2D6* catalyzed drug metabolism. The frequency of *CYP2D6* PM (1.2%) and *CYP2D6* duplication (26%) identified in this study is lower as compared to previous report [34].

In the present study, patients with NM/IM (41.7%) and NM/NM diplotypes (31.4%), all belonging to NM phenotypes had lower endoxifen concentrations (<5.9 ng/mL). Recently, it was reported that the activity of *CYP2D6* is impacted not only by genetic variations within the *CYP2D6* gene, but also by regulatory mechanisms including genetic polymorphisms in the enhancer regions or differential expression of transcription factors, which can modulate the rate of translation of mRNA into protein [35]. Genotype panels typically interrogate a limited number of SNPs identifying more commonly observed *CYP2D6* alleles and provide limited information on gene copy number and structural variants [35]. It is therefore possible that a patient carries allele(s) that was not tested for and that may contribute to the variability seen in these groups. In addition, the impact environmental factors on *CYP2D6* catalyzed drug metabolism cannot also be underestimated [34]. Moreover, other individual-specific and biologic factors may also modulate *CYP2D6* expression levels or enzyme activity and contribute to the variability *CYP2D6* enzymatic activity. Emerging evidence showed the contribution of the microbiome on overall health and its role in drug metabolism and response [36]. A recent study also provided evidence that nutritional status, specifically fasting, alters P450-mediated drug metabolism in animal studies [37]. However, the extent a person's microbiome, nutritional and fasting status influences CYP activity and *CYP2D6* metabolizer status, intra- and interindividual variability, in particular, remains to be investigated in future studies. Notably, the low level of endoxifen detected in large proportion of patients with predicted NM phenotypes in our study may suggest the need for therapeutic drug monitoring (TDM) of endoxifen in addition to genotyping for the common functional *CYP2D6* variant alleles.

Cytochrome P450 oxidoreductase (*POR*) is the only electron donor for all microsomal cytochrome P450 monooxygenases (CYP) responsible for oxidation of more than 80% of drugs [38]. In our study, we observed that *POR*28* carriers were associated with low plasma endoxifen level, although the effect is moderate ($p = 0.035$). Previous reports indicated that genetic variants of *POR* (e.g., *POR*28* (A503V)) were implicated in the impaired activity of *CYP2D6* and *CYP3A4* [39,40], possibly contributing to genetic variability in drug metabolism.

Apart from tamoxifen activating enzymes, genetic variants in drug transporters have been reported to influence pharmacokinetics and play a role to a variable degree in the clinical outcome of tamoxifen treatment and to confer treatment resistance for many anticancer drugs [41]. Results of the present study did not show significant effect of *ABCB1c.3435C>T* on tamoxifen and its metabolites level. However, it was observed that carriers of *G* allele for *ABCB1c.4036A>G* had a marginally lower $MR_{E/NDM}$ (0.025 vs. 0.036; $p = 0.054$) compared to the wild type allele (*ABCB1*, *allele A*) indicating that polymorphism of *ABCB1 ABCB1c.4036A>G* could influence exposure to endoxifen. Interestingly,

among *ABCB1c.4036G* carriers, improved predictive effect of the variability in endoxifen concentration (43%) and endoxifen formation from NDM (65.2%) was observed by *CYP2D6* diplotype. A previous study demonstrated that *ABCB1* influences the systemic exposure of tamoxifen and its active metabolites, 4-HT and endoxifen [16]. Moreover, several other studies also substantiated the impact of *ABCB1* in relation to clinical outcome of tamoxifen. *ABCB1* polymorphism was associated with risk of recurrence or disease free survival (DFS), in breast cancer patients on tamoxifen treatment [16,42,43].

Variant alleles of *CYP2C9*, *CYP2C19* and *CYP3A5* could alter tamoxifen metabolism and exposure to its metabolites especially formation of 4-hydroxy-tamoxifen [1]. However, no evidence of association was observed between *CYP2C9*, *CYP2C19* and *CYP3A5* genotypes and plasma concentrations of tamoxifen or its metabolites. Our finding is substantiated with previous studies [6,11,33]. On the other hand, other studies have reported lower plasma concentrations of 4-HT and endoxifen among carriers of *CYP2C9*2* or **3* alleles [3,12]. The disparity in the results could be attributed to the difference in sample size or the variation in allele distributions.

In addition of the significant effect of *CYP2D6* genotype, variations in glucuronidation activity of the *uridine 5′-diphosphate-glucuronosyltransferase (UGT)* also seem to play a role in maintaining optimal levels of both 4-hydroxy-tamoxifen and endoxifen. Modified glucouronidation could affect half-lives of circulating tamoxifen and its metabolites thereby influencing the effectiveness of active metabolites in the treatment of breast cancer [44]. In the present study, patients with *C* allele for *UGT2B15*c.1568A>C (rs4148269) showed a lower plasma concentrations of tamoxifen, compared to those with wild type genotype. Although we could not determine plasma levels of tamoxifen-glucouronide, our finding may suggest increased glucouronidation of tamoxifen. Higher glucouronidation activity of *UGT2B15*c.1568A>C (rs4148269) genotype was also reported against endoxifen and 4-hydroxytamoxifen [44] and against oxazepam [45].

On the other hand, our result did not show significant variations in tamoxifen and its metabolites concentration with *UGT2B15*2* (c.253G>T, rs1902023). Similarly, no correlation was detected between this polymorphism and both hydroxylated tamoxifen metabolites and their corresponding glucouronide product [12,44]. However, *UGT2B15*2* (c.253G>T, rs1902023) was demonstrated to be a determinant of glucouronidation of oxazepam [45,46] and lorazepam [47] in the human liver, suggesting a substrate-dependent effect of this polymorphism.

Our study has some limitations. We present a significant impact of *CYP2D6* and a marginal effect of *POR* and *ABCB1* on the pharmacokinetics of tamoxifen and its metabolites. However, the sample size included in our study was not powered enough and might be a reason for lack of significant association of *POR* and *ABCB1* genotypes with tamoxifen and its metabolites PK. In addition, association of plasma concentration of the active tamoxifen metabolites to clinical outcome was not assessed. The treatment of cancer in patients with comorbidities can be challenging as these individuals are underrepresented in clinical studies [48]. In addition to the *CYP2D6* genetics that much of the controversy around tamoxifen therapy has focused, drug-drug interactions (DDI) involving *CYP2D6* in polypharmacy for co-morbidities markedly increases the complexity of tamoxifen metabolism and efficacy. The concurrent use of *CYP2D6* inhibitors and tamoxifen would be expected to substantially reduce endoxifen concentrations, and there is clinical evidence to support this expectation [6,9,49]. For example, NMs who were also taking *CYP2D6* inhibitors had endoxifen plasma concentrations that were 58% lower than NMs not on *CYP2D6* inhibitors. Similarly, IMs on *CYP2D6* inhibitors had endoxifen concentrations that were 38% lower than IMs not on *CYP2D6* inhibitors [6]. As tamoxifen has complex pharmacokinetics, with more than a dozen drug-metabolizing enzymes and transporters involved in its disposition, enzyme inducers may increase the activity of several of these pathways, including phase II enzymes, ABC transporters, and various CYP enzymes other than *CYP2D6* [50]. There is a growing evidence that enzyme inducers can substantially alter the disposition of endoxifen, reducing tamoxifen efficacy [51].

4. Materials and Methods

4.1. Patients

This study was conducted at the radiotherapy center of Tikur Anbessa specialized hospital, Addis Ababa University, Addis Ababa, Ethiopia. A total of 81 female breast cancer patients who were on adjuvant tamoxifen were enrolled. These patients had completed a full course of chemotherapy and were on tamoxifen 20 mg/day for at least three months. None of the study participants were taking any known *CYP2D6* enzyme inhibitor as co-medication. The study protocol was approved by the Institutional Review Board (IRB) of the College of Health Sciences, Addis Ababa University (AAU) (Ref No: 011/16/2016), the Armauer Hansen Research Institute Ethical Review Committee (AAERC) (Ref No: PO26/16) and the Ethiopian National Research Ethics Review Committee (NRERC) (Ref No: 3.10/235/2017). Signed informed consent was obtained from individual study participants prior to study enrolment. Patients' demographic, clinical and tumor characteristics including age, menopausal status, performance status, weight, height, body mass index (BMI), histologic type of the tumor, degree of differentiation, tumor size, lymph node involvement, hormone receptor status and co-morbidity status were collected.

4.2. Genotyping of CYP2D6, CYP2C9, CYP2C19, CYP3A5, POR, ABCB1 and UGT2B15

Whole blood sample was collected in EDTA containing vacutainer tube and genomic DNA was isolated from peripheral leukocytes using QIAamp DNA Midi Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instruction. SNP genotyping was performed using TaqMan™ drug metabolism genotyping assay reagents (Applied Biosystems, USA) for allelic discrimination as described previously [52] with the following ID numbers for each SNP: C_27102425_10 for *CYP2D6*2* (rs16947), C_27102431_D0 for *CYP2D6*4* (rs3892097), C_11484460_40 for *CYP2D6*10* (rs1065852), C_2222771_A0 for *CYP2D6*17* (rs28371706), C_26201809_30 for *CYP3A5*3* (rs776746), C_25625805_10 for *CYP2C9*2* (rs1799853), C_27104892_10 for *CYP2C9*3* (rs1057910), C_25986767_70 for *CYP2C19*2* (rs4244285), C_27861809_10 for *CYP2C19*3* (rs4986893), C_8890131_30 for *POR*28* (rs1057868) and C_11711730_20 for *ABCB1c.4036A>G* (rs3842), C_7586657_20 for *ABCB1c*.3435C>T (rs1045642), C_27028164_10 for *UGT2B15*2* (rs1902023), C_9440184_20 for *UGT2B15*4* (rs4148269). The genomic DNA samples were amplified in 96-well plates on QuantStudio™ 12K Flex Real-Time PCR system (Applied Biosystems, Life Technologies Holding, Singapore). The final volume for each reaction was 10 µL, consisting of TaqMan™ fast advanced master mix (Applied Biosystems, Waltham, MA, USA), TaqMan™ 20× drug metabolism genotyping assays mix (Applied Biosystems) and genomic DNA. The PCR conditions consisted of an initial step at 60 °C for 30 s, hold stage at 95 °C for 10 min and PCR stage for 40 cycles step 1 with 95 °C for 15 and step 2 with 60 °C for 1 min and after read stage with 60 °C for 30 s. The characterized SNPs were selected on the basis of their potential to influence the functionality of enzymes to affect the disposition and transport of tamoxifen or its metabolite.

4.3. Copy Number Variation

The *CYP2D6* gene copy number for the patient DNA samples was determined by TaqMan™ Copy Number Assay (Life Technologies, California, USA) according to the manufacturer's recommendations. The genomic DNA samples were amplified in 96-well plates on QuantStudio™ 12K Flex Real-Time PCR system (Applied Biosystems, Life Technologies Holding, Singapore) using comparative $\Delta\Delta C_T$ method. *CYP2D6* exon 9 (TaqMan™ Copy Number Assay ID: Hs00010001_cn), and intron 6 (TaqMan™ Copy Number Assay ID: Hs04502391_cn) were used as primers. RNase P (ID: 431683) was used as the internal control for copy number analysis (Life Technologies). All the samples were run in triplicate. The final volume for each reaction was 10 µL, consisting of TaqMan™ Genotyping Master Mix (Applied Biosystems), TaqMan™ Copy Number Assay mix (20×, Applied Biosystems), TaqMan™ Copy Number Reference Assay mix (20×, Applied Biosystems). The PCR conditions consisted of an initial step at 60 °C for 30 s, hold stage at 95 °C for 10 min and PCR stage for 40 cycles step 1 with 95 °C for 15 and

step 2 with 60 °C for 1 min and after read stage with 60 °C for 30 s. Relative quantification of *CYP2D6* gene copy number was performed using CopyCaller™ Software (Life Technologies). Genomic DNA samples with known *CYP2D6* gene copy number (0, 1, 2, 3 and 4 *CYP2D6* gene copies) obtained from a previous study [15] were used as controls.

4.4. Quantification of Plasma Tamoxifen and Its Metabolites

Blood samples were collected in EDTA tube, centrifuged at 2500 *g* for 10 minutes, and the separated plasma was stored at −80 °C until analysis. Tamoxifen (Tam) and its major metabolites N-desmethyltamoxifen (NDM), (*Z*)-4-hydroxytamoxifen (4-HT), and (*Z*)-endoxifen (*E*) were quantified in the TDM Laboratory, Department of Clinical Pharmacology, Karolinska Institutet (Stockholm, Sweden), by ultra-high-performance liquid chromatography (UHPLC) followed by electrospray tandem mass spectrometry (LC-MS/MS). Briefly, 400 µL internal standard solution (containing 2 ng/mL of *Z*/*E*-endoxifen-d_5, and 4-OH-tamoxifen-d_5, and 20 ng/mL of N-desmethyl-tamoxifen-d_5, and tamoxifen-d_5) was added to 200 µL of thawed plasma samples and mixed for 10 minutes in a micro plate shaker (Vx-2400 Multitube mixer, Troemner, Philadelphia, USA), followed by centrifugation at 3400 rpm at 4 °C for 10 min. The supernatant was then transferred to new glass vial and evaporated to dryness using vacuum centrifuge. The residue was dissolved in 50 µL methanol over a microplate shaker for 10 min and the resulting solution was then centrifuged at 3400 rpm for 10 min at 4 °C. Ten microliter (10 µL) of the solution was injected into the LC system (Dionex Ultimate 3000, UHPLC focused, Thermo Scientific, California, USA) coupled with a mass spectrometer (TSQ Quantiva, Thermo Scientific). Separation occurred on an Accucore C-18 column (150 × 2.1 mm, Thermo Scientific). The mobile phase for gradient elution consisted of solution A (10 mmol/L ammonium formate with 0.005% formic acid in water) and solution B (10 mmol/L ammonium formate with 0.005% formic acid in 99% methanol). The LC conditions were: column oven temperature of 40 °C, flow rate of 0.50 mL/min, autosampler temperature of 10 °C. Detection was in positive ion mode using electrospray ionization and monitored using Trace Finder software (Thermo Scientific). Under this condition, the retention times were 3.25, 3.26, 3.38, 4.03, and 4.11 min for E-endoxifen, Z-endoxifen, 4-OH-tamoxifen, N-desmethyltamoxifen, and tamoxifen, respectively. Calibration curves were constructed by plotting the ratio of the area of the compound and the internal standard against the standard analyte concentration. Three quality control (QC) samples involving, (i) low-level QC [QCL] (0.3 ng/mL of E and 4-HT and 3 ng/mL of NDM and Tam, (ii) mid-level QC [QCM] (8 ng/mL of E and 4-HT and 80 ng/mL of NDM and Tam), and (iii) high level QC [QCH] (80 ng/mL of E and 4-HT and 800 ng/mL of NDM and Tam) were also included at each run.

4.5. Genotype Based Phenotype Assignment

Phenotype classification from genotype was based on the recent Clinical Pharmacogenetics Implementation Consortium (CPIC) guideline for *CYP2D6* and tamoxifen therapy [32]. Functional status of each allele was assigned an activity score (AS) value ranging from 0 to 1 [0 for no function (*4, *5), 0.5 for decreased function (*10, and *17) and 1.0 for normal function alleles (*1, and *2)]. The combination of any two alleles represents the patient's diplotype. *CYP2D6* activity score (AS) was then determined as the sum of the values assigned to each allele of the diplotype. Accordingly, *CYP2D6* AS was translated into patient's phenotype status as poor metabolizer, PM (AS = 0), intermediate metabolizer, IM (AS = 0.5), normal metabolizer, NM (AS = 1.5–2) and ultra-rapid metabolizer, UM (AS > 2). Patients with AS of 1 were classified as a *CYP2D6* normal metabolizer or intermediate metabolizers (NM or IM).

4.6. Statistical Analysis

Descriptive statistics were computed to explore the demographic characteristics, clinical profiles, genotype frequencies of participants. *Chi*-square test was used to compare the observed and expected genotype frequency according to Hardy–Weinberg equilibrium. The plasma concentrations of tamoxifen

and its active metabolites were described as median with interquartile range (IQR). Interpatient variability (IIV) of plasma endoxifen and endoxifen/N-desmethyltamoxifen metabolic ratio ($MR_{E/NDM}$), a marker for *CYP2D6* metabolic activity, was described by coefficient of variation (%COV). ANOVA or independent *t*-test was used to determine the association of log transformed concentrations of tamoxifen and its metabolites and $MR_{E/NDM}$ across *CYP2D6* diplotypes, phenotypes, non *CYP2D6* genotypes (*CYP2C9*, *CYP2C19*, *CYP3A5*, *POR*, or *ABCB1c.4036A>G*), and other patient specific factors (i.e., age, BMI, menopausal status, etc). Pearson correlation coefficient was determined to assess the magnitude of association between tamoxifen and its metabolites. The impact of *CYP2D6* diplotype and phenotype to explain the absolute concentration of endoxifen or $MR_{E/NDM}$ was assessed using linear regression. Adjusted coefficient of determination (adjusted R^2) was used to define the proportion of the variance in endoxifen concentration and $MR_{E/NDM}$ that could be predictable from *CYP2D6* diplotype and phenotype. The data were analyzed using SPSS for Windows (version 21.0, IBM Corporation, New York, USA). Graphs were prepared using *ggplot2* package in R (version 3.5.1) [53].

5. Conclusions

In conclusion, we report a high rate of low plasma endoxifen level and wide between-patient variability in plasma concentration of endoxifen, the main therapeutically active metabolites of tamoxifen. *CYP2D6* genotype and its activity score is a significant predictor of plasma endoxifen exposure. Tamoxifen therapy guided by *CYP2D6* genotyping in clinical practice may assist treatment decision-making for optimal patient benefit. Therapeutic drug monitoring is also recommended for those with normal metabolizer phenotypes. The marginal effect of *POR* and *ABCB1* on tamoxifen and its metabolites PK found in this study needs further investigation in a larger sample size. Despite the simplified AS system recommended by CPIC for genotype-phenotype translation, our study signals further refinement of the approach, probably considering other sources of variability such as *POR* and *ABCB1*. Future studies are also recommended to investigate the impact of the *CYP2D6* genetic variations on the clinical outcome of breast cancer in Ethiopian patients.

Author Contributions: Conceptualization, J.H.A., E.M. and E.A.; methodology, J.H.A., E.M. and E.A.; software, J.H.A.; validation, E.A.; formal analysis, J.H.A. and E.A.; investigation, J.H.A. and E.A.; resources, A.F., A.A., R.H. and E.A.; data curation, J.H.A.; writing—original draft preparation, J.H.A.; writing—review and editing, J.H.A., E.M., A.F., A.A., R.H. and E.A.; visualization, J.H.A.; supervision, E.M., A.A., R.H. and E.A.; project administration, A.A., R.H. and E.A.; funding acquisition, J.H.A., A.A. and R.H.

Funding: This research was supported by Armauer Hansen Research Institute (AHRI) through the BSPP Program, a grant obtained from Sida-Ethiopia Bilateral Program.

Acknowledgments: The authors would like to extend their sincere appreciation to Armauer Hansen Research Institute (AHRI) for the financial assistance required for the study.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Vitamin D Status and Association of *VDR* Genetic Polymorphism to Risk of Breast Cancer in Ethiopia

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Received: 28 December 2018; Accepted: 22 January 2019; Published: 29 January 2019

Abstract: Emerging evidence associates vitamin D deficiency and vitamin D receptor (*VDR*) genetic variations with risk for breast cancer. This study investigated the prevalence of vitamin D deficiency and its association with tumor characteristics and the implications of *VDR* genetic variations for risk of breast cancer in Ethiopia. This unmatched case–control study involved 392 female breast cancer patients and 193 controls. The plasma 25-hydroxyvitamin D ($25(OH)D_3$) level was quantified in chemotherapy-naïve (N = 112) and tamoxifen-treated patients (N = 89). Genotyping for the *VDR* common variant alleles rs7975232 (*Apa*I), rs2228570 (*Fok*I), and rs731236 (*Taq*I) was done. Eighty-six percent of the patients were vitamin D deficient (<50 nmol/L). Chemotherapy-naïve breast cancer patients had a higher prevalence of vitamin D deficiency (91.9% vs. 78.3%) compared to the tamoxifen-treated group ($p < 0.001$). The prevalence of severe vitamin D deficiency (<25 nmol/L) was significantly higher in chemotherapy-naïve (41.1%) than tamoxifen-treated (11.2%) patients. Vitamin D deficiency was not significantly associated with tumor characteristics or *VDR* genotype. The rs2228570 *GG* genotype was associated with increased risk of breast cancer (OR = 1.44, 95% confidence interval = 1.01−2.06). Our result indicates that rs2228570 might be a moderate risk factor for breast cancer development in the Ethiopian population. The high prevalence of severe vitamin D deficiency in treatment-naïve breast cancer patients indicates the need for nutritional supplementation of vitamin D at the time of chemotherapy initiation.

Keywords: vitamin D deficiency; *VDR*; genetic variations; breast cancer; Ethiopia

1. Introduction

Vitamin D has long been known for its physiological role in calcium balance and for being responsible for increased intestinal absorption of calcium, magnesium, and phosphate [1]. It plays a crucial role in the proper functioning of the immune, muscle, and nervous systems [2,3], as well as in controlling normal cell growth [2]. In addition, vitamin D is reported to have anticancer activities against many cancer types, including breast cancer [4]. Reports from epidemiologic [5,6] and mechanistic studies [7] have demonstrated that vitamin D inhibits cancer cell proliferation, induces apoptosis, and decreases angiogenesis.

In humans, most of the vitamin D is generated from sunlight-mediated conversion of dehydrocholesterol in skin to form cholecalciferol (Vit D_3). As Vit D_3 has little biological activity, it undergoes two hydroxylation reactions to become biologically active. The first hydroxylation occurs in the liver, catalyzed by several Cytochrome P450 enzymes, such as *CYP2R1, CYP27A1, CYP2D25*, and *CYP2J3*, and gives rise to 25-hydroxyvitamin D_3 (25(OH)D_3). A further hydroxylation in the kidney by the action of *CYP27B1* converts 25(OH)D_3 to 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$, calcitriol), the hormonally active form of vitamin D [8]. The bioactive 1,25-$(OH)_2D_3$ functions by binding to a nuclear vitamin D receptor (*VDR*) to regulate gene transcription [9]. Due to its abundance and longer plasma half-life, 25(OH)D_3 concentration is the parameter of choice for the assessment of vitamin D status [10].

A number of variables, including latitude, season, time of day, atmospheric components, clothing, sun screen use, and skin pigmentation influence the amount of UVB radiation entering the skin, thereby affecting vitamin D production. Moreover, other factors such as age, gender, physical activity, and obesity, as well as chronic illnesses such as cancer, could affect the synthesis and bioavailability of vitamin D [10,11]. It has become evident that vitamin D deficiency is a major problem in many populations worldwide [12]. Although high concentrations of vitamin D have been observed in some East African ethnic groups [13,14], low serum 25(OH)D_3 levels were reported in many African populations, including Algerian pregnant women and Egyptian healthy children [15]. Similarly, high rates of vitamin D deficiency or insufficiency has been reported in Ugandan children [16] and adult patients with human immunodeficiency virus (HIV) and tuberculosis (TB) [17,18]. Previous studies in Ethiopia also showed a high prevalence of low vitamin D in men and non-pregnant women [19], school children [20], and TB/HIV co-infected people [21].

The implications of vitamin D deficiency on cancer susceptibility have been demonstrated in previous studies. Association between a low serum 25(OH)D_3 level and increased breast cancer development, risk for breast cancer recurrence, and mortality has been reported [22]. A recent meta-analysis demonstrated a protective relationship between high circulating 25(OH)D_3 level and breast cancer development in premenopausal women [23]. It was also shown that, in patients with 25(OH)D_3 level below 50 nmol/L, there is a higher predicted probability of breast cancer among African Americans compared to Hispanics [24]. A pooled analysis of randomized trials demonstrated that higher 25(OH)D_3 concentrations were associated with a decrease in breast cancer risk, with concentrations of $\geq$ 150 nmol/L being most protective [25].

Genetic variations in the *VDR* genes have also been associated to risk of breast cancer [26–29]. The link between *VDR* genetic variations and breast cancer risk is attributed to the notion that the action of vitamin D is mediated by *VDR*. In addition, both normal and breast cancer cells have been found to express the receptor [30]. The *VDR* genetic variation is also associated with better survival benefit [29,31]. The *VDR* variant alleles which have been studied include rs7975232 (A > C, commonly known as *ApaI*), rs1544410 (T > C, commonly known as *Bsm*I), rs2228570 (T > C, commonly known as *Fok*I), and rs731236 (T > C, commonly known as *Taq*I). However, reports regarding the specific relevance of *VDR* allelic variations on breast cancer risk have been inconsistent. An increased risk of breast cancer was observed with the rs1544410 *bb* or *Bb* genotype, but not with the rs2228570 genotype in Iranian [32] and Egyptian [33] female breast cancer patients. Another research finding demonstrated that, compared with homozygotes for the common rs2228570 *F* allele (*FF* genotype), *ff* homozygotes

had a higher breast cancer risk [34]. On the other hand, a population-based cohort study showed no association of single-nucleotide polymorphisms (SNPs) in the *VDR* with cancer incidence [35]. Associations were also not detected between breast cancer risk and genotype and allele frequencies of rs2228570 and rs731236 polymorphisms [36].

These discrepancies suggest the need for further exploration of the issue from various population groups. Furthermore, the prevalence of vitamin D deficiency and type of breast cancer varies between populations. Black African populations display large genetic diversity compared to Asians and Caucasians. On the other hand, information regarding the prevalence of vitamin D deficiency or insufficiency in cancer patients in the black African population is unavailable. Although abundant sunshine is available, the population is reported to be at risk of low vitamin D levels due to insufficient sun exposure associated with socio-cultural factors [19]. As most cancer patients are more likely to spend most of their time indoors, we hypothesize that cancer patients could be at a greater risk of low vitamin D levels. The genetic constitution of Ethiopians could also add pertinent information related to the role of *VDR* gene polymorphism in breast cancer susceptibility. Thus, the aim of the current study was to examine the prevalence and severity of vitamin D deficiency and the association of *VDR* polymorphism with risk of breast cancer in Ethiopia.

2. Materials and Methods

The study was conducted at the radiotherapy center (9° N, 38° E) of Tikur Anbessa Specialized Hospital (TASH), Addis Ababa, Ethiopia. An unmatched case–control study design was employed involving breast cancer patients (cases) and control groups from the general population with no history of breast cancer at the time of study enrolment (controls). The inclusion criteria for cases were adult female patients with pathologically and clinically diagnosed breast cancer. All consecutive and volunteer patients (stages I–IV) who came for the first cycle of the chemotherapy regimen (treatment/chemotherapy naïve) or those who completed their chemotherapy and were on tamoxifen adjuvant therapy (tamoxifen group) in the outpatient day-care ward of TASH were included. Controls were recruited from the general population from TASH. Exclusion criteria for participants (cases) were a prior history of vitamin D supplementation (for vitamin D assay only), pregnant or breastfeeding women, and previous neo-adjuvant chemotherapy.

The study protocol was approved by Armauer Hansen Research Institute Ethical Review Committee (AAERC) (Ref No: PO26/16), Institutional Review Board (IRB) of the College of Health Sciences, Addis Ababa University (Ref No: 011/16/2016), and National Research Ethics Review Committee (NRERC) of the Federal Democratic Republic of Ethiopia (Ref No: 3.10/235/2017). Signed informed consent was obtained from individual patients prior to participation in the study.

Patients' baseline medical records, laboratory investigations (blood counts and organ function estimates), and results of biopsy reports describing tumor characteristics such as site of tumor, degree of differentiation, tumor size, and lymph node involvement were recorded. In addition, menopausal status, Karnofsky's performance status (performance scale > 70), weight, height, body mass index (BMI), chemotherapy panel (for chemotherapy groups either as neo-adjuvant, adjuvant, or metastatic), and chemotherapy regimen were recorded.

The sample size required for a 95% two-sided confidence interval for an unmatched case–control study with 90% power to detect a risk ratio of 2 for breast cancer (associated with *VDR* genetic polymorphism) was calculated using OpenEpi^{R} software [37]. Accordingly, considering a case-to-control ratio of 2 (2 cases:1 control) (n = 548) and a 5% addition, the estimated sample size (N) was 575 (190 controls vs. 383 cases).

2.1. Genotyping

Whole blood samples were collected in EDTA tubes from all participants and genomic DNA was isolated using a QIAamp DNA Midi Kit (Qiagen GmbH, Hilden, Germany). Genotyping for the common functional variant alleles of *VDR* genes relevant to breast cancer risk—*Fok*I, *Apa*I, and

*Taq*I—was carried out using Taqman allele-specific PCR (Applied Biosystems Genotyping Assays) as described previously [38]. In brief, genotyping was performed using TaqMan® SNP genotyping assay reagents for allelic discrimination (Applied Biosystems, Waltham, MA, USA) with the following ID number for each SNP: C__12060045_20 (rs2228570, T > C), C__2404008_10 (rs731236, T > C), andC__28977635_10 (rs7975232, A > C). Genotyping was carried out using a QuantStudio 12K Flex Real-Time PCR system (Life Technologies Holding, Singapore). The final volume for each reaction was 10 µL, consisting of TaqMan® fast advanced master mix (Applied Biosystems, Waltham, MA, USA), TaqMan 40X SNP genotyping assays mix (Applied Biosystems, Waltham, MA, USA), and genomic DNA. The PCR parameter consisted of an initial step at 60 °C for 30 s, hold stage at 95 °C for 10 min, PCR stage for 40 cycles: Step 1 at 95 °C for 15 mins and Step 2 at 60 °C for 1 min, and a read stage after at 60 °C for 30 s. Genotypes were assigned using the manual calling option in the allelic discrimination application, using QuantStudio 12K Flex software (Applied Biosystems, Life Technologies, Stockholm, Sweden). The characterized SNPs were selected on the basis of their potential to influence the functionality of the vitamin D receptor, as obtained from public databases. The genomic DNA of the known genotype and two no template controls (NTCs) were run in parallel to the samples.

2.2. Vitamin D Quantification

The plasma vitamin D level was measured from a total of 201 breast cancer patients, of which 112 were treatment naïve (planned to initiate their first cycle chemotherapy) and 89 were on tamoxifen at 20 mg/day, following completion of their course of chemotherapy. The plasma vitamin D level was quantified using DiaSorin assay at the Centre for Chemical Laboratory, Karolinska University Laboratory, Solna, Sweden. Plasma vitamin D status was classified according to the recommended guideline [39]. Accordingly, severe vitamin D deficiency (SVDD) and vitamin D deficiency (VDD) were defined as circulating 25(OH)D_3 levels of < 25 nmol/L, and 25–50 nmol/L, respectively. Levels between 51 and 72.5 nmol/L were considered insufficient [21].

2.3. Statistical Analysis

Descriptive analyses are presented for the demographic and clinical characteristics.The genotype and allele frequencies were assessed by Chi-square test to compare observed and expected genotypic distributions. Nonparametric testing was used to compare plasma 25(OH)D_3 levels between chemotherapy-naïve and tamoxifen groups. One-way ANOVA was used to test the relationship between vitamin D concentration and genotype. The association between breast cancer and *VDR* polymorphisms were assessed first with χ^2 test, and then the odds ratio was estimated using logistic regression models to see the magnitude of association. The data were analyzed using SPSS software version 21.0 for Windows (IBM Corporation, NY). A p-value of < 0.05 was considered statistically significant for each test, and Bonferroni correction (as the number of hypotheses is fairly small) was then applied for multiple comparisons. Graphs were prepared using GraphPad Prism software, v.7.04 (GraphPad Software, La Jolla, CA, USA).

3. Results

A total of 585 study participants were enrolled, comprising 392 breast cancer cases and 193 controls. The study flow chart and recruitment procedure is depicted in Figure 1. Out of the breast cancer cases (n = 392), 303 were chemotherapy-naïve patients who came for their first cycle of chemotherapy, and 89 were treatment-experienced patients who had completed the full course of chemotherapy and were on tamoxifen therapy. The baseline socio-demographic, clinical, and laboratory parameters and tumor profiles of study participants are presented in Table 1. The common chemotherapy regimens planned for the chemotherapy-naïve patients (n = 392) were FAC (5-**F**lourouracil 500 mg/m^2, **A**driamycin [Doxorubicin] 50 mg/m^2, and **C**yclophosphamide 500 mg/m^2) (41.3%), AC – T (**A**driamycin 60 mg/m^2 and **C**yclophosphamide 600 mg/m^2 followed by Taxol 175 mg/m^2) (39.3%), and AC (**A**driamycin 50 mg/m^2 and **C**yclophosphamide 600 mg/m^2) (18.2%).

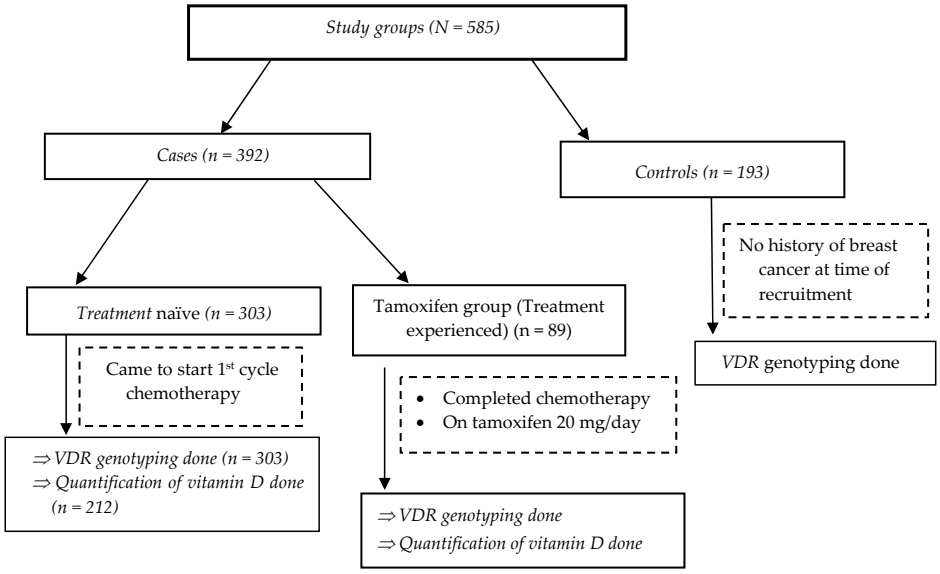

Figure 1. Study flow chart depicting study groups and participant recruitment.

Table 1. Socio-demographic, clinical, and laboratory parameters and tumor characteristics of patient participants at baseline.

Parameter		Value
Socio-demographics		
Age (years, mean ± SD) ♣		40.77 ± 10.79
BSA (m^2, mean ± SD)		1.61 ± 0.19
BMI (Kg/m^2, mean ± SD)		23.91 ± 4.61
Baseline laboratory results		
WBC ($10^3/mm^3$; median, IQR)		6.67 (2.74)
ANC ($10^3/mm^3$; median, IQR)		3.59 (2.12)
Hgb (gm/dL; median, IQR)		13.9 (1.8)
HCT (%; median, IQR)		41.35 (4.48)
PLT ($10^3/mm^3$; median, IQR)		295.5 (105)
ALT (U/L; median, IQR)		18 (14)
AST (U/L; median, IQR)		24 (11)
ALP (U/L; median, IQR)		214 (141)
SCr (mean ± SD)		0.91 ± 0.18
BUN (median; IQR)		18 + 10
Tumor characteristics		*N*, %
Site of tumor	Left	200 (51.7)
	Right	177 (45.7)
	Bilateral	10 (2.6)
Histologic type of tumor	Ductal	332 (84.7)
	Lobular	17 (4.3)
	Mixed	4 (1)
	Other	39 (10)
Degree of differentiation	Well differentiated	33 (13.9)
	Moderately differentiated	116 (48.9)
	Poorly differentiated	88 (37.1)
Lymph node involvement	Negative	52 (16.7)
	Positive	259 (83.3)
Distant metastatic site	No known distant metastasis	63 (19.1)
	Bone, skin, or lung only	189 (57.3)
	Liver, CNS, lung + other organs	78 (23.6)

♣ ALP alkaline phosphatase, ALT alanine aminotransferase, ANC absolute neutrophil count, AST aspartate aminotransferase, BUN, Blood urea nitrogen, BSA body surface area, Hgb hemoglobin, HCT hematocrit, IQR inter quartile range, PLT platelet count, SCr Serum creatinine, SD standard deviation, WBC white blood cell count.

3.1. Association of VDR Polymorphism and Breast Cancer Risk

The genotype distribution (Table 2) indicated that all the *VDR* genes in the case and control groups conformed to the Hardy–Weinberg equilibrium (HWE) (p-value > 0.05). The overall frequencies of

alleles for *rs7975232* (allele C), *rs2228570* (allele A), and *rs731236* (allele G) in Ethiopians were 0.39, 0.21, and 0.38, respectively. There was no significant difference in genotype and allele distribution between breast cancer patients (cases) and control groups ($p > 0.05$) (Table 2) except for a trend ($p = 0.078$) showing a higher frequency of the *rs2228570 G* (*F*) variant allele in cases than in controls.

Table 2. Comparison of the genotype and allele frequency distribution between breast cancer patients and healthy controls.

SNP	Genotype	Genotype Frequency by Group, *N* (%)			Allele Frequency by Group, *N* (%)			
		Cases	Controls	*p*-Value	Allele	Cases	Controls	*p*-Value
rs7975232 (*Apa*I, A > C)	AA	145 (37.5)	84 (43.7)	0.34	A	474 (61.2)	249 (64.8)	0.23
	AC	184 (47.5)	81 (42.2)		C	300 (38.8)	135 (35.2)	
	CC	58 (15)	27 (14.1)					
rs2228570 (*Fok*I, T > C)	AA	23 (5.9)	12 (6.4)	0.12	A	168 (21.5)	98 (26.2)	0.078
	AG	122 (31.3)	74 (39.6)		G	612 (78.5)	276 (73.8)	
	GG	245 (62.8)	101 (54)					
rs731236 (*Taq*I, T > C)	AA	149 (38.3)	74 (38.3)	0.33	A	481 (61.8)	230 (59.6)	0.46
	AG	183 (47)	82 (42.5)		G	297 (38.2)	156 (40.4)	
	GG	57 (14.7)	37 (19.2)					

Stratifying by variant allele carrier status, the *rs2228570 GG* (*GG* vs. *AA* + *AG*) genotype was found to be associated with breast cancer risk (OR = 1.44, 95% confidence interval = 1.01–2.05) (Table 3). The presence of this genotype could confer a 44% increase in the risk of acquiring breast cancer. However, no association was detected between homozygotes for the common *rs2228570 F* allele (*GG* genotype) and *ff* homozygotes ($p > 0.05$). Similarly, *rs7975232* (*CC vs. AA* + *AC* and *C* vs. *A*) and *rs731236* (*GG* vs. *AA* + *AG* and *G* vs. *A*) polymorphisms were not associated with the risk of breast cancer.

Table 3. Association of *VDR* polymorphisms and breast cancer risk.

	Gene	Genotype	Presence of Cancer		*p*-Value
			Yes	No	
Chi-square test	*rs7975232* (*Apa*I, A > C)	AA + AC	329 (66.6)	165 (33.4)	0.77
		CC	58 (68.2)	27 (31.8)	
	rs2228570 (*Fok*I, T > C)	AA + AG	145 (62.8)	86 (37.2)	0.04
		GG	245 (70.8)	101 (29.2)	
	rs731236 (*Taq*I, T > C)	AA + AG	332 (68)	156 (32)	0.16
		GG	57 (60.6)	37 (39.4)	

		Univariate Analysis		Multivariate Analysis	
		‡ OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value
Logistic regression	*rs2228570* (*Fok*I, T > C)				0.04
	AG + AA	1	1	1	
	GG	1.44 (1.01–2.05)	0.044	1.44 (1.01–2.06)	
	rs731236 (*Taq*I, T > C)				-
	AG + AA	1		-	
	GG	0.72 (0.469–1.14)	0.164	-	

‡ OR odds ratio, CI confidence interval.

3.2. Plasma 25(OH)D_3 Concentration and Vitamin D Status among Treatment-Naïve Versus Tamoxifen-Treated Patients

The study outcome variables were plasma 25(OH)D_3 level and vitamin D status; vitamin D deficiency (VDD) was defined as circulating levels of 25(OH)D_3 of < 50 nmol/L, and levels between 51 and 72.5 nmol/L were considered insufficient. Severe vitamin D deficiency (SVDD) was defined as a plasma 25(OH)D_3 level of < 25 nmol/L [21]. The mean plasma 25(OH)D_3 concentration among chemotherapy-naïve patients was significantly lower compared to that among the patients on tamoxifen (p-value < 0.0001, Figure 2).

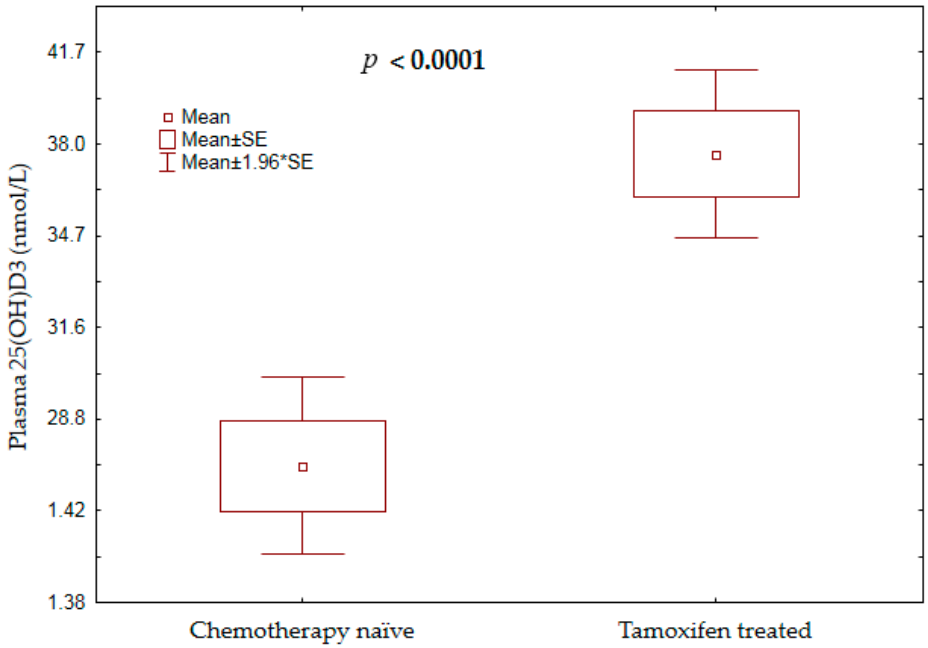

Figure 2. Comparison of mean plasma 25(OH)D_3 concentrations between chemotherapy-naïve patients and those on tamoxifen treatment.

A comparison of Vitamin D Status between chemotherapy-naïve and treatment-experienced patients (tamoxifen group) is presented in Table 4. Overall, 86% of the studied patients were vitamin D deficient with 28% being SVDD and 58% VDD (Figure 1). In the tamoxifen group, the prevalence of vitamin D deficiency was 78.3%, with SVDD and VDD accounting for 11.2% and 67.4%, respectively. On the other hand, 91.9% of chemotherapy-naïve breast cancer patients were vitamin D deficient (Table 4).

Table 4. Vitamin D status on the basis of plasma 25(OH)D_3 concentration in chemotherapy-naïve patients and in those on tamoxifen adjuvant therapy.

Vitamin D Status ♣	Chemotherapy Naïve	Tamoxifen Group	*p*-Value
SVDD (<25 nmol/L)	46 (41.1%)	10 (11.2%)	<0.001
VDD (25–50 nmol/L)	56 (50%)	60 (67.4%)	
Insufficient (51–72.5 nmol/L)	9 (8%)	12 (14.6%)	
Normal (72.5–250 nmol/L)	1 (0.9%)	6 (6.7%)	

♣ SVDD, severe vitamin D deficiency; VDD, vitamin D deficiency.

3.3. Vitamin D Status and Tumor Characteristics

Breast cancer characteristics (degree of differentiation, tumor size, lymph node involvement, or distant metastasis to liver or lung) were not significantly associated with vitamin D deficiency (p-value > 0.05, Chi-square test).

3.4. VDR Genotype with Plasma 25(OH)D_3 Concentration and Vitamin D Status

Overall, there was no significant influence of *VDR* genotype on plasma 25(OH)D_3 concentration. However, stratified by treatment group, the rs7975232 (*Apa*I) *CC* genotype was significantly associated with higher plasma 25(OH)D_3 concentration than the *AA* or *AC* genotype among the tamoxifen group (Figure 3). No such association was found in chemotherapy-naïve patients. There was also no significant association between vitamin D deficiency status and genotype or allele frequency (p-value > 0.05)

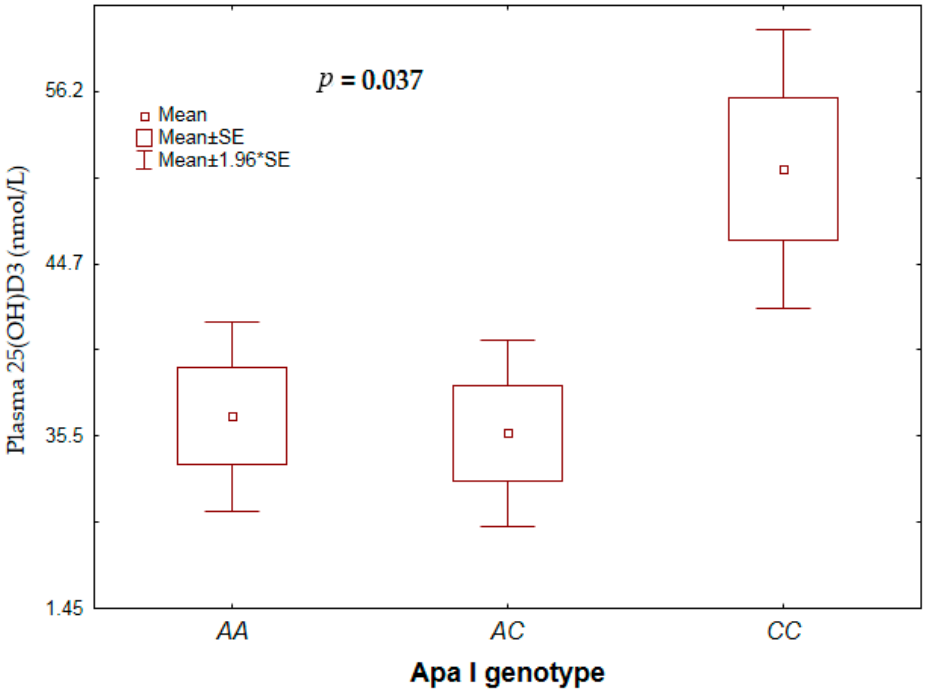

Figure 3. Association between vitamin D deficiency status and rs7975232 (*Apa*I) genotype.

4. Discussion

In recent years, several studies focusing on the effect of the vitamin D pathway have been carried out, mainly in Europeans and African Americans, and the results have generally showed the importance of vitamin D and *VDR* genetic variations in a number of clinical conditions. To the best of our knowledge, there are no published data on vitamin D status among chemotherapy-naïve and treatment-experienced breast cancer patients or on the impact of *VDR* gene polymorphisms on risk for development of breast cancer in the Sub-Saharan black African population, where there is abundant sunshine to form vitamin D.

In the present study, we have observed that vitamin D deficiency is rampant among Ethiopian breast cancer patients, with 86% of them either severely vitamin D deficient (28%) or vitamin D deficient (58%). Chemotherapy-naïve breast cancer patients had significantly lower mean vitamin D levels compared to those of the tamoxifen-treated group (Figure 2). Likewise, the prevalence of severe vitamin D deficiency was much higher among treatment-naïve patients (41%) than among those who survived to complete chemotherapy and continue long-term tamoxifen adjuvant therapy (11%). Although not proven, the results of previous studies showed that plasma vitamin D concentration could influence survival outcomes in cancer patients [40–42]. From this perspective, the studied patients may benefit from increasing their vitamin D levels through vitamin D supplementation, particularly in chemotherapy-naïve patients.

On the other hand, evidence on vitamin D level relative to cancer occurrence is inconclusive. In patients with a 25(OH)D_3 level below 50 nmol/L, higher predicted probabilities of breast cancer

have been reported among African-Americans compared to Hispanics [24]. A result of pooled analysis of two randomized trials and a prospective cohort study demonstrated that breast cancer risk was markedly lower with a higher circulating 25(OH)D_3 level (≥150 nmol/L) [25]. Similarly, the result of a meta-analysis showed a protective relationship between circulating 25(OH)D_3 level and breast cancer development in premenopausal women [23]. On the other hand, a recent nationwide, randomized, placebo-controlled trial (VITAL trial), concluded that supplementation with vitamin D did not result in a lower incidence of invasive cancer or cardiovascular events [43].

Vitamin D deficiency has been described as a problem in many countries around the world, including in Africa [12]. Vitamin D status depends largely on the production of vitamin D_3 in the skin under the influence of ultraviolet radiation and, to some extent, on vitamin D intake through the diet or vitamin D supplements [12]. Vitamin D status could also be associated with underlying health conditions and treatment modality [21,44]. Consequently, vitamin D production varies considerably around the world, across population groups, and between individuals, mainly because of wide differences in skin exposure to ultraviolet B (UVB) radiation, efficiency of cutaneous synthesis, dietary supplementation, and food fortification practices [10].

The frequent observation of low vitamin D levels, particularly in Africa and the Middle East, where abundant daily sunshine is available, may be explained by the traditional dress and avoidance of direct sunlight exposure, and multiple dietary factors as a result of specific cultural beliefs in these regions [15,45]. In Ethiopia, national or population data regarding vitamin D status in Ethiopia are non-existent, and, consequently, information on vitamin D is based on community- and health-facility-based surveys. The Ethiopian Food, Medicine and Health Care Administration and Control Authority (FMHACA) developed a directive for the import and wholesale of food supplements [46]. However, a vitamin D supplementation guideline for either the general population or groups at risk of vitamin D deficiency is unavailable. Estimating dietary intake and skin synthesis appears to be the major challenge for setting requirements. This is probably because sunshine is abundant in Ethiopia and vitamin D deficiency is thought to be unlikely to occur. Previous studies in Ethiopia reported vitamin D deficiency among school children (42%) [20], healthy non-pregnant women (84.2%), [19] and HIV (SVDD 28%) and TB/HIV co-infected patients (SVDD 57%) [21] and pulmonary tuberculosis patients [47,48]. Several factors including duration of sun exposure, chronic illness such as tuberculosis and HIV, female gender, clothing (most females in Ethiopia cover their head and most of their upper body when going outside), old age, and urban residence have been implicated in vitamin D deficiency in Ethiopia [19,47].

The prevalence of low vitamin D levels in the current study population was significantly high: 91.9% for chemotherapy naïve vs. 78.6% for the tamoxifen group. Particularly, for new patients who are about to initiate chemotherapy, the burden of subsequent vitamin D deficiency during chemotherapy treatment is expected to be very high. Although vitamin D status during chemotherapy was not investigated in the present study, other previous studies confirmed that 25(OH)D_3 levels drop considerably further in breast cancer patients on anti-tumor treatment [44,49]. The impact of chemotherapy on vitamin D levels could be severe, such that it may not even be corrected sufficiently by vitamin D supplementation. It was reported that supplementation of vitamin D3 (cholecalciferol), at 400 IU daily, was insufficient to correct chemotherapy-induced vitamin D deficiency in pre-menopausal women with breast cancer undergoing adjuvant chemotherapy [50]. In contrast, a recent study reported that *de novo* vitamin D use post-diagnosis of breast cancer was found to be associated with a reduction in breast-cancer-specific mortality [42].

A plausible mechanism for chemotherapy-induced vitamin D deficiency has been described in the literature. The anti-neoplastic drugs such as taxol are ligands for the pregnane X receptor (PXR) and thereby enhance the catabolism of 25(OH)D_3 and 1,25$(OH)_2D_3$ [51]. Anticancer chemotherapies are also known to cause gastro-intestinal toxicity [52], which can lead to reduced absorption of vitamin D from the gut [44]. Thus, vitamin D deficiency would be expected in almost all breast cancer patients receiving chemotherapy, which could lead to a greater risk of not only bone-health-related problems,

but also compromise clinical outcomes of breast cancer treatment. A recent meta-analysis revealed evidence of an association between higher blood 25(OH)D_3 concentrations and better survival in patients with colorectal cancer [53], indicating better disease prognosis and survival outcome with high circulating vitamin D.

In this study, significantly lower mean vitamin D levels were found in treatment-naïve breast cancer patients compared to those on tamoxifen. Our finding is in agreement with a previous study which demonstrated patients on tamoxifen therapy to have significantly increased serum 25(OH)D_3 levels [54]. Estrogen and selective estrogen receptor modulators (SERMs) can modulate *1-alpha-hydroxylase* activity in the kidney and facilitate the synthesis of more 1,25$(OH)_2D_3$ [55].

The *VDR* polymorphisms have been extensively explored in breast cancer risk assessment studies and their possible significance in breast cancer has been inconclusive. The *BsmI bb* or *Bb* genotype but not *Fok*I was associated with increased risk of breast cancer among Iranian and Egyptian female breast cancer patients [32,33]. However, a meta-analysis report concluded that, in the Caucasian ethnic subgroup or general population, *VDR* polymorphisms (*Fok1, Bsm1, Taq1,* and *Apa1*) were not associated with risk of breast cancer [56].

We have observed that carriers of the *VDR Fok*I *GG* (*FF*) genotype (OR = 1.44, 95% CI 1.01–2.05) were associated with risk of breast cancer. However, no association was detected between homozygotes for the common *Fok*I *F* allele (*GG* genotype) and *ff* homozygotes ($p > 0.05$). The *FokI FF* allele together with other *VDR* polymorphisms has been shown to amplify breast cancer risk in a Caucasian population [57]. In contrast to our finding, the *VDR-Fok*I *f* allele has been associated with increased risk of breast cancer in Canadians [34] and African Americans [58]. On the other hand, another study observed that there was no association between the *Fok*I polymorphism and breast cancer risk in postmenopausal women [59].

An experimental study conducted to evaluate the functional differences between *Fok*I polymorphic alleles in breast cancer cell lines demonstrated that, in response to 1α,25$(OH)_2D_3$ treatments, cell growth was inhibited by 60% in *FF* cells and 28% in *ff* cells. The induction of the vitamin D target gene *CYP24A1* mRNA was 1.8-fold higher in *FF* cells than in *ff* cells. Estrogen receptor-α protein expression was also down-regulated by 62% in *FF* cells and 25% in *ff* cells [60]. These findings suggest that the *ff* genotype may play a role in amplifying aggressive breast cancer. Results of meta-analysis also support the association of *Fok*I *ff* with breast cancer risk [30]. In the present study, the allele frequencies of the *VDR* alleles did not show evidence of significant differences between controls and cases. Consequently, the result observed in our study could not be ascertained. Moreover, the control groups were recruited from the general population and may not truly be negative for breast cancer, as some of them may develop the disease in the future. However, the *Fok*I *FF* allele frequencies are overrepresented in the cases compared to those from the general population who were free of cancer during the study enrolment.

Although the precise reasons are unknown, studies have highlighted racial disparities in cancer susceptibility and disease progression. Breast cancer tends to be diagnosed at a more advanced stage among black women than whites and, subsequently, black women experience elevated breast cancer mortality [61]. American women of African ancestry are more likely to develop breast cancer at a younger age than those with European ancestry and are more likely to have tumors with aggressive characteristics [62]. Another study also showed that more African Americans had severe vitamin D deficiency (<25 nmol/L) than European Americans with the lowest levels among those with the highest African ancestry [63]. The same study also revealed that genetic variants in the vitamin D pathway have been related to higher prevalence of estrogen receptor (ER)-negative breast cancer in African-American women [63]. In the present study, larger proportions (63.2%, mean age 40.77) of breast cancer patients were 40 years of age or below. The inherited genetic variations in the *VDR* gene may contribute in part to susceptibility to cancer at a younger age. However, evidence of an association was not detected between breast cancer characteristics and vitamin D deficiency or *VDR* genotype.

The present study investigated the severity of vitamin D deficiency among breast cancer patients in a resource-limited setting from Africa. Moreover, the study revealed pertinent information regarding the relevance of *VDR* polymorphism in a population with diverse genetic composition which has not been previously examined. However, important limitations were also identified. The small sample size could have hampered the observation of any association between vitamin D deficiency and clinico-pathology of breast cancer in Ethiopia. In addition, we used an immunoassay technique to measure vitamin D level rather than the gold-standard liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS). Consequently, due to patient-group-specific deviations, a lack of distinction between 25(OH)D_2 and 25(OH)D_3, and cross-reactivity of other vitamin D metabolites associated with the immunoassay technique, the result of vitamin D concentration could have been overestimated and misleading. Moreover, chemotherapy-induced vitamin D deficiency and the impact of low vitamin D on the clinical progression of breast cancer was not addressed. Routine laboratory testing is not done to detect the receptor status of the tumor in Ethiopia, and, consequently, association of low vitamin D level with receptor status was not possible. Moreover, data on disease progression and survival status were not incorporated to associate them with vitamin D level and *VDR* polymorphism.

5. Conclusions

In conclusion, we report a high prevalence of vitamin D deficiency in female Ethiopian breast cancer patients. Treatment-naïve patients had low levels of vitamin D compared to patients on tamoxifen. In addition, the *Fok*I *GG* genotype appears to confer an increased risk of breast cancer in Ethiopian women. Further study is recommended to see the impact of chemotherapy on the vitamin D levels of breast cancer patients undergoing treatment and the impact of vitamin D deficiency on the disease progression and clinical outcome. We recommend supplementation of vitamin D and also urge further study to set the optimum dose of vitamin D for Ethiopian breast cancer patients.

Author Contributions: J.H.A., E.M., and E.A. designed the study. J.H.A. collected the data. J.H.A. and E.A. did the genotyping. J.H.A., and E.A. analyzed the data and drafted the manuscript. J.H.A., E.A., E.M., A.F., G.Y., D.S., W.T., M.A., A.A., and R.H. were involved in the discussion of results and critical review of the manuscript. All the authors have read and approved the final manuscript.

Funding: This research was supported by Armauer Hansen Research Institute (AHRI) through the BSPP Program, a grant obtained from Sida-Ethiopia Bilateral Program (Contribution No: 5108013506).

Acknowledgments: The authors would like to extend their sincere appreciation to Armauer Hansen Research Institute (AHRI) for the financial assistance required for the study.

Conflicts of Interest: The authors declare no conflict of interest. The funding source did not have any role in the collection, analysis, or interpretation of data; in writing the paper; or in the decision to submit it.

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