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ROLE OF GENETIC POLYMORPHISMS IN DRUG METABOLIZING ENZYMES AND TRANSPORTERS IN THE TOXICITY OF DOCETAXEL IN LEBANESE BREAST CANCER PATIENTS

BY
Zainab Awada

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of the Faculty of Medicine
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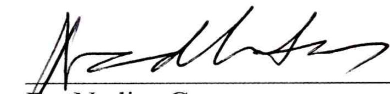

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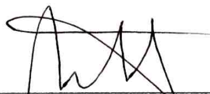

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AN ABSTRACT OF THE THESIS OF

Zainab Ibrahim Awada for Master of Science

Major: Pharmacology and Toxicology

Title: Role of Genetic Polymorphisms in Drug Metabolizing Enzymes and Transporters in the Toxicity of Docetaxel in Lebanese Breast Cancer Patients.

Introduction: Docetaxel, a drug commonly used for the treatment of advanced breast cancer, shows significant interindividual variation in its pharmacokinetic and toxicity profile. Past pharmacogenetic studies have not explained this variation. We investigated the association of genetic polymorphisms in drug metabolizing enzymes and transporters with docetaxel-induced febrile neutropenia by the novel drug metabolizing enzymes and transporters (DMETPlus) microarray platform from Affymetrix, which scans 1936 variants in 225 genes related to drug absorption, distribution, metabolism and elimination.

Material and Methods: This is a pilot case control association study. Out of 100 Lebanese breast cancer patients who received chemotherapy regimens that included docetaxel alone or docetaxel with trastuzumab, 18 developed febrile neutropenia. These were considered as cases and were age- and treatment- matched with 18 others who did not develop febrile neutropenia on docetaxel (controls). Whole blood for DNA was withdrawn, and samples were genotyped using Affymetrix DMET plus platform. Extensive chart review was performed to assess for treatment regimens and toxicity. Data analysis was performed using two sided Fisher exact test.

Results: All arrays passed the quality control metrics (QC>85%). Alleles that were not in Hardy Weinberg equilibrium were excluded. Duplicate samples revealed high reproducibility rates, and there was a high concordance between Affymetrix DMETPlus results and real time-PCR validation assays. Two variants in the ATP transporters showed significant results; for instance, 5/18 controls were found to be heterozygous for *ABCG2 C421A* (*rs2231142*), whereas no case carried this allele (P=0.045). Another variant in *ABCC2* c.1249G>A (rs2273697) was found to be statistically significant in patients who required blood transfusion after treatment with docetaxel; whereby the heterozygous genotype was present in 50% of patients who required blood transfusion and in only 22.2% of patients who did not require blood transfusion after docetaxel injection. Besides, the mutant genotype was present in 25% of patients requiring transfusion and absent in patients not requiring transfusion after docetaxel injection (P=0.024). Both SNPs have not been previously reported to be associated with docetaxel toxicity.

Conclusion: This is the first study that evaluates the effect of a large array of drug metabolizing enzymes and transporters on docetaxel toxicity in breast cancer patients. More extensive analysis, including generation of haplotype blocks and cluster analysis are currently being performed.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette family
ADME	Absorbtion, distribution, metabolism and elimination
ALT	Alanine transaminase
ANC	Absolute neutrophil count
AST	Aspartate transaminase
AUC	Area under the curve
CYP	Cytochrome P450
DMET	Drug Metabolizing Enzymes and Transporters
FAC	5-Fluorouracil, adriamycin and cyclophosphamide
FDA	Food and drug administration
FEC	5-Fluorouracil, epirubicin and cyclophosphamide
GCSF	Granulocyte colony stimulating factor
GST	Glutathione-S-transferase family
HER2	Human epidermal growth factor receptor 2
OATP1B1	Organic anion transporting polypeptide member 1B1
PCR	Polymerase chain reaction
PPAR-б	Peroxisome proliferator-activated receptor gamma
SLCO	Solute carrier organic anion transporter family
SNP	Single nucleotide polymorphism
TAC	Taxotere, adriamycin and cyclophosphamide
VKORC1	Vitamin K epoxide reductase complex subunit 1
WBC	White blood cell

I. INTRODUCTION

A. Definition of Cancer

Cancer is a large group of diseases, most of which are fatal. Cancer is caused by genetic mutations that lead to activation of oncogenes and loss of function of tumor suppressor genes. Most of cancers in adults are carcinomas, originated from epithelial cells that line body cavities and glands. Sarcomas are derived from mesenchymal tissues. Melanomas, retinoblastomas, neuroblastomas and glioblastomas arise from dividing cells in the ocular retina, neurons and neural glia respectively. Lymphomas and leukemias, sometimes referred to as the “liquid tumors”, originate from the tissues that give rise to lymphoid and blood cells (Bunz F., 2008).

B. Definition of Breast Cancer

Breast cancer is the proliferation of malignant epithelial cells lining ducts or lobules of the breast. It is the most common non-skin cancer among women, constituting around one third of all cancer cases in women (Longo D., Fauci A., Kasper D., Hauser S., Jameson L. and Loscalzo J., 2011). It is the second leading cause of cancer death in women after lung cancer. Every year, approximately 184,450 invasive breast cancer cases and 40,500 deaths from breast cancer are reported in the United States. (Jabbari S., Park C. and Fowble B., 2010).

C. Breast Cancer in Lebanon

Breast cancer is the most common cancer among women in the Arab world, including Lebanon. In the year 2004, breast cancer constituted approximately 38.2% of all cancer cases among Lebanese females. Median age at diagnosis was 52.5 years vs. 63 years in the Western countries. Age-standardized incidence rate was around 71.0 per 100,000. Age standardized incidence rate was lower than those estimated for developed countries; but, it was greater than those observed in Arab populations in the region (Salim et al., 2010, Lakkis et al., 2010).

D. Risk Factors of Breast Cancer Development

A number of factors may increase the risk of developing breast cancer. These can be divided into eight categories: sex, age, family history of breast cancer, hormonal factors, proliferative breast disease, irradiation of the breast region at an early age, personal history of malignancy, and lifestyle factors (Bevers T.B., 2007).

1. Sex

Being a woman is the major risk factor for having breast cancer. Breast cancer is 100 times more frequent in women than in men (Costanza M.E., Chen W.Y., 2012).

2. Age

Increasing age is the second most important risk factor for breast cancer after sex. According to the American Cancer Society, the probability of developing breast cancer in the next 10 years is 0.05% at age 20 years. This number increases reaching 3.82% at age 60 (Bevers T.B., 2007).

3. Family history of breast cancer

Women with a family history of breast cancer, especially breast cancer in a first degree relative (mother, sister or daughter) are at increased potential of developing breast cancer. The risk increases more if more than one first degree relative had breast cancer, if breast cancer developed before menopause, or if it were bilateral. The relative risk ranges from 1.5 for post menopausal unilateral breast cancer in a first degree relative to 9 for premenopausal bilateral breast cancer in a first degree relative (Bevers T.B., 2007).

Around 5 to 10% of breast cancer cases were said to be caused by inherited mutations in breast cancer susceptibility genes, mainly *BRCA1* and *BRCA2*. It is quite necessary to identify genetically predisposed individuals because they may have 40 to 80% lifetime risk of developing breast cancer. Eventually, they require regular screening and unique prophylactic measures (Bevers T.B., 2007).

4. Hormonal Factors

There is a link between some reproductive characteristics and increased risk of breast cancer. Early menarche (before 12 years of age), late menopause (at or after 55 years of age), late age at first full term pregnancy (35 years or older), and nulliparity increase the risk of breast cancer. Oophorectomy in women younger than 35 years decreases the breast cancer risk by 60%, thus emphasizing the role of endogenous hormones in the development of breast cancer (Bevers T.B., 2007).

Increased breast mammographic density and increased bone mineral density increase the breast cancer risk, most probably because these factors are influenced by endogenous reproductive hormones levels (Bevers T.B., 2007).

The use of exogenous hormones has also been linked to increased breast cancer risk. The Women's Health Initiative randomized trial clarified the risks of hormone replacement therapy in post-menopausal women (Bevers T.B., 2007). In one arm of the trial, post menopausal women with an intact uterus were randomly assigned to receive estrogen with progesterone or placebo. The combination of estrogen and progesterone increased the breast cancer risk by 24% (Chlebowski R.T. Hendrix S.L., Langer R.D. et al., 2003). In another arm of that trial, women who underwent hysterectomy were randomly assigned to receive estrogen alone or placebo. No increase in breast cancer risk was detected with administration of estrogen alone (Anderson G.L., Limacher M., Assaf A.R., et al., 2004).

5. Proliferative breast disease

Some benign breast lesions are associated with increased risk of breast cancer. This increased risk depends on the presence of epithelial abnormalities. Most benign breast lesions do not show proliferative changes and hence do not increase breast cancer risk. Some exhibit proliferative changes without atypia and increase the breast cancer risk by two folds. Presence of proliferative changes with atypical duct or lobular hyperplasia increase breast cancer risk by 5 folds (Bevers T.B., 2007).

6. Irradiation of the breast region at an early age

Therapeutic irradiation of the breast region in pediatric cancer patients may increase the risk of developing breast cancer in survivors later in life. Patients younger than 5 years receiving radiation therapy may have a 35% increase risk of breast cancer by age 40. Thus, it is strongly recommended to start early breast cancer screening in this population (Bevers T.B., 2007).

7. Personal history of malignancy

Personal history of breast cancer increases the risk of developing subsequent breast cancer. Moreover, personal history of other types of cancer, such as ovarian or colon cancer, may increase the risk of developing breast cancer (Bevers T.B., 2007).

8. Lifestyle factors

Epidemiological studies have elicited a number of lifestyle factors to be potentially associated with an increased risk of breast cancer. Dietary fat is a possible risk factor as there is a high relationship between National per capita fat consumption and incidence in breast cancer. In addition, alcoholic intake may be associated with higher breast cancer risk. It was observed that women consuming at least one alcoholic beverage had a slight increase in breast cancer risk. Current evidence suggests that cigarette smoking does not increase breast cancer risk except possibly in slow acetylators of aromatic amines (Bevers T.B., 2007).

Epidemiological studies have shown an increased risk of breast cancer in highly educated women, possibly related to delayed childbearing and lower parity. Breast feeding may have some protective role against breast cancer in premenopausal women (Bevers T.B., 2007).

E. Types of Invasive Breast Cancer

The mammary epithelium gives rise to a wide variety of histologically diverse breast carcinomas that differ in their prognosis. The most common types include the prognostically unfavorable invasive ductal carcinoma and invasive lobular carcinoma. Some of the prognostically favorable types include tubular carcinoma, mucinous carcinoma and medullary carcinoma (Gallager et al., 1984).

1. Invasive ductal carcinoma

It is the most common carcinoma of the breast. It constitutes about 70 to 80% of all invasive breast lesions. It is also called infiltrating duct carcinoma, carcinoma of no special type, carcinoma with productive fibrosis, scirrhous carcinoma, carcinoma simplex, multinodular carcinoma and stellate carcinoma.

Invasive duct carcinoma constitutes a non-homogeneous group, as it may widely differ in histological and gross appearances. Its cells may be small or large, uniform or pleomorphic. They may be arranged in broad sheets, in nodules of varying size, or in neoplastic gland-like formulations. The gross masses may be multinodular or stellate.

According to the American Cancer Society, most of women are 55 or greater when they are diagnosed with invasive duct carcinoma. Overall survival rate is between 55% and 65% at 5 years (Gallager et al., 1984).

2. Invasive lobular carcinoma

It is also called infiltrating lobular carcinoma. It is the second most common type of breast cancer, accounting for 5 to 10% of all invasive breast cancers. In the United States, incidence rates of invasive lobular carcinoma are rising faster than those of invasive ductal carcinomas (Bleiweiss I.J., 2010).

Compared to invasive ductal carcinomas, invasive lobular carcinomas arise in older women and the usual age at diagnosis is early 60s. As a rule, they are estrogen receptor positive, with variants showing different expressions; hence there may be a strong association between postmenopausal hormonal therapy and increased risk of invasive lobular carcinomas (Bleiweiss I.J., 2010).

Invasive lobular carcinomas tend to metastasize later than invasive ductal carcinomas and spread to unusual sites such as the peritoneum, gastrointestinal tract and meninges. Old studies suggest similar prognosis for invasive lobular carcinomas and infiltrating duct carcinomas; yet, new studies suggest that outcomes may be more favorable for invasive lobular carcinomas. However, invasive lobular carcinomas exist in many variants, and some may have poorer prognosis than others (Bleiweiss I.J., 2010).

3. Tubular carcinoma

It is a rare type of breast cancer. It accounts for about 1 to 2% of invasive breast cancers. The tumor is usually small, characterized by presence of tubules infiltrating the stroma. The tubules are elongated, aligned by a single layer of cuboidal to columnar cells and diffused in dense fibrotic background. Tubular carcinoma has favorable prognosis. Metastasis and recurrence are less likely to occur (Gallager et al., 1984).

4. Mucinous carcinoma

It is a rare type of breast cancer, accounting for about 1 to 2% of invasive breast cancer cases. It is also called colloid or gelatinous carcinoma. The tumor contains large amounts of extracellular, extraluminal mucus in contact with stroma. Histologically, it consists of well-differentiated neoplastic cells forming small clusters. These clusters float in a pool of mucus (Gallager et al., 1984).

Its favorable prognosis is due to several causes. First, it consists of well-differentiated uniform cells that have low grade nuclei. Second, the amount of neoplastic cells is less than that implied by the size of the palpable mass. The mass consists predominantly of mucus, so the ratio of mucus to neoplastic cells is high. Third, the presence of tumor in a readily accessible area to palpation leads to early detection. However, sometimes mucinous carcinoma occurs in combination with invasive ductal carcinoma, and the prognosis would be that of the less favorable type (Gallager et al., 1984).

Mucinous carcinoma tends to affect women after menopause. The usual age at diagnosis is 60 years or more (Gallager et al., 1984).

5. Medullary carcinoma

It accounts for 1 to 10% of invasive breast cancers. Medullary carcinoma is a soft fleshy mass that looks like the medulla of the brain. Histologically, it consists of a

dispersed mixture of lymphocytes and poorly differentiated tumor cells (Bleiweiss I.J., 2010).

Medullary carcinoma has favorable prognosis despite its aggressive histological appearance. It doesn't grow quickly and, in most of the cases, it doesn't spread to the lymph nodes. Usual age at diagnosis is late 40s. Medullary carcinoma is more common in women who are carriers of *BRCA1* mutations (Bleiweiss I.J., 2010).

6. Inflammatory breast cancer

It is a rare and aggressive form of breast cancer. According to the US National Cancer Institute, inflammatory breast cancer constitutes around 0.5 to 2% of all breast cancers in the Unites States, but may be of greater incidence elsewhere (Taghian A., El-Ghamry M.N., Merajver S.D., 2011).

Inflammatory breast cancer is characterized by inflammatory breast appearance that is erythema and edema of around 50% of the breast. This inflammatory appearance is caused by tumor emboli in the dermal lymphatics and not by infiltration of inflammatory cells. Inflammatory breast cancer is a rapidly growing carcinoma with symptoms becoming worse within days or hours. Because of its aggressive nature, it is important to recognize symptoms early and start immediate treatment (Taghian A., El-Ghamry M.N., Merajver S.D., 2011).

7. Paget disease of the breast (PDB)

It is a rare type of breast cancer, accounting for 1 to 3% of all new cases of female breast cancer that are diagnosed every year in the United States (Sabel M.S., Weaver D.L., 2011).

PDB develops in the ducts of the nipple first; it then affects the nipple surface and the areola. The nipple and the areola usually become scaly, itchy and red. Sometimes, a bloody discharge is present. More than 97% of patients with PDB also have breast cancer, either ductal carcinoma in situ or an invasive breast cancer (Sabel M.S., Weaver D.L., 2011).

8. Adenoid cystic carcinoma (ACC)

It is a rare type of breast cancer. It is morphologically identical to ACC of the salivary glands and other organs. Unlike that of the salivary glands, it is of excellent prognosis. Metastasis and recurrence are rare. The reported incidence of axillary metastasis is less than 5% (Gallager et al., 1984).

9. Secretory carcinoma

It is an extremely rare type of breast cancer that affects largely children and adolescents. Histologically, it consists of cells with cytoplasm rich in eosinophils. The cells surround small gland-like spaces, and both the cells and the spaces contain secretory substances (Gallager et al., 1984).

Other breast carcinomas, especially infiltrating duct carcinoma, may affect the young but they don't have the favorable prognosis of secretory carcinoma (Gallager et al., 1984).

Secretory carcinoma may occur in adults, whereby they have good prognosis, but prognosis in the younger subjects is better (Gallager et al., 1984).

10. Metaplastic carcinoma

It includes a combination of poorly differentiated ductal adenocarcinoma, mesenchymal (sarcomatous), and squamous cell carcinomas. Some studies report that tumors with predominant squamous cell carcinomas are aggressive and associated with poor prognosis. Treatment of all metaplastic breast cancers is similar to other invasive breast cancers (Bleiweiss I.J., 2010).

11. Micropapillary carcinoma

This is a rare and aggressive form of breast cancer, accounting for 1% of invasive breast cancer cases. Micropapillary carcinoma tends to spread to the lymph nodes even when its size is small (Bleiweiss I.J., 2010).

12. Phyllodes tumors of the breast

They constitute less than 0.5% of all breast tumors. They differ widely in their biological behavior. They may behave as benign fibroadenomas or as high grade sarcomas. Median age at diagnosis is 42 to 45 years (Grau A.M., Chakravarthy A.B., Chugh R., 2011).

F. Treatment of Breast Cancer

Breast cancer mortality rates have been declining in the past 10 years. This is because of early detection and advances in breast cancer treatment. The available treatment options include surgery, radiation therapy, hormonal therapy and chemotherapy (Green M.C. and Hortobagye G.N., 2007).

1. Surgery

Over the past decade, the surgical procedures used to manage breast cancer have developed significantly, and the trend has moved towards less invasive procedures. Breast conserving surgery followed by radiation therapy (breast conservation therapy) is comparable to mastectomy in terms of survival rate in patients with stage I or stage II invasive breast cancer. The feasibility of breast conservation therapy depends on the relationship between tumor size and breast size. The tumor size should be small enough in relation to the breast to permit tumor excision with adequate margins and acceptable

cosmetic outcome. In addition, the use of neoadjuvant chemotherapy may decrease the tumor size sufficiently to permit breast conservation in case of large primary breast tumors. Mastectomy is performed in cases where radiation therapy is contraindicated. Patients with locally advanced breast cancer- a tumor of 5cm or larger (T3), a tumor that involves the skin or chest wall (T4), or fixed or matted axillary lymph nodes (N2)- are treated with modified radical mastectomy or radiation therapy followed by modified radical mastectomy. In addition, the use of preoperative chemotherapy may convert these patients to candidates of breast conservation therapy. Axillary staging is done by sentinal lymph node surgery i.e. surgical dissection of the first nodes to receive lymphatic drainage from a specific area of the breast and are thus most likely to contain metastases. Only patients with sentinal lymph nodes metastases undergo standard axillary lymph node dissection. Consequently, the use of sentinal lymph node surgery allows selective axillary lymph node dissection (Hunt K.K. and Meric-Bernstam F., 2007).

2. Radiation therapy

Radiation therapy plays a central role in the management of breast cancer. It has a regional role in breast conservation therapy and in the management of ductal carcinoma in-situ or early-stage invasive cancer. Similarly, patients with locally advanced breast cancer who had sufficient response to neoadjuvant chemotherapy may undergo radiation therapy followed by breast conservation surgery. Postmastectomy irradiation decreases the risk of recurrence in patients with intermediate or advanced-stage breast cancer. In addition,

patients with symptomatic metastases unresponsive to systemic agents may experience substantial palliation after irradiation (Tereffe W. and Strom E.A., 2007).

3. Hormonal therapy

Endocrine therapy was introduced in 1896, when Beatson demonstrated a decrease in breast cancer risk after oophorectomy. A number of endocrine therapies are currently used as palliative treatment for patients with hormone-receptor positive metastatic breast cancer and as adjuvant treatment for hormone-receptor positive early breast cancer.

Endocrine therapy results in palliation of disease in 50 to 60% of patients with hormone-sensitive metastatic breast cancer. Available endocrine therapies include ovarian ablation, hormonal agonists, synthetic agents and selective aromatase inhibitors (Pinder M.C. and Buzdar A.U., 2007).

a. Ovarian ablation

Ovarian ablation occurs by surgical treatment, radiation therapy or treatment with luteinizing hormone-releasing hormone (LHRH) agonists. The FDA-approved luteinizing hormone-releasing hormone is goserelin acetate. The three types of treatment result in similar response rate (Pinder M.C. and Buzdar A.U., 2007).

b. Hormonal agents

i. Progestins

Progestins are synthetic derivatives of progesterone that exert agonist effect on progesterone receptors. Their mechanism of action is unknown, but they may interrupt the ovarian pituitary axis because of their antiestrogenic properties. Megestrol acetate is the only FDA-approved progestin for treatment of advanced breast cancer. With the introduction of aromatase inhibitors, progestins are used as 4th line therapy instead of 2nd line therapy (Pinder M.C. and Buzdar A.U., 2007).

ii. Androgens

Androgens may be used in patients with metastatic breast cancer who have been treated with many endocrine agents and still have hormone-dependent disease (Pinder M.C. and Buzdar A.U., 2007).

c. Aromatase Inhibitors

In postmenopausal women, low levels of estrogens are produced in the peripheral tissues such as fat, muscle, liver and breast by aromatization of adrenal estrogens. This amount of estrogen is sufficient to stimulate the growth of estrogen dependent tumors. The aromatase inhibitors are used as treatment in post-menopausal women with breast cancer. Aromatase inhibitors can be divided into 2 categories, non-selective and selective.

The nonselective aromatase inhibitors, such as testolactone and aminoglutethimide, block aromatase enzyme and other enzymes in the cytochrome P450 family. They alter other steroid hormone levels and lead to many side effects. They have hence been replaced by selective aromatase inhibitors that have a better safety profile.

The selective aromatase inhibitors inhibit aromatase enzyme, and they hence affect only the estrogen level. These include formestane (4-hydroxy androstenedione), anastrazole, letrozole, exemestane and fadrozole. Formestane and exemestane are suicidal aromatase inhibitors. Anastrazole and letrozole are FDA approved as first line adjuvant treatment in postmenopausal women with breast cancer. In addition, they are the agents of choice for first and second line therapy for postmenopausal women with hormonally responsive metastatic breast cancer. Both agents are now administered as first line adjuvant therapy in postmenopausal women with hormone receptor-positive breast cancer. Exemestane is FDA approved for second line therapy of patients with hormone sensitive metastatic breast cancer. Because of the limited cross resistance between exemestane and other competitive aromatase inhibitors, exemestane can be used in breast cancer patients who had disease progression while receiving anastrazole or letrozole. In addition, exemestane is now used as an adjuvant therapy after 2 to 3 years of tamoxifen in women with estrogen receptor-positive breast cancer (Pinder M.C. and Buzdar A.U., 2007).

d. Antiestrogens

Antiestrogens, also called selective estrogen receptor modulators, are the preferred first line hormonal therapy for breast cancer in premenopausal women. These drugs block the action of estrogenic compounds, 17-ß-estradiol and estrone, on estrogenic receptors. They include tamoxifen, toremifene, fulvestrant and TAS-108 (Pinder M.C. and Buzdar A.U., 2007).

i. Tamoxifen

Tamoxifen is indicated in women with high risk of developing breast cancer, as it decreases the incidence of invasive and noninvasive breast cancers by 50%. It is also indicated as an adjuvant therapy of early breast cancer in premenopausal women; given for 5 years to reduce the incidence of recurrence. In postmenopausal women, there is superiority of aromatase inhibitors over tamoxifen. In addition, tamoxifen is FDA approved as a first line treatment for metastatic breast cancer in pre- and postmenopausal women, as well as in men. Because tamoxifen may act as a weak estrogen agonist in tumors that overexpress epidermal growth factor receptor or HER-2, aromatase inhibitors may be preferable to tamoxifen therapy in treatment of such tumors (Pinder M.C. and Buzdar A.U., 2007).

ii. Toremifene

Toremifene is a triphenylethylene analogue of tamoxifen. Toremifene is FDA-approved as a first line treatment of metastatic estrogen receptor positive breast cancer or

for breast cancer of unknown estrogen receptor status. Toremifene and tamoxifen demonstrate similar efficacy in treatment of metastatic hormone-sensitive breast cancer. Both tamoxifen and toremifene increase the risk of endometrial cancer (Pinder M.C. and Buzdar A.U., 2007).

iii. Fulvestrant

Fulvestrant is a pure estrogen receptor antagonist that does not stimulate the endometrium. Fulvestrant is FDA approved for treatment of postmenopausal hormone sensitive breast cancer that was not responsive to other antiestrogens (Pinder M.C. and Buzdar A.U., 2007).

iv. TAS-108

TAS-108 is a novel antiestrogenic compound with a different mechanism of action from those of other antiestrogens. It has agonist action on the estrogen receptors in the bone and cardiovascular system. Phase I studies did not report any effect of TAS-108 on the endometrium (Pinder M.C. and Buzdar A.U., 2007). A phase II study has been completed, and phase III studies are being planned (Taiho Pharma USA, Inc., 2012).

4. Chemotherapy:

a. Adjuvant vs. neoadjuvant chemotherapy

i. Adjuvant chemotherapy

The goal of adjuvant (postoperative) chemotherapy is to attack any micrometastases in stage I to III operable breast cancer, thus reducing the recurrence risk. Multiple studies have showed that adjuvant chemotherapy benefits women with operable breast cancer. Adjuvant chemotherapy is usually administered within 8 weeks after surgery. Patients should receive adjuvant chemotherapy before but not after adjuvant radiation therapy in order to reduce the risk of systemic recurrence. The standard duration of adjuvant chemotherapy (excluding trastuzumab) ranges between 3 to 6 months depending on the administered regimen (Green M.C. and Hortobagye G.N., 2007).

ii. Neoadjuvant chemotherapy

Neoadjuvant (preoperative) chemotherapy has several theoretical advantages. It may attack the tumor cells before drug resistance develops. It can decrease the size of the tumor and thus enable the use of breast conservation therapy with improved cosmetic outcome. In addition, neoadjuvant chemotherapy allows the determination of tumor sensitivity to therapy. Since the sensitivity of the tumor to chemotherapy is similar to that of the occult micrometastases, neoadjuvant chemotherapy limits the use of ineffective therapy after surgery (Green M.C. and Hortobagye G.N., 2007).

b. Chemotherapy regimens

Chemotherapy is given if the absolute neutrophil count is greater than 1500/µl and platelet count is greater than 100 * 10^3/µl. Chemotherapy regimens commonly prescribed for the treatment of breast cancer are shown in table 1 (Green M.C. and Hortobagye G.N., 2007). They include:

i. Classic regimens

These include:

- 5-fluorouracil 500mg/m^2 administered intravenously, cyclophosphamide 500 mg/m^2 administered intravenously every 21 days for 6 cycles, with doxorubicin 50mg/m^2 administered as continuous IV infusion over 72 hours (FAC) or epirubicin 100mg/m^2 (FEC). Doxorubicin is infused over a prolonged period of time in order to permit administration of a higher cumulative dose with a lower peak level. This is because it is reported that the risk of doxorubicin-induced cardiac damage decreases when its systemic peak levels are lower.
- Cyclophosphamide 500mg/m^2 can also be administered intravenously with methotrexate 50mg/m^2 and 5-fluorouracil 500mg/m^2 administered intravenously (CMF): This combination is reserved for patients in whom anthracyclines such as doxorubicin are contraindicated. Non-anthracycline containing regimen is inferior to anthracycline containing regimen in terms of survival rate and risk of recurrence (Green M.C. and Hortobagye G.N., 2007).

ii. Trastuzumab-based regimens

Trastuzumab is a monoclonal anti-*HER2/neu* antibody that targets *HER2/neu* overexpressing breast cancer cells. The addition of trastuzumab to the chemotherapy regimen improves the disease-free survival in patients with early breast cancer *HER2/neu* positive breast cancer. In addition, trastuzumab improves both disease-free survival and overall survival in patients with *HER2/neu* overexpressing metastatic breast cancer (Green M.C. and Hortobagye G.N., 2007).

iii. Bevacizumab-based regimens

Bevacizumab is a monoclonal antibody that targets vascular endothelial growth factor (VEGF). Bevacizumab is used in combination with chemotherapy for the treatment of metastatic breast cancer. A combination of bevacizumab and paclitaxel is used at M.D Anderson as first line therapy for metastatic breast cancer. The addition of bevacizumab to paclitaxel improved the response rate and disease-free survival in patients with metastatic breast cancer as compared to paclitaxel alone. However, this combination resulted in more severe adverse events, including neuropathy, proteinurea and hypertension (Green M.C. and Hortobagye G.N., 2007).

iv. Regimens containing anthracyclines and taxanes (paclitaxel or docetaxel)

It is hypothesized that tumor cells vary in their ability to resist drugs. If this is the case, then the administration of drugs of different mechanisms of action, such as taxanes

and anthracyclines, decreases drug resistance and increases tumor cell kill. In addition, since taxanes and doxorubicin target cells at different phases, cytotoxicity is enhanced.

Taxanes include paclitaxel (Taxol) and docetaxel (Taxotere). Paclitaxel activity acts only on the mitosis phase, so paclitaxel is administered every week or 2 weeks. However, docetaxel can interfere with the mitosis, synthesis and G2 phases, so it is given every 3 weeks. Taxanes are indicated as first line treatment against metastatic breast cancer for patients previously treated with anthracyclines or for patients with conditions that preclude administration of anthracyclines. In addition, taxanes are used as second line therapy for patients with metastatic breast cancer who did not benefit from 5-fluorouracil, doxorubicin and cyclophosphamide regimen.

Furthermore, the use of taxanes in addition to anthracycline-based therapy is used in patients with poor prognosis- young patients, patients with hormone receptor-negative breast cancer, patients with nodal involvement or patients with poorly differentiated tumor cells (Green M.C. and Hortobagye G.N., 2007).

Commonly prescribed regimens containing taxanes include:

- Four cycles of doxorubicin and cyclophosphamide followed by four cycles of taxanes: This treatment regimen improves the survival rate as compared to doxorubicin and cyclophosphamide alone, especially when given in women with estrogen receptor-negative tumors (Green M.C. and Hortobagye G.N., 2007).

- Four cycles of 5-fluorouracil, cyclophosphamide with doxorubicin or epirubicin followed by paclitaxel 80mg/m2 weekly for 12 weeks or four cycles of docetaxel

100mg/m2 every 3 weeks: This chemotherapy regimen is used with trastuzumab for the treatment of stage-I and stage II *HER2/neu* positive breast cancer. In addition, it is prescribed for patients with node-positive stage II breast cancer or with locally advanced breast cancer (Green M.C. and Hortobagye G.N., 2007).

- Docetaxel 75mg/m2, doxorubicin 50mg/m2 and cyclophosphamide 500mg/m2 administered every 3 weeks for 6 cycles: This regimen is used for early stage breast cancer; but it necessitates the use of growth factors (Green M.C. and Hortobagye G.N., 2007).

G. Docetaxel

1. Mechanism of action

Docetaxel is an antineoplastic agent that binds to free tubulin and promotes its polymerization into stable microtubules while inhibiting its depolymerization. This leads to cell cycle arrest during mitosis followed by apoptosis (http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/201525s002lbl.pdf).

2. Dosage and Administration

In patients with locally advanced or metastatic breast cancer, the recommended docetaxel dose is 60 mg/m^2 to 100 mg/m^2 given intravenously over 1 hour every 3 weeks. Docetaxel should not be given if the absolute neutrophil count is less than 1500 cells/mm^3

and the platelet count is less than 100,000 cells/mm^3. Patients who receive 100mg/m^2 of docetaxel dose injection and experience febrile neutropenia or severe cutaneous reactions should have the dosage adjusted from 100mg/m^2 to 75mg/m^2. If these reactions persist, the dosage should be further reduced to 55mg/m^2 or the treatment should be discontinued.

In patients with operable node-positive breast cancer, the recommended dose of docetaxel injection is 75 mg/m^2 given over 1 hour after doxorubicin 50 mg/m^2 and cyclophosphamide 500mg/m^2 every 3 weeks for 6 cycles. Frequent blood counts should be done before every docetaxel dose injection. Docetaxel injection should not be administered if the neutrophil count is below 1500 cells/mm^3 and the platelet count is less than 100,000 cells/mm^3(http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/201525s002lbl.pdf).

Patients who are given combination therapy with docetaxel injection and experience febrile neutropenia (defined as fever with an absolute neutrophil count below 500 cells/mm^3) should receive Granulocyte-colony stimulating factor (GCSF) for all subsequent cycles of docetaxel. If febrile neutropenia continues to occur in the subsequent cycles of docetaxel, patients should remain on GCSF and should have their docetaxel dose reduced to 60mg/m^2. In patients who continue to experience febrile neutropenia, the treatment should be discontinued and the patients should be shifted to another treatment regimen (http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/201525s002lbl.pdf).

All patients should receive oral corticosteroids such as dexamethasone 16 mg/day for 3 days starting 1 day before docetaxel administration in order to reduce the incidence

and severity of fluid retention and hypersensitivity reactions (http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/201525s002lbl.pdf).

3. Adverse reactions

The most common adverse reactions associated with docetaxel therapy are infections, neutropenia, anemia, febrile neutropenia, hypersensitivity, thrombocytopenia, neuropathy, dyspnea, loss of or distortion of taste, constipation, anorexia, nail disorders, fluid retention, general fatigue, pain, nausea, vomiting, diarrhea, mucositis, alopecia, skin reactions and myalgia (http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/201525s002lbl.pdf) (Table 2).

The most serious adverse effects of docetaxel are:

a. <u>Toxic death</u>

Docetaxel administered to breast cancer patients at 100 mg/m^2 was associated with death possibly related to treatment in 2% of patients with metastatic breast cancer having normal liver function. In addition, docetaxel dosed at 60 mg/m^2 lead to treatment-related mortality in 0.6% of breast cancer patients with normal liver function and in 3 of 7 patients with abnormal liver function. Most of the deaths occurred due to sepsis (http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/201525s002lbl.pdf).

b. Neutropenia

All patients given 60 mg/m^2 to 100mg/m^2 of docetaxel experience neutropenia (<2000 neutrophils/mm^3). Grade 4 neutropenia (<500mg/mm^3) occurs in 85% of patients given 100mg/m^2 and 75% of patients given 60 mg/m^2. Frequent blood cell counts should be performed so that docetaxel dose can be adjusted. Docetaxel should not be reinfused except if the neutrophils recover to a level >1500 cells/mm^3 and the platelets recover to a level > 100,000 cells/mm^3. The incidence of febrile neutropenia is dose-dependent. Febrile neutropenia occurred in around 12% of patients receiving 100 mg/m^2 docetaxel, but it was very uncommon in patients receiving 60mg/m^2 of Docetaxel (http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/201525s002lbl.pdf).

c. Hypersensitivity reactions

Severe hypersensitivity reactions characterized by generalized erythema, hypotension and/or bronchospasm, or in very rare cases fatal anaphylaxis, have been reported even in patients premedicated with 3 days of corticosteroids. Severe hypersensitivity reactions require discontinuation of docetaxel infusion and administration of appropriate therapy.

Mild hypersensitivity reactions such as flushing or localized skin reactions may occur within few minutes after initiation of docetaxel infusion. Discontinuation of therapy is not required in this case.

All patients should be premedicated with an oral corticosteroid such as dexamethasone 16mg per day for 3 days starting 1 day before docetaxel administration in order to reduce the severity of hypersensitivity reactions. Patients should be observed closely for hypersensitivity reactions during docetaxel injection infusion, especially during the first and second infusions (http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/201525s002lbl.pdf).

d. Fluid retention

Severe fluid retention has been reported in patients given docetaxel infusion. Fluid retention generation occurs in a two-step process. Capillary hyperpermeability occurs between the second and the fourth cycle resulting in congestion of the interstitial space by proteins and water. This is followed by insufficient lymphatic drainage starting from the fifth cycle (Behar et al. 1997). When fluid retention occurs, peripheral edema starts and may become generalized with a median weight gain of 2 kg. After the last infusion of docetaxel, fluid retention is completely, but sometimes slowly, reversible with a median of 16 weeks from the last docetaxel infusion.

All patients receive oral corticosteroids before each docetaxel dose to reduce the incidence and severity of fluid retention (http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/201525s002lbl.pdf).

H. Interindividual Variability in the Pharmacokinetics of Docetaxel

Docetaxel is not absorbed in the gastrointestinal tract, and it is hence given as an intravenous infusion. *In vitro* studies showed that 94% of docetaxel is bound to proteins, mostly to α1-acid glycoprotein, albumin and lipoproteins. Dexamethasone does not alter the protein binding of docetaxel. The bulk of docetaxel is metabolized and excreted through the bile in the feces indicating that urinary excretion is minimal (http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/201525s002lbl.pdf). Docetaxel enters the liver cells via the most efficient hepatocellular influx carrier, solute carrier organic anion transporter family member 1B3 (SLCO1B3) also known as organic anion-transporting polypeptide 1B3 (OATP1B3). Inside the liver cells, docetaxel is metabolized by CYP3A4 and CYP3A5 to inactive hydroxylated metabolites. The primary route of elimination of the parent drug and hydroxylated metabolites is hepatobiliary excretion by the membrane localized energy-dependent drug efflux transporters ABCB1, ABCG2, ABCC1 and ABCC2 (Oshiro C., McLeod H., Carrillo M. et al., 2010 and Longo et al., 2010) (Figure 2).

Docetaxel treatment is associated with a wide interindividual variability in its pharmacokinetic parameters; with an up to tenfolds variability in clearance, even in patients with normal liver function. This wide variation in docetaxel clearance may contribute to the interindividual variability in drug therapeutic response and toxicity. It has been observed that a 50% decrease in docetaxel clearance increases the odds of experiencing docetaxel induced grade 4 neutropenia by 3 (Baker et al., 2009 and Longo et al., 2010). In addition, in a pharmacokinetic study done on 24 patients receiving docetaxel

at a dose of 100mg/m^2 administered as IV infusion, patients had a mean under the curve of docetaxel of 3.1 ± 0.9 h.mg/l (fig. 1) (Rosing et al., 2000).

Causes of these interindividual variations may include:

1. Age

The clearance of docetaxel appears to be greater in children than in adults. For instance, pediatric patients on 55-75mg/m2 of docetaxel administered by intravenous infusion of 1 h every 3 weeks had a mean docetaxel clearance of 33.2 l/h/m^2; however elderly patients receiving 40 to 145 mg/m^2 of docetaxel via intravenous infusion for 1 h every 3 weeks had a mean docetaxel clearance of 20.2 l/h/m^2 (Clarke et al., 1999).

When compared to adult patients aged < 65 years, elderly patients aged ≥ 65 years have unaltered docetaxel plasma pharmacokinetics; yet elderly patients tend to be more sensitive to docetaxel-induced neutropenia (Tije *et al.* 2005).

2. Gender

Females have 35% lower clearance of docetaxel when compared to males (Longo *et al.* 2010).

3. Ethnicity

The mean docetaxel clearance appeared to be lower in Asians (15.3 +/- 4.0 l/h/m2) when compared to the value reported for Whites and Japanese (22 l/h/m2). However, this difference in docetaxel clearance may be caused by the small sample size, assay variation and differences in sampling time points. Furthermore, neutropenia and febrile neutopenia appeared more significantly in Asians when compared to westerns. This observation is consistent with the lower docetaxel clearance in Asians and with the finding that Asians may have lower CYP3A activity than whites (Goh *et al*. 2002).

4. Hepatic impairment

Patients with hepatic dysfunction have reduced docetaxel clearance and increased risk of docetaxel induced grade 3 or grade 4 neutropenia. Therefore, it is recommended to reduce docetaxel dose in patients with hepatic impairment. In addition, patients with bilirubin > upper limit of normal should not receive docetaxel. Patients with mild to moderate liver impairment having alkaline phosphatase > 2.5 * upper limit of normal concomitant with AST and/or ALT >1.5 * upper limit of normal had docetaxel clearance lowered by an average of 27%, leading to an increase in systemic exposure (AUC) by 38%. These patients should not receive docetaxel. Note that liver metastasis, in absence of liver dysfunction is not associated with decreased docetaxel clearance (http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/201525s002lbl.pdf, Clarke et al., 1999).

5. Genetic polymorphisms

It has been proposed that the inherited differences in metabolism and excretion could explain, at least in part, the variable pharmacokinetics and pharmacodynamics of docetaxel. In addition, the population differences in allele frequency could explain the interethnic variations in docetaxel disposition and toxicity. (Longo et al., 2010)

I. Pharmacogenetics of Breast Cancer

Great heterogeneity exists in a patient's response to cytotoxic drugs. As a result, several strategies have been developed to identify more homogenous groups. Currently, breast cancer treatment depends on the histological grading, tumor size, nodal involvement, presence or absence of metastasis, hormone receptor status, and *HER2*/*neu* overexpression. Still, patients with the same clinical and pathological characteristics show variable responses to the same chemotherapeutic regimen. For example, only 25 to 30% *HER2*/*neu* overexpressing patients are responsive to trastuzumab and lapatinib, drugs that specifically target Her2/*neu* expressing cells (Longo et al., 2010).

It is believed that pharmacogenetics, the study of inherited variations in the DNA sequence influencing drug response, may enable oncologists to stratify the treatment regimens to patients according to their genetic profile. Most of the chemotherapeutic drugs have a narrow therapeutic index and genetic polymorphisms leading to substantial changes

in drug elimination result in patients reacting differently to the same dose, calculated on the basis of body surface area. The most common type of genetic polymorphism is the presence of single base diversity in the DNA sequence termed single nucleotide polymorphism (SNP). Some SNPs result in variability in drug elimination. The segregation of patients on the basis of genotype profiling may identify more homogenous subgroups of patients by differentiating those more likely from those less likely to benefit from therapy (Longo et al., 2010).

J. Effect of Genetic Polymorphisms in Drug Metabolizing Enzymes and Transporters on the Pharmacokinetics and Pharmacodynamics of Commonly Used Drugs in Breast Cancer Treatment

Drug metabolism and transport are two major routes of elimination for most drugs. Drug metabolism is mediated by two sets of reactions, phase I and phase II. Phase I reactions are most frequently catalyzed by the cytochrome P450 system. Phase II reactions are conjugation reactions catalyzed by sulfotransferases, UDP-glucoronosyltransferases, glutathione-S-transferase, N-acetyl transferases, and methyltransferases. Drug metabolism may result in inactive, active or toxic metabolites. The role of drug metabolism is to transform the drug from a lipophilic into a hydrophilic form readily eliminated from the body. Drug transport is mediated by two main families of transporters, ATP-binding cassette of transporters and solute carriers. Drug transporters facilitate the export of drugs

from healthy tissue and mediate their elimination from the body (Brunton L.L., Lazo J.S. and Parker K.L., 2006).

Drug metabolizing enzymes and transporters are involved in the metabolism and transport of many cytotoxic agents. The following is a description of drug metabolizing enzymes and transporters involved in the pharmacokinetic route of commonly used cytotoxic agents.

1. Tamoxifen

Tamoxifen is metabolized by CYP3A4/5 to its major metabolite, N-desmethyltamoxifen and by CYP2D6 into its minor metabolite, 4-hydroxytamoxifen. Both N-desmethyltamoxifen (by CYP2D6) and 4-hydroxytamoxifen (by CYP3A4/5) are metabolized to 4-hydroxy-N-desmethyltamoxifen (endoxifen). Both 4-hydroxytamoxifen and endoxifen are 100 times more potent than tamoxifen, but the plasma level of endoxifen is up to 14-fold higher than 4-hydroxytamoxifen. Tamoxifen metabolites are inactivated through conjugation by sulfotransferases such as SULT1A1 or by UDP-glucoronosyl transferases such as UGT1A8, UGT1A10, UGT2B7, UGT2B15 and UGT2B17. In addition, isomerization of trans 4-hydroxytamoxifen to its weakly estrogenic cis isomer may occur by CYP1B1, CYP2B6 and CYP2C19 (Desta Z., Nguyen A., Flockhart D., et al. 2011).

Many of the tamoxifen metabolizing enzymes are polymorphic with more than 80 allelic variants having been described in *CYP2D6*, the key enzyme catalyzing N-

desmethyl tamoxifen to endoxifen. Many of these variants lead to increased, decreased or absent enzyme activity resulting in ultrarapid, intermediate or poor metabolizers respectively. The variants *CYP2D6*3, CYP2D6*4, CYP2D6*6* result in absent enzyme activity. The frequency of these variants varies interethnically. Goetz *et al.* reported an association between *CYP2D6*4* genetic polymorphism and worse relapse free and disease free survival in postmenopausal breast cancer patients treated with tamoxifen. The variant *CYP2D6*10* that occurs in 38% to 70% of Asians and *CYP2D6*17* that occurs in 20 to 34% of Africans result in decreased enzyme activity and intermediate metabolism. A Korean study reported that patients homozygous for the *CYP2D6*10* variant had lower plasma levels of tamoxifen metabolites and shorter time to progression compared to carriers of the wild-type allele (Lim et al, 2007). Gene duplication or multiduplication of functional alleles results in increased enzyme activity and ultrarapid metabolizer phenotype, thus better survival when compared to extensive metabolizers or intermediate metabolizers. Therefore, these patients are likely to benefit from tamoxifen use before taking aromatase inhibitors (Longo et al., 2010 and Tan et al., 2008).

2. Anthracyclines

The disposition of anthracyclines involves various drug metabolizing enzymes and transporters that may contribute to interindividual variability. The anthracyclines doxorubicin and epirubicin undergo phase I reduction to doxorubicinol and epirubicinol by carbonylreductases (CBR1 and CBR3) in the liver and aldoketoredustase (AKR1A) in the heart tissue. Both drugs enter the cells by SLC2A16 and are effluxed out by ATP-binding

cassette of transporters including ABCB1, ABCC1, ABCC1, ABCG2 and v-ral simian leukemia viral oncogene homolog A (RalA) binding protein 1 (RALBP1). Epirubicin also undergoes phase II conjugation reactions, predominantly by UDP-glucoronsyltransferase 2B7 (Thorn C.F., Oshiro C., Marsh S., 2011).

Fan *et al.* studied the relationship between carbonyl reductases CBR1 and CBR3 genetic polymorphisms and doxorubicin pharmacokinetics and pharmacodynamics. The study revealed a correlation between the two common *CBR3* variants (CBR311G>*A* and *CBR3 730G>A*) and doxorubicin pharmacokinetics and pharmacodynamics. The *CBR3 11G>A* variant was related to a lower reduction of doxorubicin to doxorubicinol, greater tumor reduction and hematological toxicities, while *CBR3 730 G>A* variant was related to increased reduction of doxorubicin to doxorubicinol and had no effect on toxicity. The *CBR3 11A* variant is found in 57% of the Chinese population and may contribute to the doxorubicin-induced hematologic toxicity observed in Chinese (Tan et al., 2008).

Anthracyclines are converted to organic oxidation products that contribute to more cellular damage, and glutathione-S-transferase catalyses the reduction of these secondary oxidation products. Ambrosone *et al.* studied the relationship between variants in glutathione-S-transferase gene and clinical outcome of doxorubicin chemotherapy; they found that carriers of null *GSTM1* or *GSTT1* genotypes had reduced mortality (Tan et al., 2008).

Among the ATP binding cassette of transporters, the *ABCB1* gene is one of the most investigated. Many focused on the relationship between *ABCB1* variants (*1236C>T,*

2677G>T/A and *3435C>T*) that seem to impair ABCB1 substrate transport and anthracycline disposition. Kafka *et al.* reported that *ABCB1 3435T* variant was associated with better response to anthracyclines. Another study in Asian breast cancer patients reported that *ABCB1 c.1236-2677-3435 CC-GG-CC* haplotypes may lead to lower doxorubicin exposure than the *CT-GT-CT* and *TT-TT-TT* haplotypes (Tan et al., 2008).

3. Cyclophosphamide

Cyclophosphamide is a prodrug that is predominantly converted by the hepatic cytochrome P450 (CYP) isoenzymes (CYP2B6 and CYP3A4 with CYP2A6, CYP2C9 and CYP2C19 having minor contributions) to the active cytotoxic form, 4-hydroxycyclophosphamide. Cyclophosphamide undergoes a minor metabolic pathway whereby it is converted mainly by CYP3A4 with minor contributions of CYP2B6 to 2-dechloroethylcyclophosphamide and the neurotoxic agent, chloroacetaldehyde (Shukla S., Nguyen A., Thorn C. et al, 2011).

4-hydroxycylophosphamide interconverts to aldophosphamide that undergoes spontaneous (non-enzymatic) elimination reaction to the clinical cytotoxic agent, phosphoramide mustard and the toxic agent, acrolein. Aldophosphamide is oxidized to the inactive carboxyphosphamide mainly by aldehyde dehyrogenase 1H1 (ALD1H1) with aldehyde dehydrogenase 3A1 (ALD3A1) and aldehyde dehydrogenase (ALD5A1) having minor contributions. Multiple cyclophosphamide metabolites can undergo phase II

conjugation by GSTP1 and GSTA1 resulting in the formation of various conjugates (Shukla S., Nguyen A., Thorn C. et al, 2011).

Bray et al. investigated the influence of genetic polymorphisms in various pathway genes on clinical response of breast cancer patients treated with doxorubicin and cyclophosphamide. The *ABCB1 2677A, CYP2B6*2, CYP2B6*4, CYP2B6*8 and CYP2B6*9* alleles were related to worse outcome. *CYP2B6*5* was related to greater incidence of dose delay (indicative of more toxicity) and may be associated with longer progression free survival, both suggesting cyclophosphamide activation (Bray et al., 2010).

4. 5-fluorouracil

Although 5-fluorouracil is a commonly prescribed anticancer agent, serious adverse drug reactions including grade 3 or grade 4 mucositis, thrombopenia and leucopenia are a major clinical problem. Certain genetic polymorphisms in pathway genes have been linked to more severe drug-induced toxicity (Van Kuilenberg et al., 2003).

5-fluorouracil is catabolized to dihydrofluorouracil by dihydropyimidine dehydrogenase. Dihydrofluorouracil is then converted by dihydropyrimidinease to fluoro-beta-ureidopropionate that is converted to fluoro-beta-alanine by beta-ureidopropionase (UPB1). 5-fluorouracil is activated by conversion to 5-fluorodeoxyuridine monophosphate that inhibits thymidylate synthase (TYMS). The conversion of 5-fluorouracil to its active metabolite, 5 fluorodeoxyuridine monophosphate, is mediated by thymidylate

phosphorylase and thymidine kinase or fluorouridine monophosphate and ribonucleotide reductase (Thorn C.F., McLeod H., Carrillo M.W. et al., 2011).

Genetic polymorphisms in dihyropyrimidine dehydrogenase, the rate limiting enzyme in 5-fluorouracil catabolism, have been linked to 5-fluorouracil toxicity. *The G to A* mutation in the GT 5' splice recognition site of intron 14 in the *dihydropyrimidine dehyrogenase gene* has been correlated with dihydropyrimidine dehydrogenase deficiency and more toxic side effects of 5-fluorouracil (Raida et al. ,2001).

Van Kuilenberg *et al.* reported a relationship between dihydropyrimidinease deficiency and 5-fluorouracil toxicity. Carriers of a missense mutation *833G>A* in exon 5 in *dihydropyrimidinease gene* had partial dihydropyridinease deficiency and eventually less capacity to degrade 5-fluorouracil and dihyrofluorouracil and more serious drug related side effects (Van Kuilenberg et al., 2003).

Pullarkat *et al.* reported that genetic polymorphisms of thymidylate synthase, the enzyme responsible for catalyzing the conversion of deoxyuridylate to deoxythymidylate, also determine response and toxicity to 5-fluorouracil chemotherapy. 5-fluorouracil is converted to its active metabolite 5-fluorodeoxyuridylate (5-fdUMP) that inhibits thymidylate synthase enzyme. The human *thymidylate synthase* promoter is polymorphic, having either double (S) or triple repeats (L). Individuals homozygous for the triple repeat variant (L/L) had higher thymidylate synthase expression and less response rate to 5-fluorouracil compared to those homozygous for the double repeat variant (S/S) in the

tumor tissue. In addition, individuals homozygous for the triple repeat variant (L/L) had less side effects to 5-fluorouracil (Pullarkat et al., 2001).

Several ATP-binding cassette transporters seem to contribute to 5-fluorouracil resistance including ABCG2, ABCC3, ABCC4 and ABCC5, because these multidrug resistance proteins were upregulated in 5-fluorouracil resistance (Thorn C.F., McLeod H., Carrillo M.W. et al. 2011).

K. Effects of Polymorphisms in Genes Encoding Drug Metabolizing Enzymes and Transporters Involved in Docetaxel Disposition on Docetaxel Treatment Outcome

Docetaxel enters the liver cells via the most efficient hepatocellular influx carrier, solute carrier organic anion transporter family member 1B3 (SLCO1B3) also known as organic anion-transporting polypeptide 1B3 (OATP1B3). Inside the liver cells, docetaxel is metabolized by CYP3A4 and CYP3A5 to inactive hydroxylated metabolites. The primary route of elimination of the parent drug and hydroxylated metabolites is hepatobiliary excretion by the membrane localized energy-dependent drug efflux transporters ABCB1, ABCG2, ABCC1 and ABCC2 (Oshiro C., McLeod H., Carrillo M. et al., 2010 and Longo et al., 2010) (Figure 2).

The pharmacogenetics of docetaxel metabolizing enzymes and transporters was investigated in several small scale studies that resulted in conflicting results. These studies and conflicting results are summarized in tables 3 and 4.

Multiple reasons may explain these conflicting results. These include difference in sample size, various tumor types, different treatment regimens (single agent vs. combination of drugs interacting with docetaxel) and ethnicity (haplotype structure). Furthermore, there may be other genes that may play a role and were not investigated (Longo et al., 2010).

L. Influence of Genetic Polymorphisms in Drug Metabolizing Enzymes and Transporters, not Known to be Involved in Docetaxel Disposition, on Docetaxel Treatment Outcome

Iwao-koisumic *et al.* identified an association between *Glutathione-S-Transferase1* and *CYP1B1* genetic polymorphisms- though not known to be involved in docetaxel pharmacokinetics- and docetaxel resistance (Longo et al., 2010). In addition, Sissung *et al.* reported that *CYP1B1*3* polymorphism is likely associated with poor prognosis in patients treated with docetaxel. The decreased efficacy of taxanes in *CYP1B1*3* carriers is likely due to the counteraction between *CYP1B1*3* byproducts and docetaxel effect on microtubules. CYP1B1 oxidizes 4-hydroxyestradiol into estradiol-3,4-quinone that inhibits tubulin polymerization. This reaction forms more readily by the protein encoded by *CYP1B1*3* allele. In addition, an *in vitro* interaction was shown to occur between *CYP1B1*3* byproducts and docetaxel structure (Sissung et al., 2008). As for *GSTP1*, Tran *et al.* identified an association between *GSTP1 *A/*B* genotype and excessive hematologic toxicity. Febrile neutropenia occurred in 31.6% of *GSTP1*A/*B*

carriers, while the frequency of occurrence was only 3.7% in *A/*A carriers and 0% in **A/*C, *B/*B* and *B/*C carriers. It has been reported that docetaxel triggers the production of reactive oxygen species. Glutathione-S-transferase plays an important role in inactivation of endogenous toxic compounds (such as unsaturated aldehydes, quinines, epoxides and hydroperoxides) formed during oxidative stress. Genetic polymorphisms leading to *GSTP1 *B* and **C* alleles was found to be associated with decreased GSTP1 activity. Because *GSTP1* is expressed in most tissues including the bone marrow, it can be hypothesized that the result of less active GSTP1 due to presence of genetic polymorphism leads to more docetaxel cytotoxic effect on malignant cells and more severe side effects on nonmalignant cells including hematopoietic cells (Tran et al., 2006).

M. Choice of Affymetrix DMET Plus Platform

The previously described studies identified the relationship between most important polymorphisms in pathway genes and docetaxel pharmacokinetics and pharmacodynamics. However, it is important to assess for the functional significance of additional polymorphisms in pathway genes involved in docetaxel metabolism and transport such as *CYP3A4: 554C>G (*16)*, *CYP3A4: 670C>T (*17)* and many others. In addition, docetaxel is not known to be metabolized by CYP1B1 or GSTP1, yet *CYP1B1*3* and *GSTP1 *A/*B* genetic polymorphisms were linked with poor prognosis and febrile neutropenia respectively (Tran et al., 2006, Sissung at al., 2008) (Table 5). Consequently, it is important to screen for genetic polymorphisms in drug metabolizing enzymes and

transporters not known to be involved in docetaxel pathway in addition to those involved, using highly multiplexed genotyping platforms in order to identify possible relationships between polymorphisms and treatment outcome. The available highly multiplexed genotyping platforms include genome wide scanning approaches and pathway-based approaches.

The genome wide scanning approach is a discovery-driven approach that uses a high density SNP array to identify the most significant SNPs associated with the disease risk or treatment outcome. Because genome-wide association arrays scan hundreds of thousands of SNPs, most of the nominally significant SNPs passing the initial scan are false positives. In order to keep the true positives and eliminate the false positives, validation should be done. Genome-wide study design is a two-study design whereby discoveries are made using a high density SNP array and SNPs that pass a certain significance level will be scanned in the next stage of validation. The first whole-genome scanning projects in cancer are the National Cancer Institute's Cancer Genetic Markers of Susceptibility (CGEMS) project. The CGEMS project study design includes the initial scanning step in 1200 cases and 1200 controls. SNPs that pass a significance level of $p<0.05$ will be investigated in four successive independent validation steps; each requiring 200 cases and 2000 controls. Given the high cost and sample size required for genome-wide studies, they are many times impractical. So far, no pharmacogenetics study has been completed using genome-wide scanning approaches (Wu X., Gu J. and Spitz M.R., 2008 and Sissung et al., 2010).

The pathway-based approach is a hypothesis-driven approach that uses prior knowledge of genes and polymorphisms functions. It can detect associations between polymorphisms and drug pharmacokinetics and treatment outcome. The pathway-based approach detects the combined effects of a panel of polymorphisms that interact in the pathway and/or different pathways. An example of a low- to mid- scale pathway-based genotyping platform is the drug metabolizing enzymes and transporters (DMET) plus platform form Affymetrix. Because the DMET Plus interrogates fewer variants than the genome-wide association study, it requires smaller sample size than the genome-wide study. In addition, the DMET Plus platform genotypes most of the allelic variants in genes encoding drug metabolizing enzymes and transporters, whereas the genome-wide association study scans random SNPs in the genome, so it may miss important SNPs in pathway genes. Furthermore, the DMET Plus is particularly useful in studying the pharmacogenetics of docetaxel, because all studies reported that patients treated with docetaxel showed variable pharmacokinetics, and multiple genes influence this variability. Consequently, the Affymetrix DMET Plus platform was chosen in our study for exploring genetic polymorphisms in pathway genes affecting docetaxel treatment outcome in breast cancer patients (Wu X., Gu J. and Spitz M.R. 2008 and Sissung et al. 2010).

N- Affymetrix DMET Plus Platform

1. Description

The Drug Metabolizing Enzymes and Transporter (DMET) plus platform scans 1936 single-nucleotide polymorphisms (SNPs) in 225 genes related to drug absorption, distribution, metabolism and elimination. It can unravel previously unknown associations between polymorphisms in absorption, distribution, metabolism and elimination genes and drug disposition and efficacy (Sissung et al., 2010).

2. Selection of genes

Major academic, pharmaceutical industry and genomic technology representatives in the pharma absorption, distribution, metabolism and elimination (ADME) consortium selected the DMET plus genes on the basis that many of these genes contribute to absorption, distribution, metabolism and elimination of US FDA approved drugs. The DMET Plus array scans 95% (45/47) of the phase I enzymes, 93% (74/80) of the phase II enzymes, 98% (51/52) of the drug transporters, in addition to 52% (24/46) of "other genes" such as *Vitamin K epoxide reductase complex subunit 1* (*VKORC1*), *thymidylate synthase* (*TYMS*), *prostaglandin I2 synthase* (*PTGIS*) and *3-hydroxy-3-methylglutarylCoA reductase* (*HMGCR*) and 31 genes involved in intracellular processes that contribute to absorption, distribution, metabolism and elimination of drugs (Table 6). Genes were selected if they were identified as "very important pool (VIP)" genes based on pharmacogenomics knowledge base (pharmGKB) evaluation.

The DMET Plus array captures various types of polymorphisms including copy number variations, insertion/deletion, biallelic and triallelic SNPs. It is capable of identifying haplotypes among 779 polymorphisms in 61 genes selected by pharma ADME consortium as they significantly influence drug metabolism (Sissung et al., 2010).

3. Strengths of DMET Plus

The DMET plus platform scans alleles involved in ADME of therapeutic agents; therefore it can be used to study the effects of genetic variants on variations in pharmacokinetics and pharmacodynamics of these agents. It can be specifically applied in phase I clinical trials to determine polymorphisms affecting pharmacokinetics and/or pharmacodynamics of investigational agents. The identified polymorphisms would then allow us to stratify subgroups of non responders or toxic responders from normal responders, and this information could be used to adjust the therapeutic dose for Phase II and Phase III clinical trials.

The most useful aspect of DMET Plus technology is its hypothesis-driven pathway based nature. By selection of genes involved in (ADME) pathways of drugs, the DMET Plus platform investigates less allelic variants than the large scale genotyping arrays allowing for less false positive results (type 1 errors) and greater power advantage. Till now, no study has investigated all or most of the variants in the genome vs. therapeutic outcome. The reason is that such a study needs a large number of patients and is very expensive. However, the DMET plus technology scans most of the SNPs in ADME genes,

in addition to some SNPs in target genes, so it is considered as low- to mid-scale genotyping platform and requires a smaller number of patients than large scale platforms to reveal clinically meaningful results. Furthermore, the DMET plus platform allows us to identify SNPs and haplotypes related to drug response and to understand previously unknown pathways that may influence treatment outcome of investigational agents especially when dealing with multigenic drugs (Sissung et al., 2010).

4. Limitations of DMET Plus

The DMET plus array cannot be used in prospective studies to make clinical decisions as it is not FDA approved yet. While it can be used to obtain clinical variants that are associated with drug response and then validate these variants using FDA approved genotyping methods, it cannot be used to make dose adjustments or to recommend alternative therapies. Besides, the DMET Plus platform does not scan genetic polymorphisms in pathways related to environmental exposure that may have an important effect on drug disposition. Additionally, since it does not scan genes encoding drug targets (with the exception of some such as (*VKORC1*)), the DMET Plus is impractical in cases where target genetic polymorphisms affect significantly therapeutic outcome (e.g. influence of polymorphisms in vascular endothelial growth factor pathway on bevacizumab therapy). A final weakness is that all the DMET Plus studies done so far required large validation sets to translate the results into clinical decisions. The DMET Plus technology cannot be used in many Phase I clinical studies as they include a small

cohort of patients, a drawback associated with all mid to large scale genotyping platforms (Sissung et al., 2010).

O. Application of DMET Plus Platform in the Literature

Caldwell *et al.* applied the DMET technology on a large cohort of patients (N=497) treated with warfarin. The *P* value of only one SNP, rs2108622, that represents a polymorphism in *CYP4F2*, was well below the threshold for significance of $4.0 * 10^{-5}$. The investigators reported that patients with 2 TT alleles require approximately 1 mg/day more warfarin than patients with 2 CC alleles. Interestingly, CYP4F2 was not previously known to be involved in warfarin metabolism (Caldwell et al., 2008).

The DMET 1.0 assay was also used by Varenhorst *et al.* who explored the effects of genetic polymorphisms in 6 genes (*CYP2C19, CYP2C9, CYP2B6, CYP3A4, CYP3A5* and *CYP1A2*) on plasma concentration of the active metabolite, and pharmacodynamics of clopidogrel and prasugrel. The study was done on 98 patients with coronary artery disease receiving either clopidogrel or prasugrel. Authors reported that reduced function *CYP2C19* results in reduced plasma concentration of active clopidogrel metabolite and reduced clopidogrel response as compared to normal function CYP2C19; but this had no effect on prasugrel active metabolite and therapeutic response (Varenhorst et al., 2009)

Di Martino *et al.* used the DMET Plus microarray genotyping platform to explore genetic polymorphisms in drug metabolizing enzymes and transporters in association with

irinotecan-induced gastrointestinal toxicity in metastatic colorectal cancer patients. The pilot study was done on 26 colorectal cancer patients receiving irinotecan based therapy. Nine patients experienced gastrointestinal toxicity and the remaining 17 were controls without gastrointestinal toxicity. The study identified three SNPs in *ABCG1, ABCC5* and *OATP1B1/SLCO1B1* transporter genes (rs425215, rs562 and rs2306283 respectively) associated with irinotecan-induced gastrointestinal toxicity. Results were limited to those in which the *P*-value was ≤ 0.05. This was the first study to report a relationship between genetic variations in *ABCG1* and *ABCC5* and irinotecan toxicity (Di Martino et al., 2011).

P. Application of DMET Plus Platform in the Literature on Docetaxel-Treated Patients

To our knowledge, only one study was conducted by Deeken *et al.*, whereby the DMET technology was used to study the effect of genetic polymorphisms in drug metabolizing enzymes and transporters on the efficacy and toxicity of docetaxel alone (n=14) or in combination with thalidomide (n=33) in men with castration-resistant prostate cancer (Deeken et al., 2010).

The study reported that 10 SNPs in three genes: *peroxisome proliferator-activated receptor- (PPAR-б), sulfotransferase family cytosolic 1C member 2 (SULT 1C2)* and *carbohydrate (chondroitin 6) sulfotransferase 3 (CHST3)* were related to the therapeutic outcome of docetaxel. Since effects of allelic variations in *PPAR-б* gene were observed only in patients receiving both docetaxel and thalidomide, it seems that genetic polymorphisms in *PPAR-б* influence the efficacy of the antiangiogenic agent, thalidomide.

In addition, 11 SNPs in eight genes (*spastic paraplegia 7 (SPG7)*, *carbohydrate (chondroitin 6) sulfotransferase 3 (CHST3*), *cytochrome P450 family 2 subfamily D polypeptide 6 (CYP2D6)*, N-acetyltransferase 2 (NAT2), *ATP-binding cassette subfamily C member 6 (ABCC6)*, *ATPase* Cu^{++} *transporting alpha polypeptide (ATP7A)*, *cytochrome P450 family 4 subfamily B member 1 (CYP4B1)* and *solute carrier family 10 member 2 (SLC10A2)* were associated with treatment-induced adverse events such as constitutional symptoms (fatigue, myalgia, allergic reactions), cardiovascular events (arrhythmia, chest pain/ ischemia), neurological symptoms (dizziness, syncope, hallucinations, seizure), hematological events (neutropenia, infection, anemia, thrombocytopenia, bleeding) metabolic symptoms (electrolyte disturbances, hepatic dysfunction), gastrointestinal symptoms (constipation or diarrhea) and pulmonary symptoms (dyspnea) (Deeken et al., 2010) (Table 6).

This study had however few limitations, basically:

- It was a small scale study; hence results need validation by large scale studies.
- Because the regimen includes, in most of the patients, a combination of docetaxel and thalidomide and because none of the above genes is known to be involved in the metabolism or disposition of docetaxel or thalidomide, it is difficult to identify whether the polymorphisms in the novel candidate genes affect the disposition of docetaxel or thalidomide or both.
- The enzymes and transporters that are reported to affect treatment outcome are not known to be involved in the disposition of thalidomide, docetaxel, and/or their metabolites. Therefore, there is a strong possibility that these results are false positives.

Although the *P*-value was reduced to <0.01, further research is needed to validate these associations and explore the mechanism behind them (Deeken et al., 2010).

- The study reported that there is a strong relationship between some polymorphisms in drug metabolizing enzymes and transporters and the treatment outcome of docetaxel and/or docetaxel and thalidomide; but this was not clearly described, and data was not shown.
- All types of toxicities were lumped in the study.
- The authors used an earlier version of the DMET array (DMET 1.0).

II. AIMS OF THE STUDY

Breast cancer is the most common cancer among women. It is the second leading cause of cancer death in women after lung cancer (Jabbari S., Park C. and Fowble B., 2010). Among the cytotoxic drugs used for the treatment of breast cancer, docetaxel is particularly indicated in breast cancer patients with poor prognosis. It is also indicated either alone as first line or second line treatment after 5-fluorouracil, doxorubicin and cyclophosphamide regimen for metastatic breast cancer (Green M.C. and Hortobagye G.N., 2007). As described before, the pharmacokinetics of docetaxel were shown to vary widely between patients. This interindividual variability is associated with significant differences in treatment outcome and unpredictable toxicity (Baker *et al.* 2009). Causes of this variability are still undefined; yet, genetic polymorphisms in genes encoding drug metabolizing enzymes and transporters may play a role in the variability in pharmacokinetic parameters and treatment outcome of docetaxel. As studies have associated different genetic polymorphisms in several drug metabolizing enzymes and transporters with toxicity to docetaxel, and as several drug metabolizing enzymes and transporters are involved in docetaxel disposition, innovative approaches such as the drug metabolizing enzymes and transporters (DMET) Plus microarray platform are strongly required to clarify docetaxel pharmacogenetics and to monitor drug usage.

The study is done on 36 carefully chosen breast cancer patients who received docetaxel with or without trastuzumab: 18 “cases” who had febrile neutropenia after

docetaxel treatment and 18 age and treatment matched “controls” who did not have febrile neutropenia after docetaxel treatment.

We aimed to explore the correlation of genetic polymorphisms in drug metabolizing enzymes and transporters with docetaxel-induced febrile neutropenia in Lebanese breast cancer women. Other toxicity endpoints were also interrogated.

III. METHODOLOGY

A. Choice of Samples

We enrolled 36 breast cancer patients receiving docetaxel with or without trastuzumab, 18 had febrile neutropenia after docetaxel treatment and 18 age and treatment matched controls did not have febrile neutropenia after docetaxel treatment (Table 8). Sample size estimation was based on Deeken *et al.* study (sample size=47; 33 on docetaxel and thalidomide and 14 on docetaxel alone). It was also based on two other studies: Tsai *et al.* who reported an association of *ABCB1 2677G/C* polymorphism with febrile neutropenia in a sample of 59 Taiwanese women with breast cancer receiving TEC treatment of whom 6 had developed febrile neutropenia, and Tran *et al.* who reported an association of *GSTP1*A/*B* with febrile neutropenia in a sample of 58 French patients with different types of solid tumors of whom 6 developed febrile neutropenia. Note that our samples were matched by treatment type and docetaxel dose.

Although docetaxel is associated with many side effects including hematologic, neurosensory and gastrointestinal symptoms, febrile neutropenia was chosen as the primary endpoint because it is the most objective endpoint that has clinical implications and that can be reliably retrieved from the medical charts. We genotyped for those who developed febrile neutropenia on docetaxel or docetaxel with trastzumab because we wanted to focus on docetaxel only and not on a combination of drugs such as FEC or FAC. Note that trastuzumab is not usually associated with myelosupression and is not known to be eliminated by drug metabolizing enzymes and transporters.

Three genomic DNA controls were run with the samples. They served as positive controls to detect assay performance in case the processed samples were of marginal DNA quality. Two random samples were run in duplicates to assess for assay reproducibility.

B. Experimental Methodology

1. DNA isolation

DNA was isolated from blood using the Qiagen DNA blood kit according to the manufacturer's recommendations. Sample concentration was determined by nanodrop spectrophotometer. All genomic DNA samples were normalized to a single concentration of 60ng/µl using Tris EDTA buffer (Burmester J.M., Sedova M., Shapero M.H., and Mansfield E., 2010).

2. Genotyping using Drug Metabolizing Enzymes and Transporters (DMETPlus) Array from Affymetrix

a. General Description

The DMET Plus run includes a pre-amplification step whereby the markers of interest having nearby pseudogenes are selectively amplified using the multiplex PCR kit from Qiagen. Then, the pre-amplified markers join the other markers in the DMET Plus assay flow. Genomic sequences that contain the polymorphisms of interest are preferentially amplified by molecular inversion probe amplification. The amplified genomic sequences are then labeled and hybridized to the DMET Plus array whereby

genotyping is obtained using single color detection format (http://media.affymetrix.com/support/downloads/manuals/dmet_user_guide.pdf).

b. Protocol

Step 1: Part of the genomic DNA was amplified by multiplex PCR (mPCR) using the multiplex PCR kit from Qiagen.

Step 2: The remaining genomic DNA, in addition to mPCR product undergo the molecular inversion probe amplification.

Step 3: PCR amplification: Amplification by polymerase chain reaction was carried out using a universal primer pair and Titanium taq polymerase from Clontech.

Step 4: PCR product was fragmented at the second cleavage site (X2) by endonucleases releasing the tag from the genomic DNA portion.

Step 5: Each sample was labeled with biotin and loaded on a DMET Plus array. The samples were incubated overnight in an oven for each tag sequence to hybridize to its complementary sequence on the array.

Step 6: The arrays were washed and stained with SAPE stain solution in the Genechip Fluidics Station 450 from Affymetrix.

Step 7: Detection: Scanning was performed with Affymetrix Genechip scanner 3000 7G (http://media.affymetrix.com/support/downloads/manuals/dmet_user_guide.pdf). Four intensity values for each probe were generated; 2 for the expected allelic bases and 2

for the non-allelic bases. The two values for the expected allelic bases determine whether the sample is homozygous or heterozygous for a given SNP. The two values for the non-allelic bases were compared to those for the expected allelic bases to determine signal noise ratio (SNR) for the probe. A base call was made by the DMET console software if the SNR is ≥ 3 (Hardenbol P., Baner J., Jain M. et al., 2003).

c. Molecular Inversion Probe Technology

The molecular inversion probe genotyping is an efficient technology for large scale single nucleotide polymorphism analysis. It uses molecular inversion probes with only one probe required for every SNP locus. As shown in figure 3, each probe consists of 7 segments: 2 unique homology regions that recognize the genomic sequences surrounding the SNP (H1 and H2), 2 primer regions (P1 and P2) common to all probes, one unique tag region (probe barcode) complementary to a sequence on Affymetrix Genflex tag array, and 2 common cleavage sites (X1 and X2) for amplification and post amplification processing respectively. The tags have a similar melting temperature (Tm) and base composition, so that they amplify and hybridize under the same conditions. Furthermore, they exhibit minimal cross reaction with each other and with sequences on the array (Hardenbol P., Baner J., Jain M. et al., 2003).

The use of molecular inversion probe technology involves several steps:

i. Annealing step

A mixture of genomic DNA and molecular inversion probes is denatured by heat and brought to the annealing temperature. The recognition sequences at each terminus of the probe (H1 and H2) hybridize to complementary sites around the SNP location on the genome. This permits the formation of a circular structure with a gap opposing the SNP site.

ii. Gap filling step

Unlabelled dNTPs are added and DNA polymerase selectively extends the complementary nucleotide. Then, DNA ligase ligates only correctly hybridized DNA to form a covalently closed structure. In reactions where the inserted nucleotide is not complementary to the gap, probes remain linear.

iii. Exonuclease selection

Single-stranded specific exonucleases are added to selectively digest linear structures. Then, exonucleases are deactivated by heat.

iv. Probe release

Uracil-N-glycosylase is added to depurinate uracil residues in the first cleavage site of the probe. The mixture is then heated to cleave the probe at this abasic site and release it from the genomic DNA, resulting in an enzymatically inverted probe.

v. PCR Amplification

PCR reagents, including a universal primer pair, are added. Eventually, only probes circularized in the gap fill reaction are amplified (Hardenbol P., Baner J., Jain M. et al. 2003and Dumaual C., Miao X., Daly T.M. et al. 2007).

The molecular inversion probe technology has several advantages

- Only one primer pair is used, hence decreasing the possibility of primer dimers.
- It allows the use of small amounts of DNA. In our assay, around 0.5 ng of genomic DNA is required per SNP.
- The reaction is highly specific due to several factors. First, probe genomic complexes occur at a probe concentration that does not favor nonspecific interaction between probes. Second, the highly specific gap fill enzymes, DNA polymerase and DNA ligase, insert the correct nucleotide and seal only perfectly hybridized DNA respectively. Third, the tag sequences are selected with minimal cross reaction with each other and with features on the array, hence resulting in highly specific hybridization (Hardenbol P., Baner J., Jain M. et al., 2003).

C. Quality control metrics

1. Call rates

Call rates were made automatically by the Affymetrix Genechip analysis software. Samples were included in the analysis only if they had more than 85% of total variants successfully genotyped.

2. Hardy-Weinberg Equilibrium (HWE)

Hardy-Weinberg Equilibrium model states that both allele and genotype frequencies remain constant in a population. This model assumes random mating, no mutation, no migration or emigration, infinite population size and no selective pressure for or against a selective genotype. To assure that our sample is representative of the population, testing deviation from the Hardy-Weinberg principle was performed using Pearson's chi-squared test, using the observed genotype frequencies obtained from the data and the expected genotype frequencies obtained using the Hardy-Weinberg principle. Alleles that were not in Hardy-Weinberg equilibrium were excluded from the analysis.

3. SNP validation and concordance rates

Genotyping data for the SNPs that had already been run on several of our samples using LightSnip or lightmix assays on the lightCycler real-time PCR from Roche, were compared with Affymetrix data in order to determine concordance rates. The SNPs are _CYP2B6*4_ 785A>G rs2279343, _CYP2B6*5_ 1459C>T rs3211371, _CYP2B6*6_ 516G>T

rs3745274*, GSTP1* 6624A>G rs1695*, GSTT1* (473bp deletion) and *GSTM1* (210 base pair deletion).

4. Statistical Analysis:

Based on data shown in tables 4, 5 and 6, genetic polymorphisms in pathway genes known to be involved in docetaxel disposition (*CYP3A4, CYP3A5, SLCO1B3, ABCB1*, *ABCC1*, *ABCC2* and *ABCG2*) or known to influence docetaxel treatment outcome (*CYP1B1* and *GSTP1*) were included in the analysis regardless of percentage of valid genotypes for every variant. *P*-values were obtained using Fisher-exact two sided test. Statistically significant variants should pass the threshold of significance (*P*=0.05). The primary endpoint was febrile neutropenia. Analysis of additional endpoints such as the need for blood transfusion, reduction in White Blood Cells Count, or reduction in Absolute Neutrophil Count was also attempted.

IV. RESULTS

A. Baseline Demographics

The mean age in the febrile neutropenia group was 44.17 +/- 9.769 years, and the mean age in the non-febrile neutropenia group was 47.17 +/- 8.8 years. 6 (33.3%) "case" patients and 7 (38.9%) "control" patients received docetaxel alone after 3 cycles of 5-fluorouracil, epirubicin, and cyclophosphamide or after 4 cycles of adriamycin and cyclophosphamide. The remaining patients were on docetaxel and trastuzumab. As expected, given that "case" and "control" patients were age and treatment matched, there were no significant differences in age and treatment regimen between "case" and "control" patients (Table 9); thus eliminating their influence on variability in treatment outcome between both groups.

B. Quality Control metrics

Genotyping was performed for the total of 36 samples on Affymetrix Drug metabolizing enzymes and transporters (DMETPlus) platform. All samples had a call rate of > 85% that represents % of variants successfully genotyped. Duplicate samples showed 99% repeatability. Genomic controls showed high call rates indicating that the samples were successfully run on Affymetrix. Concordance testing with Real time-PCR results revealed 100% concordance for *CYP2B6*4*, *CYP2B6*6*, *GSTP1*, *GSTM1* and *GSTT1* and 92.1% concordance for *CYP2B6*5* (Table 10). Noteworthy, *CYP2B6*5* polymorphism

was not successfully genotyped by Affymetrix DMETPlus platform as it had a QC of only 43.9% (% of samples having valid genotypes for *CYP2B6*5*).

A percentage of 87.3 of the variants in genes known to be involved in docetaxel pharmacokinetics (*CYP3A4, CYP3A5, SLCO1B3, ABCB1, ABCC1, ABCC2* and *ABCG2*) or known to affect docetaxel treatment outcome (*CYP1B1* and *GSTP1*) were within Hardy-Weinberg equilibrium. Variants outside HWE were excluded from the analysis.

C. Outcome

The need for granulocyte colony stimulating factor (GCSF) was statistically significantly different in the "case" vs. "control" group (Table 11), and this was expected as GCSF is usually given in cases of febrile neutropenia. Note that controls who received prophylactic GCSF injection with every docetaxel dose were deliberately not chosen as controls, as they may have developed febrile neutropenia if no GCSF injection had been received. In contrast to GCSF, the need for red blood cells transfusion or erythropoietin was not statistically significant in the "case" group vs. "control" group (Table 11), so we elected to also study the effect of genetic polymorphisms in drug metabolizing enzymes and transporters on the need for red blood cells transfusion or erythropoietin. Complete blood count 7-10 days after the first docetaxel cycle was available for 16 patients in the "case" group and for only 7 patients in the "control" group. The absolute neutrophil count (ANC) was available for only 13 patients in the "case" group and for only 7 "control"

patients (Table 11). Based on the sample size (n=23 or n=20), we elected not to analyze the effect of genetic polymorphisms on the reduction in WBC or ANC.

D. Pharmacogenetic Influence on Docetaxel-Induced Febrile Neutropenia

The correlation between docetaxel-induced febrile neutropenia and genetic polymorphisms in pathway genes involved in docetaxel disposition (*CYP3A4, CYP3A5, SLCO1B3, ABCB1*, *ABCC1*, *ABCC2* and *ABCG2*); or genetic polymorphisms in drug metabolizing enzymes and transporters known to affect docetaxel treatment outcome (*CYP1B1* and *GSTP1*) was studied. Analysis revealed a statistically significant result with the *ABCG2* rs2231142 polymorphism. All of the samples were successfully genotyped for this allele (QC=100%). 5 (27.8%) of "control" patients were carriers of the polymorphic allele in the *ABCG2* rs2231142 whereby no "case" patient carried this allele (Fig. 4). The polymorphism decreased the risk of developing febrile neutropenia in patients on docetaxel (P=0.045), an association that has not been previously reported.

E. Pharmacogenetic Influence on Need for Red Blood Cells Transfusion or Erythropoietin

The relationship between the need for transfusion and genetic polymorphisms in *SLCO1B3, ABCB1*, *ABCC1, ABCC2, ABCG2, CYP3A4, CYP3A5, CYP1B1* and *GSTP1* was also studied. All samples (n=36) were included; whereby 4 received Red Blood Cells

transfusion or erythropoietin, and the rest did not receive Red Blood Cells transfusion or erythropoietin. A statistically significant result in *ABCC2* c.1249G>A (V417I) "rs2273697" was obtained. Only 31 samples were successfully genotyped for this SNP (QC= 85.4%). Half of the patients requiring blood transfusion (2 out of 4) were carriers of the heterozygous genotype in *ABCC2* rs2273697, whereas only (6 out of 27) 22.2% of patients not receiving transfusion carried this heterozygous genotype. The mutant genotype (two mutant alleles) of *ABCC2* rs2273697 was present in 25% of patients receiving transfusion and absent in patients not requiring transfusion (p=0.024) (fig. 5). Consequently, *ABCC2* rs2273697 increases the need for blood transfusion in patients treated with docetaxel, an association that has not been previously reported.

V. DISCUSSION

Although multiple studies have been done previously to clarify the genetic determinants of docetaxel toxicity, the pharmacogenomics of docetaxel was not fully elucidated. To understand the relationship between genetic polymorphisms in pathway genes and docetaxel toxicity, we incorporated in a primary analysis the genetic polymorphisms in pathway genes known to be involved in the metabolism (*CYP3A4* and *CYP3A5*) and transport (*SLCO1B3*, *ABCB1*, *ABCC1*, *ABCC2*, *ABCG2*) of docetaxel, or known to affect docetaxel clinical outcome (*CYP1B1* and *GSTP1*). Two polymorphisms, *ABCG2* rs2231142 and *ABCC2* rs2273697 were significantly associated with the incidence of docetaxel toxicity. *ABCG2* rs2231142 polymorphism was associated with a decreased risk of developing febrile neutropenia on docetaxel, whereas *ABCC2* rs2273697 polymorphism was associated with an increased risk of developing docetaxel-induced hematological toxicity. No study has reported an association between *ABCG2* rs2231142 or *ABCC2* rs2273697 with docetaxel toxicity.

A. *ABCG2 C421A* Polymorphism Influence on ABCG2 Transport Activity and on Docetaxel Treatment Outcome

ABCG2 is located on the chromosome 4q22, and it belongs to the subfamily G of ATP binding cassette of transporters. *ABCG2* is expressed in physiologically important

tissues e.g., lung, gut, liver and kidney. In addition, it has an important role in maintaining the barrier function of blood–brain barrier, blood–cerebral spinal fluid barrier, blood–testis barrier and the maternal–fetal barrier and placenta (Leslie E.M., Deeley R.G., Cole S.P. 2005). ABCG2 is involved in the efflux of docetaxel from the hepatocytes into the bile canaliculi. (Oshiro C., McLeod H., Carrillo M. *et al.*, 2010 and Longo *et al.*, 2010). In our study, *ABCG2 C421A* polymorphism was associated with decreased risk of developing febrile neutropenia on docetaxel.

ABCG2 C421A polymorphism is a missense mutation in the 5th exon of the gene *ABCG2*, and it results in the substitution of Glutamine for Lysine (Campa D., Butterbach K., Slager S. L., *et al.* 2011). Many studies have been done to study the effect of this polymorphism on the transporter activity, expression and cellular localization. All the studies suggest that this polymorphism is associated with a lower *ABCG2* expression and hence increased drug accumulation (Kondo *et al.* 2004, Morisaki *et al.* 2005 and Imai Y., Nakane M., Kage K. *et al.* 2002). Therefore, carriers of the polymorphic allele are expected to have decreased docetaxel efflux and more toxicity from docetaxel; which is inconsistent with our results. However, knowing that the functional studies were done in cell lines that might not reflect the human physiology, and knowing that they showed inconsistencies regarding the effect of the polymorphism on the transporter ATPase activity (Kondo C., Suzuki H., Itoda M. et al. 2004, Mizuarai S., Aozasa N., Kotani H. *et al.* 2004 and Morisaki K., Ozvegy-Laczka C., Polgar O. *et al.* 2005); validation of the effect of *ABCG2 c.C421A* polymorphism on ABCG2 activity and expression is mandatory.

B. ABCC2 C421A Polymorphism Effect on ABCC2 Transport Activity and on Docetaxel Treatment Outcome

ABCC2 is located on the chromosome 10q24 and consists of 32 exons and spans 69 kb, and it is a member of the superfamily C of ATP-binding cassette of transporters (Cascorbi I., Haenisch S. et al., 2010). ABCC2 is widely expressed at the apical membrane of physiologically important epithelia such as placental trophoblasts, brain endothelial cells, hepatocytes, kidney-proximal tubules and small intestine (Sandusky G.E., Mintze K.S., Pratt S.E. et al., 2002 and Konig J., Nies AT., Cui Y. et al., 1999). ABCC2 is involved in the transport of docetaxel and its hydroxylated metabolite from the hepatocytes into the bile canaliculus (Oshiro C., McLeod H., Carrillo M. *et al.*, 2010 and Longo *et al.*, 2010). Consequently, docetaxel clearance is reduced in the presence of reduced function of ABCC2. In addition, Rougier *et al*. reported that docetaxel clearance was reduced in patients with increased concentration of bilirubin in serum (Rougier P., Adenis A., Ducreux M. *et al.* 2000). Dysfunction in *ABCC2* transporter is known to be responsible for Dubin–Johnson syndrome, characterized by conjugated hyperbilirubinemia (Keitel V., Kartenbeck J., Nies A.T. *et al.* 2000 and Tsujii H., Konig J., Rost D. et al., 1999). Both reduction of docetaxel clearance and elevation of serum bilirubin concentration could be caused by the reduced function of ABCC2 as a result of functional polymorphism in *ABCC2* gene. In our study, *ABCC2 G1249A* polymorphism was shown to increase significantly docetaxel-induced hematological toxicity. Bilirubin data was not consistently collected, and hence could not be analyzed.

ABCC2 G1249A polymorphism is a non-synonymous SNP in exon 10 of *ABCC2* gene (Val417Il). Schwabedissen *et al*. showed that *ABCC2 G1249A* polymorphism is associated with decreased mRNA expression in human preterm placentas, but it had no statistically significant effect in term placentas. Sample size was small (26 preterm and 32 term placentas), so confirmation of the functional effect of *ABCC2 G1249A* polymorphism in a larger sample is needed (Schwabedissen *et al*. 2005).

The effect of *ABCC2* polymorphisms on docetaxel-induced leukopenia was studied by Kiyotani *et al.* and Baker *et al.*; but no association was found between *ABCC2 G1249A* polymorphism with docetaxel-induced leukopenia (Kiyotani K., Mushiroda T., Kubo M. *et al.* 2008 and Baker *et al.* 2009*)*. Knowing that Kiyotani *et al.* study was done on 84 Japanese patients (28 with docetaxel-induced grade 3 or grade 4 leukopenia/neutropenia and 56 without docetaxel-induced adverse events) and that Baker et al. study was done on 92 white patients, the inconsistency with our results may be due to the small sample size and interethnic variability.

None of the other SNPs in pathway genes reported to affect docetaxel pharmacodynamics (table 4 and 5) were shown to have significant associations with docetaxel toxicity in our sample of patients. This is probably because of our small sample size. In addition, to our knowledge, no other study reports an association between our reported SNPs and docetaxel toxicity, but because they are involved in docetaxel disposition, they may be true positive results. To eliminate any possibility that these results are false positives, the effect of these reported SNPs on docetaxel toxicity should be confirmed in a series of validation studies. Furthermore, the exact mechanism behind the

effect of these SNPs on the transporters is not fully understood, so in vitro studies are required to understand the physiology behind this association.

C. Limitations

This study had several limitations. Most importantly, our sample size was small. As a result, many SNPs involved in the analysis were found to be monomorphic or with a very low minor allele frequency. Plus, our study was a pilot study, so no power analysis was done. Based on our results, power analysis is possible for further confirmatory studies. Furthermore, this study is based on retrospective chart review, so we were not able to have a complete data for every patient regarding several variables such as liver function test and other toxicities including gastrointestinal or neurosensory adverse events etc…Blood was not withdrawn from the patients at known time points, so we did not have the docetaxel concentration time curve for every patient, and we could not study the influence of the polymorphism in the pathway gene on the area under docetaxel concentration time curve. In addition, few confounders may have interfered with our results; but knowing that the study was designed with age-, docetaxel dose- and chemoregimen- matched cases and controls; we expect interindividual variability to be minimal.

VI. CONCLUSION

Our study showed that *ABCG2 c.1249 G>A* polymorphism (rs2231142) decreased the risk of developing febrile neutropenia from docetaxel and *ABCC2 c. 421C>A* polymorphism (rs2273697) increased the risk of developing docetaxel-induced hematological toxicity. Further studies and more advanced analysis is currently being done on the effect of genetic polymorphisms in drug metabolizing enzymes and transporters not involved in docetaxel metabolism and transport. Cluster analysis and haplotype analysis will also be attempted. Irrespective of the results with our classical and further advanced analyses, confirmation of the results is recommended using large sample size.

FAC	5-fluorouracil 500mg/m^2 IV days 1 and 4 Doxorubicin 50mg/m^2 IV day 1 Cyclophosphamide 500mg/m^2 IV day 1 Cycle is repeated every 21 days for 6 cycles
FEC	5-fluorouracil 500mg/m^2 IV days 1 and 4 Epirubicin 75mg/m^2 day 1 Cyclophosphamide 500mg/m^2 IV day 1 Cycle is repeated every 21 days for 6 cycles
FAC-Docetaxel	5-fluorouracil 500mg/m^2 IV days 1 and 4 Doxorubicin 50mg/m^2 IV day 1 Cyclophosphamide 500mg/m^2 IV day 1 Cycle is repeated every 21 days for 4 cycles. Followed by docetaxel 100mg/m^2 IV every 3 weeks for 4 cycles
FEC-Docetaxel	5-fluorouracil 500mg/m^2 IV days 1 and 4 Epirubicin 75mg/m^2 day 1 Cyclophosphamide 500mg/m^2 IV day 1 Cycle is repeated every 21 days for 4 cycles Followed by docetaxel 100mg/m^2 IV every 3 weeks for 4 cycles
FAC-Docetaxel + Trastuzumab	This regimen is identical to the FAC-docetaxel regimen above except that tratuzumab 2mg/kg weekly is added beginning with the first dose of docetaxel. Trastuzumab is continued for 1 year.
TAC	Docetaxel 100mg/m^2 IV day 1 Doxorubicin 50mg/m^2 IV day 1 Cyclophosphamide 500mg/m^2 IV day 1 Cycle is repeated every 21 days for 6 cycles.

Table 1. Chemotherapy regimens commonly used to treat breast cancer (Green M.C. and Hortobagye G.N., 2007).

ADVERSE REACTION		%
Hematologic		
Neutropenia	Neutrophils count <500 cells/mm^3	86
Leucopenia	White Blood cells count <1000 cells/mm^3	44
Thrombocytopenia	Platelets <100,000 cells/mm^3	9
Anemia	Hemoglobin <11g/dl	94
	<8g/dl	8
Febrile Neutropenia		12
Death	Septic	1
	Non-Septic	1
Infections	Any	22
	Severe	6
Fever in Absence of Infection	Any	35
	Severe	2
Hypersensitivity Reactions		
Regardless of Premedication	Any	18
	Severe	3
With 3-day Premedication	Any	15
	Severe	2
Fluid Retention		
Regardless of Premedication	Any	60
	Severe	9
With 3-day of Premedication	Any	64
	Severe	7
Neurosensory	Any	58
	Severe	6
Cutaneous	Any	47
	Severe	5
Nail Changes	Any	41
	Severe	4
Gastrointestinal	Severe	6
Stomatitis	Any	52
	Severe	7
Alopecia		74
Asthenia	Severe	15
Myalgia	Severe	2

Table 2. Summary of Adverse Reactions in Breast Cancer Patients Receiving Docetaxel at 100mg/m2 (http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/201525s002lbl.pdf).

Study	Type of cancer	Treatment regimen	Sample size	Genes studied
Bosch et al., 2006	Breast, prostate, lung and other cancers	Docetaxel alone or in combination[1]	92	*CYP3A4, CYP3A5 and ABCB1*
Tran et al., 2006	Breast, prostate, lung and other cancers	Docetaxel[2]	58	*CYP3A4, CYP3A5, ABCB1, GSTM1, GSTT1, GSTM3* and *GSTP1*
Baker et al., 2009	Breast, prostate, lung and other cancers	Docetaxel either alone or with Capecitabine, Cyclophosphamide, Doxorubicin, Trastuzumab or other medications	92	*SLCO1B3, ABCB1, ABCC2, CYP3A4* and *CYP3A5*
Tsai et al., 2009	Breast cancer	TEC* for 6 cycles	59	*CYP3A4, CYP3A5* and *ABCB1*
Kiyotani et al., 2008	Lung, breast, esophageal and other cancers	Docetaxel alone or in combination[1]	84	*CYP3A4, CYP3A5, ABCB1, ABCC2, SLCO1B3, NR1I2, NR1I3*
Sissung et al., 2008	Prostate cancer	Docetaxel alone or with thalidomide and estramustine or with prednisone	52	*CYP1B1*

Table 3. Data representing different articles that studied the effects of genetic polymorphisms in drug metabolizing enzymes and transporters on pharmacokinetic parameters and/or treatment outcomes of docetaxel.

TEC*: Taxotere, Epirubicin and Cyclophosphamide.

Docetaxel alone or in combination[1]: The combination regimen was not specified in the study.

Docetaxel[2]: It was not mentioned whether docetaxel wsa given alone or with other chemotherapeutic agents.

Gene	Polymorphism	rs #	Effect on docetaxel Pharmacokinetics	Pharmacokinetic Reference	Effect on docetaxel Pharmacodynamics	Pharmacodynamic Reference
CYP3A4	*-A392G*	rs2740574	No effect Polymorphism increased docetaxel clearance by 62%. Haplotype CYP3A4/5 *2 carriers had increased clearance by 64%.	Bosch 2006 Tran, Baker Tran, Baker	No effect	Tran 2006
CYP3A5	*A6986G*	rs776746	The wild type allele was associated with increased docetaxel clearance by 49%. No effect	Baker 2009 Tran 2009	No effect CYP3A5*1/*3 carriers had more incidences of fever, pleural effusions and febrile neutropenia than *3/*3 carriers.	Tran 2006 Tsai 2009
ABCB1	*C3435T*	rs1045642	No effect	Tran, Baker	TT carriers had increased risk of grade3 neutropenia. CC carriers had increased risk of neutropenia.	Tran 2006 Tsai 2009
	G2677T/A	rs2032582	No effect	Tran, Baker	No effect GG carriers had increased risk of febrile neutropenia.	Tran 2006 Tsai 2009
	C1236T	rs1128503	No effect TT genotype decreases docetaxel clearance by 25%.	Baker 2009 Bosch 2006	No effect	Tsai 2009
ABCC2	*G1249A* G101620771C	rs2273697 rs12762549	No effect N/A	Baker 2009 N/A	No effect Polymorphism associated with severe leukopenia	Kiyotani Kiyotani
SLCO1B3	IVS12-5676A > G	rs11045585	N/A	N/A	Polymorphism associated with severe leukopenia	Kiyotani

Table 4. Effect of genetic polymorphisms in genes known to be involved in docetaxel disposition on docetaxel pharmacokinetics and pharmacodynamics.

Gene	Polymorphism	rs #	Effect on docetaxel Pharmacokinetics	Reference	Effect on docetaxel Pharmacodynamics	Reference
GSTT1	Wild type Absent allele	rs2266636	No effect	Tran 2006	No effect	Tran 2006
GSTM1	Wild type Absent allele	rs74837985	No effect	Tran 2006	No effect	Tran 2006
GSTM3	*A310C*		No effect	Tran 2006	No effect	Tran 2006
GSTP1	*A313G*	rs1695	No effect	Tran 2006	GSTP1*A/*B carriers had more hematologic toxicity than *A/*A carriers.	Tran 2006
	A313G+C341T		No effect	Tran 2006	No effect	Tran 2006
	C341T	rs1138272	No effect	Tran 2006	No effect	Tran 2006
CYP1B1	*CYP1B1*3*	rs1056836	N/A	N/A	Poor prognosis	Sissung

Table 5. Effect of genetic polymorphisms in genes not known to be involved in docetaxel disposition on docetaxel pharmacokinetics and pharmacodynamics.

Gene	Polymorphism	rs #	Effect on docetaxel Pharmacodynamics
ABCC6	*G3803A*	rs2238472	Role in toxicity
PPAR-6	*A48189G* *G73444A* *G35369806A* *C89676T* *G57191A*	rs6922548 rs2016520 rs1883322 rs3734254 rs7769719	Related to therapeutic outcome if both docetaxel and thalidomide were given.
SULT 1C2	*G108994808C*	rs1402467	Role in efficacy
CHST3	*C50388T* *T50998C* *G53895A* *A53643G* *G52587A* *G52895A* *C50471T*	rs4148943 rs4148947 rs12418 rs730720 rs4148950 rs1871450 rs4148945	Role in therapeutic outcome and toxicity.
Spastic paraplegia 7 (SPG7)	*T43319C* *G50524A*	rs2292954 rs12960	Role in toxicity
CYP2D6	*2539-2542del*	rs72549353	Role in toxicity
NAT2	*G590A*	rs1799931	Role in toxicity
ATP7A	*G2299C*	rs2227291	Role in toxicity
CYP4B1	*C517T*	rs4646487	Role in toxicity
SLC10A2	*C26469T*	rs2301159	Role in toxicity

Table 6. Effect of genetic polymorphisms in pathway genes on docetaxel pharmacodynamics according to Deeken et al. study (Deeken et al., 2010).

Phase I enzymes (N=47)			**Phase II enzymes (N=80)**			**Drug transporters (N=52)**		**Other (N=46)**	
CYP1A1	CYP4F2	ADH1A	DYPD	NAT1	UGT2A1	SLC5A6	SLCO2B1	ABP1	ORM1
CYP1A2	CYP4F3	ADH1B	FMO1	NAT2	UGT2B4	SLC6A6	SLCO3A1	AHR	ORM2
CYP1B1	CYP4F8	ADH1C	FMO2	NNMT	UGT2B7	SLC7A5	SLCO4A1	AKAP9	PNMT
CYP2A6	CYP4F11	ADH4	FMO3	NQOL	UGT2A11	SLC7A7	SLCO5A1	ALB	PON1
CYP2A7	CYP4F12	ADH5	FMO4	SULT1A1	UGT2B15	SLC7A8		AOX1	PON2
CYP2A13	CYP4Z1	ADH6	FMO5	SULT1A2	UGT2B17	SLC22A1		ARNT	PON3
CYP2B6	CYP7A1	ADH7	FMO6	SULT1A3	UGT228	SLC22A2		ARSA	POR
CYP2B7	CYP7B1	ALDH1A1	GSTA1	AULT1B1	UGT8	SLC22A3		CBR1	PPARD
CYP2B7P1	CYP8B1	ALDH2	GSTA2	SULT1C1		SLC22A4		CBR3	PPARG
CYP2C8	CYP11A1	ALDH3A1	GSTA3	SULT1C2		SLC22A5		CDA	PTGIS
CYP2C9	CYP11B1	ALDH3A2	GSTA4	SULT1E1		SLC22A6		CES2	RALBP1
CYP2C18	CYP11B2	CHST1	GSTA5	SULT2A1		SLC22A7		CROT	RPL13
CYP2C19	CYP17A1	CHST2	GSTM1	SULT2B1		SLC22A8		DCK	RXRA
CYP2D6	CYP19A1	CHST3	GSTM2	SULT4A1		SLC22A11		EPHX1	SEC15L1
CYP2E1	CYP20A1	CHST4	GSTM3	TPMT		SLC22A12		EPHX2	SERPINA7
CYP2F1	CYP21A2	CHST5	GSTM4	UGT1A1		SLC22A14		FAAH	SETD4
CYP2J2	CYP24A1	CHST6	GSTM5	UGT1A3		SLC28A1		G6PD	SPG7
CYP2S1	CYP26A1	CHST7	GSTO1	UGT1A4		SLC28A2		HMGCR	TBXAS1
CYP3A4	CYP27A1	CHST8	GSTP1	UGT1A5		SLC28A3		HNMT	TPSG1
CYP3A5	CYP27B1	CHST9	GSTT1	UGT1A6		SLC29A1		MAT1 A	VKORC1
CYP3A7	CYP39A1	CHST10	GSTT2	UGT1A7		SLC29A2		METTL1	XDH
CYP3A43	CYP46A1	CHST11	GSTZ1	UGT1A8		SLCO1A2		NR1I2	
CYP4A11	CYP51A1	CHST13	MAOA	UGT1A9		SLCO1B1		NR1I3	

Table 7. Genes included in the Affymetrix DMET Plus platform (Di Martino et al., 2011).

Case	Chemo regimen	dxl dose	Age (yrs)	Stage	Grade	Control	Chemo regimen	dxl dose	Age (yrs)	Stage	Grade
1	TH6	100	40	IIIB	3	1	TH6	100	46	IIIA	3
2	FEC3TH3	100	36	IV	3	2	FEC3TH3	100	48	IIA	3
3	TH4	100	58	IIIA	3	3	TH6	100	66	I	3
4	AC4T4	75	38	IIA	1	4	AC4T4	75	45	IIB	2
5	FEC3TH3	100	43	I	2	5	FEC3TH3	100	49	I	1
6	FEC3TH3	100	31	IIB	3	6	FEC3TH3	100	40	IIB	3
7	FEC3T3	100	38	IIA	1	7	FEC3T3	100	39	IIA	2
8	FEC3T3	100	51	IV	2	8	FEC3T3	100	51	IIA	3
9	FEC4T4	100	54	IIB	3	9	FEC3T4	100	51	IIIA	1
10	FarmoC4T4	100	34	IIIA	2	10	AC4T4	100	37	IIB	2
11	FEC3TH3	100	54	IIA	2	11	FEC3TH3	100	50	IIIA	3
12	FEC3TH3	100	45	IIB	3	12	FEC3TH3	100	52	I	3
13	FEC3TH3	100	40	IIB	3	13	FEC3TH3	100	33	I	3
14	FEC3TH3	100	48	III	3	14	FEC3TH3	100	50	IIA	1
15	FEC3TH3	100	69	I	3	15	FEC3TH3	100	65	IIB	3
16	FEC3T3	100	39	IIIB	3	16	FEC3T3	100	43	N/A	2
17	FEC3TH3	100	41	IIA	2	17	FEC3TH3	100	48	IIA	2
18	FEC3TH3	100	36	IIA	3	18	FEC3T3	100	36	IIB	2

Table 8. Baseline chemotherapy regimen, docetaxel dose, age, tumor stage and grade of patients who developed febrile neutropenia on docetaxel and their age- and treatment matched- controls not developing febrile neutropenia on docetaxel.

Dxl: docetaxel, FEC3T3: 3 cycles of 5-fluorouracil, eprubicin and cyclophosphamide followed by 3 cycles of taxotere, FEC3TH3: 3cycles of 5-fluorouracil, epirubicin and cyclophosphamide followed by 3 cycles of taxotere and herceptin, TH6: 6 cycles of taxotere and herceptin, TH4: 4 cycles of taxotere and herceptin, FarmoC4T4: 4 cycles of farmorubicin and cyclophosphamide followed by 4 cycles and taxotere, AC4T4: 4 cycles of adriamycin and cyclophosphamide followed by 4 cycles of taxotere

Patient Characteristics		Febrile neutropenia (N=18)	Non-febrile neutropenia (N=18)	*P*-value
Age (years)	Mean ± SD	44.17 ± 9.769	47.17 ± 8.8	0.34[*]
Chemo regimen	Docetaxel alone	6 (33.3)[*]	7 (38.9)[*]	1.00[#]
	Docetaxel with trastuzumab	12 (66.7)[*]	11 (61.1)[*]	
Stage	I	2 (11.1)[*]	4 (23.5)[*]	0.472[#]
	II	9 (50.0)[*]	10 (58.8)[*]	
	III	5 (27.8)[*]	3 (17.6)[*]	
	IV	2 (11.1)[*]	0 (0)[*]	
Grade	1	2 (11.1)[*]	3 (16.7)[*]	0.812[#]
	2	5 (27.8)[*]	6 (33.3)[*]	
	3	11 (61.1)[*]	9 (50)[*]	

Table 9. Comparison of baseline age, chemotherapy regimen, tumor stage and grade among “case” patients who developed febrile neutropenia on docetaxel and “control” patients who did not develop febrile neutropenia on docetaxel.

[*]: N (%)

Polymorphism	rs number	Nucleotide change	QC of the variant	Concordance with Real Time-PCR
*CYP2B6*4*	rs2279343	785A>G	100%	100%
*CYP2B6*5*	rs3211371	1459C>T	43.9%	92.1%
*CYP2B6*6*	rs3745274	516G>T	92.7%	100%
GSTP1	rs1695	6624A>G	100%	100%
GSTM1	N/A	210bp del	100%	100%
GSTT1	N/A	473bp del	95.1%	100%

Table 10. Percentage of samples run on Affymetrix Drug Metabolizing Enzymes and Transporters (DMETPlus) platform having valid genotypes for CYP2B6*4, CYP3B6*5, CYP2B6*6, GSTP1, GSTM1 and GSTT1 (QC), and concordance with Real Time-PCR results.

Case	WBC count before first docetaxel injection (cells/mm3)	WBC count 7-10 days after first docetaxel injection (cells/mm3)	ANC before first docetaxel injection (cells/mm3)	ANC after 7-10 days of the first docetaxel injection (cells/mm3)	Control	WBC count before first docetaxel injection (cells/mm3)	WBC count 7-10 days after first docetaxel injection (cells/mm3)	ANC before first docetaxel injection (cells/mm3)	ANC after 7-10 days of the first docetaxel injection (cells/mm3)
1	14000	3000	10080	450	1	11700	n/a	8775	n/a
2	8500	1800	5100	414	2	4300	1000	3440	290
3	11900	5100	9996	3570	3	7300	4700	3212	1269
4	3700	5300	925	2809	4	3600	n/a	1476	n/a
5	7800	500	6240	0	5	3300	n/a	1815	n/a
6	11500	1500	9890	90	6	6700	2180	4489	545
7	9700	700	9021	0	7	4400	n/a	1936	n/a
8	6400	2100	5248	84	8	17400	n/a	14964	n/a
9	15200	1800	12616	54	9	1700	n/a	1190	n/a
10	4800	n/a	3216	n/a	10	5400	n/a	4914	n/a
11	5400	1200	4104	n/a	11	13300	2400	11172	198
12	15000	1300	13200	65	12	11300	n/a	9604	n/a
13	3500	n/a	2310	n/a	13	6100	1900	5185	133
14	8700	1600	6351	32	14	15700	2200	13816	572
15	18100	400	15747	n/a	15	13900	n/a	12093	n/a
16	2500	600	1350	n/a	16	18100	n/a	14661	n/a
17	17700	800	14691	32	17	5100	7300	n/a	n/a
18	12500	1800	10250	198	18	5100	n/a	2448	n/a
Average	9827	1843	7796	599		8577	3097	6775	501

Table 11. Comparison of the white blood count before first docetaxel injection and after 7-10 of the first docetaxel injection among case patients who developed febrile neutropenia on docetaxel and control patients who did not develop febrile neutropenia on docetaxel.

ANC: absolute neutrophil count

WBC: white blood cells

Outcome		Febrile neutropenia (N=18)	Non-febrile neutropenia (N=18)	*P*-value
Need for GCSF		15/18 (83.3)	2/18 (11.1)	0.000#
Need for red blood cells transfusion or erythropoietin		3/18 (16.7)	1/18 (5.6)	0.603#
NCI grade of hemoglobin 7-10 days after first docetaxel injection	1	13/16 (81.2)	6/7 (85.7)	1.00#
	2	3/16 (18.8)	1/7 (14.3)	
NCI grade of white blood cells count 7-10 days after first docetaxel injection	1	3/16 (18.8)	2/7 (28.6)	0.097#
	2	1/16 (6.2)	3/7 (42.9)	
	3	7/16 (43.8)	2/7 (28.6)	
	4	5/16 (31.2)	0/7 (0)	
NCI grade of absolute neutrophils count 7-10 days after first docetaxel injection	1	2/13 (15.4)	1/7 (14.3)	0.075#
	2	0 (0)	1/7 (14.3)	
	3	0 (0)	2/7 (28.6)	
	4	11/13 (84.6)	3/7 (42.9)	
NCI grade of platelets count 7-10 days after first docetaxel injection	1	15/15 (100)	7/7 (100)	1.00#
Reduction of white blood cells count after 7-10 days of the first docetaxel injection (%)	Mean ± SD	75.35 ±33.4	53.35±45.63	0.207*
Reduction of absolute neutrophils count after 7-10 days of the first docetaxel injection (%)	Mean ± SD	72.46 ±83.53	69.52 ±52.07	0.934*

Table 11. Need for neupogen injection or red blood cell transfusion or erythropoietin and occurrence of myelosuppression after docetaxel injection in patients with febrile neutropenia compared to patients not developing febrile neutropenia. P-value* was obtained using t-test, and other P-values# were obtained using Fisher-exact two sided test.

GCSF: granulocyte colony stimulating factor

National Cancer Institute (NCI) hematological toxicity criteria:

Toxicty grade	1	2	3	4
WBC (cells/mm3)	≥3000	2000-2999	1000-1999	<1000
Platelet (cells/mm3)	≥75000	50000-74999	25000-49999	<25000
Hemoglobin (g/dl)	≥10	8.0-10.0	6.5-7.9	<6.5
Granulocytes(cells/mm3)	≥1500	1000-1499	500-999	<500

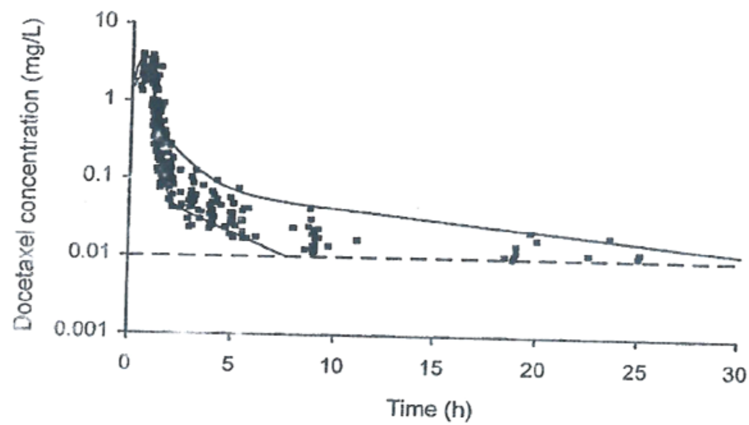

Figure 1. Concentration-versus-time curve of 24 patients after a 1 hour infusion of 100 mg/m^2 docetaxel (Rosing et al., 2000).

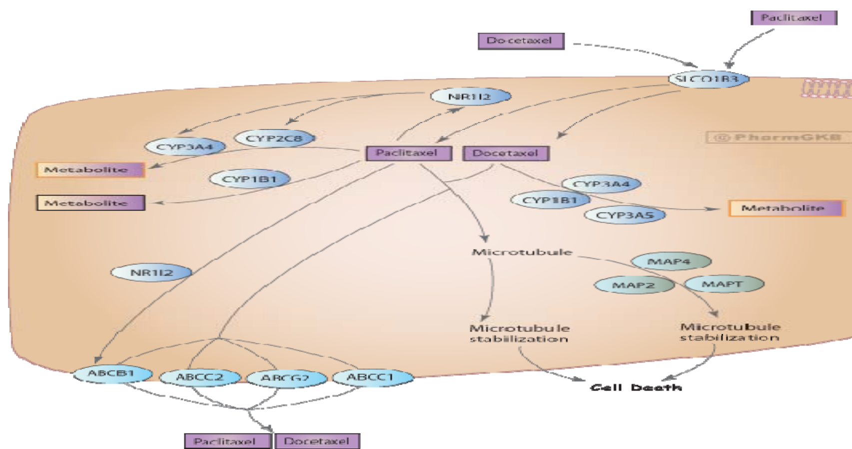

Figure 2. Pharmacokinetic pathway of Docetaxel (Oshiro C., McLeod H., Carrillo M. et al., 2010).

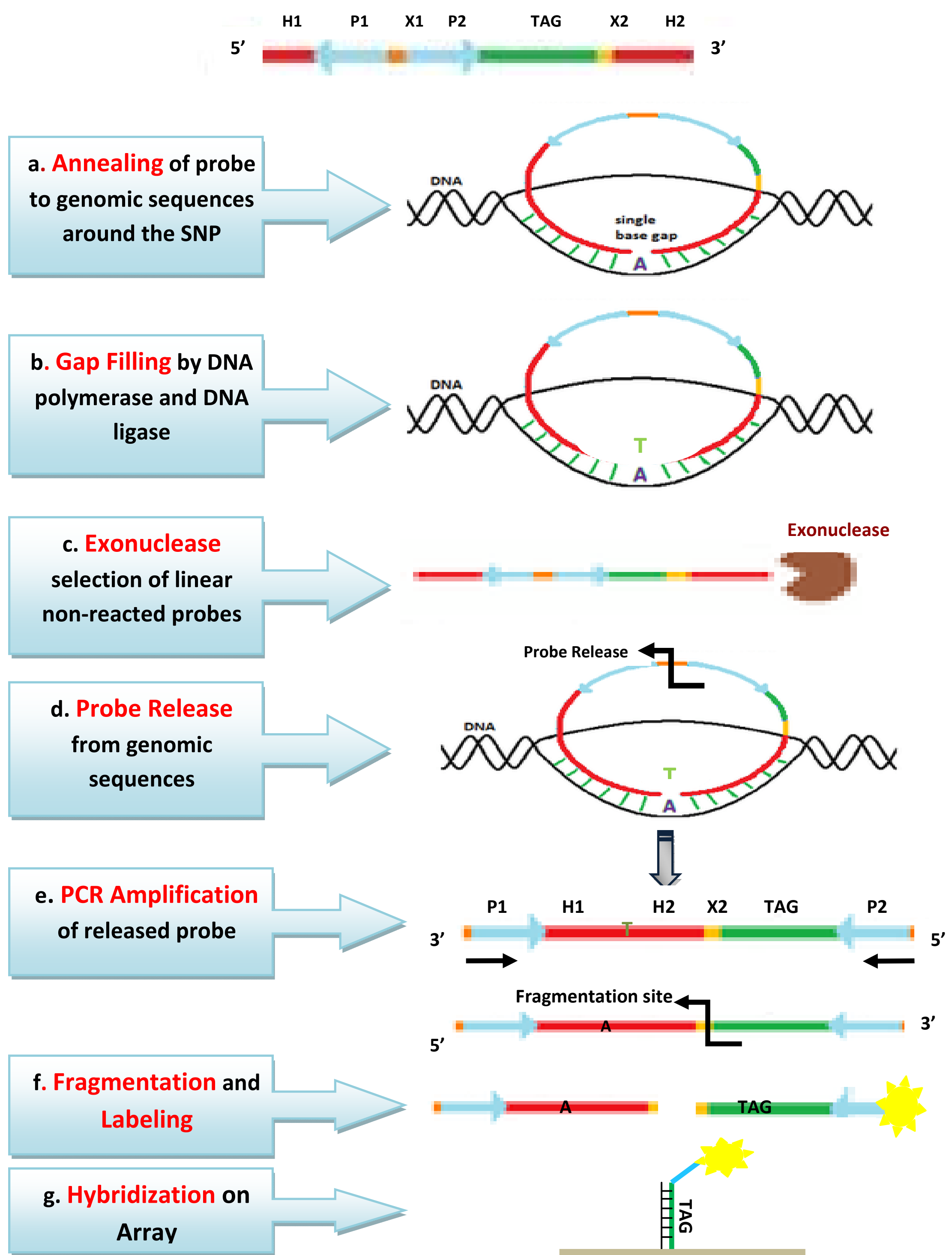

Figure 3. Amplification of DNA targets by applying molecular inversion probe technology (Hardenbol P. 2003).

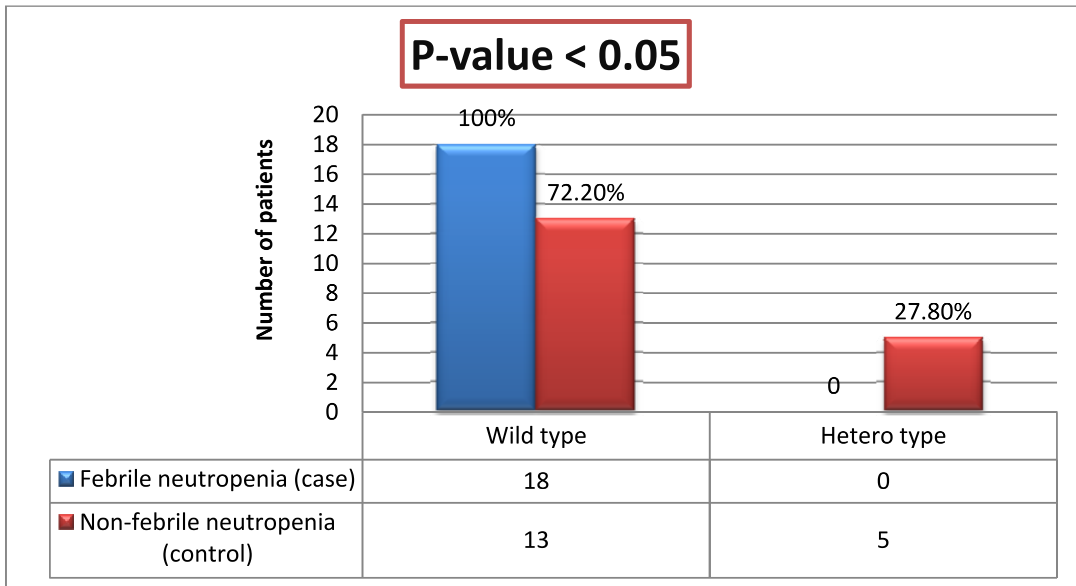

Figure 4. Bar graph comparing the genotypes distribution of ABCG2 rs2231142 among patients who developed febrile neutropenia (cases) and patients who did not develop febrile neutropenia (controls) after treatment with docetaxel.

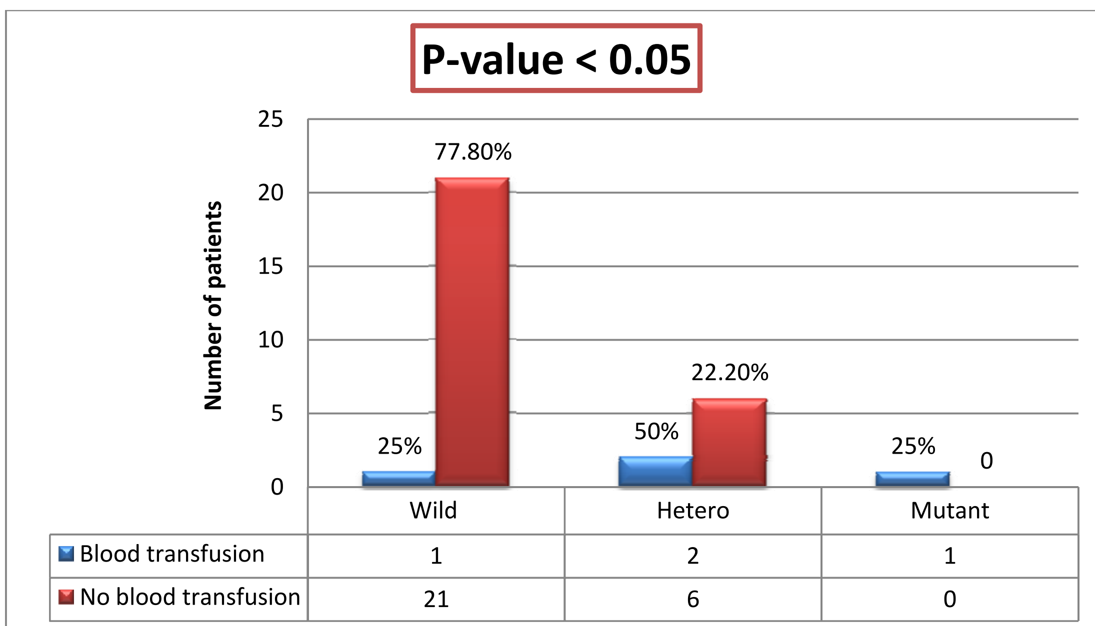

Figure 5. Bar graph comparing the genotype distribution of ABCC2 rs2273697 among patients requiring transfusion and patients not requiring transfusion after treatment with docetaxel.

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