THE IMPACT OF *CYP3A4* AND *CYP3A5* POLYMORPHISMS ON ANASTROZOLE'S PHARMACOKINETICS AND PHARMACODYNAMICS IN POST-MENOPAUSAL BREAST CANCER PATIENTS

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by

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$(NH_4)H_2PO_4$	Ammonium dihydrogen phosphate
ACN	Acetonitrile
AFLP	Amplified fragment length polymorphisms
AI	Aromatase inhibitors
AJCC	American Joint Committee on Cancer
ANAS-*d*12	Deuterium-labeled anastrozole
ANOVA	Analysis of variance
ATAC	Arimidex, Tamoxifen, Alone or in Combination
AUC	Area under the curve
BLAST	Basic Local Alignment Search Tool
BMI	Body mass index
CH3COONH4	Ammonium acetate
CI	Confidence interval
COMPAS	Compliance in Adjuvant treatment of primary breast cancer Study
CTCAE	Common Terminology Criteria for Adverse Events
CV	Coefficient of variation
CYP	Cytochrome P450
DHPLC	Denaturing high performance liquid chromatography
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate
ECD	Electron capture detection

EDTA	Ethylenediamine tetraacetic acid
ER	Oestrogen receptor
FDA	Food and drug administration
FID	Flame ionization detection
FSH	Follicle stimulating hormones
GC	Gas chromatography
gMAF	Global allele frequency
HER2	Human epidermal growth factor receptor
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
HRT	Hormone replacement therapy
IQR	Interquartile range
IS	Internal standard
IU	International unit
LC MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
LH	Luteinizing hormone
LOD	Limit of detection
LOQ	Limit of quantitation
MD	Mood disturbances
MeOH	Methanol
$MgCl_2$	Magnesium chloride
mL	Millilitre
MSAE	Musculoskeletal adverse events
NaOH	Sodium hydroxide

NCI	National Cancer Institute
NS	Not significant
OR	Odds ratio
PCR	Polymerase chain reaction
PDA	Photo diode array
PTFE	Polytetrafluoroethylene
QC	Quality control
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
UHPLC	Ultra-high performance liquid chromatography
SD	Standard deviation
SN	Signal-to-noise
SNP	Single Nucleotide Polymorphism
SPE	Solid phase extraction
SSCP	Single strand conformation polymorphism
$t_{1/2}$	Half life
T_a	Annealing temperature
TBE	Tris-boric acid-EDTA
TEA	Triethylamine
TEA	Triethylamine
Tis	Carcinoma *in situ*
T_m	Melting temperature
TNM	Tumour, lymph node, metastasis
UGT	UDP-glucuronosyl transferase
UPLC	Ultra-performance liquid chromatography

UV	Ultraviolet
VD	Vaginal dryness
VS	Vasomotor symptoms

KESAN POLIMORFISMA *CYP3A4* DAN *CYP3A5* KE ATAS FARMAKOKINETIK DAN FARMAKODINAMIK ANASTROZOLE DI KALANGAN PESAKIT-PESAKIT KANSER PAYUDARA PASCAMENOPAUS

ABSTRAK

Kanser payudara adalah kanser yang kedua paling kerap di antara semua jenis kanser dan paling biasa berlaku di kalangan wanita. Anastrozole merupakan salah satu ubat barisan hadapan pilihan untuk rawatan kanser payudara dan dipercayai lebih unggul berbanding dengan tamoxifen. Walau bagaimanapun, sebahagian besar pesakit-pesakit yang dirawat dengan anastrozole mengalami keberulangan laku kanser payudara atau pun terjadinya kesan-kesan mudarat ubat yang teruk. Kebolehubahan antara pesakit ini adalah disebabkan oleh beberapa faktor seperti variasi genetik. Anastrozole secara umumnya dimetabolisme oleh enzim-enzim CYP3A4 dan CYP3A5. Tujuan kajian ini adalah untuk menentukan kesan polimorfisme genetik *CYP3A4* dan *CYP3A5* ke atas farmakokinetik dan farmakodinamik anastrozole di kalangan pesakit kanser payudara wanita. Sejumlah 94 pesakit kanser payudara wanita pasca-menopaus telah direkrut untuk kajian ini. Data-data demografi sosial dan pembolehubah-pembolehubah klinikal telah direkodkan dan sampel-sampel darah telah dikumpul untuk pemerolehan DNA, sukatan aras hormon dan aras anastrozole dalam serum. Pengenotipan *CYP3A4*18A* dan *CYP3A5*3* telah dilakukan dengan menggunakan kaedah tindak balas berantai polimerase-polimorfisme cebisan pemotongan panjang (PCR - RFLP) konvensional, manakala pengenotipan *CYP3A4*4*, *CYP3A4*18B* dan *CYP3A4*22* pula menggunakan kaedah

PCR-RFLP multipleks. Kepekatan anastrozole dalam serum telah ditentukan dengan menggunakan kaedah kromatografi cecair resolusi pantas (UHPLC) yang baru dibangunkan berserta prosedur ekstraksi fasa pepejal ringkas. Kajian kami melaporkan bahawa *CYP3A4*18B* G>A berkerapan yang tinggi (0.48) di kalangan penduduk Malaysia manakala *CYP3A4*18A* T>C dan *CYP3A5*3* A>G masing-masing muncul dalam kekerapan rendah (0.03) dan tinggi (0.64) di kalangan penduduk Malaysia. Tiada alel-alel varian *CYP3A4*4* dan *CYP3A4*22* dikesan di kalangan subjek. Pesakit-pesakit yang mempunyai *CYP3A4*18B* G>A dan *CYP3A5*3* A>G homozigot masing-masing mempunyai paras anastrozole dalam serum yang rendah dan tinggi berbanding dengan mereka yang mempunyai varian-varian jenis liar dan heterozigot. Kaedah multipleks PCR-RFLP untuk pengesanan serentak *CYP3A4*4 A>G*, *CYP3A4*18B* G>A dan *CYP3A4*22 C>T* tersebut diaplikasikan untuk pengenotipan kesemua subjek. Kaedah UHPLC yang baru dibangunakan tersebut menunjukkan lienariti yang baik antara julat kepekatan 20 dan 1600 ng/mL. Purata kepersisan untuk anastrozole adalah 88.17% dengan limit kuantifikasi sebanyak 20 ng/mL. Pembolehubah-pembolehubah seperti umur pesakit dan jangka masa semenjak permulaan rawatan anastrozole adalah masing-masing berhubung kait dengan risiko yang lebih tinggi untuk berlakunya simptom-simptom vasomotor dan gangguan-gangguan ragam dan/atau kekeringan faraj/dispareunia. Tiada hubung kait yang ketara di antara polimorfisme-polimorfisme genetik *CYP3A4* dan *CYP3A5* dan farmakodinamik anastrozole. Alel *CYP3A4*18B* G>A dan *CYP3A5*3* A>G boleh digunakan sebagai biomarker yang penting dalam mempengarahi metabolisme anastrozole di kalangan pasakit kanser payudara pascamenopaus di masa hadapan.

THE IMPACT OF *CYP3A4* AND *CYP3A5* POLYMORPHISMS ON ANASTROZOLE'S PHARMACOKINETICS AND PHARMACODYNAMICS IN POST-MENOPAUSAL BREAST CANCER PATIENTS

ABSTRACT

Breast cancer is the second most frequent cancer among all cancer types and is by far the commonest cancer in women. Anastrozole is one of the first line drugs of choice in the treatment of breast cancer and is believed to be superior to tamoxifen. However, a significant proportion of patients treated with anastrozole experienced recurrences of breast cancer or developed severe adverse drug reactions. This inter-patient variability is attributed to a number of factors such as genetic variations. Anastrozole is predominantly metabolized by CYP3A4 and CYP3A5 enzymes. The objective of this study was to determine the impact of *CYP3A4* and *CYP3A5* genetic polymorphisms on anastrozole's pharmacokinetics and pharmacodynamics in post-menopausal breast cancer women. A total of 94 postmenopausal breast cancer women were recruited for this study. Patients' socio-demographic data and clinical variables were recorded and blood samples were collected for DNA acquisition, hormonal and anastrozole serum levels. Genotyping of *CYP3A4*18A* and *CYP3A5*3* was performed using the conventional polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), while that of *CYP3A4*4*, *CYP3A4*18B* and *CYP3A4*22* was carried out by a novel multiplex PCR-RFLP method. Serum anastrozole concentration was determined by an ultra-high performance liquid chromatography (UHPLC) method using a simple solid-phase extraction procedure. Our study reported that *CYP3A4*18B* G>A has a high frequency (0.48) among Malaysians and that *CYP3A4*18A* T>C and *CYP3A5*3*

A>G occur in low (0.03) and high (0.64) frequencies respectively among Malaysians. No variant alleles of *CYP3A4*4* and *CYP3A4*22* were detected among all the subjects. Patients homozygous for *CYP3A4*18B* G>A and *CYP3A5*3* A>G had lower and higher anastrozole serum levels respectively compared to those having the respective wild types or heterozygous variants. The multiplex PCR-RFLP method for the simultaneous detection of *CYP3A4*4 A>G*, *CYP3A4*18B* G>A and *CYP3A4*22 C>T,* was applied in genotyping of all the subjects. The newly developed UHPLC method demonstrated a good linearity over concentration ranges of 20 – 1600 ng/mL. The mean recovery for anastrozole was 88.17% with a limit of quantitation of 20 ng/ml. Variables such as patients' age and time since commencement of anastrozole therapy were associated with higher risk of developing vasomotor symptoms and mood disturbances and/or vaginal dryness/dyspareunia respectively. No significant association was established between *CYP3A4* and *CYP3A5* genetic polymorphisms and anastrozole's pharmacodynamics. The detected *CYP3A4*18B* G>A and *CYP3A5*3* A>G alleles may serve as an important biomarkers of altered anastrozole metabolism in breast cancer patients receiving anastrozole in future.

CHAPTER ONE

INTRODUCTION

1.1 Background

Globally, breast cancer is the second most common cancer and by is far the most frequent cancer in women with estimated 1.3 million cases and approximately 500,000 deaths reported annually (WHO, 2015). However, in terms of mortality, it ranks fifth as a result of fairly favourable prognosis (Ferlay *et al.*, 2015). In general, cancer can be regarded as a genetic disease (Workman, 2002; Gilbertson, 2011). Its well-known complex interactions between an individual's genome and the environment play a crucial role in the development of breast cancer (Hankinson *et al.*, 2004; Song *et al.*, 2011; Forman *et al.*, 2015). Both oestrogen biosynthesis pathway and oestrogen receptors are important therapeutic targets for breast cancer in which prolonged exposure to oestrogen has been implicated in the aetiology of breast cancer (Key *et al.*, 2002; Brown and Hankinson, 2015).

Physiologically, oestrogen plays a key role in the regulation of mammary gland development (Lamote *et al.*, 2004; Musumeci *et al.*, 2015). Two ligand-dependent transcription factors designated as oestrogen receptor alpha (ERα) and oestrogen receptor beta (ERβ) are the major transducers of oestrogen physiological effect. ERβ are expressed in approximately 70% of breast tumours, while the majority of breast tumours co-express both ERα and ERβ (Dotzlaw *et al.*, 1997; Fuqua *et al.*, 2003; Huang *et al.*, 2015). ERα stimulates the growth of breast cells while ERβ exerts the opposite effect by enhancing anti-proliferative and pro-apoptotic functions (Liu *et al.*, 2002; Paruthiyil *et al.*, 2004; Strom *et al.*, 2004).

The rate-limiting and final step of oestrogen biosynthesis is catalysed by an enzyme called the aromatase (CYP19A1) (Simpson *et al.*, 1994; Gennari *et al.*, 2011). The source of oestrogen varies significantly depending on the menopausal status of a woman. In pre-menopausal women, the principal source of oestrogen is the ovary while in post-menopausal women (when the production of oestrogen by the ovary ceases) oestrogen is synthesized by a number of extra-gonadal locations such as the adipose tissue, breast, brain, liver, and muscle (van Landeghem *et al.*, 1985; Simpson *et al.*, 1994; Gennari *et al.*, 2011; Lonning *et al.*, 2011).

Until recently, tamoxifen has been the drug of choice as an adjuvant therapy for both pre- and postmenopausal women with oestrogen receptor-positive early breast cancer (Montemurro *et al.*, 2015; Li and Shao, 2016). Although tamoxifen is still an indispensable therapeutic option in both pre- and post-menopausal women with breast cancer (Pan and Chlebowski, 2014), its long term use has raised concerns owing to its association with potentially life-threatening adverse effects such as increasing incidence of endometrial cancer, thromboembolism and cerebrovascular events (Braithwaite *et al.*, 2003; Fisher *et al.*, 2005; Lewis, 2007; Perez, 2007; Ryden *et al.*, 2016). In addition, some proportion of women with breast cancer can be primarily resistant to tamoxifen or may, in due course become resistant to it even if they previously expressed high levels of oestrogen receptors (Normanno *et al.*, 2005; Zilli *et al.*, 2009; Hayes and Lewis-Wambi, 2015). These recent concerns provided justification for introducing alternative endocrine therapies for treatment of hormone-responsive postmenopausal breast cancer and inhibition of aromatase has become a prevailing current of thought in the treatment of these cases (Wood *et al.*, 2003; Normanno *et al.*, 2005; Zilli *et al.*, 2009; Li and Shao, 2016). Consequently, a

number of aromatase inhibitors (AIs) have been developed to either serve as alternative to or be used following a few years of tamoxifen treatment and the current guidelines recommend the use of third-generation AIs (anastrozole, exemestene and letrozole) which are highly specific to the aromatase enzyme and have fewer adverse effects when compared with previous generations of AIs (Fabian, 2007; Goldhirsch *et al.*, 2009; NCCN, 2012).

Anastrozole, which is a non-steroidal third-generation aromatase inhibitor is an achiral triazole derivative known as 2,2' [5-(1H- 1,2,4-triazol- 1-ylmethyl)- 1,3-phenylene]bis(2-methylpropiononitrile) and has been reported to suppress plasma oestradiol optimally when administered at 1 to 10 mg/day with both doses capable of suppressing the oestradiol completely (Plourde *et al.*, 1994; Wood *et al.*, 2003). The mechanism of action of anastrozole is by inhibition or inactivation of aromatase with consequent inhibition of conversion of androgens to oestrone and oestradiol in peripheral tissues as well as in a few sites of the central nervous system (Simpson, 2003; Wood *et al.*, 2003).

Anastrozole is a well-established drug of choice for a variety of clinical settings ranging from breast cancer chemoprevention to treatment of postmenopausal women with early-stage breast cancer in both the adjuvant setting and advanced-stage disease (Chumsri, 2015). In the Arimedex, Tamoxifen Alone or in Combination trial (ATAC), anastrozole was shown to be more efficacious and less toxic (Cuzick *et al.*, 2010) than tamoxifen. It was on this basis that anastrozole was approved by the US Food and Drug Administration in 2002 for use in the adjuvant setting to treat women with early-stage endocrine-sensitive breast cancer and is therefore currently

considered as the first-line drug of choice for this indication (Behan *et al.*, 2015). Anastrozole has shown some encouraging results in the initial therapy setting (Forbes *et al.*, 2008), following two to three years of tamoxifen (Boccardo *et al.*, 2005; Jakesz *et al.*, 2005; Kaufmann *et al.*, 2007) and as in the extended adjuvant after five years of tamoxifen (Jakesz *et al.*, 2007).

The primary site of anastrozole clearance is in the liver (Ingle *et al.*, 2010a) where it is oxidized by CYP3A4 to form hydroxyl anastrozole, which may further be glucuronidated to hydroxyl anastrozole by UGT1A4, alternatively, it can also be directly glucuronidated to anastrozole *N*-glucuronide and the conjugation reaction is mainly catalyzed by UGT1A4 and to a lesser degree by UGT2B7 and UGT1A3 (Kamdem *et al.*, 2010; Lazarus and Sun, 2010). Anastrozole is also metabolised to some extent by CYP3A5 and to a negligible extent by CYP2C8 (Kamdem *et al.*, 2010).

The current trend in personalized treatment include among other approaches genetic testing to investigate a patient's ability to effectively metabolize drugs which have resulted in improved dosing of medications for many disease conditions (PMC, 2011). Genetic polymorphisms affect metabolism causing either increased drug toxicity or decreased efficacy of not only the drug but its metabolites (Vogel *et al.*, 2013). It has recently been reported that sequencing analysis of UGT1A4 promoter (non-coding) region from the liver specimens of 96 human subject demonstrated the presence of four SNPs variants of varying frequencies i.e., rs77588960 (0.07), rs11876575 (0.13), rs2074746 (0.08) and -219C>T (0.16) in which interestingly three of these SNPs (rs11876575, rs2074746 and -219C>T exhibited significant

association with anastrozole glucuronidation (Edavana *et al.*, 2013). However, to date, no data on the impact of *CYP3A4* and *CYP3A5* single nucleotide polymorphisms (SNPs) on anastrozole's pharmacokinetics and pharmacodynamics exists.

1.2 Problem statement

Although it has been clearly demonstrated that anastrozole is superior and more efficacious than tamoxifen (Cuzick *et al.*, 2010), significant proportion of patients experience a recurrence of their disease (Ingle *et al.*, 2010a). In addition, there is a high incidence of inter-individual variability with respect to tolerability to an extent that adverse effects like musculoskeletal complaints results in withdrawal of some patients from treatment (Ingle *et al.*, 2010a; Lombard *et al.*, 2016). This variability is believed to be due to a number of factors including potential inter-patients differences with respect to anastrozole pharmacokinetics and/or pharmacodynamics possibly due to their genetic variability (Edavana *et al.*, 2013). It can therefore be conceived that the genetic variability in the genes that encode the drug target (aromatase) or drug metabolizing enzymes (CYP3A and UGT1A) could play a vital role in determining individual's responses to anastrozole.

1.3 Research objectives

General objective:

To investigate the influence of *CYP3A4* and *CYP3A5* single nucleotide polymorphisms on anastrozole-associated adverse events and serum levels of anastrozole in post-menopausal breast cancer patients.

Specific objectives

The specific objectives are to:

1. Develop a novel multiplex PCR-RFLP method for simultaneous genotyping of *CYP3A4*4, CYP3A4*18B* and *CYP3A4*22* alleles in breast cancer patients
2. Determine the allelic frequencies of *CYP3A4*4, CYP3A4*18A, CYP3A4*18B, CYP3A4*22 and CYP3A5*3* in breast cancer patients
3. Develop a newly validated HPLC detection method for anastrozole measurement in serum
4. Determine the impact of *CYP3A4*4, CYP3A4*18A, CYP3A4*18B, CYP3A4*22* and *CYP3A5*3* polymorphisms on anastrozole's pharmacokinetics and pharmacodynamics

1.4 Research hypothesis

It was hypothesized that the allelic and genotypic frequencies of *CYP3A4*4, CYP3A4*18A, CYP3A4*18B, CYP3A4*22* and *CYP3A5*3* among Malaysian breast cancer patients would vary from those commonly reported in the western countries and that these alleles may influence patients' response to anastrozole treatment. To our knowledge, this is the first study to investigate the potential role of *CYP3A4* and *CYP3A5* genetic polymorphisms on inter-patient variability in response to treatment with anastrozole.

CHAPTER TWO

LITERATURE REVIEW

2.1 Breast cancer

2.1.1 What is breast cancer?

As simply defined by the American Cancer Society, breast cancer refers to a malignant (cancerous) tumour capable of invading the surrounding tissues or metastasizing to distant parts of the body (ACS, 2014).

2.1.2 Breast cancer incidence

Since 1990, in spite of the significant reduction in breast cancer mortality rates (by 2.2% each year) in the developed countries (Toriola and Colditz, 2013), breast cancer persists as the most common malignant disease in women globally, with 1.3 million newly diagnosed cases and approximately 500,000 mortality annually (WHO, 2015). The yearly reported number of new cases had doubled over the last three decades (UK, 2013). The increased incidence is attributed to a number of factors which include longer life expectancy, improved detection techniques, altered reproductive patterns, higher prevalence of obesity globally and westernization of developing countries (ACS, 2014; WHO, 2015).

In contrast to developed countries, where the incidence has stabilized or even reduced (Ravdin *et al.*, 2007; Fontenoy *et al.*, 2010; Gompel and Plu-Bureau, 2010),

the breast cancer incidence has accelerated in most Asian countries (including Malaysia) in the last two decades (Hirabayashi and Zhang, 2009; Pathy *et al.*, 2011; GLOBOCAN, 2012). The local data has revealed that breast cancer is the most commonly occurring malignancy in Malaysian women (Ibrahim *et al.*, 2012). In fact, Asian women have higher tendencies to be diagnosed at advanced stage of the disease when compared with their counterparts from the industrialized western Nations (Miao *et al.*, 2014) where awareness is higher. For example, it has been reported that an estimated 10 – 20% of Asian breast cancer women would present with *de novo* advanced-stage breast cancer that has already metastasized, when compared with only 3 – 5% in the developed European Nations and the United States of America (Chopra, 2001; Sant *et al.*, 2004; Tan *et al.*, 2005; Yip *et al.*, 2006; Lim *et al.*, 2007). Another intriguing finding is that Asian women tend to have larger tumour size and metastatic lesions often involving multiple locations (Agarwal *et al.*, 2007) when detected.

2.1.3 Pathogenesis and aetiological factors

Breast cancer is a complex heterogeneous disease consisting of several entities with multiple histological and clinical features arising as a result of the interactions between the environment and an individual's genetic makeup thus leading to mutations in the genes that are involved in regulation of cellular growth and functions (Borresen-Dale *et al.*, 2010). Approximately 80% of breast cancers diagnosed affect women between 50 to 69 years old (Kaminska *et al.*, 2015). The complexity of aetiology and pathogenesis of breast cancer has made it so unpredictable that only 20 to 30% of newly detected cases of breast cancer can be

traced to the various risk factors reported to be associated with its development (Kaminska *et al.,* 2015). The commonest identifiable aetiological factors implicated in the pathogenesis of breast cancer are age, genetics, past history of breast diseases, positive history of cancer in first-degree family, early age at menarche, late age (after 35 years of age) at birth of first child, diet, alcohol consumption, obesity, lifestyle, physical inactivity, endocrine factors and age at menopause (Bland, 1987; Tavani *et al.,* 1999; Ali and Coombes, 2002; Kaminska *et al.*, 2015).

2.1.3.1 Age

Although the incidence of breast cancer is low before 20 years of age, the incidence rate progressively increases with age and it has been estimated that by the age of 90 years; 10% of women are affected (Russell RC, 2000). This is in line with the observation that reproductive hormones produced by the ovaries and the adrenal glands play important role in the pathogenesis of breast cancer; especially due to existing evidences that cancers which do not respond to hormones will not display any observable change in their incidence during the female reproductive age (Abdulkareem, 2013). Moreover, it is understood that both early age at menarche and late age at menopause contribute to the continuous and prolonged exposure to the detrimental effects of steroid hormones, which are believed to collaborate with other factors such as genetic and environment to promote breast cancer development (Aguas *et al.,* 2005).

2.1.3.2 Gender

Breast cancer is extremely rare in males (Russell RC, 2000) and is believed to be due to differences in hormonal exposure since it has been observed that male breast cancer also expresses oestrogen, progesterone and androgen receptors and men with klinefelter's syndrome have been reported to show increased odds of having breast cancer (Murphy *et al.*, 2006).

2.1.3.3 Genetic factors

Women with family history of breast cancer have increased chance of developing breast cancer when compared to the general population; and it has been documented that only about 5% of breast cancers are associated with a specific mutation (Russell RC, 2000). Dumitrescu and Cotarla have summarized the findings of a meta-analysis of 52 epidemiological studies which showed that 12% of breast cancer women have had at least one affected relative while 1% has had one or more relatives with breast cancer (Dumitrescu and Cotarla, 2005).

Previously, a hereditary factor was suspected to play a role in susceptibility to breast cancer (Ford and Easton, 1995). Nevertheless, subsequent investigations revealed that between five to ten per cent of all breast cancers are caused by germline mutations in high-penetrance breast cancer susceptibility genes which include *BRCA1, BRCA2,* and *p53*; which make an individual more susceptible to hereditary breast cancer (Dumitrescu and Cotarla, 2005). The *BRCA1* and *BRCA2* genes are located on the long arm of chromosomes 17 and13 respectively and it has been

reported that gene-positive individuals have approximately 80% chance of developing breast cancer (Duncan *et al.*, 1998; Russell RC, 2000; Winter *et al.*, 2016).

2.1.3.4 Endocrine factors

2.1.3.4.1 Endogenous

Breast cancer is more frequently seen in women with infertility and those who have not breast-fed their infants (Abdulkareem, 2013). On the other hand, a woman that had a term pregnancy at an early age particularly if she had a late menarche and early menopause (factors that reduce the duration of exposure to oestrogen) has significantly reduced risk of breast cancer (Russell RC, 2000). Similarly, a woman with high parity (also believed to minimise prolonged exposure to oestrogen) has half the risk of having breast cancer when compared to a nulliparous woman (Russell RC, 2000). This is thought to be as a result of low circulating oestrogens during pregnancy.

2.1.3.4.2 Exogenous

Hormone replacement therapy (HRT) is an established risk factor for breast cancer particularly among current users of oestrogen and progestin for at least five years or above (Aguas *et al.*, 2005). However, the HRT also has its own merit in relieving vaginal dryness and itching, tension headaches, mood disturbances, minimising the risk of osteoporosis and pathological fractures among other conditions

(Abdulkareem, 2013). The use of oral contraceptives has also been linked to a modest risk of breast cancer (Aguas *et al.*, 2005).

2.1.3.5 Diet and alcohol

An increased risk of breast cancer has been observed with diets low in phyto-oestrogens and heavy alcohol consumption (Russell RC, 2000; Dumitrescu and Cotarla, 2005). Similarly, diets rich in 35 – 40% of fat in calories (as seen with most western foods) increase the risk of breast cancer development. This is believed to be due to the presence of high cholesterol levels which is a precursor in oestrogen biosynthesis (Aguas *et al.*, 2005).

2.1.3.6 Lifestyle and physical activity

The hormonal levels of plasma may be influenced by a combination of dietary factors along with exercise (Abdulkareem, 2013). These two factors either independently or together can affect a woman's body mass index and it has been observed that obesity is a risk factor for breast cancer among post-menopausal women (Aguas *et al.*, 2005). The likely explanation for this is the fact that fat deposits in adipose tissues tend to increase the circulating levels of oestrogens that are sourced from cholesterol (Abdulkareem, 2013).

2.1.4 Breast cancer classification

Recent advances in molecular researches have changed the way breast cancer has been traditionally viewed as a single disease entity (Reis-Filho and Pusztai, 2011). To date, it is perceived as a collection of diseases with diverse anatomical characteristics, with variable clinical responses to therapy and prognosis (Sotiriou and Pusztai, 2009; Reis-Filho *et al.*, 2010; Taherian-Fard *et al.*, 2015). Therefore, in general, breast cancer classification falls into five systems of classification (Figure 2.1), with the two most common forms of such classifications discussed below (sections 2.1.4.1 and 2.1.4.2).

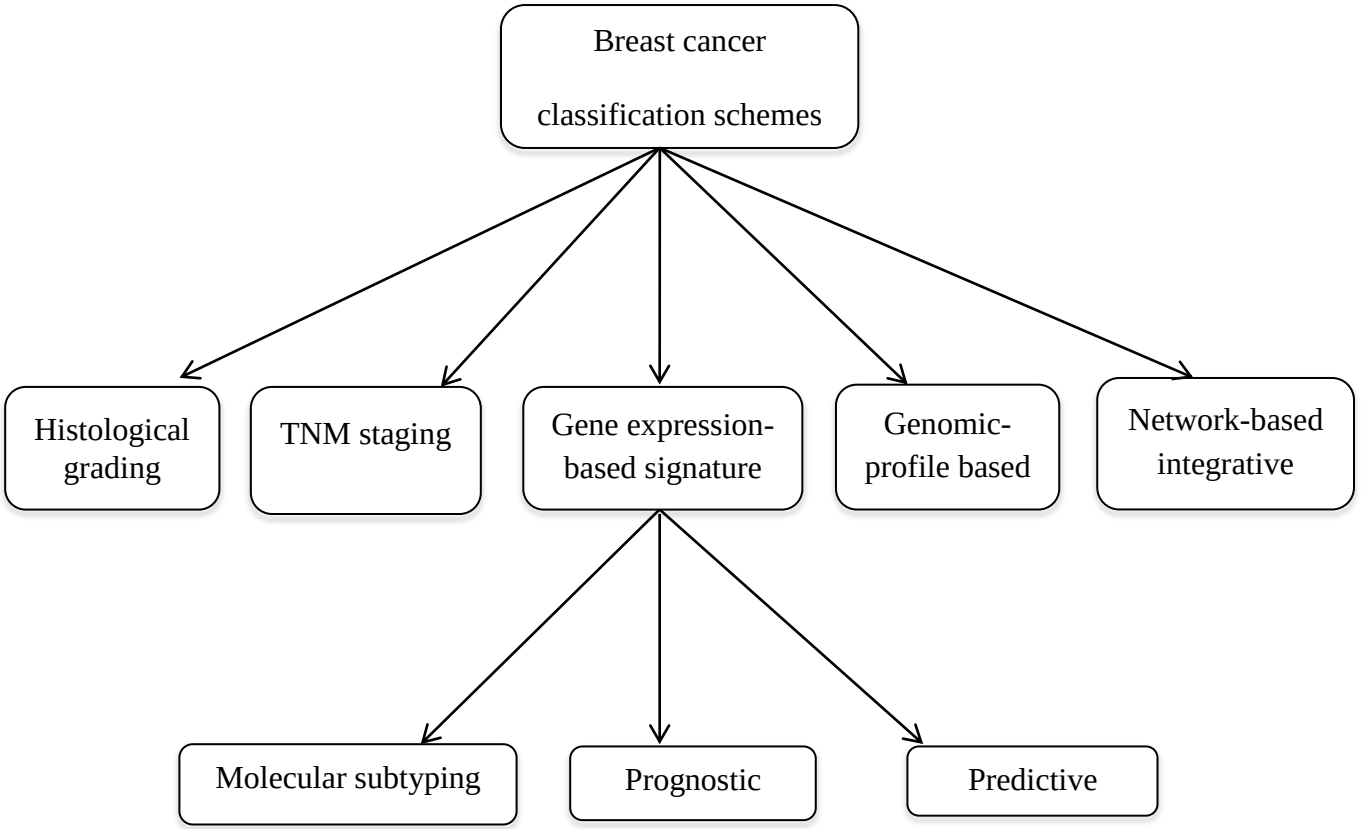

Figure 2.1: Histopathological, anatomical, expression and genomic schemes for classification of breast cancers. Reproduced from Taherian-Fard *et al.*, (2015) with permission by RightsLink.

2.1.4.1 Histological grading of breast cancer

The most widely used grading system in breast cancer is the modified Bloom-Richardson score (Taherian-Fard *et al.,* 2015). This grading system utilises microscopic features of the tumour's malignant cells relative to normal cells. It typically grades the tumour into grade 1-4. Briefly, Grade 1 tumour possesses cells that are very similar to the normal breast tissue and grade 2 tumour cells exhibit mild variation from the normal cells. On the contrary, Grades 3 and 4 tumours exhibit high dissimilarity with the normal breast tissue; such tumour cells have high proliferative capacity and metastasize faster than low-grade tumours (Meyer *et al.*, 2005).

2.1.4.2 TNM staging

The American Joint Committee on Cancer (AJCC) staging system is commonly employed to classify breast cancers (AJCC, 2014). The system uses three main features in the staging and include primary tumour (T), regional lymph nodes (N), and distant metastasis (M) collectively referred to as TNM classification. Based on these characteristics, breast cancer is grouped into five main stages (0 to IV) (Table 2.1)

Table 2.1: TNM staging of breast cancer

Stage	Tumour size (T)	Nodes involvement (N)	Distant metastasis (M)
0	Carcinoma *in situ* (Tis)	None	None
I	Present but < 2.0 cm in its greatest dimension	None	None
IIA	Present but < 2.0 cm in its greatest dimension	Metastasis to movable ipsilateral axillary lymph node	None
IIB	From 2 to5 cm in its greatest dimension	Either localized or spread to 1 – 3 axillary lymph nodes	None
IIIA	At < 5 cm or none	Spread to 4 – 9 axillary lymph nodes, fixed or matted	None
IIIB	Tumour of any size with direct extension to chest wall or skin	Either localized or spread to axillary lymph nodes	None
IIIC	Any size	metastasis in 10 or more axillary lymph nodes or in infra-clavicular lymph nodes, or clinically apparent ipsilateral internal mammary lymph node (s) in the presence of one or more positive axillary lymph node (s)	None
IV	Any size	Either localized or metastasis to nearby lymph nodes	

2.1.5 Oestrogens and aromatase enzyme in breast cancer

One of the major physiological roles of oestrogen is regulation of mammary gland development (Lamote *et al.*, 2004; Musumeci *et al.*, 2015). Oestrogen receptors (ER) designated as ERα and ERβ are the main transducers of oestrogen biological activity. The ERβ is expressed in approximately 70% of breast tumours, while the bulk of breast tumours co-express both ERα and ERβ (Dotzlaw *et al.*, 1997; Fuqua *et al.*, 2003; Huang *et al.*, 2015). Interestingly, when ERα displays a growth stimulatory effect on breast cells, ERβ manifests the opposite effect by stimulating the anti-proliferative and pro-apoptotic activities (Liu *et al.*, 2002; Paruthiyil *et al.*, 2004; Strom *et al.*, 2004).

The key enzyme involved in oestrogen biosynthesis is the aromatase which catalyses the final reaction in oestrogen formation (Gennari *et al.*, 2011). The source of oestrogen varies significantly depending upon the menopausal status of a woman. In pre-menopausal period, the chief source of oestrogen is the ovary. Conversely, in post-menopausal period (when the production of oestrogen by the ovary ceases) oestrogen is synthesized in several locations including the adipose tissue, breast, brain, liver, and muscle (Simpson *et al.*, 1994; Simpson, 2003; Gennari *et al.*, 2011; Lonning *et al.*, 2011). Consequently, the aromatase enzyme has a direct role on *in situ* oestrogen formation in the breast (Yue *et al.*, 1998; Geisler, 2003) and is believed to play a vital part in the proliferation of breast cancer cells (Utsumi *et al.*, 1996; Chen *et al.*, 2009).

2.1.6 HER2 and breast cancer prognosis

Approximately 15-20% of all breast cancer tumours exhibit over-expression of human epidermal growth factor receptor (HER-2; neu; c-erB-2), a proto-oncogene that is linked to a poor prognosis and resistance to chemotherapy and hormonal treatment of breast cancer (Slamon *et al.*, 1987; De Placido *et al.*, 1998; Ross and Fletcher, 1998; Sun *et al.*, 2015). The advent of anti-HER-2 therapy by using trastuzumab (herceptin), which binds to the HER2 extracellular domain, has effectively improved the outcome of HER2 positive breast cancer (De Laurentiis *et al.*, 2005; Guarneri *et al.*, 2010; Ahmed *et al.*, 2015). However, as with other therapeutic agents, the incidence of trastuzumab-related adverse events especially cardiac toxicity is worrisome (Guglin *et al.*, 2009; Tarantini *et al.*, 2012; Advani *et al.*, 2015; Bregni *et al.*, 2015). Interestingly, attention has shifted to elucidating the solution to this adverse event, since a recent meta-analysis has revealed that *HER2* 655 A > G polymorphism is associated with higher odds of developing trastuzumab-associated cardiac toxicity (Gomez Pena *et al.*, 2015). This phenomenon implies that in future this variant allele can be utilized to predict patients with higher risk of getting cardiac toxicity when receiving trastuzumab.

2.2 Anastrozole

2.2.1 History

The treatment of pre-menopausal breast cancer by bilateral oophorectomy was first introduced by Sir George Beatson in 1896 (Beatson, 1896). The 1950s and 60s witnessed focus on development of non-surgical alternatives to the surgical approaches in the treatment of breast cancer which include use of glucocorticoids, androgens and oestrogens (Lipsett and Pearson, 1957; Lipsett *et al.*, 1957; Segaloff *et al.*, 1963; Manni *et al.*, 1977).

Another strategy of direct inhibition of adrenal steroid synthesis was suggested by Ralph Cash which he thought could be an effective alternative to surgical removal of adrenals that was commonly used at that time to treat post-menopausal breast cancer (Cash *et al.*, 1967). His suggestion was based on the fact that aminoglutethemide could block cholesterol side chain cleavage. The inhibitory effects of aminoglutethemide on the adrenals necessitated the use of replacement glucocorticoid, and therefore dexamethasone was selected for this purpose. However, it was soon observed that the efficacy of dexamethasone is affected by aminoglutethemide administration (Santen *et al.*, 1974). To overcome this obstacle, hydrocortisone was substituted because it has no significant interaction with aminoglutethemide (Santen, 1981; Santen *et al.*, 1990).

An earlier chance meeting by Siiteri and Santen led to the understanding that aminoglutethemide could effectively block the aromatase levels in the whole body of post-menopausal women (Santen *et al.*, 2009). Prior to the meeting, in an *in vitro* study, Sitteri had previously shown in an *in vitro* study in 1969 that aminoglutethemide was capable of blocking aromatase (S Bolton, 1969) and was also conversant with the reports of Schwarzel and colleagues on AIs (Schwarzel *et al.*, 1973). Sitteri and his colleagues later suggested that aminoglutethemide's mechanism of action was most probably through inhibition of aromatase in breast cancer and they opined that further investigations were needed to elucidate this (MacDonald *et al.*, 1967). This hypothesis was then tested and it was found that there was 95 – 98% aromatase inhibition in postmenopausal breast cancer women (Santen *et al.*, 1978). This observation shifted the emphasis on the inhibitory activity of aminoglutethemide against aromatase and resulted in its subsequent classification as a "non-selective first generation" aromatase inhibitor (Cocconi, 1994; Dowsett and Coombes, 1994; Reddy, 1998; Goss and Strasser, 2001; Mokbel, 2002; Rose, 2003; Lonning, 2004; Gibson *et al.*, 2007).

As a result of the severe adverse effects associated with aminoglutethemide and failure to minimise them despite many efforts (Harris *et al.*, 1983; Harris *et al.*, 1984; Dowsett *et al.*, 1985; Stuart-Harris *et al.*, 1985), the need for selective aromatase inhibitors became necessary which led to the introduction of formestane (Coombes *et al.*, 1984; Dowsett *et al.*, 1987; Dowsett *et al.*, 1989; Dowsett and Coombes, 1994; Chen *et al.*, 2002). However, subsequent investigations revealed that formastane did not effectively block aromatase to be superior to aminoglutethemide and therefore

more efficacious inhibitors were sought (Coombes *et al.*, 1984; Perez Carrion *et al.*, 1994; Geisler and Lonning, 2005).

Identifying and appreciating the potential contributions of AIs in breast cancer treatment, a significant number of pharmaceutical companies took interest and considerably contributed to the discovery and development of more potent selective steroidal and non-steroidal AIs (Santen *et al.*, 2009); this consequently gave rise to the emergence of fadrozole (CGS 16949A) which was the first agent to be categorized as second-generation aromatase inhibitor (AI) (Steele *et al.*, 1987). Nevertheless, there was a setback when it was incidentally found to have inhibitory effects on aldosterone (Demers *et al.*, 1990; Trunet *et al.*, 1992). With more advances in research, the pharmaceutical companies explored structure/function evaluation and animal models among other strategies to develop the two new and popular non-steroidal (anastrozole and letrozole) and one steroidal (exemestane) AIs (Santen *et al.*, 2009) all of which received FDA approval and were demonstrated to have high potency and superior efficacy than aminoglutethemide, formestane and fadrozole and also possess less adverse drug reactions than aminoglutethemide and fadrozole (Goss and Strasser, 2001). Eversince the discovery of these important agents (AIs), the use of anastrozole as an alternative endocrine therapy for treatment of hormone-responsive postmenopausal breast cancer has become a prevailing approach in the treatment of these cases (Zilli *et al.*, 2009; Li and Shao, 2016).

2.2.2 Indication and dosage

Until recently, tamoxifen has been the main adjuvant therapy for both pre- and postmenopausal ER+ early breast cancer cases (Montemurro *et al.*, 2015; Li and Shao, 2016). Although tamoxifen remains a valuable therapeutic option in both groups of patients (Pan and Chlebowski, 2014), its long term use was reported to be linked with potentially life-threatening adverse events such as high incidence of endometrial cancer, thromboembolism and cerebrovascular complications (Braithwaite *et al.*, 2003; Fisher *et al.*, 2005; Lewis, 2007; Perez, 2007; Ryden *et al.*, 2016). Besides this, certain cases of breast cancer can be primarily resistant to tamoxifen or may, in due course acquire resistance to the anti-oestrogen even if they formerly expressed significant amount of oestrogen receptors (Normanno *et al.*, 2005; Zilli *et al.*, 2009; Hayes and Lewis-Wambi, 2015).

As a result of the aforementioned concerns, the introduction of alternative endocrine therapies for treatment of endocrine-sensitive postmenopausal breast cancer was justifiable in making the blockade of aromatase activity a prevailing current approach in the treatment of these cases (Li and Shao, 2016). This therefore led to development of several AIs to either serve as an alternative to tamoxifien or to be used following some years of using tamoxifen. The use of third-generation AIs (anastrozole, exemestene and letrozole) is recommended by the current treatment guidelines since these agents are highly specific and efficient in blocking the aromatase activity and with relatively less adverse effects when compared with previous generations of AIs (Fabian, 2007; NCCN, 2012).

Anastrozole has been well recognized as the drug of choice for adjuvant treatment of both early- and advanced-stage postmenopausal breast cancer (Ingle and Suman, 2005; Ingle, 2006). It has also been investigated in prevention of breast cancer among women at high risk of having the disease (Ingle, 2005). In terms of efficacy and adverse effects, anastrozole was compared with tamoxifen in the "Arimedex, tamoxifen alone or in combination trial (ATAC)" and was more effective but associated with lesser adverse events when compared with tamoxifen (Forbes *et al.*, 2008). Consequently, its use as adjuvant in the treatment of women with early-stage hormone-responsive breast cancer was approved in 2002 by the US Food and Drug Administration and is currently considered as the first-line drug of choice in the adjuvant setting (Behan *et al.*, 2015). Interestingly, anastrozole was demonstrated to yield good results in the initial treatment setting (Forbes *et al.*, 2008), following two to three years of tamoxifen (Boccardo *et al.*, 2005; Jakesz *et al.*, 2005; Kaufmann *et al.*, 2007) and even in the extended adjuvant therapy following five years of tamoxifen therapy (Jakesz *et al.*, 2007).

In spite of the fact that anastrozole has been confirmed to be more effective than tamoxifen (Forbes *et al.*, 2008), a significant population of patients still have breast cancer recurrence (Ingle *et al.*, 2010a). Moreover, a considerable inter-patient variability in terms of toxicity has been observed to a level that adverse events such as musculoskeletal symptoms results in patients' withdrawal from treatment (Lombard *et al.*, 2016; Sahin *et al.*, 2016). This variation is partly attributed to inter-individual variability resulting from genetic variations that lead to differences in anastrozole's pharmacokinetics and/or pharmacodynamics (Abubakar *et al.*, 2014).