

The Role of Estrogen Receptor α and the Androgen Receptor in Human Breast Cancer

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY



Shalini Jindal

Dame Roma Mitchell Cancer Research Laboratories,
Discipline of Medicine, School of Medicine, Faculty of Health Sciences
The University of Adelaide and Hanson Institute

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ॐ भूर्भुवः स्वः

ॐ तत्सवितुर्वरेण्यं भर्गो देवस्य
धीमहि धियो यो नः प्रचोदयात् ।

‘We meditate on the splendor of the Creator;
Who has manifested the Worlds;
Who alone is worthy of Worship;
Who is the embodiment of Knowledge and Light;
Who is the remover of all duality and ignorance;
May He awaken us.’

SHALINI JINDAL
Dedication

Dedicated to the anchor of my life, my dear husband

Sudeep

And my adorable children

Aadil & Advika

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Abstract

The androgenic signalling axis interacts with other major growth pathways in breast cancer, such as estrogen receptor (ER) signalling, and is of renewed interest due to the promise of exploiting the pathway for therapeutic benefit. The effects of signalling via the androgen receptor (AR) are pleiotropic, and there is evidence *in vitro* and *in vivo* that it can both promote and inhibit proliferation of breast epithelia, largely depending on ER expression and activation. Given this complexity, the effect of pathway modulation in individual women with breast cancer remains unclear.

Therefore, the purpose of the studies undertaken in this thesis was to establish baseline parameters in terms of tissue expression of AR and apply them to meaningful clinical scenarios to better establish which population of patients might benefit from androgen pathway-targeting therapies. In the first part of the study, dual-labelling immunofluorescence was performed on a tissue microarray (TMA) containing normal breast and an array of malignant tissues representing tumour progression. AR was expressed more frequently than ER, and AR+ER-cells comprised one third of the total epithelial cell population. 26.6% of the total epithelial population were AR+ER+, 37.5% AR-ER-, and a minor proportion AR-ER+ (2.8%). There were no significant differences in AR expression (either alone or co-localised) between primary and nodal metastasis lesions, and expression remained constant in *in situ*, invasive, and metastatic disease. AR and ER expression therefore show remarkable but stable intratumoural heterogeneity, with implications for how individual cells might respond to therapy within the tumour population as a whole.

The second part of this thesis aimed to firmly establish: a) the prognostic value of AR in two independent cohorts of patients with primary breast cancer and with long-term follow-up, and b) criteria for measurement of the biomarker to pave the way for biomarker measurement in androgen-therapy trials. AR was an independent prognostic factor in two independent cohorts of primary breast cancers tested with different antibodies, and ROC analysis established that the optimal cut-point of AR positivity was 78%. Patients with high AR expression had approximately two-fold reduced risk of cancer-related death in both cohorts, and AR expression was significantly associated with ER expression. Patients with equal or high AR:ER ratios had the best 10-year overall survival of over 80%. Although unlikely to add much to existing prognostic algorithms and approaches, establishing a simple and robust diagnostic test with an appropriate cut-point will expedite studies using androgen pathway-targeting

therapies.

Finally, the third part of this thesis explored the hypothesis that some of the risk of breast cancer associated with increased breast density might be associated with AR expression. Although AR expression was higher in malignant than benign disease, it was not associated with breast density; breast density is likely to be more related to cumulative exposure to estrogen and drive the underlying pathogenesis.

The data presented in this thesis open up several further avenues for investigation, including a robust immunohistochemical assay that can be used in prospective clinical trials and a quantitative immunofluorescence double-staining methodology that can be applied to large clinical cohorts with documented clinical outcomes to help reveal the significance and relative contributions of the co-expressing AR/ER subpopulations to breast cancer pathogenesis and progression. AR expression needs to be investigated in suitable dynamic models of disease progression in order to establish exactly how different populations of cells within the tumour interact and change over time and in response to therapy. These data provide the starting point for these more advanced studies.

SHALINI JINDAL
Declaration

THESIS DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution to Shalini Jindal and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis when deposited in the University Library being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

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Shalini Jindal

Date:

ABBREVIATIONS

17-OHP	17 alpha-hydroxyprogesterone
ACTH	Adrenocorticotrophic hormone
ADH	Atypical ductal hyperplasia
ALH	Atypical Lobular hyperplasia
AR	Androgen receptor
BSSA	BreastScreen South Australia
CDK	Cyclin-dependent kinase
DBD	Deoxyribonucleic acid binding domain
DCIS	Ductal carcinoma <i>in situ</i>
DFS	Disease free survival
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone-sulphate
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
ER	Estrogen receptor
ER α	Estrogen receptor alpha
ER β	Estrogen receptor beta
FFPE	Formalin-fixed paraffin-embedded
FSH	Follicle stimulating hormone
HER2	Human epidermal growth factor receptor-2
HREs	Hormone response elements
HRT	Hormone response therapy
HSPs	Heat shock proteins
IDC	Invasive ductal carcinoma
IGFR-1	Insulin-like growth factor receptor-1
KO	Knockout
LBD	Ligand binding domain
LCIS	Lobular carcinoma <i>in situ</i>
LDL	Low density lipoprotein
LH	Luteinising hormone

SHALINI JINDAL
Abbreviations

MAPK	Mitogen-activated protein kinase
MBD	Mammographic breast density
NR	Nuclear receptor
NTD	Amino-terminal transactivation domain
OCP	Oral contraceptive pill
OS	Overall survival
PBS	Phosphate buffer solution
PR	Progesterone receptor
RNA	Ribonucleic acid
TDLU	Terminal duct-lobular unit
TMA	Tissue microarray
WHI	Women's Health Initiative

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I would like to dedicate this thesis to my family for their trust and confidence in me. Ma and Pa have enduringly nurtured values and principles in me that have made me capable of taking on the responsibility of this PhD program. Big thanks Rachit, Manvi, and Raghav for the prompt IT support for my research work. Last but not least, this thesis would not have been possible without the unfaltering and unconditional support of my husband Sudeep. Inspiration and encouragement from him has been endless over these years, especially when precious family moments were threatened. My pride rests in my son Aadil and my daughter Advika whose smiles and cuddles have always been with me even while pursuing my research, and they have been my motivation to complete this work to the best of my ability.

Finally, this work has reached its present shape and presentation with the blessings of Almighty God.

1. The relationship between estrogen and androgen receptors in breast cancer: a literature review

1.1 Introduction

Breast cancer is the most common form of invasive cancer in women, accounting for nearly 25% of invasive cancers in women globally and 28.1% of new cancers in Australian women [1]. Breast cancer is responsible for 6.4% of all cancer deaths and 14.7% of cancer deaths in women [1]. The lifetime risk of breast cancer in Australian women up to the age of 75 years is one in eleven [1], and as high as one in eight in American women [2]. While there have been impressive increases in five-year survival rates for women with breast cancer over the past few decades, largely due to the influence of adjuvant hormone therapies and early detection via population screening, the management of late-stage or metastatic disease remains a challenge.

Therefore, there is a need to identify new ways of manipulating breast cancer biology for therapeutic benefit. The use of hormone manipulation has revolutionised the management of breast cancer, primarily through an in-depth understanding of the biology of the estrogen receptor (ER) and the progesterone receptor (PR) and their roles in archetypal sex steroid signalling pathways. However, the role of androgen signalling in breast cancer is less well understood, even though androgens are important hormones in women and there is evidence to suggest that this key signalling pathway intersects with estrogen signalling [3]. Therefore, the purpose of this review is to present the background on sex steroid hormone receptors and signalling in health and disease and provide the foundation for understanding ER and androgen receptor (AR) expression in the different clinicopathological contexts that are the focus of this thesis.

1.2 The Human Breast

1.2.1 Anatomy and histology of the breast

The female breast is a modified apocrine gland that produces and secretes milk following full-term pregnancy in order to nourish the offspring. The female breast, which is typically larger in volume than the male breast, contains a complex parenchyma containing ducts and lobules surrounded by supporting stroma and fat containing the nerves, ligaments, arteries,

veins, and lymphatics [4]. A general overview of breast anatomy and microanatomy is shown in **Figure 1.1**. The female breast extends from the second to the sixth rib in the mid-clavicular line, lying over the pectoralis major, serratus anterior, and external oblique muscles [4]. It is innervated by the peripheral nervous system via the anterior and lateral cutaneous branches of the 4th, 5th, and 6th intercostal nerves, with the thoracic spinal nerve 4 (T4) innervating the nipple-areola complex. Blood vessels supply the breast tissue while lymphatic vessels drain lymphatic fluid into the axillary lymph nodes located in the upper chest and armpit [4].

At the microanatomical level (**Figure 1.1**), the breast is composed of glandular ducts and lobules that are embedded in fatty tissue and supported by fibrous connective tissue and suspensory ligaments, known as Cooper's ligaments [5]. The breast is divided into 12-15 major lobes, each of which contain a main duct that branches repeatedly to form a number of terminal ducts. Each terminal duct and its associated lobule is called a terminal duct-lobular unit (TDLU; [6]).

The glandular tissue of the breast is composed of at least two distinct types of epithelial cells: luminal epithelial cells and myoepithelial cells (**Figure 1.1**). Ducts are lined by luminal epithelial cells (which can be cuboidal or columnar) and are surrounded by myoepithelial cells [6, 7]. The luminal and myoepithelial cells arise from luminal-restricted and myoepithelial-restricted progenitor cells, respectively [8, 9].

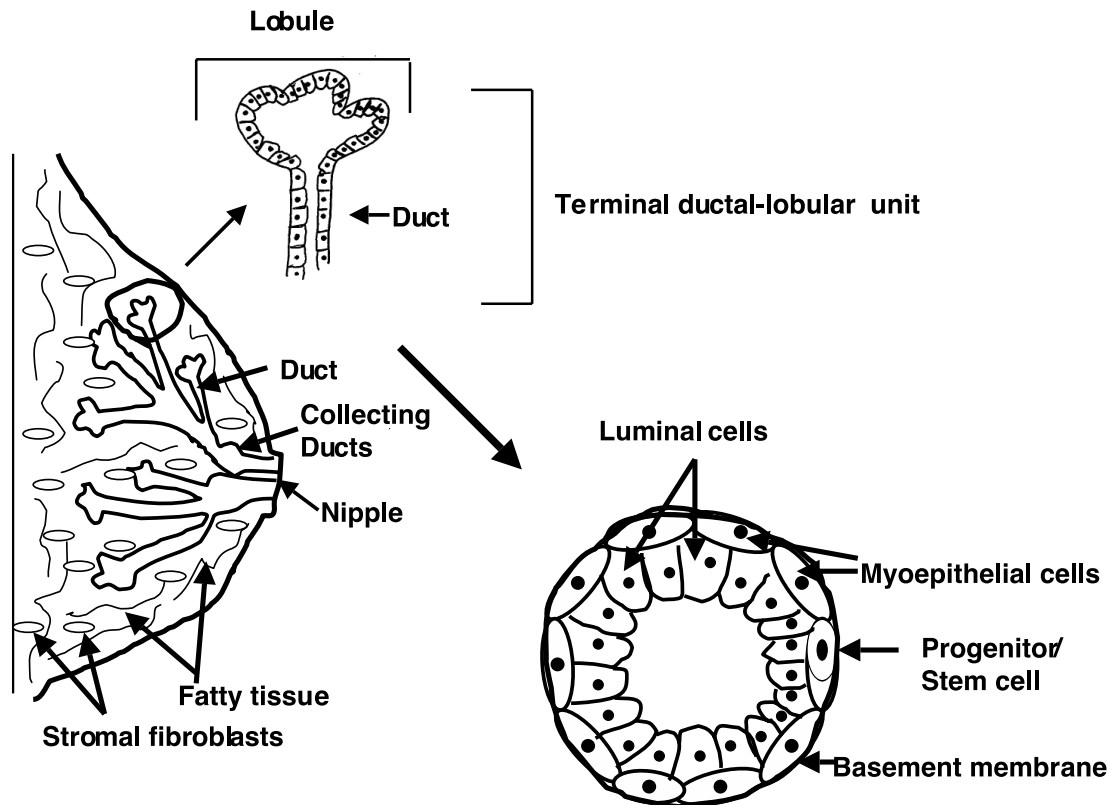


Figure 1.1. Anatomy of the breast (Adapted from Dimri et al., 2005 [6]).

The breast is a modified apocrine gland containing a branching network of ducts that terminate in lobules (the milk-secreting unit of the breast). At its simplest level, the duct is a bilayer of luminal epithelial cells surrounded by myoepithelial cells.

1.2.2 Synthesis and role of sex steroid hormones and their receptors in the female breast

The ovaries and the adrenal glands secrete endogenous sex steroid hormones (i.e., estrogens, progestogens, and androgens), and they regulate the development and morphology of the breast by controlling tissue proliferation and differentiation. The most important sex steroid hormones with respect to breast development are estrogens (estriol (E3), estrone (E1), and 17β -estradiol (E2)), androgens (testosterone, 5-alpha-dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), DHEA-sulphate (DHEA-S), androstenedione (A4), and progestogens (progesterone (P4) and 17 alpha-hydroxyprogesterone (17-OHP)). Glucocorticoids (cortisol and aldosterone) also influence breast development but are not considered sex steroid hormones. Androgens are more commonly associated with male reproductive biology and are essential for the development and maintenance of male reproductive organs [10]. However, androgen hormones also circulate in women, albeit at lower

concentrations than in men, and DHEA, DHEA-S, androstenedione, testosterone, and DHT are all found in the female circulation [11].

Synthesis of sex steroid hormones in women mainly occurs in the ovaries and adrenal glands and starts with the enzymatic conversion of cholesterol. Steroidogenesis is homeostatically regulated by the pituitary hormones, follicle-stimulating hormone (FSH), luteinising hormone (LH), and adrenocorticotrophic hormone (ACTH). The steroid hormone synthesis pathway is shown in **Figure 1.2**. Briefly, progesterone, 17 alpha-hydroxyprogesterone, the inactive precursor androgens DHEA and DHEA-S (often called the pro-androgens), and androstenedione (A4) are the first products of the steroid synthesis pathway and are synthesised via a chain of enzymatic reactions as shown in **Figure 1.2**. This is followed by conversion of DHEA and androstenedione to 5-androstene-3 β , 17 β -diol, and testosterone by 17 β -hydroxysteroid dehydrogenase (encoded by the *17 β -HSD* gene) and 3 β -HSD. In peripheral tissues including the breast, 5-alpha reductases type 1 and 2 (encoded by *SRD5A1* and *SRD5A2*) convert testosterone to DHT, the androgen with the greatest ligand binding affinity for androgen receptors. DHT may be metabolized to 5-alpha-androstane-3 alpha, 17 β -diol, and androsterone by the enzyme 17 β -HSD (Henderson et al, 2003). Estrone and 17 β -estradiol are synthesised by the aromatization of the androgens androstenedione and testosterone by the enzyme aromatase [12].

In pre-menopausal women, sex hormone synthesis occurs via two main pathways: (1) production of steroid hormones by the ovarian tissue and adrenal glands, and (2) synthesis of steroid hormones from circulating precursor hormones in peripheral tissues, including the breast. However, in post-menopausal women or women who have had an oophorectomy, steroid synthesis mainly occurs within the adrenal gland and peripheral tissues. Although estrogen and progesterone production is dramatically diminished following the menopause, post-menopausal ovaries can still synthesise androgenic and, to a lesser degree, estrogenic steroids. As observed by Brodowski et al. [13], ovarian tissue homogenates and serum concentrations of estradiol, testosterone, and androstenedione remain raised in women up to five years after the menopause, but decrease significantly thereafter. Within the adrenal glands and peripheral tissues of pre- and post-menopausal women, circulating precursor sex steroid hormones can be converted to active estrogenic and androgenic steroids [14].

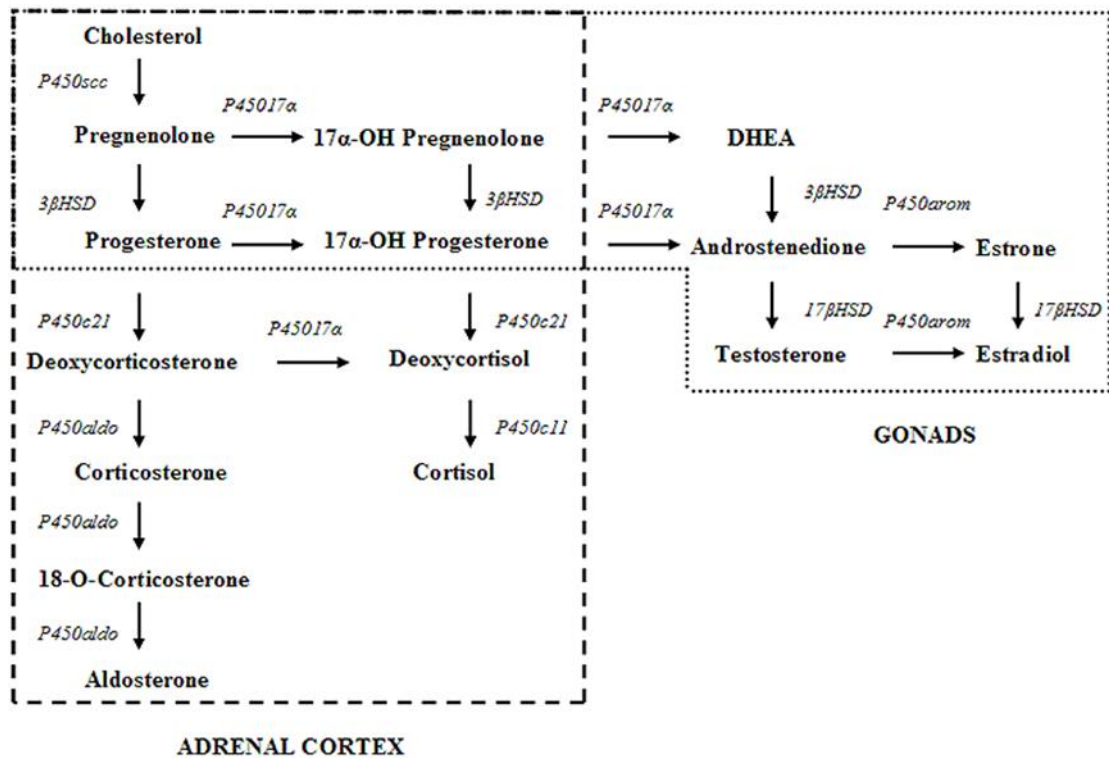


Figure 1.2. General steroid hormone synthesis pathways, including synthesis of sex steroid hormones.

Genes that encode enzymes responsible for each step are italicised (Adapted from [15]).

1.2.2.1 The steroid nuclear receptor super-family

Estrogens, progestogens, and androgens exert their biological function in target tissues by binding to specific intracellular receptors, namely the estrogen receptor-alpha ($ER\alpha$) and -beta ($ER\beta$), progesterone receptor (PR), and androgen receptor (AR), respectively. These receptors belong to the nuclear receptor (NR) superfamily and function as ligand-induced transcription factors that regulate gene expression [16] and control a range of biological processes such as cellular proliferation and differentiation, organ development, reproduction, homeostasis, and metabolism [17].

All the sex steroid receptors ($ER\alpha$, $ER\beta$, PR, and AR) possess a structure characteristic of NRs. They are comprised of an amino-terminal transactivation domain (NTD), a central DNA-binding domain (DBD), a hinge region, and a carboxy-terminal ligand-binding domain (LBD; [18]). The NTD modulates transactivation of the receptors by interacting with co-regulatory factors, the DBD binds to DNA, and the LBD binds ligand to induce receptor activation. In the absence of hormones, steroid receptors exist in a non-active form associated with heat shock proteins (HSPs) and other cellular chaperones [18, 19]. Upon binding to their specific hormone, these sex steroid receptors undergo a conformational change that initiates dissociation from HSP complexes, receptor dimerisation and phosphorylation, and translocation to the nucleus, where they bind to hormone response elements (HREs) within the regulatory regions of target genes. This process involves interaction with co-activators or co-repressors, chromatin remodelling complexes, and the basal transcriptional machinery, depending on context. Ultimately this activity results in transcriptional regulation of target genes, which can be up- or down-regulated in the process [18-20].

1.2.2.2. Role of estrogen and the ER in normal female growth and development

The three major circulating estrogens are 17 β -estradiol (E2), which is the most potent hormone, and its less potent metabolites, estrone (E1) and estriol (E3). The majority of estrogen production occurs in mature, reproductive age ovaries, and to a lesser extent in the adrenal glands, adipose tissue, and brain. After the menopause, ovarian production of estrogen sharply declines, and the adrenal cortex and ovaries secrete mostly androgens that are converted into estrogens in peripheral tissues by aromatase enzymes [21]. Estrogens are involved in several physiological and developmental processes including the growth, differentiation, and function of female reproductive tissues, but they also exert important actions in other organ systems, such as bone, liver, cardiovascular system, and brain. A decline in circulating estrogen, such as during the menopause, is commonly associated with a rise in low-density lipoprotein (LDL), hot flushes and night sweats, and increased bone loss [22]. To counteract these symptoms, women are sometimes prescribed hormone replacement therapy (HRT) containing either an estrogen alone or an estrogen and progestogen in combination [23]. Aberrant estrogen signalling has been described in a number of human diseases such as cancer, osteoporosis, Parkinson's disease, and schizophrenia [24-26].

ER α was identified in the late 1950s and the gene was cloned and sequenced from MCF-7 human breast cancer cells in 1986 [27]. Ten years later, ER β was identified and cloned [28, 29]. While ER α and ER β are present in many of the same tissues, there are reported differences in organ and tissue distribution and expression [30, 31]. ER α is mainly expressed in the uterus, ovary (theca cells), bone, prostate (stroma), white adipose tissue, kidney, testis (Leydig cells), epididymis, breast (epithelium), liver, skeletal muscle, and various regions of the brain, while ER β is mainly expressed in prostate (epithelium), ovary (granulosa cells), lung, testis (Leydig cells), epididymis, bone marrow, salivary gland, regions of the brain, urogenital tract, and intestinal epithelium [30, 31]. Estrogen is a critical regulator of breast epithelial cell proliferation, differentiation, and apoptosis in multiple tissues acting via ER α , ER β , or both. Girls who are deficient in the aromatase enzyme cannot convert androgens to estrogens and hence do not develop breasts at puberty; however, estrogen replacement therapy results in normal breast development [32]. The importance of ERs *in vivo* has been established in knockout (KO) mouse studies, where ER α has been shown to be essential for normal mammary gland development and function, whereas ER β is important for the terminal differentiation of the mammary gland [33]. ER α is expressed in 7-10% of luminal epithelial cells in the normal human breast and its expression fluctuates with the menstrual cycle [34, 35]. In contrast, ER β

expression is expressed in 80-85% of luminal cells and does not fluctuate during the menstrual cycle [36, 37].

Breast epithelial cell proliferation is highest during the luteal phase of the menstrual cycle in premenopausal women [11]. E2 causes expression of hormone-responsive genes that drive cell cycle progression and inhibit apoptosis. In ER-positive MCF-7 human breast cancer cells (used as a model to understand the action of estrogen), E2 stimulates proliferation at the molecular level by inducing a G1- to S-phase transition [38] via up-regulation of c-myc and cyclin D1, with subsequent activation of cyclin-dependent kinases (CDKs, in particular CDK2) and phosphorylation of the retinoblastoma (Rb) protein [39, 40]. E2 also has 'extra-genomic' effects (that is, independent of nuclear ER-mediated transcription) [41-43]. Specifically, ER α interacts with several membrane-bound or cytoplasmic proteins including c-Src, the p85 subunit of phosphoinositol 3-kinase (PI3K), caveolin 1, modulator of non-genomic activity of ER (MNAR) [42, 44], epidermal growth factor receptor (EGFR), insulin-like growth factor receptor 1 (IGFR1), and HER2 [45]. It rapidly activates PIP2-phospholipase C and the mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways [41, 44, 45]. E2 is also a potent inhibitor of apoptosis via regulation of expression of several anti-apoptotic proteins such as Bcl-2 [46].

1.2.2.3. Role of progesterone and the progesterone receptor in normal female breast tissue

The role of progesterone in human breast development is far less well defined than for estrogen [47]. Studies on PR knockout (KO) mouse models suggest that progesterone induces lobuloalveolar development rather than ductal elongation [48]. Progesterone plays a similar role to estrogen in the human breast and stimulates TDLU formation and expansion during puberty and pregnancy. However, this has not been directly demonstrated, perhaps because it is almost impossible to study human breast tissue during these stages of development.

Progesterone has two receptor isoforms, PRA and PRB, which, like ER, are members of the steroid/thyroid hormone nuclear receptor superfamily. Similar to the putative opposing effects of ER α and ER β , progesterone has two receptor isoforms, PRA and PRB [49], and PRA has been shown to be capable of activating transcription. The two isoforms initiate different gene expression profiles and PRB mainly mediates the effects of progesterone on mammary gland development, at least in the mouse [50].

The antibodies used in early immunohistochemical studies were not isoform specific. In spite of this, PR is present in around 15–30% of luminal epithelial cells and is not expressed

in other cells types in the breast [51]. Dual-label immunofluorescence staining, similar to the techniques exploited in this thesis, have shown that all PR-expressing cells also express ER α ; however, they are separate from, but often adjacent to, cells in the cell cycle (as measured by immunohistochemical detection of the proliferation marker, Ki67) [51-53]. This important separation of steroid receptor expression and proliferation has been confirmed in both the human breast and mouse mammary glands [54]. Therefore, estradiol and/or progesterone appear to have an indirect effect on proliferation in normal breast epithelial cells, with receptor-containing cells postulated to secrete positive or negative paracrine and/or juxtacrine growth factors that drive the proliferative activity of nearby luminal cells that lack these receptors [55]. This mechanism might help explain how the breast epithelium becomes sensitive to steroid hormones, such that proliferation can only occur after prolonged exposure to high levels of steroid hormones, which then allows accumulation of paracrine growth factors. This would allow context-specific proliferation, such as during pregnancy, but suppress proliferation at other times [56].

1.2.2.4 Androgens in breast development

Androgens are sex steroids that are primarily involved in the development and maintenance of male characteristics. In females, androgens play an important role in regulating the functions of the reproductive tract and the development and maintenance of bone mass and muscle strength [57-59]. Androgen production in females begins at puberty, and the serum level of testosterone is estimated to be approximately 1.3 nM in pre-menopausal adult women [60-62]. During the menstrual cycle, circulating testosterone peaks mid-cycle at 1.8 nM before falling to 1.2 nM at the start of the cycle [63]. As noted above, androgens are synthesised from cholesterol in the ovaries and the adrenal glands, and circulating steroid precursors can be converted into active androgens in peripheral tissues including breast, liver, kidney, and adipose tissue.

The role of androgens as male hormones is well known, but many female tissues, including the breast, also possess functional AR signalling, and androgenic hormones circulate in women as well as men [64]. Most of these are metabolised into estrogenic hormones, but circulating androgens also act directly as, or can be metabolised into, AR agonists. Circulating testosterone and locally-acting 5 α -dihydrotestosterone (DHT) are both endogenous AR ligands. While circulating estradiol levels reach a nadir during the follicular phase of the menstrual cycle, testosterone levels stay constant, a period that corresponds with the highest rate of

apoptosis in the breast epithelium [11]. After the menopause, circulating estradiol levels decrease 10-fold but testosterone levels decrease only 1.5-fold [65].

In humans, the effect of androgens on the proliferation of breast epithelial cells *in vivo* has predominantly been inhibitory [66, 67]. Involutional changes and reduced breast size are observed in female to male transsexuals treated with testosterone esters and in female users of androgenic anabolic steroids [68]. In addition, congenital adrenal hyperplasia, which is characterised by excess levels of androgens, results in suppression of breast development [69]. *Ex vivo*, human breast tissue explants cultured with testosterone and/or DHT show inhibited proliferation and increased apoptosis in the epithelium, and E2-stimulated proliferation and cell survival are opposed in an AR-dependent manner [70].

The importance of androgen signalling for normal mammary gland development has been illustrated using AR KO mouse models. Yeh et al. [71] generated an AR KO mouse by deleting exon 2 of the *AR* gene, which encodes the N-terminus of the DNA-binding domain (DBD). At four weeks, the AR KO mice showed a 30-50% reduction in ductal extension in the mammary gland and a 50% reduction in proliferation compared to wild-type mice. By 8 – 20 weeks, AR KO mice demonstrated reduced secondary and tertiary branching. In another AR KO mouse model generated by deleting exon 1 of the *AR* gene, which encodes the AR-NTD [72], day 3 lactating mammary glands had reduced ductal branching and elongation. Given the putative inhibitory effects of androgens on mature mammary glands, these findings appear counter-intuitive. However, since AR is expressed in the mammary gland *in utero* [73], it is possible that neonatal exposure to androgens is required for normal breast development. Alternatively, AR KO mice might possess reduced estrogen signalling, a hypothesis that is supported by E2 treatment of four week old ovariectomised mice, which induced expression of the estrogen-regulated genes *Efp* and *Hgf* in wild-type mice, but expression of these genes was halved in the AR KO mice [71]. This indicates that the AR KO mouse phenotype may be due to an indirect, rather than a direct, effect of AR signalling blockade, perhaps via crosstalk between these two pathways. With this in mind, Peters et al. [74] investigated whether development of the murine mammary gland could be altered by stimulating or suppressing AR signalling *in vivo*, and showed that stimulation of androgen signalling from mid-puberty to 12 weeks of age inhibited ductal branching in mice but not thereafter, while inhibition of androgen signalling had no effect up to 12 weeks but increased ductal branching thereafter. Since AR expression increased after 12 weeks in the absence of changes in ER expression, it seems that mammary growth and development is dependent on the homeostatic balance of endogenous

hormones, their receptors, and their interplay. Taken together, these studies demonstrate that androgen signalling pathways contribute to normal mammary gland development and suggest that estrogen and androgen signalling pathways interact in the normal mammary gland in a time- and context-dependent fashion.

1.3 Breast Cancer

1.3.1 Risk factors for breast cancer

1.3.1.1 Genetic risk factors

The most important susceptibility genes in the context of familial breast cancer are *BRCA1* and *BRCA2* on chromosome 17 and 13, respectively [75, 76]. Mutations in these genes account for nearly 25 - 40% of familial breast cancer, 5% of total breast cancers, and 20% of ovarian cancers [77, 78]. A meta-analysis of 22 population- and hospital-based studies showed that the risk of breast cancer at age 70 in *BRCA1* and *BRCA2* mutation carriers was 65% and 45%, respectively [79]. Other inherited cancer syndromes, such as Li-Fraumeni syndrome (*TP53* (tumour protein p53) mutations), Cowden disease (*PTEN* (phosphatase and tensin homolog) mutations), Peutz-Jeghers syndrome (*STK11/LKB1* (serine/threonine kinase 11) mutations), and hereditary diffuse cancer syndrome (*CDH1* (cadherin 1; E-cadherin) mutations) also increase the risk of breast cancer [80]. Although hereditary breast cancer accounts for only 5 - 10% of all cases, less than 25% of the hereditary cases are attributed to germline mutations in the breast cancer susceptibility genes identified to date [81, 82].

1.3.1.2. General hormonal factors

Prolonged lifetime exposure to estrogen is linked to an increased risk of developing breast cancer [83, 84], whilst reduced exposure is believed to be protective [85]. Factors that increase the number of menstrual cycles in a woman's lifetime such as early menarche, nulliparity, and late menopause are believed to prolong exposure to estrogen and therefore increase risk [86-88]. Longer periods of lactation decrease the total number of ovulatory cycles and are therefore protective [89-91]. The risks associated with postmenopausal hormone replacement therapy (HRT) remain contentious (see below); while the risk of breast cancer due to HRT appears to be relatively small, some studies have shown that long-term use increases the risk of developing breast cancer [92]. This risk appears to be dependent on race/ethnicity, BMI, breast density, and the point at which HRT was introduced [93, 94]. Collins et al. [95] reviewed further evidence on the risks associated with specific postmenopausal hormone use and found that the average

estimate of risk of invasive breast cancer with estrogen use was 0.79 (95% confidence intervals (95% CIs) = 0.61–1.02) in four randomised trials representing 12,643 women. The average breast cancer risk with estrogen–progestin use was 1.24 (95% CIs = 1.03–1.50) in four randomised trials involving 19,756 women. More recent epidemiological studies have shown higher risk: 1.18 (95% CIs = 1.01–1.38) with current use of estrogen alone and 1.70 (95% CIs = 1.36–2.17) with current use of estrogen–progestin.

1.3.1.3 Endogenous hormones

Sex steroid hormones play a significant role in the aetiology of breast cancer [56, 96]. The effects of estrogen on cancer risk are multifactorial; as well as the overall effect on DNA replication, estrogen may be converted to quinone derivatives that react with DNA to remove purine bases, thereby applying a mutagenic effect [97, 98]. Pro-androgens may increase the risk of breast cancer by influencing cell growth and proliferation and by being converted to estrogen, which in turn promotes cell division [99]; this is normally associated with hypertrophy [100, 101]. With respect to estrogen itself, the estrogens produced by adipose cells in the breast tissue may be more significant than serum estrogen for the development of breast cancer in postmenopausal women. Indeed, it has been hypothesised that most estrogens first act locally before entering the circulation in postmenopausal women. As a result, circulating estrogen levels in postmenopausal women point to an estrogen effect, but this has not been confirmed [102]. The concentration of estrogen has been found to be higher in the breast tissue of women with breast cancer and, after the menopause, aromatisation of androgens to estrogens in adipose tissue is one of the most important sources of estrogen in the circulation and peripheral tissues [103].

The role of progestin in the development of cancer is less clear [104]. The significance of the progesterone proliferative signal for the development of breast tissue and in the development of breast cancer has been demonstrated in the PR KO mouse [105]. In humans, breast cell proliferation is maximal during the luteal phase when progesterone levels peak [106–108], although PR expression does not appear to occur in the proliferating cells but in adjacent cells, suggesting a paracrine mechanism [51, 105]. Animal and *in vitro* studies have demonstrated that progesterone has a significant influence on breast physiology and is hypothesised to alter breast cancer risk [109, 110].

1.3.1.4. Exogenous hormones

Over the past thirty years, there has been immense interest in the effect of exogenous hormones on the risk of cancer in women. It is believed that exogenous steroid hormones also influence cell proliferation and thereby increase the risk of hormone-dependent cancers such as breast cancer. Oral contraceptives and hormone replacement therapy are two forms of exogenous hormones that have been extensively studied [111].

As discussed above, although there are conflicting data, HRT is generally associated with an increased risk of breast cancer. A meta-analysis of over 160,000 women concluded that among recent and current users of HRT, the risk of breast cancer increases with increased duration of use [112]. Women who had used HRT in the preceding five years had a 2.3% increased risk of breast cancer compared to women who stopped using HRT for over five years [112]. The Women's Health Initiative (WHI) established that among nearly 5000 hysterectomised women, administration of conjugated equine estrogen for an average of 6.8 years did not increase the risk of invasive breast cancer compared to women who were administered a placebo [113]. Similarly, Simpson [102] demonstrated that a low level of estrogen in the serum of postmenopausal women was not associated with the concentration of estrogen in a breast tumour. As mentioned earlier, it was hypothesised that breast cancer development was due to local production of estrogen rather than circulating estrogen. It has been suggested that the risk of breast cancer is higher in postmenopausal women using combined estrogen-progestin hormone therapy (EPT) [112, 114, 115]. The Heart and Estrogen/Progestin Replacement study of nearly 2700 women with coronary artery disease found a small but non-significant increase in the prevalence of breast cancer among EPT users [115]. Although the breast cancer risk associated with EPT is poorly understood, this small increase cannot be ignored.

The use of the oral contraceptive pill (OCP) has also been implicated in the development of breast cancer [116]. A meta-analysis of 54 studies representing nearly 150,000 women demonstrated a moderate increase in the risk of breast cancer among women who used the OCP. The relative risk of breast cancer was 1.24 in current OCP users and 1.16 in recent users, with risk disappearing 10 years after cessation of contraceptive use. The age at first use was also significantly associated with subsequent breast cancer, and further studies are required to assess the long-term effect of early use of oral contraceptives [112].

Between 2001-2002 and 2005-2006, there were significant reductions in the incidence of breast cancer (of up to 22%) in many US and European populations. Decreased rates of breast

cancer were greatest for 50- to 60-year-old women (those most likely to be current users of HRT), affected the number of ER+ and PR+ cancers (those most strongly associated with HRT use), and were greatest in women with the highest pre-decline prevalence in HRT use with the sharpest decline in use [117]. There is now substantial evidence to support the hypothesis that the decline in the incidence of breast cancer is in large part attributable to the sudden drop in HRT use, not least following publication of the WHI and Million Women studies [94]. Nevertheless, the problem of how to advise women contemplating HRT remains. Women with menopausal symptoms still require medical management, so new therapeutic options need to be explored [118].

1.3.1.5 Breast density

Breast density is a measure of the extent of radio-dense fibroglandular tissue in the breast and was first linked to breast cancer risk in the 1970s [119]. Quantitative analyses have since established that women with increased breast density are four to six times more likely to develop breast cancer than women with less dense breast tissue [120-122], to the extent that measuring breast density might be useful way to triage or prioritise the frequency of screening of women for breast cancer [123, 124]. Increased breast density may be due to genetic factors, increased levels of exposure to growth factors, increased exposure to estrogen, or elevated serum prolactin levels [125, 126]. A meta-analysis has reported a strong linear trend between increasing risk of breast cancer and increasing percentage breast density [127]. The mechanism by which breast density affects breast cancer risk is not fully understood. However, since breast cancers originate from glandular epithelial cells and breast density is, in part, a measure of epithelial tissue in the breast, it is postulated that increased breast density provides a greater number of cells at risk of uncontrolled proliferation [128].

1.3.2 Breast carcinogenesis and biomarkers

1.3.2.1 Pre-malignant lesions

A pre-malignant lesion is a morphologically altered tissue containing genetic abnormalities that confer a greater than normal risk of malignant transformation. The best characterised pre-malignant breast lesions are atypical ductal hyperplasia (ADH), atypical lobular hyperplasia (ALH) with or without ductal involvement by cells of ALH (DIALH), lobular carcinoma *in situ* (LCIS), and ductal carcinoma *in situ* (DCIS). Unfolded lobules and atypical ductal hyperplasia are sometimes considered early pre-malignant lesions [129-132]. Simple cysts, uncomplicated fibroadenomas, stromal fibrosis, and sclerosing adenosis are not associated with a clinically significant increased risk of breast cancer [133]. Studies have

demonstrated that premenopausal women who have been diagnosed with ALH have four- to five-times increased risk of developing bilateral breast cancer [137-137], but postmenopausal women with ALH are at reduced risk of breast cancer compared to their premenopausal counterparts [136, 138]. LCIS lesions are associated with double the risk of breast cancer compared to ALH [139, 140]. Although DIALH was initially believed to increase risk to a similar degree as LCIS [141], a later study with a longer period of follow-up reported that this increased risk was not statistically significant [138]. ADH is of slightly lower risk than ALH, conferring a relative risk of cancer about three to four times that of the general population [135, 136]. DCIS is a localized premalignant lesion and increases risk of subsequent breast cancer, especially if not excised, such that the 20-year breast cancer-specific mortality in DCIS patients is over 3% [142].

1.3.2.2 Pathogenesis and classification of breast cancer lesions

Breast carcinogenesis is believed to be a multi-step process [134, 143]. The specific models for breast carcinogenesis have evolved as different concepts, such as the presence of stem cells and telomeres, have been reported (see **Figure 1.3**). The classical model suggests that breast cancer arises from benign breast lesions with cellular atypia (ADH, ALH), progresses to carcinoma *in situ*, and finally to invasive carcinoma (**Figure 1.3** and [137, 144]). This hypothesis is supported by molecular studies that demonstrate altered expression of cell cycle and apoptosis-related proteins in both invasive carcinomas and pre-malignant lesions [145, 146]. Similarly, identical genetic alterations have been observed in invasive cancer and pre-malignant lesions [129, 132, 147-149]. Some studies have suggested that ADH, ALH, and *in situ* disease are precursors of carcinogenesis since they are more frequently found in breasts with invasive cancer [150, 151]. Epidemiological studies have demonstrated that the risk of breast cancer increases with increasing severity of morphological changes of breast lesions [124, 137, 143, 152]. Breast cancers are classified as non-invasive (*in situ*; ductal carcinoma *in situ*, lobular carcinoma *in situ*) and invasive (invasive ductal carcinoma, invasive lobular carcinoma, inflammatory breast cancer, male breast cancer, Paget's disease of the nipple, and phyllodes tumours of the breast).

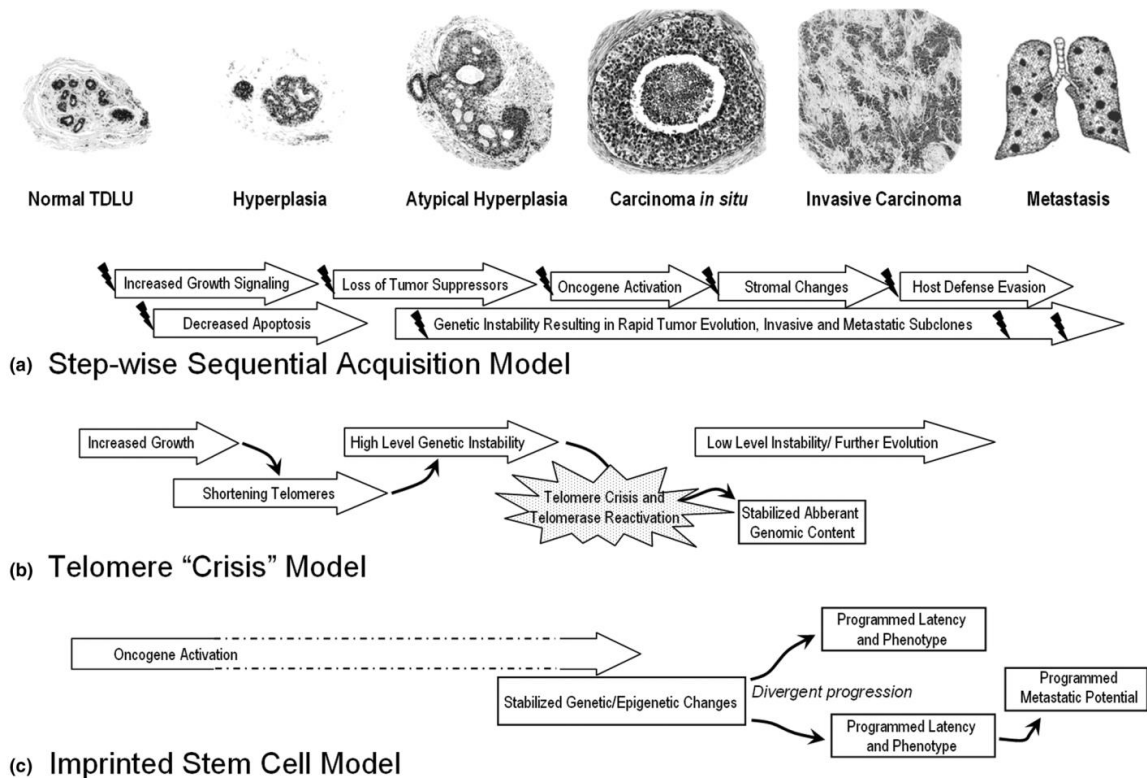


Figure 1.3. Different models of breast cancer progression (from Damonte et al. [153]).

1.3.2.3 Prognostic and predictive factors

A prognostic factor may be defined as a measurable variable that is associated with the natural history of the disease and can be used to determine the probability of recovery or disease recurrence [154]. This is in contrast to predictive factors, which are defined as measurable variables that are associated with response to a given therapy [154]). Some factors are both prognostic and predictive, such as ER α . The following sections describe some important prognostic and predictive factors in breast cancer.

1.3.2.4 Estrogen and progesterone receptors as biomarkers

ER α (often just written as ER) and PR are the best studied biomarkers in breast cancer, and ER α and PR expression in invasive breast carcinoma is both prognostic and predictive. The 5-year disease free survival (DFS) in women with ER α -positive tumours is about 74% and overall survival (OS) 92%, whereas the 5-year DFS in women with ER α -negative tumours is only 66% and OS 82% [155].

It has been established that the expression of ER α or PR in the primary tumour is predictive of probability of benefit from adjuvant therapy. The use of adjuvant tamoxifen for

five years reduces the risk of recurrence and mortality in patients with ER-positive tumours to 47% and 26%, respectively [156]. This equates to a 5.6% reduction in absolute mortality for patients with lymph node-negative disease and a 10.9% reduction in patients with node-positive disease. In the latter study, five years of adjuvant tamoxifen also reduced the risk of contralateral breast cancer by 47% in women with ER+ disease. However, these benefits of tamoxifen were not observed in patients with ER-negative tumours [156].

1.3.2.5 Human epidermal growth factor receptor 2 (HER2) as a biomarker

The HER2/neu (c-erbB-2) proto-oncogene encodes a transmembrane glycoprotein with intrinsic tyrosine kinase activity homologous to the epidermal growth factor receptor (EGFR) [157]. It is amplified and/or overexpressed in approximately 20% of human breast cancers [158]. Overexpression is associated with increased tumour aggressiveness, increased rates of recurrence, and increased mortality in node-positive patients, while its influence in node-negative patients is variable [159-162].

However, HER2 status is now well established as a biomarker for directing HER2-targeting therapies, for example to trastuzumab or, more recently, its derivative Kadcyla (ado-trastuzumabemtansine) [163, 164]. Trastuzumab has been so successful in the treatment of HER2-positive breast cancer that clinical outcome of these patients now resemble those with HER2-negative disease [165].

HER2 overexpression alters responses to both chemotherapy and endocrine therapy. Early data in the pre-trastuzumab era reported improved treatment outcomes in HER2-positive women on adjuvant cyclophosphamide, adriamycin, and 5-fluorouracil (CAF) or adjuvant anthracycline [166-168], and HER2 overexpression was linked to resistance to alkylator-based chemotherapy [169, 170]. The influence of HER2 on the response to endocrine therapy is complex, but there is good preclinical and clinical data to suggest that HER2-positive breast cancers that express ER are resistant to endocrine therapy due to pathway crosstalk, although recent data suggest that some HER2+ER+ tumours continue to be driven by ER signalling and, therefore, remain responsive to endocrine therapy [171].

1.3.3 Hormones and breast carcinogenesis

1.3.3.1 The role of estrogens and ER in malignant breast growth

The role of estrogen in breast carcinogenesis has long been established. Beatson [172] first observed the reduction in breast tumour growth in 1896 following bilateral oophorectomy and following surgical removal of the adrenal or pituitary glands in women with metastatic

breast cancer [173, 174]. Since the synthesis of sex steroid hormones in women primarily occurs in the ovaries or adrenal glands via the hypothalamic-pituitary axis, these early observations strongly implicated estrogen in the development of malignant breast growth.

Dao et al. [175] demonstrated that 70% of human breast tumours obtained from women treated with estrogen prior to surgery had increased DNA replication, and thereby established the stimulatory role of estrogen in malignant breast growth. In addition, many *in vitro* studies have supported the significance of estrogen in malignant breast cell growth. Studies have demonstrated that growth of MCF-7 xenografts *in vivo* is stimulated by E2 and that treatment with the selective estrogen receptor modulator (SERM) tamoxifen inhibits E2-stimulated tumour growth [175-177].

However, the action of E2 seems to be context specific. Physiological levels of E2 can induce apoptosis in long-term estrogen-deprived breast cancer cells or those that have been exhaustively treated with anti-estrogens [178-182]. These data are particularly interesting because high-dose estrogen therapy was used as a treatment for post-menopausal patients with metastatic breast cancer from the 1940s [183] until the introduction of the safer SERM tamoxifen in the 1970s [184].

Nevertheless, E2 is a potent mitogen that stimulates cellular proliferation and prevents cell death in breast cancer cells through activation of the ER. The ERs are crucial mediators of estrogenic function and have important roles in carcinogenesis [185]. Therefore, both estrogen synthesis and the action of the ER have been exploited as targets for therapy to treat hormone-dependent breast cancer. ER β expression is often decreased or absent during carcinogenesis [185], whereas ER α expression is increased, suggesting a possible tumour suppressor role for ER β . ER α promotes proliferation, whereas ER β has been shown to suppress ER α -mediated transcriptional activity and decrease expression of ER α target genes, e.g., pS2, cyclin D1, and PR [186, 187]. In addition, ER β promotes anti-proliferative and pro-apoptotic functions, as well as decreasing motility [188-191]. The balance between ER β and ER α is altered during tumorigenesis [36, 192], and this may contribute to the pathogenesis of breast cancer.

Approximately 70% breast cancers express ER α , which is used clinically as a predictor of response to endocrine therapies such as tamoxifen [156]. Tumours that express both ER α and PR show the greatest benefit from endocrine therapy [193]. In general, ER α expression is associated with lower tumour grade, longer DFS, and better OS [193]. In contrast, loss or reduction of ER α is associated with poorer prognosis, increased incidence of metastasis, and

increased recurrence [194]. How best to use ER β in the management of breast cancer is still uncertain, and it is not currently used as a clinical diagnostic biomarker. Loss of ER β has been associated with a more invasive phenotype, poorer survival, and tamoxifen resistance [195]. Conversely, ER β has also been shown to have negative prognostic value [196] and be associated with higher histological grade [197].

As noted above, both ERs are phosphorylated at multiple sites, with serine 118 being the most important activating phosphorylation site on ER α , which results in ER-induced gene transcription [198, 199]. ER α -ser118 phosphorylation has been associated with improved outcome in tamoxifen-treated tumours, probably by indicating pathway activation [200], while phosphorylation of ER β at serine 105, which might also be an indicator of ligand-independent receptor activation, is associated with improved survival [201]. In spite of their theoretical use as markers of pathway activation, the clinical usefulness of phosphoprotein-related markers has been hampered by their sensitivity to degradation during specimen handling and processing [202]. Altered expression of ER co-factors has also been reported during breast carcinogenesis; for instance, SRC-3 has been reported to be overexpressed in breast cancer and contributes to endocrine resistance, perhaps by reducing the antagonist activity of tamoxifen [198, 200].

The expression of ER α is also regulated by DNA methylation. Hypermethylation of the ER α promoter is associated with a decrease in mRNA levels, and inhibition of DNA methyltransferases reactivates ER α expression [203]. Crosstalk between ER and growth factor pathways may also contribute to breast carcinogenesis since growth factors can activate ERs independently of hormone, e.g., EGF has been shown to activate both ER α and ER β via MAPK phosphorylation [204, 205]. Constitutive activation of the PI3K pathway and overexpression of HER2 also activate ER α [206]. This crosstalk may provide alternative growth pathways for tumours and contribute to resistance to therapy.

1.3.3.2 The role of progesterone and PR in malignant breast growth

With respect to the role of progesterone in breast carcinogenesis, there are now some data to suggest that exogenous synthetic progestins taken in the form of combined HRT increases the risk of postmenopausal breast cancer to a greater extent than use of estrogen replacement therapy alone [96, 207]. This increased risk declined markedly soon after discontinuation of combined hormone therapy and was unrelated to changes in frequency of mammography [208].

Several studies have shown that PR is expressed in pre-malignant and pre-invasive lesions and that it increases with increasing atypia [209]. PRA and PRB ratios alter during carcinogenesis, with PRA predominating [210]. Similar to the action of AR and ER, or ER α and ER β , PR isoform ratio is likely to have an impact on breast carcinogenesis. Several studies have shown that PR is expressed in pre-malignant and pre-invasive lesions and that it increases with increasing atypia, and PR expression is generally regarded as a marker of intact ER α function [209].

1.3.3.3 The role of androgens and the androgen receptor in malignant breast growth

In the AR-positive breast cancer cell lines MCF-7, T-47D, and ZR-75-1, physiological concentrations of DHT have predominantly been shown to inhibit both basal and E2-induced proliferation [211-218]. In contrast, proliferation of the AR-negative breast cancer cell lines MDA-MB-231 and BT-20 was not affected by treatment with DHT [214], indicating that AR expression is required for inhibition of breast cancer cell proliferation by androgens. This finding is supported by the observations that the anti-proliferative effects of DHT and the steroid precursor DHEA were reversed by the anti-androgen hydroxyflutamide [212-214]. Collectively, these studies demonstrate that androgens can inhibit proliferation of breast cancer cells via an AR-mediated mechanism.

In addition to the inhibitory effects of androgens on breast cancer cell lines, androgens also inhibit the proliferation of breast cancer cells *in vivo* [215-217]. Treatment of N-methyl-N-nitrosourea (MNU)-induced mammary tumours in rats with DHEA (100nM serum level) reduced tumour incidence from 100% to 40% and significantly delayed the average time of onset of tumour growth from 54 days to 99 days [217]. Similarly, studies using the ZR-75-1 xenograft model in athymic mice demonstrated that DHT treatment completely blocked E2-induced tumour growth, and the addition of flutamide reversed the inhibitory effect of DHT, indicating an AR-mediated effect [216].

One of the mechanisms by which androgens may inhibit the growth of breast cancer cells is by induction of apoptosis [218, 219]. Treatment of MCF-7, T-47D, and ZR-75-1 breast cancer cell lines with DHT induced apoptosis 1.5-fold compared to control cells [218]. In ZR-75-1 cells, the inhibitory effect of DHT on the expression of the anti-apoptotic protein Bcl-2 was reversed by the anti-androgen, supporting an AR-mediated mechanism of inducing apoptosis [219]. In addition to the apoptotic effect of androgens, studies in MCF-7 cells have shown that DHT (1-10nM) treatment for 6-7 days arrests cells in the G1 phase of the cell cycle

and decreases the percentage of cells in S-phase [215, 218]. Collectively, the results from these studies indicate that the inhibitory effects of androgens on breast cancer cell lines occurs, at least in part, by arresting cell growth and inducing cell death.

Various studies have reported either increased or decreased levels of circulating androgens in patients with breast cancer compared to healthy controls. These variable findings may relate to the difficulty in accurately measuring androgen levels in women. Moreover, since some studies have demonstrated that the levels of androgens in breast cancer tissues of postmenopausal women are significantly higher compared to serum levels [220, 221], the relevance of serum androgen levels is unclear. Reduced serum androgen levels have been reported in premenopausal women with breast cancer or benign breast disease compared to healthy controls [222, 223]. In a similar way, a prospective study observed subnormal urinary androgen levels in women who subsequently developed breast cancer [224]. In contrast, numerous studies have demonstrated that increased serum and urinary testosterone levels are associated with an increased risk of breast cancer in premenopausal [225] and postmenopausal women [226-230]. A potentially confounding issue for the analysis of serum androgen levels and a women's risk of breast cancer is that increased serum E2 levels often accompany increased serum testosterone levels in postmenopausal breast cancer patients [227-230]. This finding indicates that the increased risk of breast cancer in postmenopausal women attributed to higher testosterone levels may be due to increased E2 levels.

Historically, androgens have been used as a hormonal therapy for advanced breast cancer [231-234], with an efficacy of tumour suppression comparable to that of tamoxifen [235, 236]. Additionally, combination therapy with tamoxifen and the androgen fluoxymesterone was significantly more effective than tamoxifen alone for the treatment of advanced breast cancer and resulted in increased DFS [235, 236].

An AR-deficient (AR^{-/-}) MCF-7 cell line has been developed to assess the importance of AR in the growth of breast cancer cells. The AR^{-/-} MCF-7 cells, which were generated by targeted deletion of AR in the MCF-7 (ER α and AR positive) cell line, demonstrated reduced proliferation compared to wild-type MCF-7 cells [71]. A reporter gene assay showed that E2-induced activity of endogenous ER was reduced in AR^{-/-} MCF-7 cells compared to wild-type MCF-7 cells. Treatment of MCF-7 cells with an AR small-interfering (si)RNA also reduced the proliferative potential of the cells and expression of the estrogen regulated gene c-myc [71], indicating that the reduced proliferation observed in AR-negative MCF-7 cells is due to reduced

estrogen signalling. This finding contradicts the assertion that AR is growth inhibitory in breast cancer but does highlight the intimate relationship between androgen and estrogen signalling. The two pathways clearly need to be measured together and in different tissue contexts, such as different tumour grades, to fully appreciate their influence on tumour phenotype.

The AR is expressed in approximately 80-90% of primary breast tumours [237-242]. Moreover, AR is the only sex steroid receptor expressed in approximately 10-20% of primary breast cancers [237, 240, 243] and in approximately 25% of metastatic cancers [237, 244]. The significance of AR expression in breast tumours is illustrated by the observed association between AR expression and histological grade [240, 243]. Well differentiated tumours (grade 1) exhibit the highest AR expression (95-79% positivity), moderately differentiated tumours (grade 2) show 75% positivity, and poorly differentiated tumours (grade 3) exhibit the lowest AR expression (63-51% positivity) [240, 243].

AR expression is associated with ER α expression in primary and metastatic breast tumours [239, 241, 242] and, like ER α expression, is associated with longer disease free survival and overall survival [245-247]. Expression of AR in ER α -negative breast cancers has been shown to be significantly associated with DFS and OS [248]. Taken together, these studies are consistent with the hypothesis that the level of both ER α and AR are critical determinants of breast cancer cell growth.

1.4 The interaction between estrogen and androgen signalling

An interaction between the ER α -LBD and AR in the presence of E2 and the synthetic androgen, mibolerone [249], has been observed using yeast and mammalian two-hybrid assays, suggesting that AR and ER α have the potential to directly interact. Furthermore, co-localisation of ER α and AR was shown in COS-1 cells by fluorescence microscopy [250]. While this supports the possibility that the two receptors may interact, a direct interaction between full-length ER α and full-length AR in breast cancer cells was later refuted [251]. The latter study investigated emerging evidence that the balance between ER α and AR signalling is a critical determinant of growth in the normal and malignant breast [214], and concluded with evidence that, rather than directly interacting, AR could bind to a subset of estrogen-response elements (EREs) and thereby prevent activation of ER target genes that mediate the stimulatory effects of 17 α -estradiol on breast cancer cells.

There is also evidence to suggest that signalling through AR can inhibit the activity of ER α . In CV-1 cells, a monkey kidney fibroblast-like cell line, the E2-induced (10nM) activity of ectopically expressed ER α was significantly inhibited by transfection with AR in the presence of the synthetic androgen mibolerone [249]. Furthermore, overexpression of AR in MCF-7 cells inhibited the E2-induced (100nM) activity of endogenous ER α in the absence of an AR agonist [215]. While both of these studies suggest that AR can inhibit ER α activity, there are some limitations to these studies. Firstly, Panet-Raymond et al. [249] did not use a breast cancer cell line and, due to divergent expression of co-regulators in different cell lines, a different effect of AR on ER α activity might be observed in breast cancer cell lines. In a study by Ando et al. [215], a supra-physiological concentration of E2 was used to activate the ER α . Furthermore, Panet-Raymond et al. [249] required an AR-agonist to observe an inhibitory effect of AR on ER α activity but Ando et al. [215] did not require an AR-agonist.

Since ARs are expressed in the majority of breast cancers, it is reasonable to hypothesise that androgens are directly involved in breast carcinogenesis. In addition, due to the wide heterogeneity of breast cancers, AR may exert divergent effects depending on context. For example, while AR may prevent ER activity in some cancers, androgens, by binding to their receptors, might act independently to produce tumours with specific clinical behaviours [252]. Studies using clinical samples have shown that a number of poorly differentiated breast carcinomas are ER α -negative and progesterone receptor (PR)-negative but AR-positive, or patients with AR-positive tumours have better DFS [253]. Recently, AR expression in a tumour has been considered as an indicator of lower malignant potential; this provides a new range of therapeutic targets for poorly differentiated cancers [254].

1.5 Conclusions

This chapter presents an overview of the role of sex steroid hormones in normal breast development and the role that they play in the risk and subsequent development of breast cancer. We have focussed on the main sex steroid hormones – estrogen, progesterone, and androgens - to highlight the similarities in their mode of action and that they exist as a complex set of interacting signalling pathways. This lays the foundation for measurement of AR and ER in human disease for the purpose of better stratifying patients for novel therapies.

1.6 Gaps in our existing knowledge

From the preceding discussion, it is clear that androgens are of increasing relevance to the breast cancer community both in terms of a need to understand their basic biological function and how best to apply this knowledge to the diagnosis and treatment of breast cancer. The successes and failures of the use of other targeted agents in breast and other cancers have indicated that specific populations need to be defined on the basis of accurate measurement of biomarkers in tissues, such as ER or HER2 in breast cancer for tamoxifen/aromatase inhibitors and trastuzumab, respectively. As attention has turned from the initial excitement of the observed clinical efficacy of some targeted therapies to how to treat recurrent or resistant disease – and how it arises – it is evident that there is a clear need for tissue receptor measurements to be robust, accurate, and take heterogeneity (both between individuals but also within tumours) into account.

Most of these data are lacking for AR in breast cancer, and the situation is likely to be even more complex for AR signalling because of the observed pleiotropism in its mechanism of action, particularly with respect to ER expression (i.e., antagonistic in ER+ disease and agonistic in ER- disease). Since the *in vitro* data indicate that these pathways may act together in the same cell, it would be useful to know exactly how frequently the receptors are expressed in the same cells in primary breast cancers, but this information is currently lacking. However, identifying co-expression of different proteins in the same cell at the same time in a quantitative manner requires non-standard pathological techniques such as immunofluorescence, and the measurements need to be made in a biologically meaningful cohort of tissues such as those representing tumour progression.

Although many studies have indicated that AR expression is prognostic for women with breast cancer, the data are conflicting, methodologies vary, and cut-points for ‘high’ and ‘low’ expression still need to be defined. Many of these studies are limited by small sample size and lack of validation in independent cohorts. There is still an urgent need to acquire high-quality and robust baseline prognostic data and establish independently validated criteria for the measurement of the AR for clinical diagnostic use.

1.7 Objectives

The purpose of the studies undertaken in this thesis was to establish baseline parameters in terms of tissue expression of AR and apply them to meaningful clinical scenarios, given the current excitement in using androgen-based endocrine therapy and the known interaction with ER α signalling. This thesis has, therefore, three main objectives:

1. To establish whether quantitative assessment of mammographic breast density can be used to predict the existence of breast cancer and whether mammographic breast density is related to ER α and AR expression.
2. To measure the co-expression of ER α and AR in normal breast epithelium and in a range of malignant tissues representing progression from pre-invasive to invasive and nodal metastatic disease.
3. To definitively establish whether AR is prognostic in women with breast cancer and determine exactly what level of expression constitutes 'high' and 'low'. In addition, since AR and ER α signalling interact at the molecular level, understanding how relative levels of AR and ER α contribute to outcome will be investigated.

1.8 References

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2. Mammographic density for the diagnosis of non-malignant and malignant breast disease and its relationship with estrogen and androgen receptor expression

2.1 Abstract

Background: Mammographic breast density (MBD) has consistently been associated with an increased risk of breast cancer, but its clinical utility in the population of women being screened for breast cancer has not been well established. Given that estrogen and androgen hormones exert proliferative and anti-proliferative effects in breast epithelia, respectively, their activities are likely to influence MBD. Therefore, we aimed to establish whether quantitative assessment of MBD can be used to predict breast cancer and whether MBD is related to expression of estrogen and androgen hormone receptors.

Methods: Seven hundred and twenty-three women that were screened between 1995-2005 as part of the BreastScreen South Australia (BSSA) screening programme consented to participate in the study and completed a hormonal history questionnaire. All had a core biopsy taken at the time of mammography that revealed either a non-malignant (n=533) or malignant (n=190) histopathological diagnosis. Cranio-caudal mammograms taken at the time of core biopsy and, where available, from the previous screen ('pre-core' mammogram), were digitised and the MBD calculated using a semi-quantified thresholding technique (Cumulus) in both the affected breast and the contralateral unaffected breast. Diagnostic biopsies underwent independent histopathological review and estrogen receptor alpha (ER α) and androgen receptor (AR) levels were measured by immunohistochemistry. Univariate and multivariate statistical analyses were conducted adjusting for age, BMI, number of full-term pregnancies, and family history.

Results: There were no significant differences in MBD between breasts containing non-malignant (mean 14.6% \pm 13.4%) versus malignant (13.3% \pm 12.8%) lesions at the core scan (p = 0.2). Women with malignant diagnoses were older, of younger age at menarche, slightly older at time of first full-term pregnancy, and were more likely to have used hormone replacement therapy in the preceding five years. MBD was not associated with expression of either AR or ER α in the core biopsy. Both AR and ER α were more frequently expressed in malignant compared to non-malignant breast biopsies, and high AR, high ER α , and higher age at first full-

term pregnancy were all associated with an increased likelihood of having malignant disease in multivariate analysis.

Conclusions: Quantitative measurement of MBD is unlikely to provide additional diagnostic or prognostic utility to routine radiographic assessment of mammograms in population breast screening programmes.

2.2. Introduction

Breast density is defined as the proportion of radio-dense fibro-glandular tissue and radiolucent adipose tissue present in the breast [1]. Increased breast density was identified as a risk factor for breast cancer in the 1970s [2], and several case-control studies have since been conducted using quantitative measures of mammographic breast density (MBD) that have confirmed MBD as a strong independent risk factor for breast cancer [3-5]. A high MBD, the definition of which varies, has been consistently associated with a three- to six-fold increase in breast cancer risk when compared to lower densities [6-13]. However, no studies have yet been undertaken to determine whether breast density can discriminate non-malignant from malignant breast disease and can, therefore, be used to refine the radiological diagnosis prior to biopsy.

Endogenous sex steroid hormones, particularly estrogen, induce cellular proliferation in breast tissues, and prolonged exposure or high levels are associated with higher breast cancer risk. Tamoxifen therapy, which selectively inhibits estrogen action in breast epithelial cells, has been shown to reduce MBD [14], and breast density is hypothesised to be a surrogate of cumulative exposure to sex steroid hormones [15]. Although there is conflicting evidence, several studies have shown that higher MBD is associated with tumours that express estrogen receptor α (ER α) and the progesterone receptor (PR) [16-18]. While the actions of estrogen and progesterone on breast development and carcinogenesis are well characterised because they are considered the key 'female' sex hormones, the role of androgenic sex hormones, which are also produced in females, is less well understood. Androgen hormones inhibit breast growth in men, and renewed interest in their role in breast cancer has arisen because they represent a potential avenue for therapy. Androgens act via the androgen receptor (AR) to oppose estrogen-induced proliferation of normal breast epithelial cells [19, 20] and ER+AR+ breast cancer cell lines [21-28]. Consistent with this antagonistic and growth inhibitory effect, high AR expression is associated with improved overall survival for women with breast cancer, particularly those with ER+ disease [26, 29-33]. Since the AR is the most abundant sex steroid receptor present in both

primary and secondary breast tumours [34-37], and given its known anti-proliferative effect, we therefore hypothesised that high tissue levels of AR are associated with a lower MBD and a lower risk of breast cancer.

We therefore examined mammograms and associated biopsies from a population screening cohort of women who underwent biopsy due to a mammographic suggestion of breast disease to examine the utility of MBD in the diagnosis of malignancy and the association of AR and ER α expression with breast density and malignancy.

2.3 Materials and Methods

2.3.1 Study population

Two thousand one hundred and one women had a breast core biopsy taken at BreastScreen South Australia (BSSA), a nationally accredited population breast screening assessment unit, between January 1995 and December 2005. Each had a mammotome core biopsy performed following detection of a suspicious lesion on mammography. Ethical approval (for data collection from BSSA and contacting the women) was obtained from the University of Adelaide and South Australian Department of Health Research Ethics Committees.

The inclusion criteria were a core biopsy with a definitive histopathological diagnosis of non-malignant or malignant breast disease and no evidence of malignancy during a minimum of five years of follow-up in the non-malignant group. In addition, patients were required to have an available mammogram from the time of diagnostic core biopsy, the formalin-fixed paraffin-embedded (FFPE) block of the diagnostic core biopsy available from the BSSA archives, and participants needed to be alive at the time of the study in order to give consent and complete the hormone history questionnaire (**Figure 2.1**).

Demographics and hormonal history were obtained by mailed questionnaire (**Figure 2.1**), and patient eligibility and participation are summarised in **Figure 2.2**. Overall, 848 women in the non-malignant group and 263 women in the malignant group met the inclusion criteria. Of these, 533 (63%; non-malignant group) and 190 (72%; malignant group) women provided informed written consent to participate and returned the questionnaire.



1. At what approximate age did your menstrual periods begin? (Mark only one answer)
 - Never menstruated
 - 11 years or younger
 - 12 - 13 years inclusive
 - 14 years or older
 - Don't know

2. How many times have you had a pregnancy that went to full term? (If you are currently pregnant, be sure to count this pregnancy).
 - Number of full-term pregnancies

3. How old were you at the time of your first full term pregnancy?
 - Age in years

4. What is your approximate height in cm?
 - cm

5. What is your approximate weight in kg?
 - kg

6. Have you had a hysterectomy?
 - Yes
 - No

7. Have you ever taken hormone replacement therapy (HRT)?
 - Yes, in the last five years
 - Yes, but not in the last five years
 - No, never taken HRT
 - Don't Know

8. If yes to Question 7, for how many years, approximately speaking, did you take HRT?
 - Number of Years
 - Don't Know

Figure 2.1. The hormonal history questionnaire.

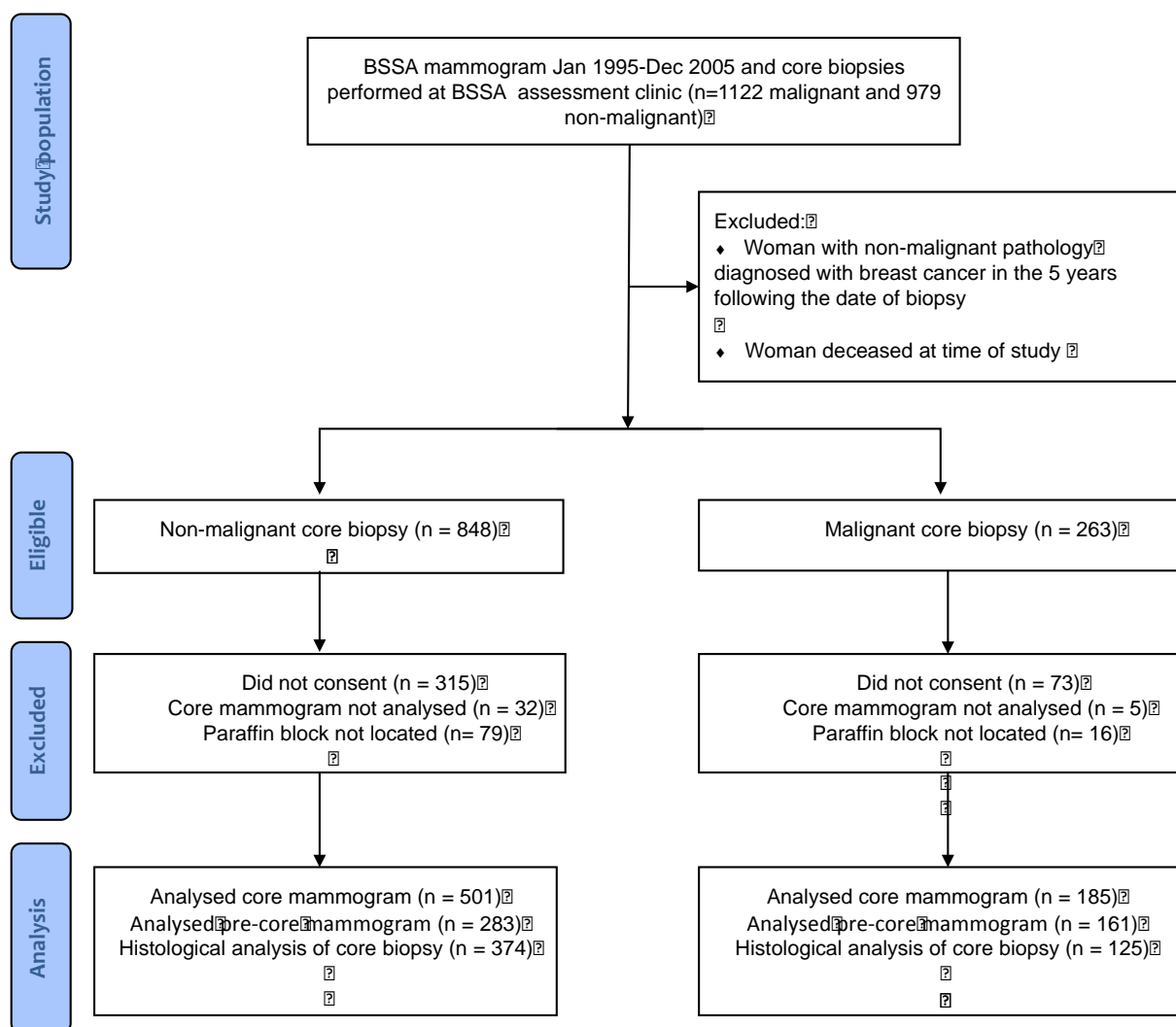


Figure 2.2. Flow diagram describing the population studied.

2.3.2. Mammography and breast density analysis

For those participants who had attended more than one round of screening, the mammogram from the screen preceding the core biopsy mammogram (the ‘core scan’) was included in the study when available and referred to as the ‘pre-core scan’. Cranio-caudal mammographic views were digitized using an Array 2905 HD Laser Film Digitizer with 100 film auto-feeder scanner (Array Corp., Tokyo). Mammograms were classified according to the Australian NBCC scoring system by BSSA radiologists at the time of mammography, and scores, in this thesis referred to as the ‘radiological grade’, were retrieved from the BSSA database and defined as: 1, no significant abnormality; 2, benign; 3, indeterminate/equivocal; 4, suspicious; and 5, malignant.

MBD, measured as the percentage of dense area in each breast, was quantified using the Cumulus semi-automated software package [38]. Cumulus uses a computer-aided interactive thresholding technique that measures the areas of breast tissue and dense tissue, from which non-dense areas and the MBD are straightforwardly derived. Briefly, the observer first selects a grey value as a threshold to separate the breast image from background in order to determine breast size. A second threshold is then selected to identify the edge(s) of the mammographically dense tissue, with the number of pixels in the digitised image within the defined areas being used to calculate areas. This method has been shown to be highly reproducible and reliable, with intra- and inter-observer errors 0.90 and 0.87, respectively (intra-class correlation coefficient) [38]. The reader performing densitometric measurements was blinded to all identifying information and mammograms were randomised into reading sets of approximately 100, ensuring that all mammograms from the same individual were measured in the same set. For an individual, the mammograms were viewed sequentially but the temporal order was unknown to the reader. This procedure has been shown to be the best method for randomisation and viewing of multiple mammograms since it reduces variation in measurement without affecting the mean [38]. A 10% random sample of repeats was included in each set and between every fifth set to test the reliability of the measurements.

Core-scans were successfully analysed for MBD in 501 (94%) and 185 (97%) cases in the non-malignant and malignant groups, respectively (**Figure 2.2**). Of these cases, MBD measurements were also successfully done in 283 (56%) and 161 (87%) pre-core scans in the non-malignant and malignant groups, respectively. The median time between the pre-core and core-scans was two years in both groups (range 1-13 years). Mammographic density was also assessed in the contralateral unaffected breast to provide 'normal' breast density data for comparison.

2.3.3 Histopathology

Histological sections were prepared and stained using haematoxylin and eosin for independent pathological review by a qualified pathologist to determine the disease type (non-malignant vs. malignant); where available, subcategorisation of non-malignant included diagnoses of fibroadenoma, ductal hyperplasia without atypia, radial scar, sclerosing lesions, sclerosing adenosis, fibrocystic disease, cyst, and papillomatosis, tumour type (ductal vs. lobular), and grade (low, intermediate, or high grade for ductal carcinoma *in situ* (DCIS) or Grade I, II, or III for invasive cancer).

2.3.4 Immunohistochemistry

Four μm formalin-fixed paraffin-embedded (FFPE) tissue sections were immunostained with AR antibodies: U407 (in-house) (26) and ER α (Santa Cruz Biotechnology). Tissues were antigen retrieved in 10 mM citrate buffer (pH 6.5) in a microwave oven before being incubated overnight with a 1:300 dilution of U407 or a 1:300 dilution of ER α in blocking buffer (5% normal goat serum in PBS) at 4°C in a humidified chamber. Positive immunoreactivity was visualised using biotinylated anti-rabbit immunoglobulins (Dako), streptavidin–peroxidase conjugate (Dako), and diaminobenzidine tetrahydrochloride (DAB). FFPE tissue sections from a prostate cancer block known to be positive for AR and a breast cancer block known to be positive for ER α were used as positive controls; the negative control was replacement of the primary antibody with PBS. Staining was quantified by a qualified pathologist. The images of the stained tissues were captured using the Hamamatsu Nanozoomer slide scanner. The percentages of AR and ER α positive nuclei were determined by blind manual counting of at least 500 malignant cells over 5 to 10 fields at x40 magnification.

2.3.5 Statistical analyses

MBDs of the pre-core and core scans were compared between unaffected (contralateral) breasts and breasts with non-malignant or malignant lesions using both matched and unmatched non-parametric tests. The Mann-Whitney U-test was used to compare differences in MBD according to receptor expression, defined as the % positive cells where any staining by IHC was considered positive. The IHC data were therefore treated as continuous variables and no cut points were applied. Univariate analyses were undertaken to examine associations between key covariates and malignancy status, MBD, and change in breast density. Multivariate logistic regression analysis was used to examine for independent predictors of malignancy status. Generalised linear models (multivariate regression analysis) were used to examine for independent predictors of malignancy analysed as categorical and continuous variables.

2.4. Results

2.4.1 MBD does not differ between non-malignant and malignant disease

In order to establish whether there is an association between breast density and malignancy, we first compared MBD values between breasts that had a non-malignant versus a malignant lesion. As expected, the radiological classification was higher in mammograms requiring a diagnostic core biopsy, compared to their pre-core scans (**Figure 2.3**). The

radiographic grade was higher in the malignant lesion than non-malignant lesion, consistent with the larger number of ‘suspicious’ (grade 4) and ‘malignant’ (grade 5) mammographic diagnoses in malignant films, compared to the high number of indeterminate/equivocal (grade 3) diagnoses in the non-malignant group (**Figure 2.3**).

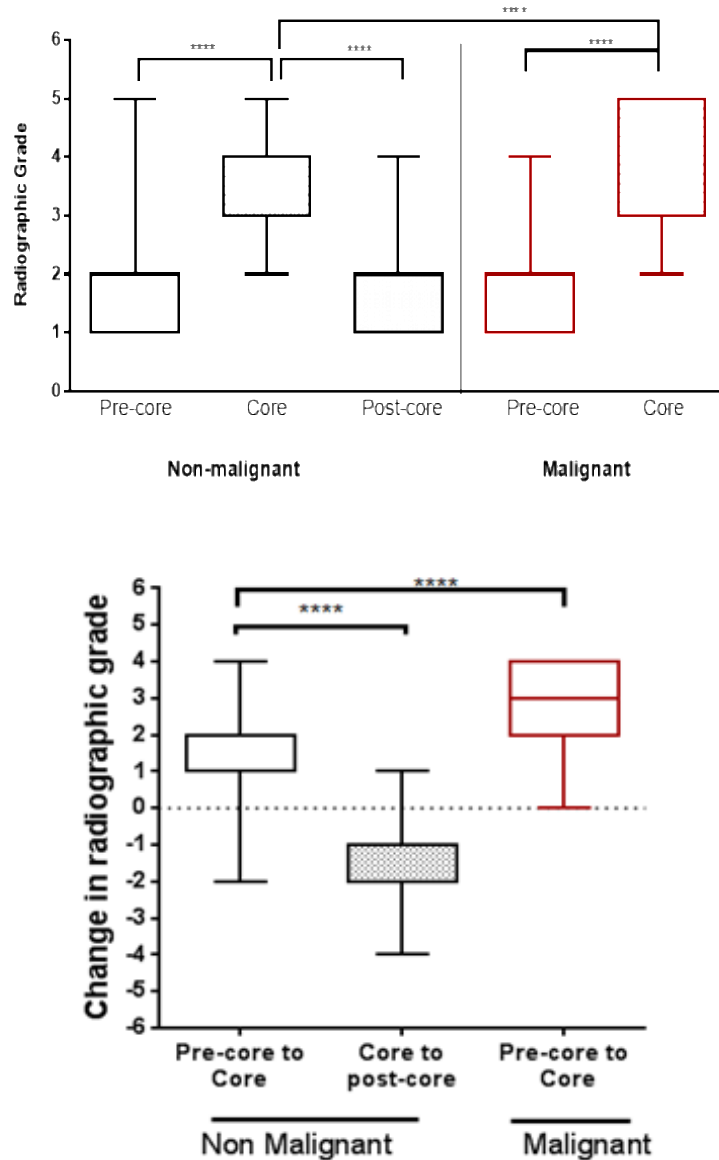


Figure 2.3. Changes in radiographic grade between rounds of mammography in the non-malignant and malignant groups. Post-core refers to mammographies performed after surgical intervention to remove the lesion.

The NBCC scoring system was used to “grade” the tumours on a scale of 1-5. The y-axis extends to 6 due to illustration of significance values.

There were no significant differences between the MBDs of breasts harbouring a non-malignant or malignant breast lesion, either at the time of diagnostic core biopsy or in those measured in pre-core mammograms (**Figure 2.4A**). There were changes between pre-core and core MBDs in a proportion of individuals in both affected and contralateral breasts, but this occurred in both non-malignant and malignant cases. The affected breast always showed an increased MBD when compared to the contralateral (unaffected) breast in women with non-malignant ($p < 0.001$) or malignant lesions ($p < 0.01$; **Figure 2.4B**).

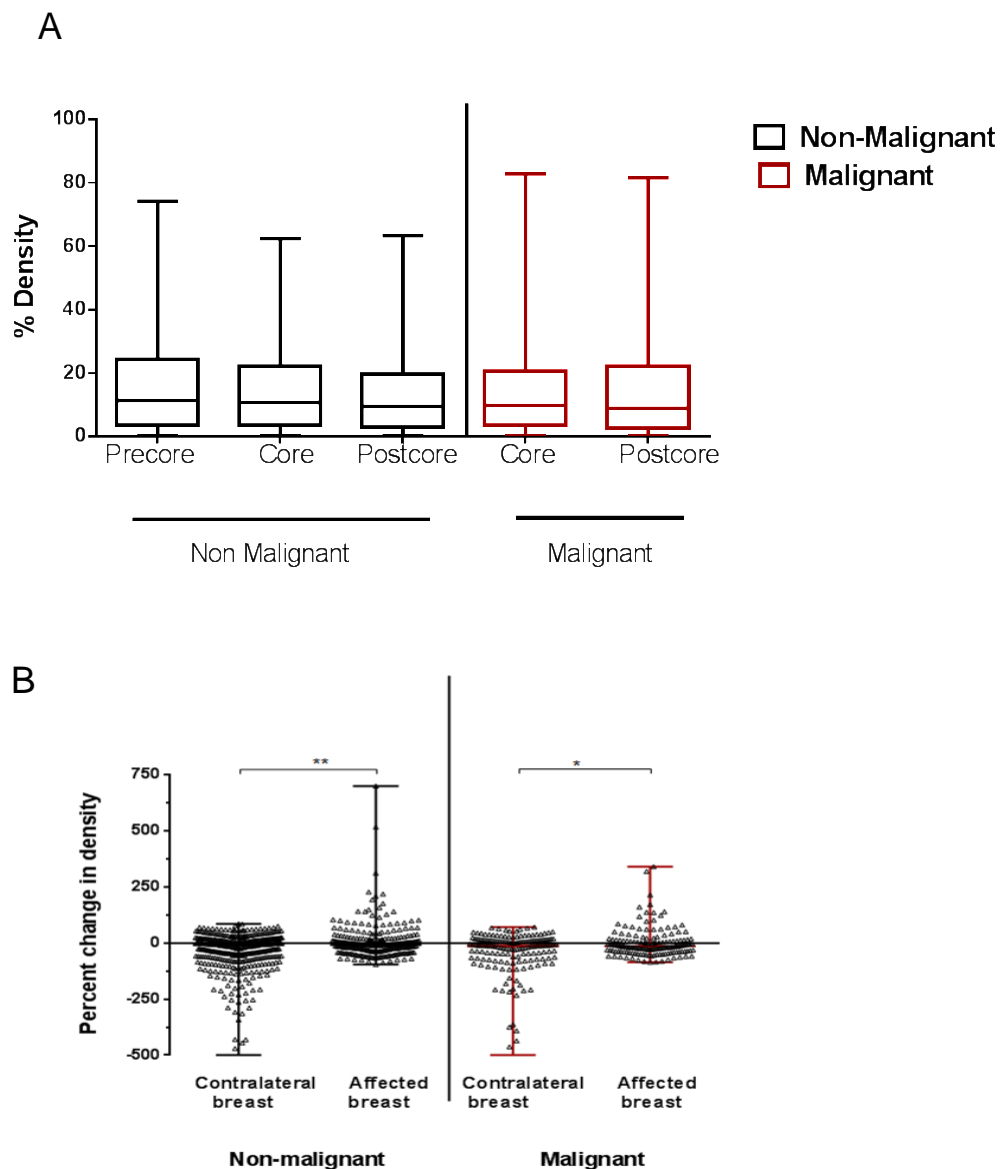


Figure 2.4. (A) Differences in mammographic density in breasts containing non-malignant and malignant breast lesions. (B) Changes in MBD in the non-affected (contralateral) and affected breasts between pre-core and core mammograms.

As expected, there were significant differences in the demographics of women with non-malignant and malignant disease (**Table 2.1**). Women with malignant diagnoses were older (60 vs. 56 years; OR 1.05 (1.03-1.07); $p < 0.0001$), were of younger age at menarche (12-13 years vs. 14 years or older; OR 3.43 (1.38-4.26); $p = 0.006$), were slightly older when they had their first full-term pregnancy (24 vs. 23 years; OR 0.96 (0.92-0.99); $p = 0.029$), and were more likely to have used hormone replacement therapy (HRT) in the preceding five years (OR 1.4 (0.84-2.3); $p < 0.0001$).

Table 2.1. Demographics and hormonal history of the patients studied

	Non-malignant (n = 501)	Malignant (n = 185)	Odds Ratio (95% CIs)	p - value
Median age at core biopsy (yrs)	56 (40-81)	60 (41-82)	1.05 (1.03-1.07)	p < 0.0001
Age group at menarche 2:3:4	93:248:143	17:110:49	3v2 2.43 (1.38- 4.26) 4v2 1.88 (1.02 v3.45)	p = 0.006
Age at 1st full term pregnancy (yrs) (FTP)	23 (15-40)	24 (17-39)	0.96 (0.92-0.99)	p = 0.029
HRT 1= used in last five years	111	24	1v 3 1.4 (0.84-2.3)	p < 0.0001
2=used, but not in last five years	131	82	2v3 0.48 (0.33-0.71)?	
3=never used	245	74		
BMI	28 (16-69)	28 (18-63)	-	NS
Weight (Kg)	72 (40-180)	72 (45-170)	-	NS
Number of FTP	2 (0-6)	2 (0-5)	-	NS
Years on HRT	8 (0.01-44)	8 (0.1-36)	-	NS
Hysterectomy N:Y	322:171	114:68	-	NS

2.4.2. MBD is not associated with AR and ER α expression

We next examined AR and ER α expression within the epithelial cells of non-malignant and malignant breast lesions using immunohistochemistry (**Figures 2.5 and 2.6**). In addition, we analysed the ratio of expression of AR and ER α , since the balance between the contributions of the two signalling pathways has been shown to be important for proliferative responses to hormones *in vitro* and *in vivo* [22, 26, 41], and breast density is, at least in part, related to epithelial proliferation. MBD was not correlated with expression of AR, ER α , or AR:ER α ratio in either non-malignant or malignant disease (R2 values all close to zero; **Figure 2.7**). Although AR:ER α ratios greater than one showed a trend towards being associated with decreased breast density in malignant breasts ($p = 0.09$), these differences were not statistically significant (**Figure 2.8**).

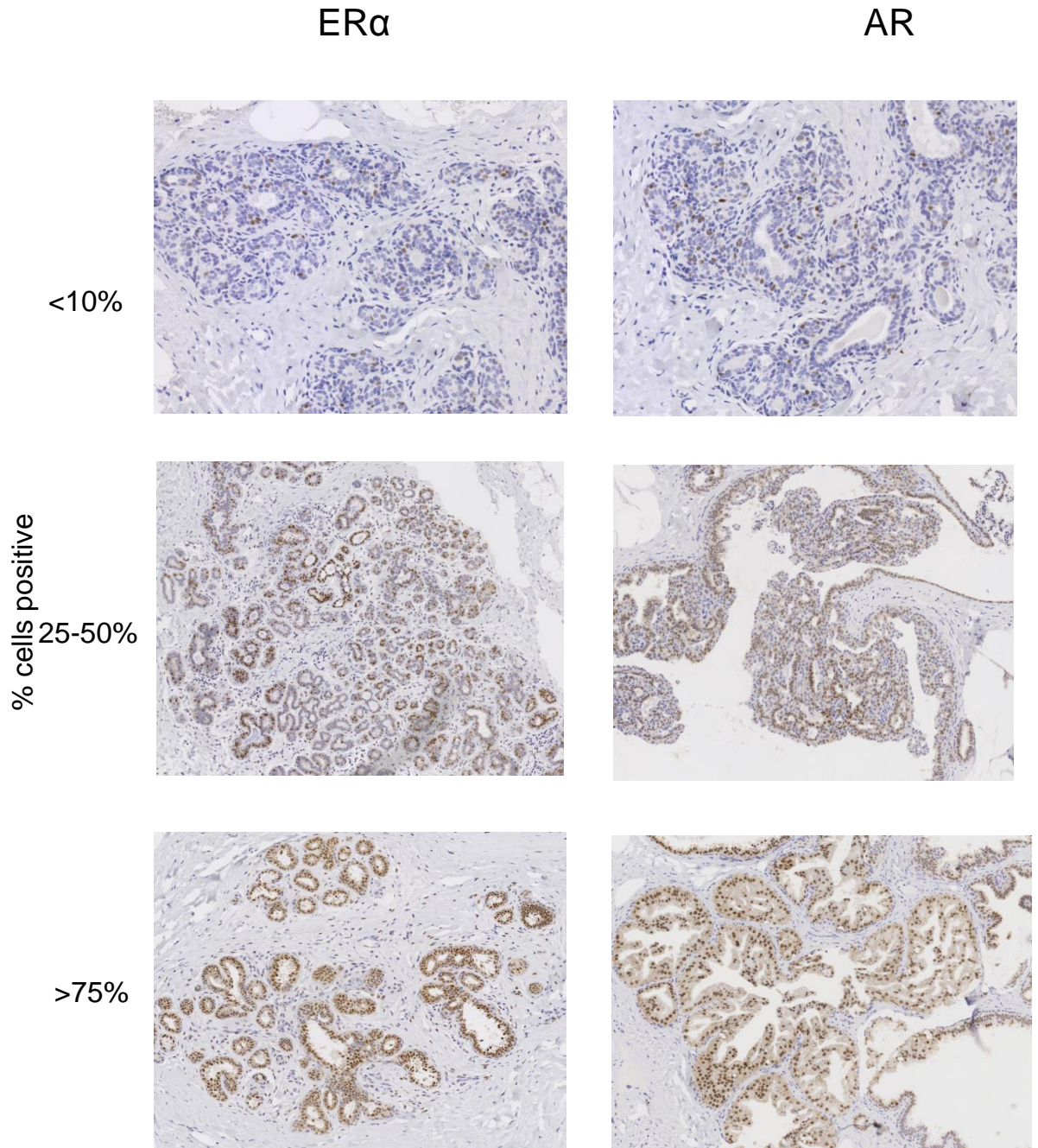


Figure 2.5. Immunohistochemistry for ER α and AR in non-malignant breast tissue (x20 magnification).

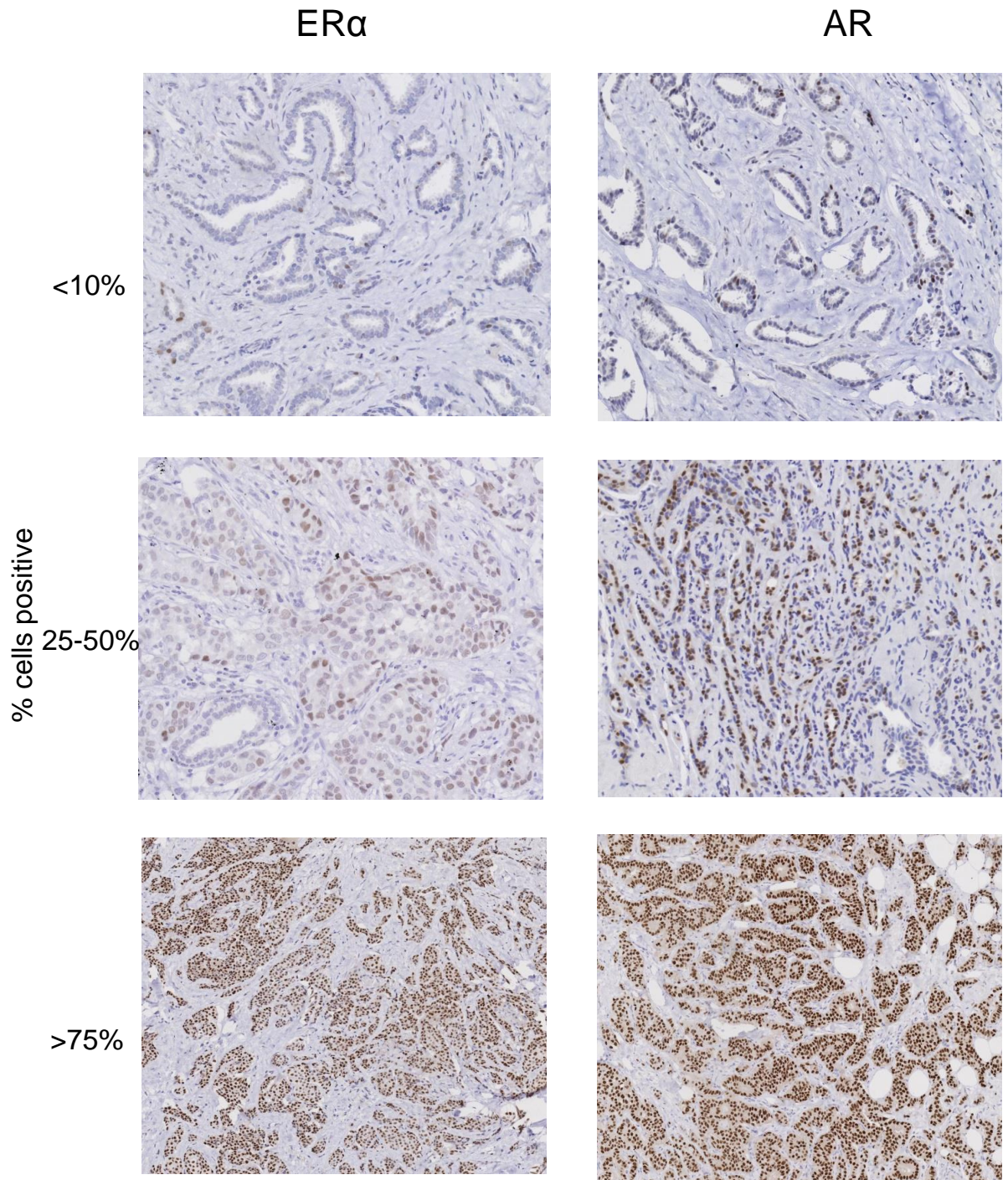


Figure 2.6. Immunohistochemistry for ER α and AR in malignant breast tissue illustrating quantitative scoring of receptor positivity (x20 magnification).

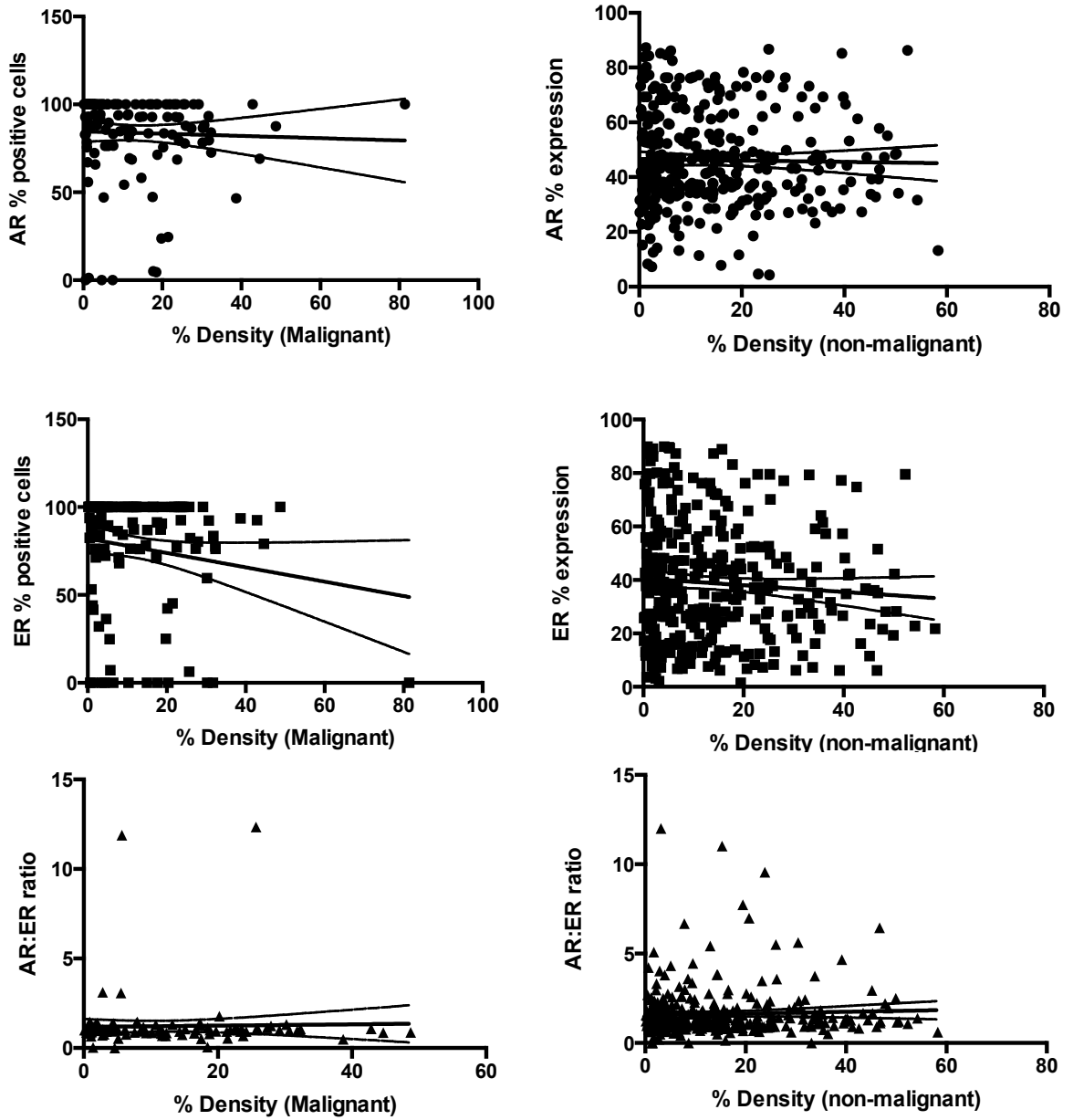


Figure 2.7. Scatter plots showing the relationship between breast density and receptor expression

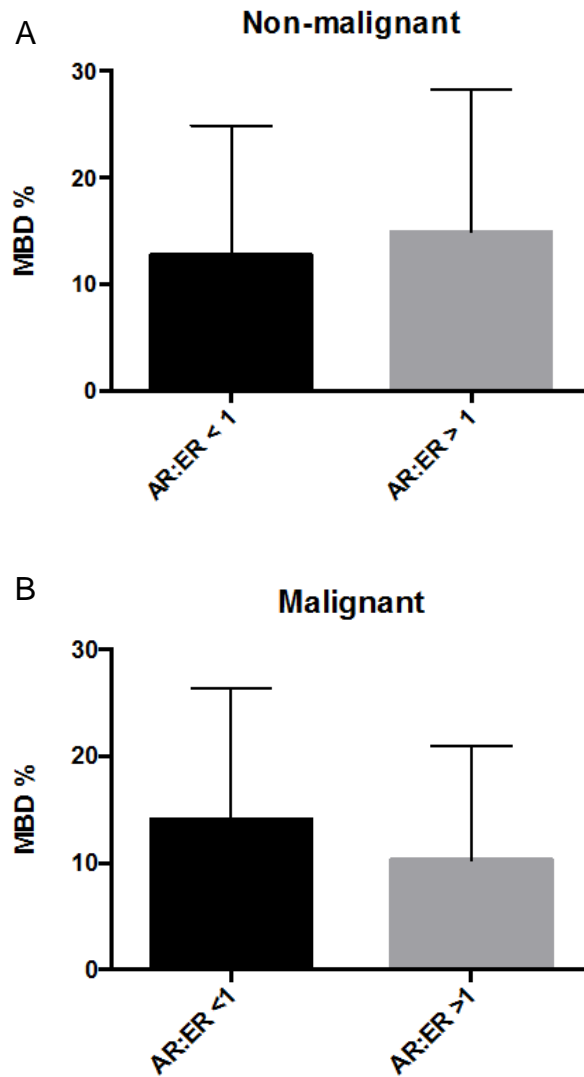


Figure 2.8. Differences in breast density (MBD) according to ratio of AR and ER expression in breasts containing (A) non-malignant and (B) malignant lesions.

2.4.3 AR and ER α expression is increased in malignant lesions

Anti-AR and ER α antibodies were used to measure receptor expression where tissue was available (**Figure 2.2**), with receptor expression measured on a continuous scale of % positive cells (where any intensity of staining was deemed positive). Both AR and ER α expression were significantly greater in malignant lesions when compared to non-malignant lesions ($p < 0.0001$; **Figure 2.9A**). AR was more frequently expressed in non-malignant lesions than ER α ($p < 0.0001$), but not in malignant lesions (**Figure 2.9A**). When receptor expression was examined with respect to radiological grade, there was a trend to increasing receptor expression with increasing grade, although this was not significant (**Figure 2.9B**).

2.4.4. Multivariate analysis

When all the variables (MBD, age at the time of biopsy, age at first full term pregnancy, age at menarche, AR, ER, HRT, and number of prior scans) were examined using multivariate analysis, AR, ER α , and age at first full-term pregnancy were all associated with an increased likelihood of having malignant disease (**Figure 2.10**).

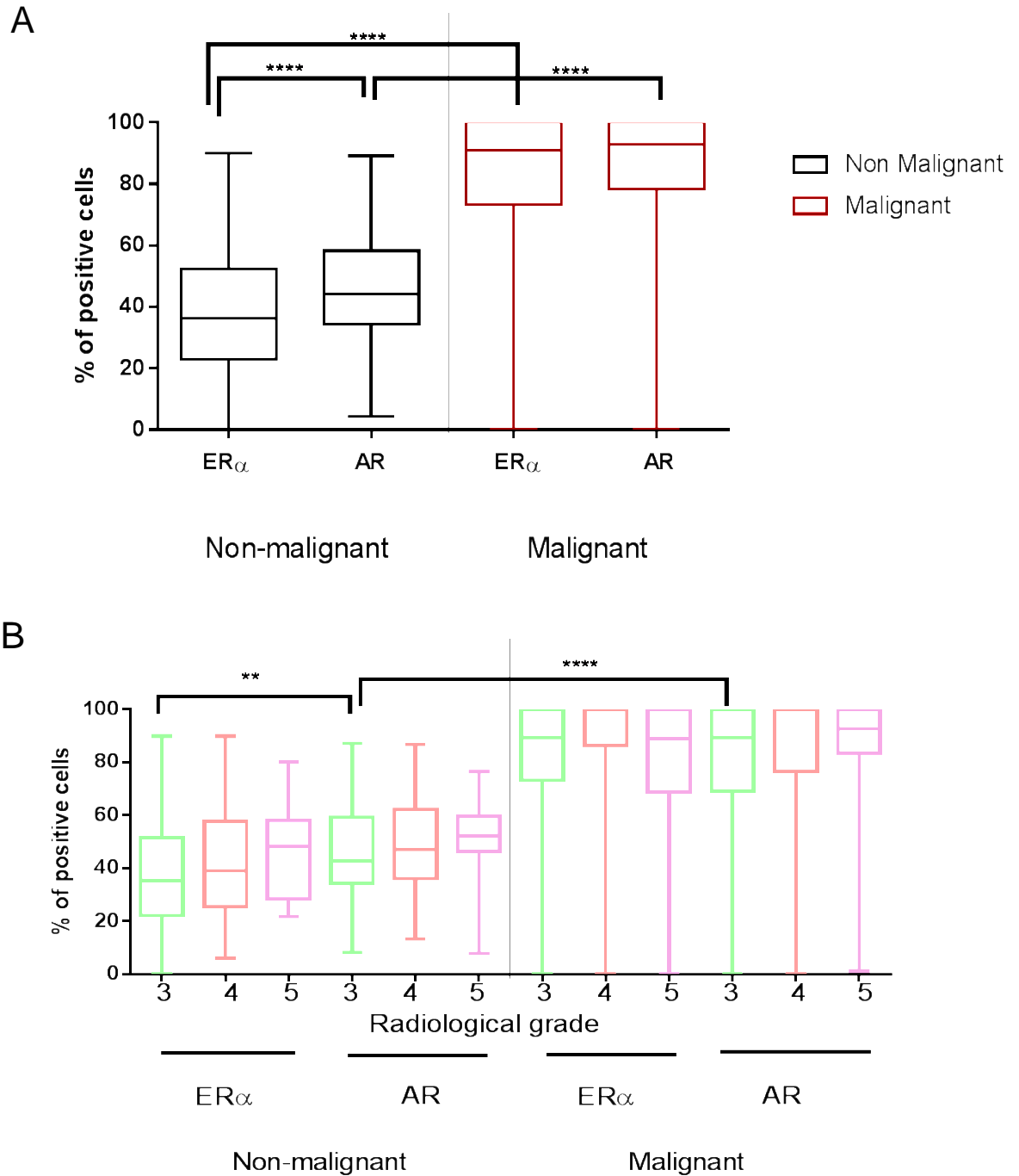


Figure 2.9. AR and ER expression in non-malignant and malignant breast lesions (A) all lesions and (B) according to NBCC scoring system.

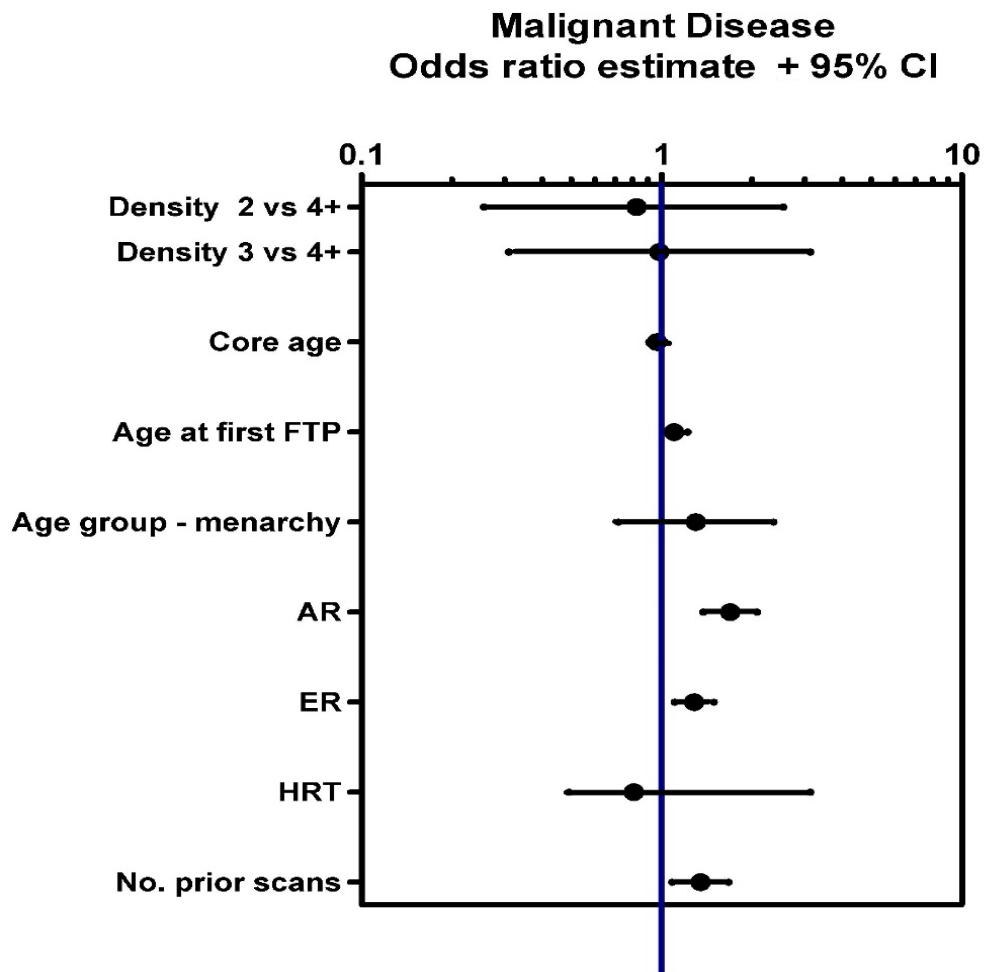


Figure 2.10. Multivariate analysis of the chances of developing malignant disease according to clinicopathological variables. The odds ratio is shown on the x-axis. AR: androgen receptor, ER: estrogen receptor, FTP: full-term pregnancy, HRT: hormone replacement therapy.

After controlling for key covariates only AR, ER, and age at first full-term pregnancy were significant predictors of malignancy status.

2.5 Discussion

Here we present, for the first time, an examination of the relationship between MBD and non-malignant and malignant pathologies of the breast. Since high MBD has consistently been associated with an increased risk of breast cancer [2-5] and is influenced by both lifetime endocrine exposure and endocrine therapy [39, 40], we postulated that MBD might provide useful additional information at the time of mammography to help distinguish non-malignant from malignant disease, since benign lesions not related to cancer are likely to be associated with lower breast density. In addition, since androgens have been shown to have an inhibitory role in breast epithelial proliferation and are antagonistic to ER α signalling [22, 26, 41], we hypothesised that AR expression or the AR:ER α ratio may be inversely related to breast density. We established that neither hypothesis was true, with MBD being the same both at the time of diagnosis and in pre-core mammograms in both non-malignant and malignant disease, and AR, ER α , and AR:ER α ratio were not associated with MBD in either group. Although MBD measurements are playing an increasing role in risk stratification of women undergoing mammography [42], these measurements do not appear to have a role in diagnostic decision-making.

There are few published data that have examined the association between MBD and benign breast disease, even though one would expect areas of increased mammographic density to be associated with the types of benign breast disease that confer an increased risk of cancer. Boyd et al. examined the association of breast density with hyperplasia, with and without atypia, and carcinoma *in situ*, and established that the relative risk (RR) of high density for usual type hyperplasia was 13.85, and 9.23 for hyperplasia with atypia or carcinoma *in situ* in women with high levels of density (>75%) [43]. This finding lends support to MBD conferring its risk via intermediate pathologies that are known to be associated with malignancy. In our study, we did not see any association between breast density and non-malignant or malignant disease, although the study was limited by the fact that the subcategorisation of all the non-malignant pathological diagnoses were not available for analysis, and therefore based on the Boyd et al. data contamination of the 'non-malignant' group with hyperplasias may have been a confounder. Our finding of significantly increased breast density in the breast containing a lesion (compared to the unaffected breast) was expected, since the lesion itself, which is likely to be radio-dense, will have contributed to the overall score, especially when large in size.

An increased lifetime exposure to estrogen is known to be associated with an increased risk of developing breast cancer [39, 40]. Using a hormonal questionnaire, here we confirmed that women with malignant diagnoses were of younger age at menarche (12-13 years vs. 14 years or older; OR 3.43 (1.38-4.26); $p = 0.006$) and slightly older when they had their first full-term pregnancy (24 vs. 23 years; OR 0.96 (0.92-0.99); $p = 0.029$), consistent with the existing literature (44). Although there are some conflicting data, HRT is generally considered to confer an increased risk of developing breast cancer [45]. In our study, women with malignancy were more likely to have used HRT in the preceding five years (OR 1.4 (0.84-2.3); $p < 0.0001$), consistent with results from a large meta-analysis of over 160,000 women that concluded that women who had used HRT in the five years preceding their diagnosis had a 2.5% increased risk of breast cancer compared to those who had stopped for five years [45]. These data provide reassurance that the cohorts studied here are representative of the population at risk.

It is well established that AR is frequently expressed at higher levels in primary breast tumours (between 53 and 85%, depending on the study) than benign breast epithelium [31, 32, 37, 46-48]. We have recently shown in two independent multinational cohorts of breast cancers (see Chapter 3), and in line with several previous studies, that patients with high AR expression have approximately two-fold reduced risk of breast cancer-related death. Intriguingly, patients with 1:1 or higher AR:ER α ratios had the best 10-year overall survival, suggesting that the relative levels of signalling of these two pathways is biologically relevant. Contrary to other reports of an association between MBD and ER α expression of the primary tumour (high MBD associated with high ER α expression) [16-18], we did not find any significant associations between receptor expression and MBD. However, the near-significant association of an AR:ER α ratio less than one being associated with a higher breast density, and vice versa, is consistent with the hypothesis that the relative levels of AR and ER α signalling might contribute to the overall proliferative capacity of the tissue, and that when the anti-proliferative effect of AR signalling is reduced, breast density increases, at least in malignant lesions.

This study was limited by its retrospective design and initial availability of a consecutive series from the BSSA database. Formal power calculations could not be performed due to the opportunistic nature of the availability of samples for IHC analysis (many were not available), the large number of patients who did not consent, and the lack of availability of mammograms in many cases. Therefore, although the starting population of screened patients was very large, the final number of biopsies examined was relatively small and therefore the statistical power of the study is likely to have been compromised.

Overall, it appears that while MBD might be a useful tool to help risk stratify the screening population, quantitative knowledge of the MBD at the time of mammography does not help diagnostic decision-making since it does not help discriminate benign and malignant lesions. Further work is required to firmly establish the association between AR and ER α signalling and the full spectrum of pathologies (benign/non-proliferating, proliferating, premalignant, and malignant lesions) associated with breast density.

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3. Intratumoral heterogeneity of colocalised androgen and estrogen receptor expression persists during breast cancer progression

3.1 Abstract

Background: It is now clear that the estrogen receptor alpha (ER α) and androgen receptor (AR) both play important roles in normal breast development. In cancer, the role of these receptors is altered. Studies have supported a balance between the stimulatory role of ER α compared to the inhibitory actions of AR; however, the extent of expression of these two receptors within the same breast epithelial cell has not been fully investigated.

Methods: A progression tissue microarray was constructed using archival tissue representing 30 non-malignant breast tissues, 90 ductal carcinoma *in situ* (DCIS), 90 invasive ductal carcinomas, and 30 lymph node metastatic deposits. The proportion of AR and ER α co-expressing cells in each AR+ER α + breast tissue was quantified using dual-label immunofluorescence and manual counting of the different AR and ER α expressing epithelial cell populations.

Results: Expression of both AR and ER α was significantly increased in malignant epithelial cells compared to non-malignant breast tissue ($p < 0.01$). The majority of increased receptor expression was due to an increased proportion of AR+ER α + co-expressing cells, with loss of the AR-ER α - cell population and preservation of an AR+ER α - population (33% of total). The relative proportions of receptor expression were relatively constant across all grades of DCIS, invasive cancer, and in metastatic disease.

Conclusions: The increase expression of AR and ER α in cancer compared to non-malignant tissue is due to an increase in the AR+ER α + co-expressing cells and a corresponding decrease in the AR-ER α - population of cells. How these distinct populations of cells change over time and in response to therapy will help establish how best to use hormone-based therapeutics.

3.2 Introduction

While measurement of the archetypal steroid receptors, estrogen receptor-alpha (ER α) and the progesterone receptor (PR), is standard clinical practice for guiding the use of endocrine therapy in women with breast cancer, there is evidence that the androgen receptor (AR), a ligand-dependent transcription factor, also has a role in the development of breast cancer and as a prognostic and predictive biomarker [1, 2]. AR has been shown to be expressed in anywhere from 4% to approximately half of ER α -negative breast cancers [3, 5], and, overall, expression of AR is associated with improved survival [3-8]. Conversely, loss of AR is associated with poor outcomes in patients with lymph node positive, triple negative disease [7, 9, 10]. Since the AR can be therapeutically targeted using both agonists and antagonists, targeting this pathway has potential to be exploited in the treatment of women with breast cancer [11, 12]. However, its exact distribution in malignant epithelium and contribution to the balance of endocrine pathway signalling has yet to be determined.

In contrast to ER α and PR, AR expression is thought to have an antagonistic effect on tumour growth and progression via an inhibitory effect on ER α pathway activity [6, 13-21]. *In vitro* studies in which AR is activated with 5- α -dihydrotestosterone or dehydroepiandrosterone sulphate (DHEAS) causes inhibition of cell proliferation in AR-positive cell lines, supporting the hypothesis that AR is growth inhibitory in breast cancer [6, 13-21], and ligand-activated AR induces cell motility via downregulation of E-cadherin [22], suggesting a possible role in invasion and metastasis. It could therefore be hypothesised that a reduction in the expression of AR may allow ER α to act unopposed and contribute to the progression of indolent in situ or invasive cancer to a more aggressive phenotype.

While there is limited evidence to suggest that AR and ER α co-localise in both normal and malignant breast epithelial cells in clinical samples [6], there has yet to be a comprehensive analysis of the co-expression of AR and ER α in individual cells during tumour progression; this parameter is important to establish, since the pathways may interact to produce the invasive phenotype and influence the clinical course of the disease. Therefore, in this study we used dual-label immunofluorescence to assess expression of AR and ER α in non-malignant breast epithelium and in pre-invasive, invasive, and metastatic breast cancer in order to assess the changes in distribution of the different co-expressing populations of epithelial cells with tumour progression.

3.3 Materials and methods

3.3.1 Tumour samples and tissue microarray construction

A tissue microarray (TMA) was constructed to represent breast cancer progression. This was an exploratory study of receptor co-localisation; therefore, thirty each of non-malignant, low, medium, and high grade ductal carcinoma *in situ* (DCIS), grade I, II, and III invasive ductal carcinoma (IDC), and metastatic tumour in lymph nodes (LN metastasis), were selected from the South Australian Health Database (**Table 3.1**) and no formal power calculations were performed. The non-malignant areas were selected from areas distal to the tumour within the same specimen. A qualified pathologist reviewed the slides, and diseased areas were selected and marked on the slides. Formalin-fixed, paraffin-embedded (FFPE) tissue blocks were retrieved from the archives. The Royal Adelaide Hospital research ethics committee approved the study protocol.

The progression TMA was constructed using the Chemicon Advanced Tissue Arrayer (ATA100; Merck Millipore, Germany) according to the manufacturer's instructions. In addition, a 'validation' TMA was constructed, consisting of 10 invasive carcinomas and 10 non-malignant tissues for optimisation of antibody staining. In order to ensure adequate representation of the tissue, a core size of 1mm was selected and cores were arrayed in duplicate with liver and spleen as orientation cores.

Table 3.1. Number of cases available for analysis.

		Cored	Available	AR+ERα+
Non-		30	24	24
malignant				
DCIS	Low grade	30	22	19
	Intermediate grade	30	24	23
	High grade	30	27	21
Invasive	Grade I	30	24	22
	Grade II	30	26	22
	Grade III	30	30	23
Metastatic		30	29	23

3.3.2 Immunofluorescence

Three μm TMA sections were cut for immunofluorescence staining. The sections were deparaffinised and antigen retrieved in a decloaking chamber. Sections were washed in deionized water and PBS followed by incubation with 0.5% pontamine sky blue (20 min), washed in deionised water and PBS before blocking in 1% BSA/PBS-0.3% Tween 20 at room temperature for one hour. Slides were then incubated overnight with optimised primary antibody concentrations. The validation TMA was used to identify the optimised antibody concentration that ensured the correct nuclear sub-cellular localisation and optimal signal to noise ratio. The primary antibody concentrations used were 1:50 for ER α (1D5, DAKO, Denmark) and 1:50 for AR (N-20, Santa Cruz Biotechnology, Santa Cruz, CA) and the secondary antibody concentrations used were 1:400 for Alexa Fluor 488 and Alexa Fluor 594. The slides were mounted using ProLong Gold Antifade with DAPI mounting medium (Molecular Probes; **Figure 3.1**).

3.3.3 Tissue imaging and quantitative analysis of ER α and AR expression

The images from AR+ER+ breast tissue samples were captured from each channel using the Hamamatsu Nanozoomer slide scanner. Quantitative analysis of ER α and AR expression was performed manually using the captured images. A minimum of 500 epithelial cells were counted in each core and the percentage positivity calculated; cases with >1% positivity were considered positive. This allowed classification into one of four categories: AR+ER α +, AR-ER α -, AR+ER α -, and AR-ER α + cells, and a proportion of total calculated for each category. In addition, the percentage of ER α or AR positive cells was calculated.

3.3.4 Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 6.0, GraphPad Software, CA). Differences between groups were examined using the Kruskal-Wallis test for non-parametric variables with application of Dunn's multiple comparison tests.

3.4 Results

3.4.1 AR and ER α expression in non-malignant and malignant tissues using dual-label immunofluorescence

Classical immunohistochemical techniques using colorimetric reagents are not suited to visualising multiple targets in the same tissue section. We therefore used double-label immunofluorescence in order to establish whether AR and ER α are expressed alone or together

in individual non-malignant or malignant epithelial cells. Representative images of non-malignant, DCIS, invasive, and metastatic disease are shown in **Figure 3.1**. AR was visualised in red, ER α in green, and the merged images highlighting co-expressing nuclei in yellow.

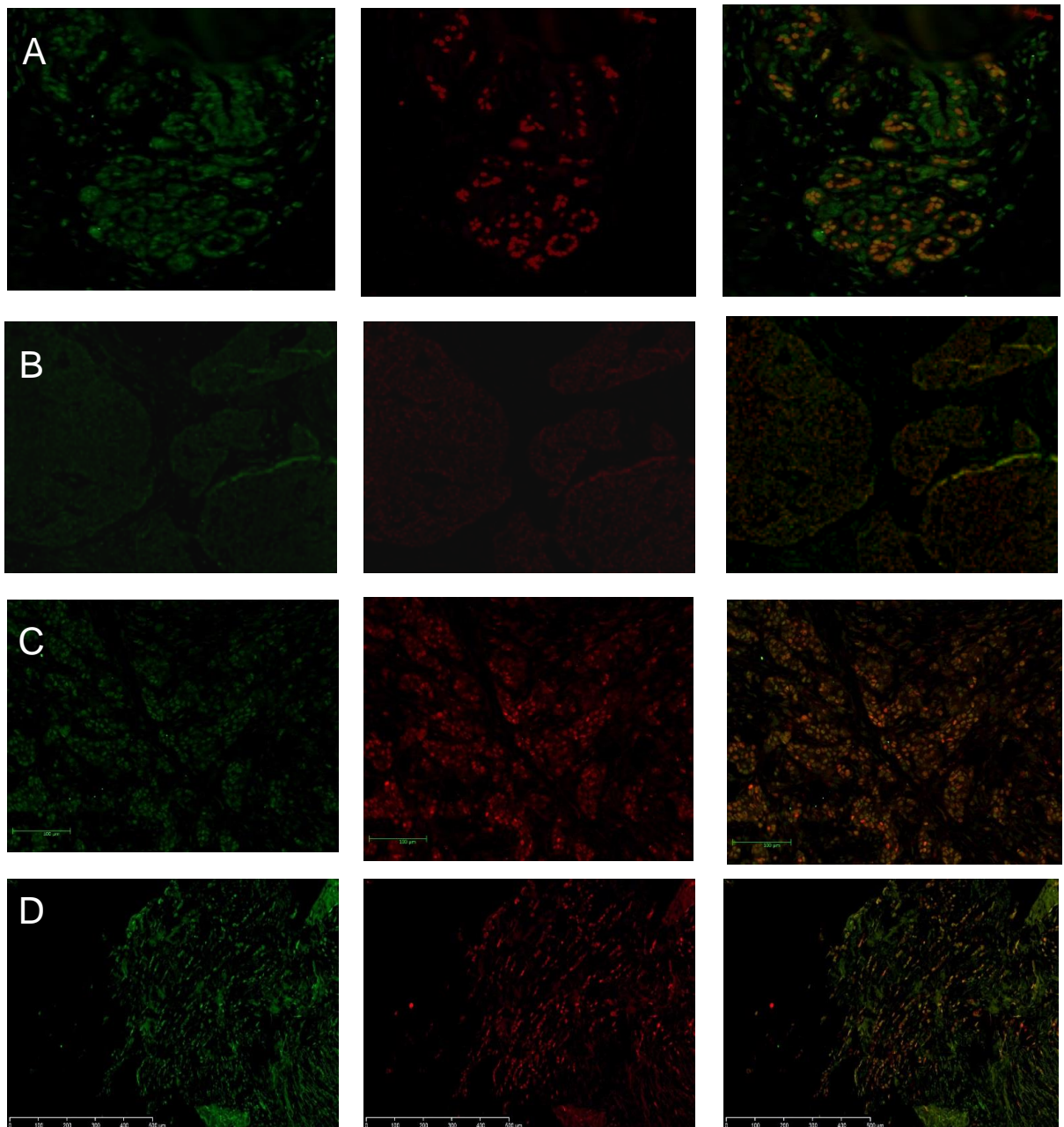


Figure 3.1. Double-label immunofluorescence staining of breast tissue.

Non-malignant (A), *in situ* (B), invasive (C), and nodal metastatic (D) breast tissue. AR (green) and ER α (red) expression were localised to the nuclei of epithelial cells. Images were acquired using the slide scanner with a x10 (A) or x20 objective (B-D).

3.4.2 AR and ER α are upregulated in malignant compared to non-malignant breast epithelial cells

In order to establish whether AR and ER α show different levels of expression in non-malignant and malignant disease, the proportion of AR⁺ and ER α ⁺ cells in non-malignant epithelium, DCIS, invasive breast cancer, and metastatic disease were first compared (**Figure 3.2**). Tumours with no expression of either AR or ER α were excluded from the analysis since the study's aim was to assess co-localisation of AR and ER α ; the number of lesions available for analysis are shown in the figure legend. AR and ER α expression in non-malignant epithelial cells was 59.8% and 29.3% per cent, respectively. All malignant lesions, including lymph node metastases, contained an increased proportion of AR⁺ and ER α ⁺ cells, ranging from 81.1% in grade III IDC to 96.9% in high grade DCIS ($p < 0.01$) for AR, and from 56.6% in grade III IDC to 84.4% in grade I IDC for ER ($p < 0.01$). Although there was a trend to decreasing expression of ER α with increasing nuclear grade (84.4%, 75.8%, and 56.6% in grade I, II, and III carcinomas, respectively), these differences were not statistically significant.

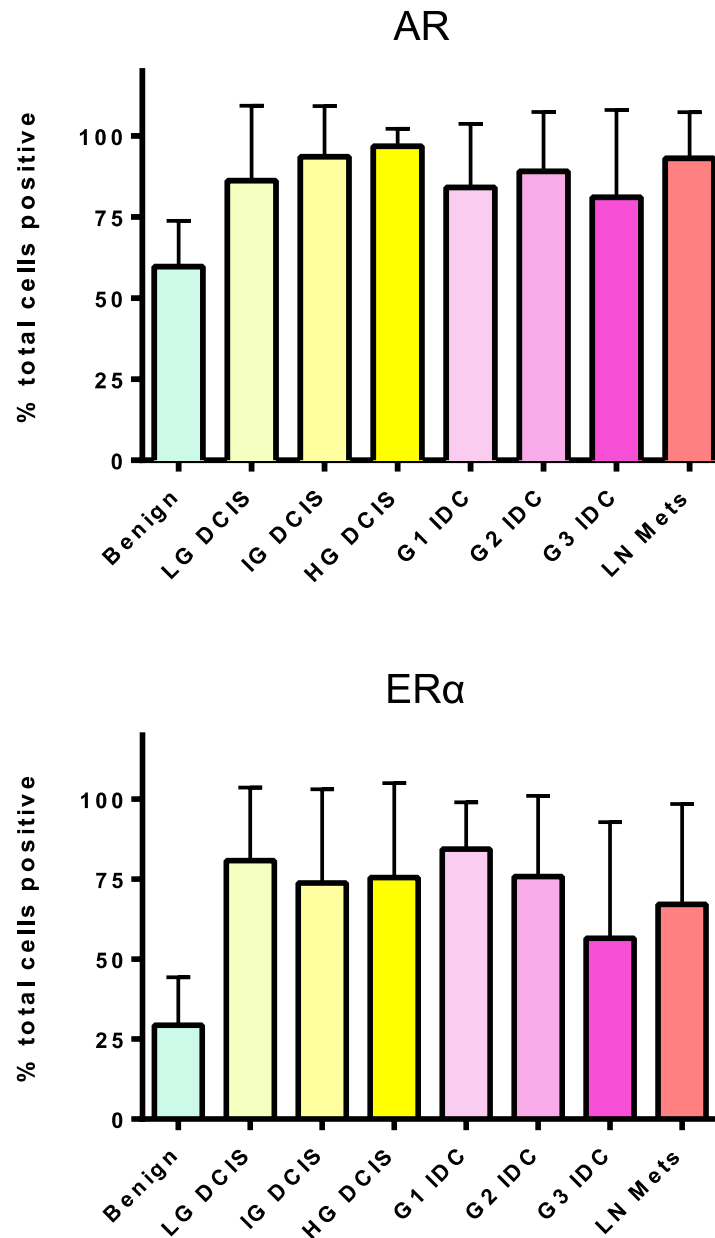


Figure 3.2. Proportion of cases showing either AR or ER α positivity during tumour progression.

Both AR and ER α are upregulated in pre-invasive, invasive, and nodal metastatic disease compared to tumour-associated normal epithelium (all $p < 0.01$). NM = non-malignant ($n=24$), LG DCIS = low grade ductal carcinoma *in situ* ($n=19$), IG DCIS = intermediate grade ductal carcinoma *in situ* ($n=23$), HG DCIS = high grade ductal carcinoma *in situ* ($n=21$), G1 IDC = grade 1 invasive ductal carcinoma ($n=22$), G2 IDC = grade 2 invasive ductal carcinoma ($n=22$), G3 IDC = grade 3 invasive ductal carcinoma ($n=23$), LN Mets = lymph node metastasis ($n=23$).

3.4.3 The proportions of the different AR and ER α co-expressing population changes from non-malignant to malignant breast tissue

We next examined how the four different AR/ER α populations changed during tumour progression (**Table 3.2, Figure 3.3**). Contributing to the overall increase in AR and ER α in non-malignant to malignant breast tissue, AR+ER α + co-expressing cells increased from 26.6% in non-malignant lesions to 71.0% in DCIS, 62.3% in IDC, and 64.7% in metastatic disease ($p < 0.001$). The proportion of AR-ER α - cells decreased from 37.5% in non-malignant breast tissue to 2.1%, 5.5%, and 4.4% in DCIS, IDC, and metastatic disease, respectively ($p < 0.0001$). Likewise, the proportion of AR+ER α - cells decreased in malignant lesions, although to a lesser extent than AR-ER α - cells (33.2% in non-malignant lesions to 21.4, 22.4, and 28.4%, respectively in DCIS, IDC, and lymph node metastases; $p < 0.05$). Although there were slight increases in the proportion of AR-ER α + cells in DCIS (5.4%) and IDC (9.7%) compared to non-malignant lesions (2.8%), these differences were not significant; however, there was a small but statistically significant decrease in AR-ER α + cells in metastatic disease compared to non-malignant epithelium (from 2.8% to 2.5%; $p < 0.05$). Although there was a trend towards a decrease in AR+ER α + and AR-ER α + cells and an increase in AR+ER α - cells with increasing nuclear grade, the differences were not statistically significant (**Figure 3.4**); in fact, the proportions of co-localised populations of cells remained remarkably constant throughout tumour progression from *in situ* to lymph node metastasis.

Table 3.2. Proportions of co-localising double-positive AR and ER α expressing cells in tumour-associated normal epithelium and breast cancer during tumour progression.

	AR+ER α +	AR-ER α -	AR+ER α -	AR-ER α +
NM	26.6 (20.0-33.2)	37.5 (31.5-43.5)	33.2 (27.4-39.0)	2.7 (1.8-3.7)
LG DCIS	68.2 (53.3-83.1)	1.2 (0-2.3)	18.0 (7.8-28.2)	12.6 (1.6-23.6)
IG DCIS	71.4 (58.0-84.7)	3.8 (-2.4-10.2)	22.3 (10.9-33.8)	2.5 (0.4-4.5)
HG DCIS	73.4 (59.6-87.2)	1.0 (0.1-1.8)	23.5 (10.0-37.0)	2.1 (0.4-4.0)
G1 IDC	70.6 (59.7-81.6)	2.0 (0.9-3.2)	13.4 (6.9-20.2)	13.8 (5.7-21.8)
G2 IDC	66.2 (54.7-77.7)	1.2 (0.1-2.4)	23.0 (11.9-33.9)	9.6 (1.4-17.9)
G3 IDC	50.7 (35.7-65.7)	13.0 (2.8-23.2)	30.4 (15.3-44.6)	5.9 (0.7-11.0)
LN Mets	64.7 (50.9-78.6)	4.4 (-1.3-10.1)	28.4 (17.2-39.7)	2.5 (-0.3-5.3)

Data are expressed as mean (95% CIs). NM = non-malignant, LGDCIS = low grade ductal carcinoma in situ, IGDCIS = intermediate grade ductal carcinoma in situ, HGDCIS = high grade ductal carcinoma in situ, IDC = invasive ductal carcinoma, LN Mets = lymph node metastasis.

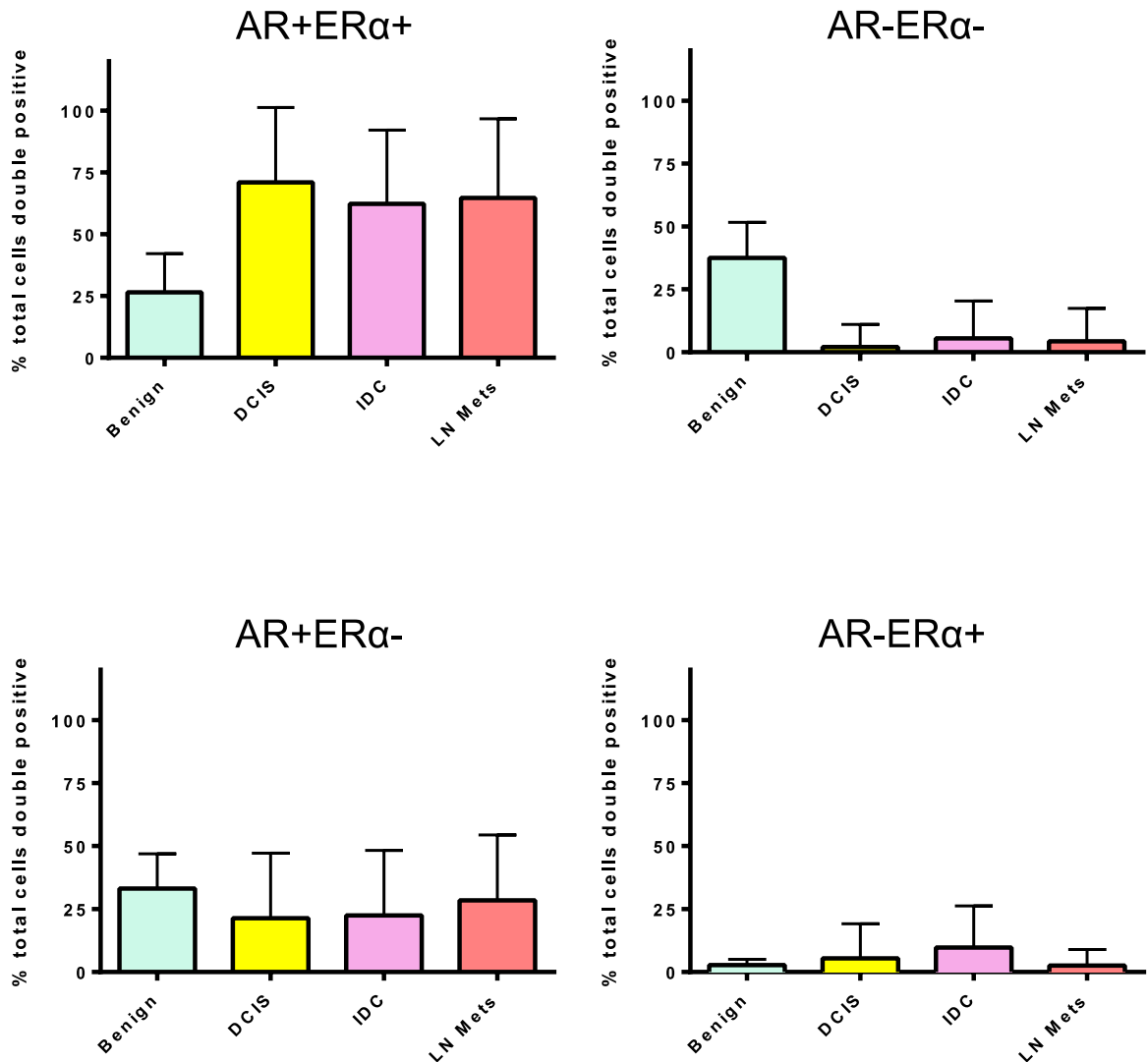


Figure 3.3. Co-localisation of AR and ERα during disease progression.

The proportion of AR+ERα+ epithelial cells is greater in malignant cells ($p < 0.001$) than non-malignant cells, while the proportion of AR-ERα- ($p < 0.0001$) and AR+ERα- ($p < 0.05$) cells is lower. The proportion of AR-ERα+ epithelial cells is significantly less in metastatic adenocarcinoma ($p < 0.05$). NM = non-malignant ($n=24$), DCIS = ductal carcinoma *in situ* ($n=63$), IDC = invasive ductal carcinoma NOS ($n=67$), LN Mets = lymph node metastasis ($n=23$). Data shown are mean +/- standard deviation.

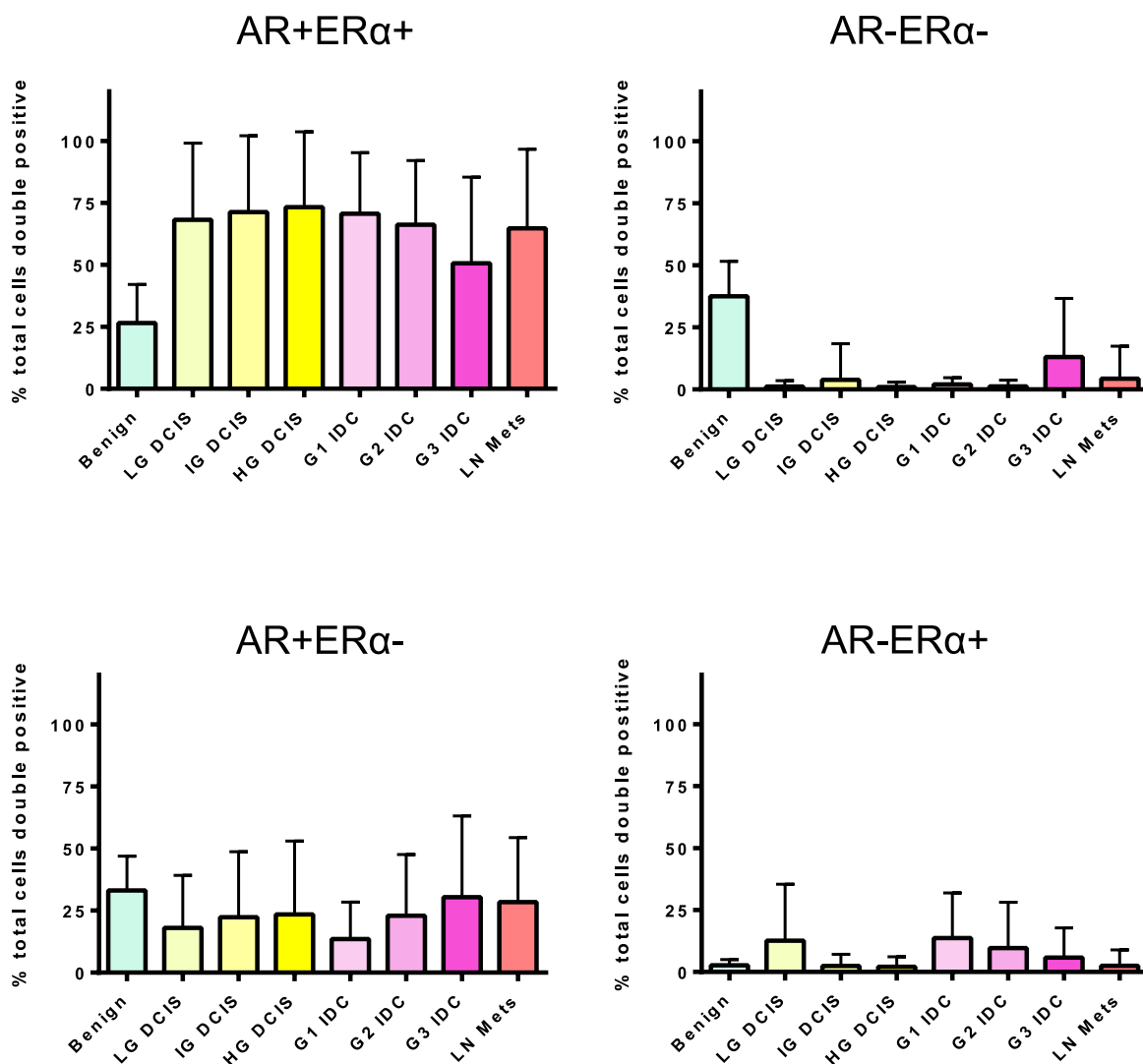


Figure 3.4. Co-localisation of AR and ER α during disease progression.

Although there is a trend towards a decrease in AR+ER α + and AR-ER α + cells and an increase in AR+ER α - cells with increasing nuclear grade, the differences are not statistically significant. NM = non-malignant (n=24), LG DCIS = low grade ductal carcinoma *in situ* (n=19), IG DCIS = intermediate grade ductal carcinoma *in situ* (n=23), HG DCIS = high grade ductal carcinoma *in situ* (n=21), G1 IDC = grade 1 invasive ductal carcinoma (n=22), G2 IDC = grade 2 invasive ductal carcinoma (n=22), G3 IDC = grade 3 invasive ductal carcinoma (n=23), LN Mets = lymph node metastasis (n=23). Data shown are mean +/- standard deviation.

3.5 Discussion

Here we present, for the first time, co-localisation of AR and ER α expression in individual epithelial cells during breast cancer progression using quantitative dual-labelling immunofluorescence. We establish that the overall changes in receptor expression in AR and ER α positive tumours can mainly be attributed to an increase in the proportion of AR+ER α + co-expressing cells, a decrease in the AR-ER α - population of cells, and maintenance (albeit to a slightly lesser degree) of the population of AR+ER α - cells. Since the balance between signalling of these pathways is a key determinant of normal and malignant cell growth, establishing which populations of cells exist in individual tumours is essential to understanding how tumours evolve in response to anti-estrogens, aromatase inhibitors, and other hormone-based therapies.

Therapeutic targeting of the AR is of renewed interest due to a flurry of recent clinical and molecular studies and the availability of androgen-based therapies [1]. Androgen treatment was historically used to treat breast cancer prior to the widespread use of tamoxifen and aromatase inhibitors in women with breast cancer [23-26]. The efficacy of targeting the AR is likely to depend on how the AR and ER α signalling pathways interact and influence cell fate in malignant cells. In normal adult breast, both AR and ER α are expressed in luminal epithelial cells, and AR is also expressed in stromal fibroblasts and adipocytes [1]; AR is consistently expressed in a higher proportion of normal human adult epithelial cells than ER α , as shown here. It therefore follows that there must be a significant proportion of AR+ER α - cells in the luminal cells of breast ducts. Here we quantified the proportions of AR and ER α co-expressing cells in non-malignant and malignant epithelium for the first time and show that, in fact, one third (33.3%) of non-malignant breast epithelial cells are AR+ER α -, with 26.6% AR+ER α + cells, 37.5% AR-ER α - cells, with a minor proportion (2.8%) of AR-ER α + cells. In malignant epithelium (DCIS, IDC, or nodal metastatic), the proportions are different, with between 21 and 28% AR+ER α - cells, 62-71% AR+ER α + cells, 2-5.5% AR-ER α - cells, and 2.5-10% AR-ER α + cells. Overall, non-malignant epithelium contains three main populations of cells (AR+ER α -, AR+ER α +, and AR-ER α -) while malignant epithelium contains two (AR+ER α - and AR+ER α +). Whether non-malignant or malignant, it is clear that AR and ER α can either act together or independently within heterogeneous epithelial populations. This raises the possibility that within the same cell, when AR and ER are co-expressed (i.e. the cell is AR+ER+), the two pathways may interact to prevent uncontrolled proliferation and that other populations of cells (such as AR+ER-) may be more likely to be resistant to treatment or, under

the right conditions, escape homeostatic control and develop into more aggressive recurrent or metastatic disease.

The question therefore arises of how these subpopulations of cells within the same tumour behave both under normal growth conditions and in the face of endocrine therapy. Studies in cell lines suggest that, depending on the presence or absence of ER α , AR can either act as a tumour suppressor or oncogenic protein (reviewed in [1]). In well-established models of luminal breast cancer in which AR is also expressed at varying degrees (MCF-7, T47D, and ZR-75-1)[14, 27, 28], the growth inhibitory effect of AR appears to be influenced by the relative levels of AR and ER α , with induction of cell cycle arrest [14, 16, 29-31] and even apoptosis [32] in ZR-75-1 and T47D cells, and more variable effects in MCF-7 cells depending on whether the AR is endogenously or exogenously expressed [13, 20]. On the other hand, in MDA-MB453 cells, which are AR+ER-, androgens stimulate proliferation [1]. There are a number of postulated mechanisms by which AR can inhibit ER α , including ER α depletion [33], inhibition of ER α -mediated transcriptional activity [34], or by competition for co-regulatory molecules [35]. We have previously demonstrated using an inducible AR model that ER α undergoes dose-dependent inhibition and that AR can bind to consensus EREs, leading to the conclusion that AR can compete with ER α for ERE binding [6]. With respect to a potential oncogenic role, AR is most likely to act in this capacity in ER α -negative cells, particularly those that fall into the 'molecular apocrine' subgroup of primary breast carcinomas [14, 36], perhaps by acting as an ER α mimetic [37]. The presence of both populations of these cells in most primary tumours raises the possibility that there is competition between the agonistic and antagonistic effects of AR in the same tumour and that this might influence tumour growth, particularly when the endocrine milieu is altered by endocrine therapy.

Although there are some data to suggest that AR expression varies between primary tumours and metastatic lesions, with a decrease in expression in the latter [38-41], we found no convincing evidence that either total AR expression, or co-localised AR expression, were altered during disease progression; in fact, receptor expression was remarkably consistent in *in situ*, invasive, and nodal metastatic disease. This is consistent with a study by Cimino-Mathews et al. [42], who showed that AR levels were maintained in matched primary and metastatic disease from the primary resection, but not those metastatic lesions that ultimately resulted in the death of the patient. However, we did not have tissue available from distant metastatic disease, and therefore the possibility of receptor expression changes at distant sites remains a possibility.

Unfortunately, in the current study, outcome data were not available to investigate the association between AR/ER α expression and clinical outcomes such as disease-free or overall survival or responses to endocrine therapy. The study was designed to be exploratory for patterns of receptor colocalisation and no formal power calculations were performed, thereby weakening the study. Furthermore, due to incomplete data (such as complete histopathological subtyping and HER2 receptor expression status), we were unable to examine AR/ER α expression in relation to these parameters. A further limitation was that co-expression was measured in a progression TMA in which tissue cores were taken from tissue distal to the tumour, which may therefore be regarded as “tumour-associated normal tissue” rather than truly normal tissue. This is important since tissue adjacent to tumours is known to harbour genetic and epigenetic changes [43]. Tissue taken from patients who were histopathologically confirmed to be disease-free or tissue from reduction mammoplasties would have been preferable. Nevertheless, AR has been reported as an independent prognostic factor in women with ER α + breast cancer in several studies [4, 6, 44-46], with higher levels of AR associated with improved disease-free survival, including in the neoadjuvant setting [46]. It should be remembered, however, that in these retrospective studies in which biomarker measurements performed on resection specimens are associated with long-term clinical outcomes, ER α + patients are likely to have been treated with endocrine therapy, and therefore these biomarkers are more likely to be more predictive of endocrine response than truly prognostic. In light of the fact that AR levels remain constant in *in situ*, invasive, and metastatic disease, the predictive power of AR as a biomarker may be more due to the differential effects of endocrine therapy on subpopulations of AR and ER α expressing cells and how this ultimately alters the balance between tumour suppressive and oncogenic subpopulations over time, rather than basal levels of receptor *per se*. Further studies, perhaps by measuring receptor expression in matched tumours pre- and post- neoadjuvant endocrine or chemotherapy, may shed light on this paradox.

In conclusion, here we demonstrate that breast tumours contain a complex mixture of AR and ER α co-expressing cells which do not markedly alter during tumour progression from *in situ* to lymph node metastasis. Effectively targeting this mixture of cells is likely to require a multi-agent approach. Understanding the dynamic changes that occur in these competing populations of cells over time, and in response to mixtures of endocrine therapy, will help establish why women respond or fail endocrine therapy and who may benefit from androgen-based therapeutics.

3.6 References

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4. The androgen receptor is an independent predictor of survival in two multinational breast cancer cohorts

4.1 Abstract

Background: There is considerable interest in the androgen receptor (AR) as a therapeutic target and a prognostic factor in breast cancer. The lack of consensus for the assessment of AR status in breast tumours has had an impact on its clinical usefulness as a prognostic factor.

Methods: AR positivity was assessed in two multinational clinically validated breast cancer cohorts (test cohort n=219; validation cohort n=418) using specific antibodies that recognise distinctly different epitopes. Receiver Operating Characteristic (ROC) analysis was used to determine the optimal cut-point for AR to be an independent predictor of overall survival (OS) with a high degree of specificity and sensitivity. OS was assessed by univariate and multivariate analyses.

Results: A cut-point of 78% nuclear positivity resulted in the best sensitivity and specificity for AR to independently predict OS in the test cohort (HR 0.41, p=0.015). This was confirmed in the validation cohort (HR 0.50, p=0.014). AR was not independent when cut-points of either 1% or 10%, analogous to those used for ER α , were applied. Patients with ER α positive tumours ($\geq 1\%$) with high AR nuclear positivity ($\geq 78\%$) had the best outcome in both cohorts (p<0.0001). Conversely, a reduced AR:ER α ratio (<0.82) or positivity for AR but not ER α resulted in a reduced OS (p<0.0001).

Conclusions: This study provides a significant advance in understanding the context for AR to be a robust independent prognostic factor for OS, thereby facilitating evaluation of AR status as an important clinical tool for the management of breast cancer.

4.2 Introduction

ER α and PR status in breast cancer informs clinical decisions regarding adjuvant hormone therapy. Most ER α positive tumours respond to anti-estrogenic treatments, including selective estrogen receptor modulators (SERMs) such as tamoxifen, or aromatase inhibitors including anastrozole, letrozole or exemestane. Use of these anti-estrogenic agents has increased breast cancer survival in women with ER α -positive disease, but treatment options following resistance to ER α -targeted therapies are limited. Recently, the role of androgens and their cognate receptor has become a major topic of interest in breast cancer [1]. Depending on the study and the cut-point used for positivity, AR expression in primary breast tumours ranges from 53-85% [2-7]. In these same studies, the frequency for ER α positivity is 40-85%, and approximately 20% of breast cancers are AR positive and ER α negative [2-7]. The current evidence is consistent with AR exerting a growth inhibitory effect in ER α positive normal and malignant breast tissues [8-10]. In support of this, non-aromatisable androgens such as flouxymesterone have demonstrated an efficacy comparable to that of tamoxifen in advanced disease [11, 12]. Currently, there is renewed interest in the clinical utility of the AR, with several trials of AR targeting agents underway (clinicaltrials.gov). However, the absence of clear guidelines for the assessment of AR status may be a major limitation in the selection of patients who may be responsive to these agents.

Higher AR levels in breast tumours are associated with increased overall survival but, as summarized in **Table 4.1**, this is not a consistent finding. The role and prognostic capacity of AR in ER α negative disease is even less clear [1]. Depending on the ER α status of the tumour, AR signalling may have dichotomous roles in breast carcinogenesis (reviewed in [1]). Since the role of AR may differ depending on hormone receptor status and tumour subtype, it is likely that AR may be either a prognostic marker or therapeutic target depending on disease context.

The lack of concordance of previous studies assessing the prognostic value of AR in breast cancer can be attributed to differences in the nature and size of the cohort, the AR antibody used, and the method of assessing AR levels. Another potentially important factor is the AR positivity cut-point used to dichotomise data sets. Until recently, the accepted clinical criterion for ER α positive status in breast cancer was the presence of ER α or PR in at least 10% of the tumour cells. In 2010, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) recommended changing the clinical guidelines for

ER α and PR positivity to 1% immunoreactive malignant cells, in part because of data supporting a therapeutic benefit of anti-estrogen therapy in women with such tumours [13]. Unlike ER α , there is no standardised cut-point for AR positivity in breast cancer tissues. In the current study, we demonstrate that AR is an independent predictor of survival in two clinically validated multinational breast cancer cohorts [14, 15]. Additionally we show that the AR to ER α ratio is an important determinant of overall survival in ER α positive breast cancer.

Table 4.1. AR is a prognostic factor in breast cancer.

Cohorts not selected on ER status								
Ref	Year	N	Sample	Ab	Cut point	Univariate	Multivariate	HR (95% CIs)
[16]	1979	292	RLB	-	≥10 fmol/mg	NS	ND	
[17]	1984	1181	RLB	-	≥5 fmol/mg	OS (P<0.001)	ND	
[18]	1986	796	RLB	-	≥5 fmol/mg	OS (P<0.05)	ND	
[19]	1990	61	RLB	-	≥10 fmol/mg	OS at 36 months (P=0.043)	ND	
[20]	1992	224	RLB	-	≥50.5 fmol/mg	Yes	MFS (P=0.001)	NA
[21]	1996	269	RLB	-	>43 fmol/mg	NS	ND	
[22]	1996	153	Frozen - WS	ARF39.3	≥10%	DFS (P=0.043)	NS	
[23]	2006	232 ^a	TMA	AR441	>10%	DFS (P=0.028)	NS	
[24]	2007	115	FFPE - WS	AR441	>10%	OS (P=0.03)	NS	
[25]	2007	1087	TMA	AR441	Allred score >3	NS	NS	
[26]	2008	488	FFPE - WS	AR441	NS	RFS (P=0.023)	NS	
[27]	2008	111	TMA	AR441	>0	OS (P = 0.01)	OS (P = 0.03)	0.46 (0.23–0.93)
[28]	2008	138	FFPE - WS	AR441	≥15%	OS (P=0.01)	NS	
[28]	2008	138	RLB	-	≥30 fmol/mg	RFS (P=0.007) OS (P=0.007)	RFS (P<0.0001) OS (P=0.003)	0.36 (0.22–0.59) 0.4 (0.21–0.71)
[29]	2009	347	RPA	NR	≥-0.085 (median)	RFS (P=0.002) OS (P=0.004)	RFS (P=0.002) OS (P=0.013)	0.53 (0.36–0.80) 0.57 (0.36–0.89)
[30]	2011	626	TMA	ARF39.4.1	Remmele score ≥3	RFS (P=0.033) OS (P=0.023)	NS	
[31]	2011	335	FFPE - WS	AR441	Allred score NR	OS (P<0.001)	OS (P<0.001)	0.31 (0.19–0.50)
[4]	2011	1467	TMA	AR441	≥1%	Yes	NS	
[32]	2012	73 ^b	TMA	AR441	≥1%	OS (P=0.004)	ND	
[3]	2012	403	FFPE - WS	AR27	≥10%	DFS (P = 0.017) OS (P = 0.034)	NS	

[33]	2013	250	NR	AR441	≥75%	DFS (P=0.0005)	DFS (P=0.005)	0.46 (0.26-0.79)
[34]	2013	109	TMA	AR441	>1%	DFS (P=0.026) OS (P=0.022)	DFS (P=0.031) OS (P=0.031)	0.24 (0.07-0.88) 0.19 (0.04-0.86)
[35]	2014	1039	TMA	AR441	≥1%	OS (P=0.002)	ND	
ER Negative Breast Cancers								
[36]	2003	69 ^c ER-	FFPE - WS	ARF39.4.1	>5%	DFS (P=0.049)	NS	
[37]	2010	226 ^d ER-PR-	FFPE - WS	AR27	≥10%	NS	NS	
[38]	2007	282 ^e TNBC	TMA	ARF39.4.1	Modified H-score ≥1%	OS (P=0.04)	NS	
[39]	2010	137 ^e TNBC	FFPE - WS	NR	Score ≥2	OS at 5 years (P=0.018)	OS at 5 years (P=0.047)	NR
[40]	2011	287 ^f TNBC	TMA	AR441	≥5%	DFS (P=0.008)	DFS (P=0.032)	0.47 (0.23-0.94)
[41]	2011	127 ^f TNBC	TMA	AR441	≥10%	OS (P=0.038)	OS (P=0.048)	NR
[42]	2012	83 ^g TNBC	FFPE - WS	AR441	>1%	NS	NS	
[43]	2013	203 ^f TNBC	FFPE - WS	AR441	H-score ≥10%	NS	ND	
[44]	2014	699 ^g TNBC	TMA	AR27	≥1%	DFS (P=0.05)	ND	
[45]	2014	173 ^f TNBC	TMA	ARF39.4.1	≥5%	OS (P=0.032)	NS	
[46]	2014	119 ^f TNBC	TMA	AR441	≥10%	NS	ND	
ER Positive Breast Cancers								
[9]	2009	157 ^d	TMA	AR-407	≥75%	RFS (P=0.011) OS (P=0.003)	RFS (P=0.003) OS (P=0.002)	0.33 (0.20-0.85) 0.22 (0.08-0.58)
[47]	2010	859 ^g	TMA	AR441	≥1%	RFS (P=0.001) OS (P<0.001)	RFS (P<0.0001) OS (P<0.0001)	0.46 (0.30-0.71) 0.26 (0.14-0.48)
[7]	2011	672 ^d	TMA	AR441	≥10%	DFS (P=0.005) OS (P=0.032)	DFS (P=0.049) NS	0.65 (0.43-1.00)
[48]	2013	543 ^g	TMA	AR441	≥1%	OS (P<0.001)	OS (P=0.003)	0.26 (0.11-0.62)
[49]	2014	798 ^g	TMA	AR441	>1%	DFS (P=0.025)	NS	
[50]	2014	192 ^h	FFPE - WS	AR441	>0%	NS	ND	

Abbreviations: FFPE, formalin fixed paraffin embedded; AR+, AR positive; AR-, AR negative; IHC, immunohistochemistry; RFS, breast cancer specific recurrence; DFS, breast cancer

specific relapse or breast cancer death; OS, overall survival or breast-cancer specific survival; MFS, metastasis free survival; NA, not available; ND, not determined; NS, not significant; Ref, reference; RPA, reverse phase protein microarray; NR, not reported; TMA, tissue microarray; WS, whole section; RLB, radio ligand binding assay; ^atumour samples from patients who developed metastatic disease; ^bgrade III cancers; ^cER cut-point >5%; ^dER cut-point \geq 10%; ^eER cut-point >0%; ^fER cut-point not reported; ^gER cut-point \geq 1%; ^hER cut-point \geq 10 pmol/mg protein; TNBC, triple negative breast cancer (ER-PR-HER2-); studies in **bold** were significant by multivariate analysis.

4.3 Materials and Methods

4.3.1 Patient Cohorts

Two independent breast cancer cohorts were studied; both represented on tissue microarrays (TMA) consisting of replicate sample cores. For detailed pathological and clinical characteristics see **Table 4.2**.

Test cohort: 219 patients with invasive ductal breast carcinoma diagnosed between 1992 and 2002 from St Vincent's Hospital, Sydney, Australia [15]. Prior approval for this TMA construction was obtained from the Human Research Ethics Committee of St Vincent's Hospital, Sydney.

Validation cohort: 418 patients with invasive breast cancer diagnosed at Vancouver General Hospital, between 1974 and 1995 [14]. TMAs were constructed at the Genetic Pathology Evaluation Centre, University of British Columbia, Vancouver, Canada with ethical approval from the institutional ethical review board.

4.3.2 Immunodetection

Tissue sections underwent microwave antigen retrieval (5 min 750W, 15 min 350W) in 10 mM citrate buffer (pH 6.5) and were incubated overnight with a 1:300 dilution of U407 or a 1:1000 dilution of AR-N20 in blocking buffer (5% normal goat serum in PBS) at 4°C in a humidified chamber. Visualisation of immunoreactivity was achieved using biotinylated anti-rabbit immunoglobulins (Dako), streptavidin–peroxidase conjugate (Dako) and diaminobenzidine tetrahydrochloride (DAB) to yield an insoluble brown deposit as described previously [9]. A whole tissue section from a breast cancer paraffin block known to be immunoreactive for AR was utilised as a positive control in both instances, and the primary antibody was omitted for the negative control. Positive immunostaining for AR was predominantly seen in the nucleus of tumour cells with use of either antibody. The percentage of AR positive tumour nuclei in each TMA core was assessed within 100-200 tumour cells by independent scorers blinded to clinical outcome in 2-4 40x high power fields. A high concordance was observed between the two observers for the training cohort ($\rho_c=0.849$, Lin's Concordance test) and an audited subset of cases in the validation cohort ($\rho_c=0.998$, Lin's Concordance test). The average AR positivity was calculated between the two independent observers for the replicate cores for the training and validation cohorts. For cases that had both AR negative and AR positive cores, only the positive cores were used to calculate the average percentage of AR positive cancer cells. Up to

10% of cases in the training and validation cohort had both positive and negative cores. ER α , PR, HER2/*neu* and Ki67 were stained and scored as described previously for the training cohort [15, 51]. For the validation cohort, ER α , PR and HER2/*neu* status had already been determined by the Genetic Pathology Evaluation Centre, University of British Columbia, Vancouver, Canada [14].

4.3.3 Statistical analyses

All analyses were performed using PAWS statistics 17 Windows software (SPSS Inc., Chicago, IL). To evaluate the relationship with clinical outcome, AR levels were analysed initially as continuous variables using univariable Cox regression analysis. Variables which were significant as continuous variables were then analysed as dichotomized values using Cox regression analysis and by ROC analysis. ROC analysis was used to dichotomize AR and Ki67 positivity. The optimal cutpoints were determined using Youden index (J), which was calculated using the formula $J = \max [\text{sensitivity} + \text{specificity} - 1]$ [52]. In addition to ROC analysis, recursive partitioning was also applied to the training cohort to select the most appropriate AR cut point [53]. In Cox regression and Kaplan-Meier analyses, relapse or death due to breast cancer was used as the endpoint to determine whether AR levels or the AR to ER α ratio are associated with relapse-free survival (RFS) or breast cancer specific overall survival (OS). Patients who died from other causes were censored on their date of death. The AR to ER α ratio was analysed as tertile groups to compare outcome for patients with tumours containing comparable levels of AR and ER α to tumours with a predominance of either receptor (AR > ER α or AR < ER α). The training and validation cohorts were combined to increase the sample size of patient groups. AR to ER α ratio tertiles were calculated to be <0.82, 0.82-1.05 and >1.05. The seventeen breast cancer patients with ER α positive tumours lacking AR were included in the group of patients with an AR:ER α ratio <0.82. Six patients with AR and ER measurements were not included in this analysis due to loss of clinical follow-up.

Table 4.2. Clinical and pathological characteristics of the patient cohorts.

Characteristic	Test Cohort (n=219)	Validation Cohort (n=418)
Median age (range) years	54.0 (24.0-87.0) years	61.60 (28.0-93.45)
Median follow-up (range) months	63.9 (0.10-147.3) months	143.5 (0.36-355.79)
Tumour grade		
Grade I	33 (16%)	84 (21%)
Grade II	78 (38%)	219 (54%)
Grade III	93 (46%)	100 (25%)
Unknown	15	15
Tumour size		
<20 mm	125 (57%)	185 (44%)
≥20 mm	86 (39%)	233 (56%)
Unknown	8	0
Nodal status		
Negative	114 (52%)	236 (56%)
Positive	105 (48%)	134 (32%)
Unknown	0	48
Hormone receptor status		
ERα negative <1%	49 (23%)	78 (21%)
ERα positive ≥1%	164 (77%)	287 (79%)
Unknown	6	53
PR negative <1%	65 (30%)	105 (39%)
PR positive ≥1%	149 (70%)	163 (61%)
Unknown	5	150
AR negative <10%	43 (20%)	59 (14%)
AR positive ≥10%	176 (80%)	318 (76%)
Unknown	0	41
AR <78%	109 (50%)	210 (56%)
AR ≥78%	110 (50%)	167 (44%)
Unknown	0	41
Her2/neu status		
Negative	171 (82%)	309 (90%)
Positive	38 (18%)	35 (10%)
Unknown	10	74
Ki67 status		
Ki67≥7.5%	86 (43%)	N/A
Ki67<7.5%	113(57%)	N/A
Unknown	20	
Recurrence		
Non-relapse	162 (74%)	N/A
Relapse	57 (26%)	N/A
Metastatic disease		
No	167 (80%)	N/A
Yes	52 (20%)	N/A

Death during follow-up		
Alive or death other causes	177 (81%)	278 (67%)
Breast cancer death	42 (19%)	136 (33%)
Lost to follow-up	0	4

4.4 Results

4.4.1 Analysis of studies investigating AR status and breast cancer outcome

To date, at least 40 studies have assessed AR status and disease outcome in breast cancer using either radioligand binding assays or immunostaining (**Table 4.1**; search conducted using the Medline database via the Ovid interface using the terms (“prognostic marker” OR “prognosis” AND “breast cancer” AND (“androgen receptor” OR “hormone receptor” OR “estrogen receptor”))). Although many studies of unselected breast cancer cohorts found that AR is prognostic for OS by univariate analysis, only 7/23 studies in unselected cohorts found that AR was an independent prognostic marker of OS in multivariate analyses (**Table 4.1**). Additional studies addressing the prognostic capacity of AR in breast tumours that lack ER α have also been conflicting, with 3/11 showing that AR is an independent prognostic factor of OS (**Table 4.1**). However, 4/6 studies in ER α positive breast cancers have identified AR as an independent predictor of outcome. Collectively, the studies to date highlight a lack of consensus on whether AR is an independent prognostic factor for breast cancer survival. Notably, there is a similar lack of consensus regarding the most appropriate cut-point (e.g. 1%, 5%, 10%, or the median) for AR positivity in breast cancer, which likely impacts the evaluation of AR as a clinically useful prognostic factor.

4.4.2 AR status is an independent predictor of overall survival in breast cancer

The majority of breast cancers in the test (80%) and validation (76%) cohorts were classified AR positive by immunostaining using the traditional cut-point for ER α positivity of 10% (**Table 4.2, Figure 4.1**).

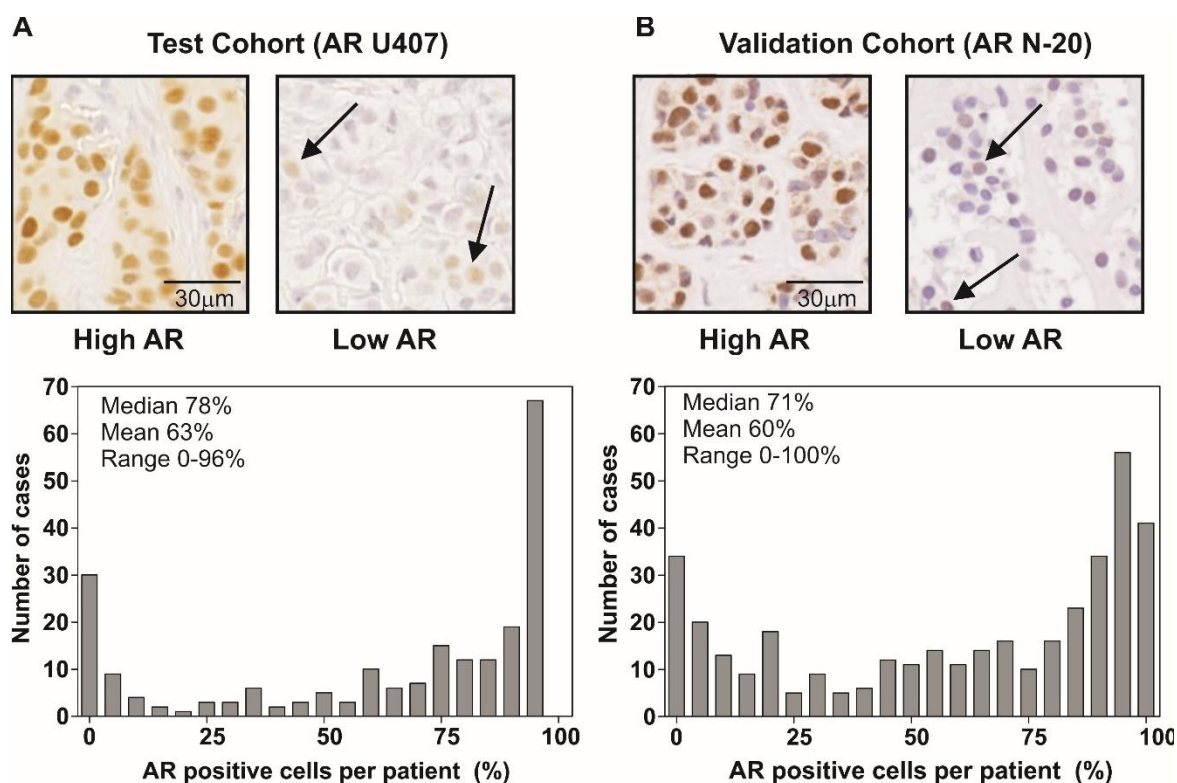


Figure 4.1. Examples of AR immunostaining.

(A) Test cohort; left panel. An example of breast cancer with uniform nuclear AR immunoreactivity of moderate intensity in 100% of tumour cells. Test cohort; right panel. An example of weak AR immunostaining in approximately 20% of the tumour cells. Frequency distribution for AR as assessed in a cohort of 219 breast cancers by visual scoring. The mean, median and range of per cent positivity immunostaining are shown. (B) Validation cohort; left panel. An example of strong AR nuclear immunoreactivity in approximately 80% of tumour cells. Validation cohort; right panel. An example of weak AR immunostaining in approximately 20% of the tumour cells. Frequency distribution for AR was assessed in a validation cohort of 418 breast tumours by visual scoring. The mean, median and range of per cent positivity immunostaining are shown. Arrows denote examples of AR positive tumour cells.

ROC analysis indicated an area under curve equal to 0.656 (95% CIs; 0.563-0.748, $p=0.002$) for the Test cohort and 0.608 (95% CIs; 0.548-0.668, $p=0.001$) for the validation cohort (**Figure 4.2A** and **Figure 4.3A**). A cut-point $>10\%$ positivity had a high specificity of 84.5% for the Test cohort and 86.4% for the Validation cohort, but a low sensitivity of 33.3% and 20% for the Test and Validation cohorts, respectively, for predicting OS. Notably, utilization of the recently adopted cut point of 1% positivity for ER α immunostaining in breast tumors as the cut-point for AR resulted in a low sensitivity of 26.2% for the Test cohort and 10% for the Validation cohort. The highest Youden index was obtained for the median value for AR

immunostaining (i.e. 78% AR positivity; Figure 1A) which resulted in a sensitivity of 77.5% and a specificity of 56.5% for predicting OS in the Test cohort (Figure 1A). The highest Youden index in the Validation cohort was also obtained with a cut point of 78% positivity which resulted in a sensitivity of 70% and a specificity of 51.5% (Figure 2A).

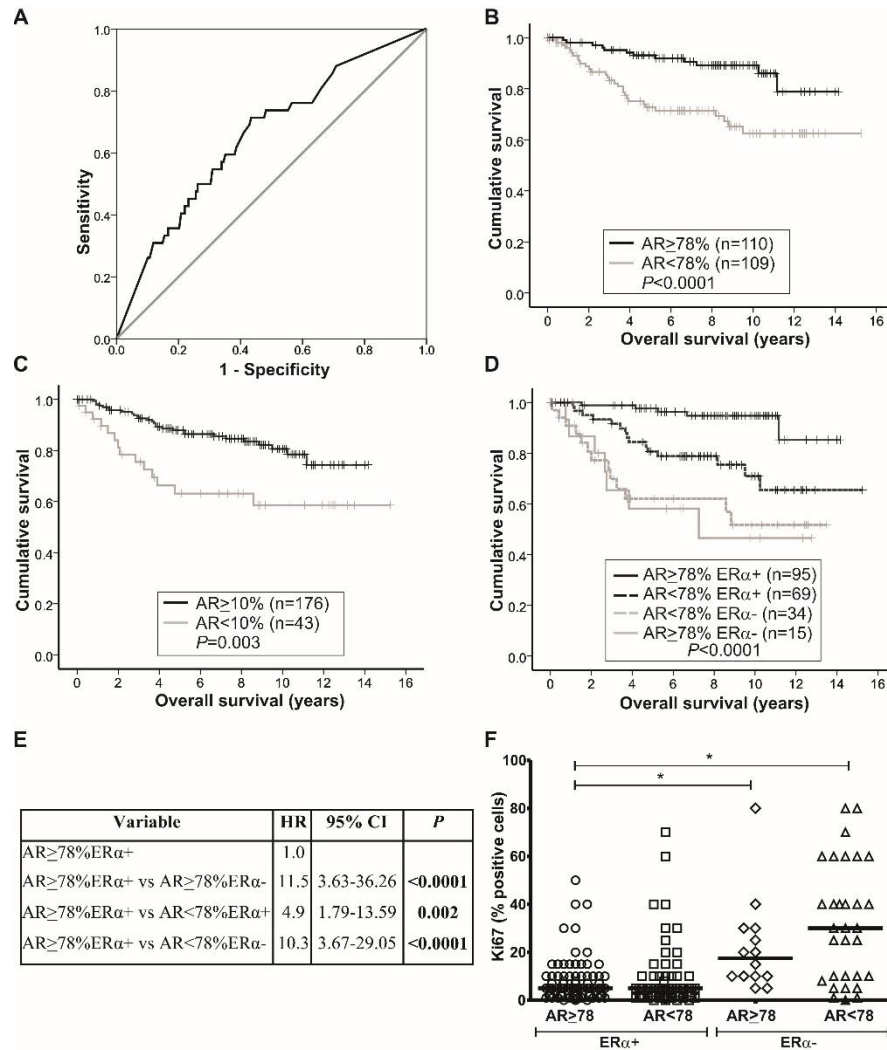


Figure 4.2. High AR expression is associated with an increased overall survival in a test cohort.

(A) Receiver operating characteristic (ROC) analysis in the test cohort identified an area under the curve of 0.656 (95% CIs; 0.563-0.748, $p=0.002$). (B) High AR ($\geq 78\%$ nuclear positivity) was significantly associated with increased overall survival (log rank statistic = 12.60, $p<0.0001$). (C) Positive AR ($\geq 10\%$ nuclear positivity) was significantly associated with increased overall survival in the test cohort (log rank statistic = 9.07, $p=0.003$). (D) Breast tumours from patients with high AR ($\geq 78\%$ nuclear positivity) and positive ER α ($\geq 1\%$ nuclear positivity) (AR $\geq 78\%$ ER α +) were significantly associated with increased overall survival (log rank statistic = 32.34, $p<0.0001$) when compared to the remaining groups (AR<78% ER α +, AR $\geq 78\%$ ER α - and AR<78% ER α -). (E) Cox regression analysis on overall survival comparing relative risk for AR and ER α sub-groups in D. (Test cohort, $n=204$). ER α +, denotes ER α $\geq 1\%$ positive cells, ER α - denotes ER α negative tumours. AR ≥ 78 denotes AR positivity greater than or equal to 78% and AR<78 refers to AR positivity less than 78%. (F) Ki67 status for the 4 subgroups in D) (AR ≥ 78 ER α +, AR<78 ER α +, AR ≥ 78 ER α -, AR<78 ER α -). The horizontal bar indicates the median value for each group. ER α - tumours had a significantly higher Ki67 positivity regardless of AR positivity (* $p<0.05$, Mann Whitney U test).

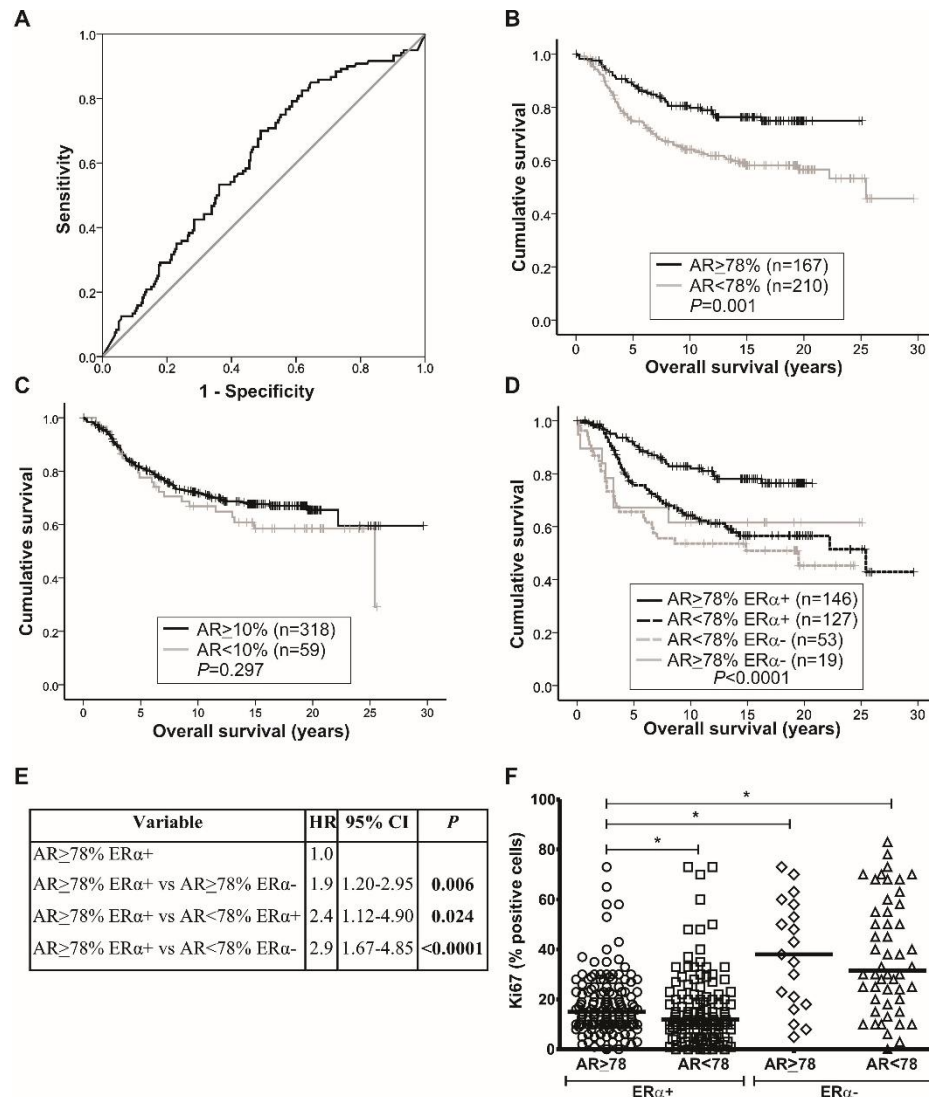


Figure 4.3. AR immunostaining and overall survival in the validation cohort.

(A) Receiver operating characteristic (ROC) analysis in the validation cohort identified an area under the curve of 0.608 (95% CIs; 0.548-0.668, $p=0.001$). (B) High AR ($\geq 78\%$ nuclear positivity) was significantly associated with increased overall survival (log rank statistic = 12.07, $p=0.001$). (C) Positive AR ($\geq 10\%$ nuclear positivity) was not significantly associated with increased overall survival (log rank statistic = 1.09, $p=0.297$). (D) Breast tumours from patients with high AR ($\geq 78\%$ nuclear positivity) and positive ER α ($\geq 1\%$ nuclear positivity) (AR $\geq 78\%$ ER α +) were significantly associated with increased overall survival (log rank statistic = 20.12, $p<0.0001$) when compared to the remaining groups (AR<78% ER α +, AR $\geq 78\%$ ER α - and AR<78% ER α). (E) Cox regression analysis on overall survival comparing relative risk in the AR and ER α sub-groups in (D) (validation cohort, $n=344$). ER α +

Using an alternative method, recursive partitioning, the optimal cut point for the Test cohort was 77%. Examples of high and low AR positivity and the frequency distributions for both cohorts are shown in **Figure 4.1**. Using 78% AR nuclear positivity to dichotomize the data, AR status was significantly associated with ER α status and breast cancer subtype in both cohorts (**Tables 4.3 and 4.4**, chi-squared test). The majority of tumours (69.4%) with AR positivity <78% were also ER α negative (i.e. <1% ER α positivity) in the test cohort; 73.6% were ER α negative in the validation cohort. A higher proportion of the basal-like breast cancer subtype had low AR positivity in both the test (79%) and validation (69%) cohorts (**Table 4.5**). Additionally, histological grade III and PR negative tumours were significantly associated with low AR positivity in the test cohort (**Tables 4.3**). A lack of association with PR in the validation cohort (**Tables 4.4**) likely results from unknown PR values for 150/418 cases (**Tables 4.2**). PR was measured in 214/219 of cases in the Test cohort (**Tables 4.2**).

Table 4.3. Association of AR immunostaining levels with clinicopathological parameters (test cohort)

Variable	AR immunostaining ^a	
	<78% n (%)	≥78 n (%)
Age		
≤55	55 (48.3%)	59 (51.7%)
>55	54 (51.4%)	51 (48.6%)
	<i>P</i> =0.686	
Tumour size		
<20	58 (46.4%)	67 (53.6%)
≥20	46 (53.5%)	40 (46.50%)
	<i>P</i> =0.330	
Histological grade		
well	13 (39.4%)	20 (60.6%)
moderate	32 (41.0%)	46 (59.0%)
poor	57 (61.3%)	36 (38.7%)
	<i>P</i>=0.013*	
Nodal status		
negative	54 (48.7%)	57 (51.3%)
positive	53 (50.5%)	52 (49.5%)
	<i>P</i> =0.892	
ERα status ^b		
negative	34 (69.4%)	15 (30.6%)
positive	69 (42.1%)	95 (57.9%)
	<i>P</i>=0.001*	
PR status ^c		
negative	46 (70.8%)	19 (29.2%)
positive	58 (38.9%)	91 (61.1%)
	<i>P</i><0.0001*	
HER-2/neu status		
negative	82 (48.0%)	89 (52.0%)
positive	20 (52.6%)	18 (47.4%)
	<i>P</i> =0.720	
Ki67 ^d		
negative	50 (44.3%)	63 (55.7%)
positive	46 (53.5%)	40 (46.5%)
	<i>P</i> =0.202	
Cancer subtype ^e		
Luminal A	59 (41.3%)	84 (58.7%)
Luminal B	9 (47.4%)	10 (52.6%)
Her2+	9 (56.3%)	7 (43.7%)
Basal-like	23 (79.3%)	6 (20.7%)
	<i>P</i>=0.001*	

^aAR status (% positive cells) as a dichotomous variable median cut point <78 vs. ≥78

^bERα status (% positive cells) as a dichotomous variable cut point <1 vs. ≥1

^cPR status (% positive cells) as a dichotomous variable cut point <1 vs. ≥1

^dKi67 positivity (% positive cells) as a dichotomous variable cut point <7.5 vs. ≥7.5

^eLuminal A classified as ERα+ or PR+ and Her2-; luminal B classified as ERα+ or PR+ and Her2+; Her2+ classified as ERα- and PR- and Her2+; basal-like classified as ERα-, PR- and Her2-. * statistically significant at *P*<0.05, chi-squared test

Table 4.4. Association of AR immunostaining levels with clinicopathological parameters (validation cohort)

Variable	AR immunostaining ^a	
	<78% n (%)	≥78 n (%)
Age		
≤55	81 (60.4%)	53 (39.6%)
>55	129 (53.1%)	114 (46.9%)
	<i>P</i> =0.194	
Tumour size		
<20	89 (52.4%)	81 (47.6%)
≥20	121 (58.4 %)	86 (41.6%)
	<i>P</i> =0.253	
Histological grade		
well	48 (66.7%)	24 (33.3%)
moderate	104 (51.2%)	99 (48.8%)
poor	51 (58.0%)	37 (42.0%)
	<i>P</i> =0.070	
Nodal status		
negative	115 (53.7%)	99 (46.3%)
positive	95 (58.3%)	68 (41.7%)
	<i>P</i> =0.403	
ERα status ^b		
negative	53 (73.6%)	19 (26.4%)
positive	127 (46.5%)	146 (53.5%)
	<i>P</i>=0.0001*	
PR status ^c		
negative	58 (61.1%)	37 (38.9%)
positive	77 (48.7%)	81 (51.3%)
	<i>P</i> =0.069	
HER-2/neu status		
negative	152 (36.7%)	130 (63.3%)
positive	17(56.7%)	13 (43.3%)
	<i>P</i> =0.848	
Cancer subtype ^d		
Luminal A	88 (50.0%)	88 (50.0%)
Luminal B	3 (30.0%)	7 (70.0%)
Her2+	5 (83.3%)	1 (16.7%)
Basal-like	24 (68.6%)	11 (31.4%)
	<i>P</i>=0.039*	

^aAR status (% positive cells) as a dichotomous variable median cut point <78 vs. ≥78

^bERα status (% positive cells) as a dichotomous variable cut point <1 vs. ≥1

^cPR status (% positive cells) as a dichotomous variable cut point <1 vs. ≥1

^dLuminal A classified as ERα+ or PR+ and Her2-; luminal B classified as ERα+ or PR+ and Her2+; Her2+ classified as ERα- and PR- and Her2+; basal-like classified as ERα-, PR- and Her2-. *statistically significant at *P*<0.05, chi-squared test

Table 4.5. Association of AR:ER α ratio groups with breast cancer subtypes (combined test and validation cohorts)

AR:ER α ratio group	Breast cancer subtype ^a			
	Luminal A	Luminal B	Her2/ <i>neu</i> +	Basal like
AR:ER α ratio 0.82-1.05	110 (98.2%)	2 (1.8%)	0 (0%)	0 (0%)
AR:ER α ratio >1.05	102 (82.9%)	19 (15.5%)	2 (1.6%)	0 (0%)
AR:ER α ratio <0.82	103 (94.5%)	6 (5.5%)	0 (0%)	0 (0%)
AR+ER α -	3 (4.9%)	0 (0%)	17 (27.9%)	41 (67.2%)
AR-ER α -	1 (3.7%)	0 (0%)	3 (11.1%)	23 (85.2%)
Total	319	29	22	64

P <0.0001 (chi-square test)

^aLuminal A classified as ER α + or PR+ and Her2-; luminal B classified as ER α + or PR+ and Her2+; Her2+ classified as ER α - and PR- and Her2+; basal-like classified as ER α -, PR- and Her2-

Kaplan-Meier analyses demonstrated that a nuclear AR positivity of $\geq 78\%$ was significantly associated with OS in both the test (**Figure 4.2B**, $p < 0.0001$) and the validation (**Figure 4.3B**, $p = 0.001$) cohorts. Patients with nuclear AR positivity $\geq 78\%$ in their tumour had approximately 2-fold reduced risk of cancer-related death in the test cohort (HR = 0.32, Cox regression analysis, $p = 0.015$, **Table 4.6**) and the validation cohort (HR = 0.51, Cox regression analysis, $p = 0.014$, **Table 4.8**). Multivariable Cox regression analysis indicated that AR immunostaining with a cut-off of 78% is an independent predictor of OS after adjusting for all other variables significant by univariable analysis in both cohorts (**Tables 4.7 and 4.9**). Kaplan-Meier and Cox regression analyses demonstrated that $\geq 10\%$ nuclear AR positivity was significantly associated with OS in the test cohort ($p = 0.003$; **Figure 4.2C**, **Table 4.6**), but not in the validation cohort ($p = 0.297$; **Figure 4.3C**, **Table 4.8**).

Table 4.6. Univariate Cox Regression analyses for disease-free survival and overall survival (test cohort).

Variable	Disease-free survival			Overall survival		
	HR	95% CIs	P value	HR	95% CIs	P value
Tumour size ^a (n=205)	2.18	1.27-3.74	0.005	2.34	1.24-4.40	0.009
Grade ^b (n=198)	4.90	1.19-20.17	0.028	3.68	0.89-15.29	0.072
Lymph status ^c (n=208)	3.29	1.82-5.94	<0.0001	2.42	1.25-4.68	0.008
ER α status ^d (n=204)	0.37	0.21-0.66	<0.0001	0.23	0.12-0.43	<0.0001
PR status ^e (n=205)	0.36	0.21-0.61	<0.0001	0.22	0.12-0.42	<0.0001
HER-2/neu status ^f (n=201)	2.75	1.56-4.84	<0.0001	3.06	1.60-5.87	0.001
Ki67 7.5 ^g (n=190)	2.73	1.57-4.73	<0.0001	3.42	1.77-6.61	<0.0001
AR status ^h (n=210)	0.992	0.98-1.00	0.015	0.99	0.98-1.00	0.001
AR status 1 ⁱ (210)	0.48	0.25-0.92	0.026	0.36	0.18-0.73	0.004
AR status 10 ^j (n=210)	0.585	0.32-1.06	0.077	0.39	0.20-0.73	0.004
AR status 78 ^k (n=210)	0.54	0.32-0.92	0.024	0.32	0.16-0.62	0.001

a = tumour size (mm) as a dichotomous variable cut point ≤ 20 vs. >20

b = tumour grade (well or moderate vs. poor)

c = lymph node status (negative vs. positive)

d = ER α status (% positive cells) as a dichotomous variable cut point <1 vs. $\geq 1\%$ positive cells

e = PR status (% positive cells) as a dichotomous variable cut point <1 vs. $\geq 1\%$ positive cells

f = HER-2/neu status (negative vs. positive)

g = Ki67 status (% positive cells) as a dichotomous variable cut point <7.5 vs. $\geq 7.5\%$ positive cells

h = AR status (% positive cells) as a continuous variable

i = AR status (% positive cells) as a dichotomous variable cut point <1 vs. $\geq 1\%$ positive cells

j = AR status (% positive cells) as a dichotomous variable cut point <10 vs. $\geq 10\%$ positive cells

k = AR status (% positive cells) as a dichotomous variable cut point <78 vs. $\geq 78\%$ positive cells

*AR status 1 and AR status 10 were not significant by multivariable analysis

Table 4.7. Multivariate analysis of all significant variables in univariate analysis (test cohort)

Variable	Disease-free survival (n=196)			Overall survival (n=174)		
	HR	95% CIs	P value	HR	95% CIs	P value
Tumour size ^a	1.53	0.80- 2.76	0.181	1.38	0.74- 2.57	0.369
Tumour grade ^b	1.25	0.29- 5.51	0.788	-	-	-
Lymph node status ^c	2.68	1.41- 5.08	0.003	1.89	0.95- 3.73	0.068
ER α status ^d	0.88	0.33- 2.50	0.797	0.52	0.18- 1.52	0.228
PR status ^e	0.64	0.22- 1.73	0.402	0.69	0.22- 2.17	0.527
HER-2/neu status ^f	1.80	0.90- 3.56	0.085	2.11	1.01- 4.38	0.046
Ki67 ^g	1.95	0.96- 3.90	0.067	1.77	0.81- 3.87	0.151
AR status 78 ^k	0.62	0.36- 1.20	0.129	0.41	0.20- 0.84	0.015

a = tumour size (mm) as a dichotomous variable cut point ≤ 20 vs. >20

b = tumour grade (well or moderate vs. poor)

c = lymph node status (negative vs. positive)

d = ER α status (% positive cells) as a dichotomous variable cut point <1 vs. $\geq 1\%$ positive cells

e = PR status (% positive cells) as a dichotomous variable cut point <1 vs. $\geq 1\%$ positive cells

f = HER-2/neu status (negative vs. positive)

g = Ki67 status (% positive cells) as a dichotomous variable cut point <7.5 vs. $\geq 7.5\%$ positive cells

h = AR status (% positive cells) as a continuous variable

i = AR status (% positive cells) as a dichotomous variable cut point <1 vs. $\geq 1\%$ positive cells

j = AR status (% positive cells) as a dichotomous variable cut point <10 vs. $\geq 10\%$ positive cells

k = AR status (% positive cells) as a dichotomous variable cut point <78 vs. $\geq 78\%$ positive cells

*AR status 1 and AR status 10 were not significant by multivariable analysis

Table 4.8. Univariate Cox regression analyses for overall survival (validation cohort)

Variable	HR	95% CIs	P value
Tumour size ^a (n=417)	1.97	1.37-2.83	<0.0001
Grade ^b (n=402)	0.89	0.60-1.34	0.585
Lymph status ^c (n=370)	2.46	1.95-3.92	<0.0001
ER α status ^d (n=364)	0.53	0.36-0.77	<0.0001
PR status ^e (n=267)	0.63	0.42-0.93	0.024
HER-2/neu status ^f (n=343)	2.05	1.25-3.35	0.004
Ki67 ^g (n=367)	1.64	1.15-2.35	0.007
AR status ^h (n=376)	0.99	0.99-1.00	0.008
AR status 1 ⁱ (376)	0.60	0.33-1.10	0.598
AR status 10 ^j (n=376)	0.79	0.50-1.23	0.298
AR status 78 ^k (n=376)	0.51	0.34-0.75	0.001

a = tumour size (mm) as a dichotomous variable cut point ≤ 20 vs. >20

b = tumour grade (well or moderate vs. poor)

c = lymph node status (negative vs. positive)

d = ER α status (% positive cells) as a dichotomous variable cut point <1 vs. $\geq 1\%$ positive cells

e = PR status (% positive cells) as a dichotomous variable cut point <1 vs. $\geq 1\%$ positive cells

f = HER-2/neu status (negative vs. positive)

g = Ki67 status (% positive cells) as a dichotomous variable cut point <16 vs. $\geq 16\%$ positive cells

h = AR status (% positive cells) as a continuous variable

i = AR status (% positive cells) as a dichotomous variable cut point <1 vs. $\geq 1\%$ positive cells

j = AR status (% positive cells) as a dichotomous variable cut point <10 vs. $\geq 10\%$ positive cells

k = AR status (% positive cells) as a dichotomous variable cut point <78 vs. $\geq 78\%$ positive cells

Table 4.9. Multivariate analysis of all significant variables in univariate analysis (Validation Cohort).

Overall survival (n=222)			
Variable	HR	95% CI	P value
Tumour size ^a	1.90	1.07-3.36	0.028
Lymph node status ^c	2.34	1.38-3.98	0.002
ER α status ^d	1.35	0.63-2.89	0.438
PR status ^e	0.59	0.30-1.14	0.116
HER-2/neu status ^f	2.72	1.34-5.48	0.005
Ki67 ^g	1.66	0.95-2.91	0.075
AR status 78 ^k	0.50	0.29-0.87	0.014

a = tumour size (mm) as a dichotomous variable cut point ≤ 20 vs. >20

b = tumour grade (well or moderate vs. poor)

c = lymph node status (negative vs. positive)

d = ER α status (% positive cells) as a dichotomous variable cut point <1 vs. $\geq 1\%$ positive cells

e = PR status (% positive cells) as a dichotomous variable cut point <1 vs. $\geq 1\%$ positive cells

f = HER-2/neu status (negative vs. positive)

g = Ki67 status (% positive cells) as a dichotomous variable cut point <16 vs. $\geq 16\%$ positive cells

h = AR status (% positive cells) as a continuous variable

i = AR status (% positive cells) as a dichotomous variable cut point <1 vs. $\geq 1\%$ positive cells

j = AR status (% positive cells) as a dichotomous variable cut point <10 vs. $\geq 10\%$ positive cells

k = AR status (% positive cells) as a dichotomous variable cut point <78 vs. $\geq 78\%$ positive cells

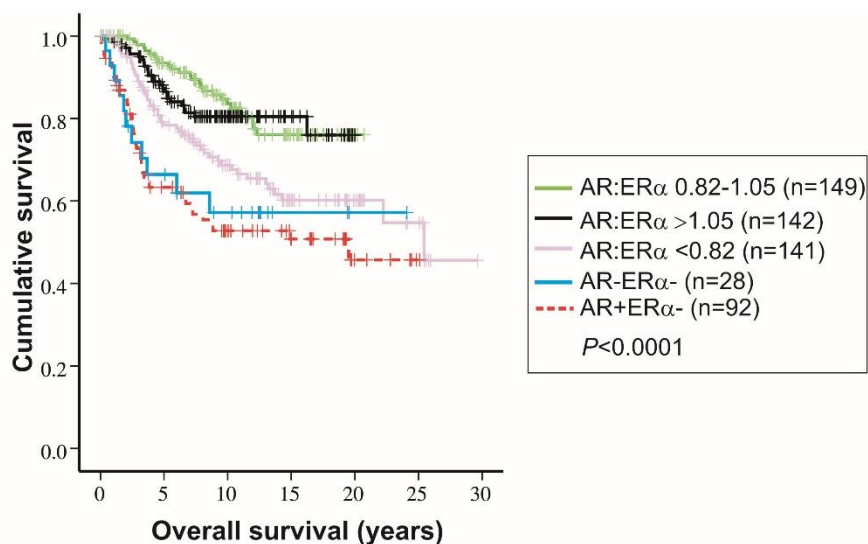
4.4.3 ER α positive breast cancers with high AR have the best survival outcome

When the test and validation cohorts were analysed on the basis of an ER α positivity cut-point of 1% nuclear immunostaining, patients with high tumour levels of AR (ER α $\geq 1\%$,

AR \geq 78%) had significantly increased OS in comparison to patients in the other 3 possible ER α and AR classifications (ER α \geq 1 and AR <78%, ER α \leq 1 and AR \geq 78%, ER α \leq 1 and AR <78%; **Figure 4.2D and 4.3D**). Patients with ER α negative disease, regardless of AR status, had an increased risk of death compared to those with ER α positive AR >78% tumours (test cohort: 11.5 and 10.3-fold risk, $p < 0.0001$, **Figure 4.2E**; validation cohort: 1.9 and 2.9-fold risk, $p = 0.006$ and $p < 0.0001$, **Figure 4.3E**). Patients with ER α positive tumours had significantly lower Ki67 positivity compared to patients with ER α negative tumours in both the test cohort (**Figure 4.1F**, Mann-Whitney U test, $p < 0.0001$) and validation cohort (**Figure 4.3F**, Mann-Whitney U test, $p < 0.0001$), irrespective of the level of AR.

4.4.4 The AR to ER α ratio is a determinant of breast cancer survival

For subsequent subgroup analyses, unbiased tertiles were calculated for the AR to ER α ratio in the combined cohorts. Patients with tumours that contained comparable levels of AR and ER α (i.e. an AR to ER α ratio approximating 1; range 0.82-1.05), or where the AR: ER α ratio was >1.05 (i.e., AR predominates over ER α), had the highest 10-year breast cancer survival outcome (83.6% and 80.5%, respectively; $p < 0.0001$; **Figure 4.4A**). Breast cancers that were negative for both AR and ER α were associated with a particularly poor outcome, with a 10-year survival rate of 57.2% (**Figure 4.4**) and a 3.2 fold increased risk of death ($p = 0.001$). In contrast patients with an AR: ER α ratio <0.82 (i.e., less AR than ER α) had a poorer survival outcome compared to patients with similar AR and ER α immunostaining levels (**Figure 4.4**, $p = 0.002$). ER α negative tumours that were positive for AR had a similarly poor survival outcome (52.8%) to those that were negative for both receptors (**Figure 4.4**) and were associated with a 3.5-fold increased risk of death ($p < 0.0001$). A significant relationship was also observed between the patient groups in **Figure 4.4** and specific breast cancer subtypes. The group of patients with AR but no ER α had a significantly higher proportion of HER2/*neu* positive tumours (17/22, 77.3%) and the basal-like breast cancer subtype (41/64, 64.1%, $p < 0.0001$ chi-square test, **Table 4.5**).



Variable	Combined Test & Validation Cohorts Overall survival		
	HR	95% CI	<i>P</i>
AR:ER α 0.82-1.05	1.00		
AR:ER α 0.82-1.05 vs AR:ER α >1.05	1.24	0.71-2.14	0.445
AR:ER α 0.82-1.05 vs AR:ER α <0.82	2.15	1.31-4.73	0.002
AR:ER α 0.82-1.05 vs AR+ER α -	3.53	2.14-5.82	<0.0001
AR:ER α 0.82-1.05 vs AR-ER α -	3.18	1.55-6.51	0.001

Figure 4.4. Overall survival in patients with different AR:ER α ratios.

Subgroups were separated into tertiles. Patient groups include those with an AR:ER α ratio approximating 1 (i.e. 0.82-1.05), AR:ER α ratio >1.05, AR:ER α ratio <0.82, AR+ ER α -, AR-ER α - (log rank statistic = 37.19, *P* < 0.0001). In the Cox regression analysis relative risk of death in the different AR:ER α ratio groups was compared to the patient group with an AR:ER α ratio approximating 1.

4.5 Discussion

The current study provides compelling evidence that AR is an independent and robust predictor of breast cancer specific overall survival if appropriate assessment criteria are applied. Moreover, measurement of AR levels in addition to ER α may permit more precise prediction of breast cancer outcome. Numerous studies have investigated AR as a prognostic factor in this disease; however, the findings have been inconsistent. Of the previous studies that assessed the association between AR and patient outcome, only 30% (2 radioligand binding, 4 immunostaining studies, and 1 protein array of 23 studies in total) found that the AR is an independent prognostic factor for OS in unselected breast cancer cohorts. While methodological differences are likely to have contributed to the inconsistent findings, application of an arbitrary cut-point to dichotomise AR was a common feature and, therefore, a potential limitation in assessing the prognostic value of AR. In the present study, ROC analysis was used to demonstrate that, rather than the traditional cut-point of 10% positivity or the more recently adopted cut-point of 1% for ER α , a relatively high cut-point of 78% AR positivity was required to achieve the best sensitivity and specificity for independent prediction of breast cancer survival in two independent, well characterized breast cancer cohorts. This finding is of particular clinical relevance given the current interest in targeting AR in breast cancer, with several clinical trials already underway or completed (ClinicalTrials.gov identifiers NCT00468715 (bicalutamide in advanced breast cancer), NCT01597193 (enzalutamide alone or in combination with endocrine therapy), NCT00755885 (abiraterone in advanced/metastatic breast cancer), and NCT01889238 (enzalutamide; AR+, ER/PR/HER2-)). However, such trials are constrained by the absence of guidelines for selecting patients on the basis of AR status who are likely to benefit from this therapeutic approach. Our findings suggest that assessment of AR status with an optimised AR assay and application of a validated cut-point may enable better selection of patients who are likely to respond to these hormonal interventions.

Previous studies [9, 10] have shown that AR functions as an inhibitor of proliferation in ER α -positive breast cancer, potentially by acting as a transcriptional modifier of ER α [9, 54]. For this anti-proliferative effect to occur, AR must be co-expressed with ER α in breast tumours and, ideally, be present at greater or comparable levels. In support of this, previous studies from our laboratory have found AR typically to be co-expressed with ER α in individual breast cancer cells [9], that AR positivity is comparable to or greater than ER α in the majority of malignant breast tissues [1], and that optimal inhibition of ER α transcriptional activity by AR requires a

ratio of AR to ER α of approximately 4:1 [9]. Consistent with these earlier findings, we now show that AR had the greatest prognostic significance for overall survival in ER α -positive breast cancer when AR and ER α levels were at least comparable or AR was in excess of ER α . These findings support the notion that the balance between the estrogen and androgen signalling pathways is critical for the growth and survival of breast cancer cells. Assessment of AR status together with ER α could provide further prognostic information for women with breast cancer providing the appropriate criteria for assessing AR positivity are applied. A relationship between AR and PR status was also observed for the test cohort, but not the validation cohort, most likely because PR was unknown in 36% of cases. Nevertheless, even if this is a real difference between the two cohorts, it does not preclude AR being an independent predictor of OS in both cohorts providing the objectively determined optimal cut-point of 78% is used.

When stratified by ER α status, cancers in our cohorts that lacked this receptor had a significantly higher proliferation rate regardless of AR status, consistent with previous observations [55, 56]. Moreover, there was no statistical difference in overall survival rates between AR positive and AR negative cases for the ER α negative subgroup, both being associated with the poorest overall survival rates. This finding is consistent with a study by Hu et al. [4] showing that postmenopausal women with ER α negative breast cancers had poor breast cancer survival irrespective of AR status. *In vitro* studies have demonstrated dichotomous proliferative effects of androgens, ranging from stimulatory to inhibitory in different ER-negative molecular breast cancer subgroups [1], which may explain why AR expression alone does not robustly predict outcome in ER α -negative breast cancers. Indeed, ER α negative breast cancers are a highly diverse molecular group and to definitively dissect the influence of AR on disease outcome in these cancers will require stratification of cancers into separate molecular subgroups in much larger clinical cohorts to provide sufficient statistical power. However, a recent Phase II clinical trial in AR+ER-PR- metastatic breast cancer patients showed that the use of the AR antagonist bicalutamide did show some clinical benefit [57]. It is interesting to note that 4 of the 5 patients that had a clinical benefit response to bicalutamide had an AR positivity >80%; however, the AR distribution for the patients that did not respond was not shown.

In summary, AR status is an independent predictor of breast cancer survival providing that an objectively determined optimal cut-point for AR positivity is employed. Defining the optimal cut-point for ER α as a prognostic or predictive factor in breast cancer has been an ongoing process, with continuing debate regarding the functional significance of a 1% cut-point

for ER α positivity and, in particular, whether tumours with 1-9% ER α positivity are endocrine responsive [58, 59]. Similarly, it will be critical to assess the applicability and significance of an objectively obtained cut-point for nuclear AR positivity in prospective clinical studies. Such studies are warranted given the biological basis for using a different cut-point for AR that differs to that used for ER α . Our finding in two independent breast cancer cohorts objectively defined a cut-point of 78% nuclear AR positivity could independently predict overall survival. However, given the known problems with technical and intra- and interobserver variability when measuring biomarkers in the clinical setting, a consensus on scoring methods or newer quantitative technologies may be useful to standardise biomarker development [60].

We propose that assessment of AR status together with the traditional assessment of ER α will provide additional valuable prognostic information for women with breast cancer providing appropriate criteria for assessing AR positivity are applied. Furthermore, it will be important to establish an objectively defined cut-point for AR status in future clinical trials of AR targeting agents in breast cancer.

4.6 References

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5. Discussion

The role of androgens and their cognate receptor in breast cancer is of renewed and growing interest [1]. The androgenic signalling axis is not only interesting from the perspective of how it interacts with other major growth regulatory pathways in breast cancer, such as estrogen receptor (ER) signalling, but also due to the promise of exploiting the pathway for therapeutic benefit. This interest has recently culminated in several clinical trials that either directly or indirectly target signalling via the androgen receptor (AR) (ClinicalTrials.gov identifiers NCT00468715, NCT01597193, NCT00755885, and NCT01889238). Although the approach taken in all of these trials is – perhaps intuitively – to inhibit cell growth by decreasing endogenous testosterone levels (in the case of abiraterone acetate) or to directly inhibit the AR via use of non-steroidal antagonists (bicalutamide, enzalutamide), both historical studies and more recent mechanistic research show that the cellular response to modulation of androgen signalling is pleiotropic and context dependent and can, more often than not, be growth inhibitory. For example, the breasts of female to male transsexuals treated with testosterone esters and female abusers of anabolic steroids undergo involution [2], and women with congenital adrenal hyperplasia (in which excess androgens are produced) have suppressed breast development [3].

Knockout mouse studies convincingly show that mammary development is reduced (either directly or indirectly) by disruption of the AR [4, 5], and testosterone and DHT inhibit proliferation and promote apoptosis in human breast tissue explants [6]. Prior to the advent of tamoxifen, testosterone or synthetic analogues were used effectively to treat breast cancer [7-10], and later in combination with tamoxifen with measureable therapeutic benefit [11, 12]. There is a raft of *in vitro* and *in vivo* data to support an inhibitory effect for androgens via the AR, particularly in ER-positive tissues [13-15]. Therefore, it is by no means clear-cut that inhibition of the AR will have a positive effect in women with breast cancer, nor is it clear who would benefit from agonising rather than antagonising the pathway.

The observed heterogeneity in clinical response to any targeted therapeutic used in cancer therapy suggests that modulation of cell signalling pathways can have both intuitive and counterintuitive cellular responses, depending on the underlying genotype and feedback, feed-forward, and cross-talk between signalling pathways; in other words, cancers and the signalling that drives them are inherently complex [16]. This has led to years of *post hoc* study and analysis of the subgroups of patients who are likely to respond to particular therapies based on the molecular characteristics of individual tumours, and has given birth to a whole era of

personalised medicine [17]. Ideally, these studies would be conducted prior to clinical trials in order to tailor therapy to groups of patients most likely to benefit (and in doing so spare unnecessary morbidity and mortality due to side-effects), but only recently have the tools been available to deconvolute the complexity of individual tumours. Although clear to pathologists for many years that individual cancers can look different (both histologically and at the molecular level), it is only recently that the importance of intratumoural heterogeneity is being appreciated as a significant obstacle to therapeutic success [18]. Therefore, the purpose of the studies in this thesis was to establish baseline parameters in terms of tissue expression of AR and apply them to meaningful clinical scenarios, given the current flurry of interest in using androgen-based endocrine therapy and the known interaction with ER signalling.

Understanding whether or not AR and ER are expressed in the same cell at the same time is essential to establishing how individual cells might respond to androgen or estrogen-based therapy, either alone or in combination. It is well established that AR is frequently expressed in primary breast tumours (between 53 and 85%, depending on the study), and consistently more frequently than expression of ER [19-24]. It therefore follows that a proportion of cells must be AR+ but ER-, but the exact proportions of cells expressing the receptors alone or together had not been established at the commencement of this thesis. My first successful experiment in dual labelling of AR and ER α in malignant breast tissue was published [25], and this finding was later confirmed by an independent study [26]. Using dual-labelling immunofluorescence of normal breast epithelium and an array of malignant tissues representing tumour progression, AR was found to be expressed more frequently than ER α at all stages of progression and there was, as predicted, a significant proportion of AR+ER α - cells, comprising one third of the total normal or malignant epithelial cell population. In addition, approximately one third of cells were AR+ER α +, 37% AR-ER α -, and a very minor proportion were AR-ER α + (3%). Although there was no significant difference in expression of AR (either alone or among the sub-populations of AR and ER α expressing cells) between primary and nodal metastatic lesions and between *in situ*, invasive, and metastatic disease, this was not completely unexpected. Firstly, it has previously been shown that AR levels were the same between primary and metastatic disease at the time of resection (but not in late metastases) [27]; secondly, the metastases in the current study were lymph node metastases and not distant metastases; lymph node metastases have been shown in several studies to have the same phenotype as the primary tumour [28]; thirdly, the numbers in the groups were relatively small and therefore may have been underpowered to identify small differences; and finally, although

the counts of positive nuclei were quantitative, the decision to call a nucleus positive or negative was binary (i.e. any expression was considered positive); therefore, the abundance of protein expression was not taken into account, and may be relevant given the dose-dependent nature of AR response [9].

Therefore, it is unlikely that AR is driving the metastatic process, at least in early metastases. However, given the intratumoural heterogeneity and the pleiotropism of AR action, any selective pressure in the form of endocrine therapy (targeting AR or ER) is likely to produce complex outcomes, with different cell populations potentially responding differently to ER inhibition or AR agonists or antagonists. For instance, the AR+ER α - population could conceivably proliferate in response to an androgen, and the AR+ER α + population be inhibited or undergo apoptosis, all within the same tumour. This, of course, fails to take into account the fact that the cells do not exist in isolation but as an interacting community responding to paracrine signals (for instance, ER does not usually co-localise with markers of proliferation, at least in the normal breast [29-31], and therefore signalling in one cell may have unpredictable consequences in a neighbouring cell. Future studies might use *in vivo* labelling, *ex vivo* tissue modelling, or exploit the neoadjuvant clinical model (i.e. compare molecular characteristics in tissues before and after therapy) in order to address these questions. More extensive testing of late, distant metastatic disease would need to be undertaken in order to establish whether any sub-clones emerge over time and in the face of the selective pressure of therapy. Furthermore, the quantitative immunofluorescence method could be extended to take pixel intensity into account instead of just using a binary read-out; some commercial platforms exist in order to do this in tissue specimens [32].

Although these data suggest that responses to androgen-based therapies are likely to be complex, and perhaps even more complex than response to therapies (which are mainly, but not exclusively, anti-proliferative) which target the ER, targeted therapies are remarkably effective in spite of heterogeneity. However, there are lessons to be learnt from measurement of ER and HER2 in breast cancer for patient selection for therapy: patients need to be selected on the basis of suitably specific and sensitive biomarkers in order to minimise both overtreatment and undertreatment, and these biomarkers need to be robust, validated, standardised, and show similar results in fully independent cohorts [33]. To this end, the second part of this thesis aimed to definitely establish: a) the prognostic value of AR in two independent cohorts of patients with primary breast cancer and with long-term follow-up and b) criteria for measurement of the biomarker, to pave the way for biomarker measurement in androgen-therapy trials. This study

was particularly necessary since previous studies have been conflicting, possibly due to the statistical power of the studies, different methods of assaying for AR, short follow-up in some studies, and criteria for defining positive and negative status (see Chapter 4). In this regard, even for well-established biomarkers such as ER, there have been very recent changes in the definition of an 'ER-positive' patient from 10% positive cells to 1% positive cells [34]. The field of biomarker analysis and validation is notoriously difficult, to the extent that guidelines have been produced to assist with reporting (REMARK [35]).

AR is shown herein (Chapter 4) to be an independent prognostic factor in two completely independent cohorts of primary breast cancers and, as opposed to using an arbitrary cut-point, ROC analysis was used to establish the optimal cut-point of 78% AR positivity. Although the pathway to clinical validation of an immunohistochemical biomarker can be hampered by the use of different antibodies on different cohorts (if they have dubious or variable specificity or affinity), in this study the use of an in-house antibody (test cohort) and commercial antibody (validation cohort) strengthened the results, since the results were replicated in the validation cohort meaning that the biomarker is likely to be robust and reproducible under a wide range of conditions. Although immunofluorescence may yield more data with respect to be able to determine co-localisation and more accurate quantification, immunohistochemistry was chosen in this part of the project since it is widely used in every histopathology laboratory and therefore more likely to be a useful clinical assay.

Patients with high AR expression had approximately two-fold reduced risk of cancer-related death in both cohorts, and AR expression was significantly associated with ER α expression. However, intriguingly and in-line with the hypothesis that the relative levels of AR and ER signalling have an impact on phenotype, those patients with equal or high AR:ER α ratios had the best 10-year overall survival. Although not quite as 'low-risk' as those patients stratified as low risk with gene expression based risk stratification tools such as Oncotype DX (around 90% ten-year overall survival for the low-risk group [36]), patients with equal or high AR:ER α ratios had an OS of over 80%. More importantly, the good prognosis of these patients might suggest that their 'inhibitory' AR pathways are intact and they might benefit from an androgen agonist approach (with traditional endocrine therapy to target ER) rather than an AR antagonist. Furthermore, AR-negative tumours had higher proliferation rates and AR expression did not affect the (poor) outcome in ER α -negative tumours, suggesting that in the absence of ER α , AR may retain its function but have uncertain or even growth-promoting effects (AR co-localises with Ki67 in this context [1,19], which might indicate that antagonist

approaches are more appropriate in ER-negative tumours. However, it should be noted that AR overexpression may not be universally associated with good prognosis depending on clinical course of the disease (see also **Table 4.1B**); for instance, a recent *in vitro* study has shown that in AR/ER α overexpressing but tamoxifen-resistant cells, a dual inhibitory approach might be necessary in the resistant setting [37].

The results presented here pave the way for new, prospective clinical trials that take the biology and expression of AR into account and put it into the context of existing stratification approaches, such as ER α expression. Although unlikely to add much to existing prognostic algorithms and approaches, establishing a simple and robust diagnostic test with an appropriate cut-point will expedite studies using androgen pathway-targeting therapies.

To further investigate how the AR signalling axis might contribute to observable pathological phenotypes, the third part of the thesis explored the hypothesis that since AR signalling is associated with tissue turnover and anti-proliferative responses, especially during development, some of the risk associated with breast density might be associated with AR expression. As expected, AR expression was higher in malignant disease than benign disease, as seen throughout the three studies conducted in this thesis; it was therefore strongly associated with the malignant phenotype. However, AR expression was not associated with breast density under any circumstances, suggesting that the existing theory that breast density is more associated with cumulative exposure to estrogen might predominate as the underlying pathogenesis. Others have found that breast density is associated with ER α expression in the primary tumour [38-40], and although we did not find this to be the case, there was a trend towards an AR:ER α ratio less than one being associated with higher breast density and vice versa. This is consistent with the hypothesis that the relative levels of AR and ER signalling might influence the overall proliferative capacity of the tissue, and that when the anti-proliferative effect of AR signalling is reduced, breast density increases, at least in breast tissues harbouring malignant lesions. Together with the result presented in Chapter 4 regarding AR:ER α ratio and clinical outcome, these are strong supportive data to suggest that the relative levels of AR and ER signalling are biologically important, and consistent with the *in vitro* data.

Although the starting population of screening patients was large, the final number of biopsies analysed was relatively small, which may have weakened the power of the study. Furthermore, the detail of the benign histopathological diagnoses was not available, and therefore associations between AR expression and specific lesions and pathogeneses could not be established. It would be fascinating to know to what extent AR is expressed in benign lesions

and how AR signalling contributes to the development of benign or very early pre-neoplastic lesions.

In conclusion, the data presented in this thesis open up several further avenues for investigation. Application of the quantitative immunofluorescence double staining methodology to large clinical cohorts with documented clinical outcomes is likely to help reveal the significance and relative contributions of the co-expressing AR/ER α subpopulations to breast cancer pathogenesis and progression. If dominant sub-clones appear in late metastatic disease, these may be more specifically targeted and indicate that biopsy of the metastasis will be necessary, rather than basing the decision-making on primary tumour characteristics. There need to be further studies on the effects of both AR agonists and antagonists in pre-clinical models to establish exactly which tumour subtypes are likely to respond to various therapies; this will be facilitated by molecular profiling techniques, such as gene expression profiling and next generation sequencing, which could also be applied to specific AR/ER-expressing subpopulations in order to establish exactly what the role of AR is in different environments and contexts. Finally, AR expression needs to be investigated in suitable dynamic models of disease progression in order to establish exactly how different populations of cells within the tumour interact and change over time and in response to therapy. These data provide the starting point for these more advanced studies.

5.1 References

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