The Role of Androgens in Breast Cancer and a Lectin ELISA for cerbB-2/HER-2

MD THESIS

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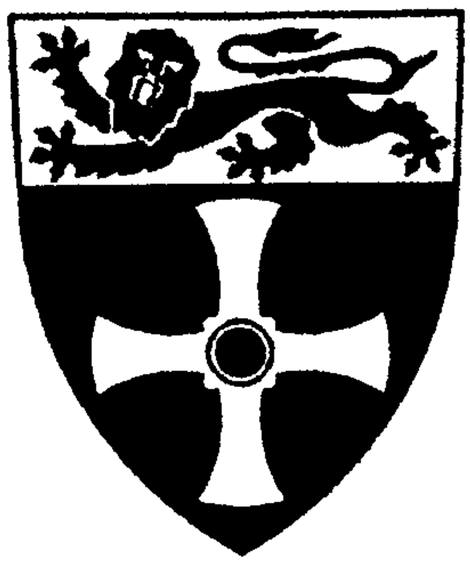

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Introduction

1.1 Steroid Hormones

Endocrinology is the science, which describes how cells communicate via the sending of chemical messengers termed hormones. There are three classes of hormones based on their chemical structure: peptide or protein hormones, steroid hormones and amino acid-related hormones.

There are six families of steroid hormones. They are oestrogens, androgens, progestins, mineralocorticoids, glucocorticoids and vitamin D. Bile acids are structurally related to cholesterol and therefore constitute a seventh member of the steroid family. All of these steroids are derived from cholesterol.

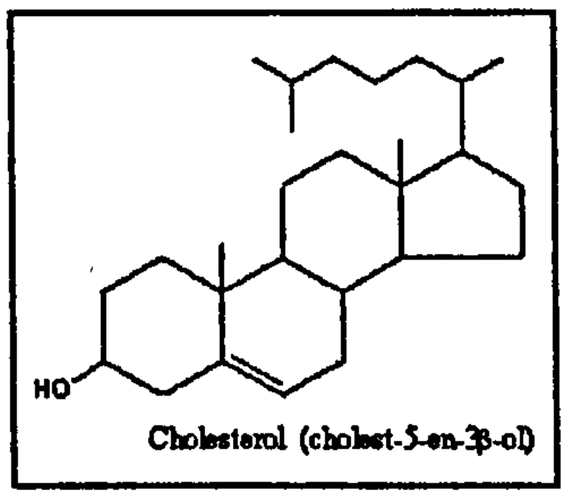


Figure 1.1: Structure of Cholesterol

Cholestane the fully saturated ring structure of cholesterol gives rise to the parent ring structures of mammalian steroid hormones oestrane, androstane, pregnane and cholane.

The principal tissues of synthesis of the five classical steroid hormones (oestrogens, androgens, progestins, glucocorticoids and mineralocorticoids) are the adrenal cortex, ovaries and the testis. The metabolic pathway for the conversion of cholesterol into steroid hormones is outlined below.

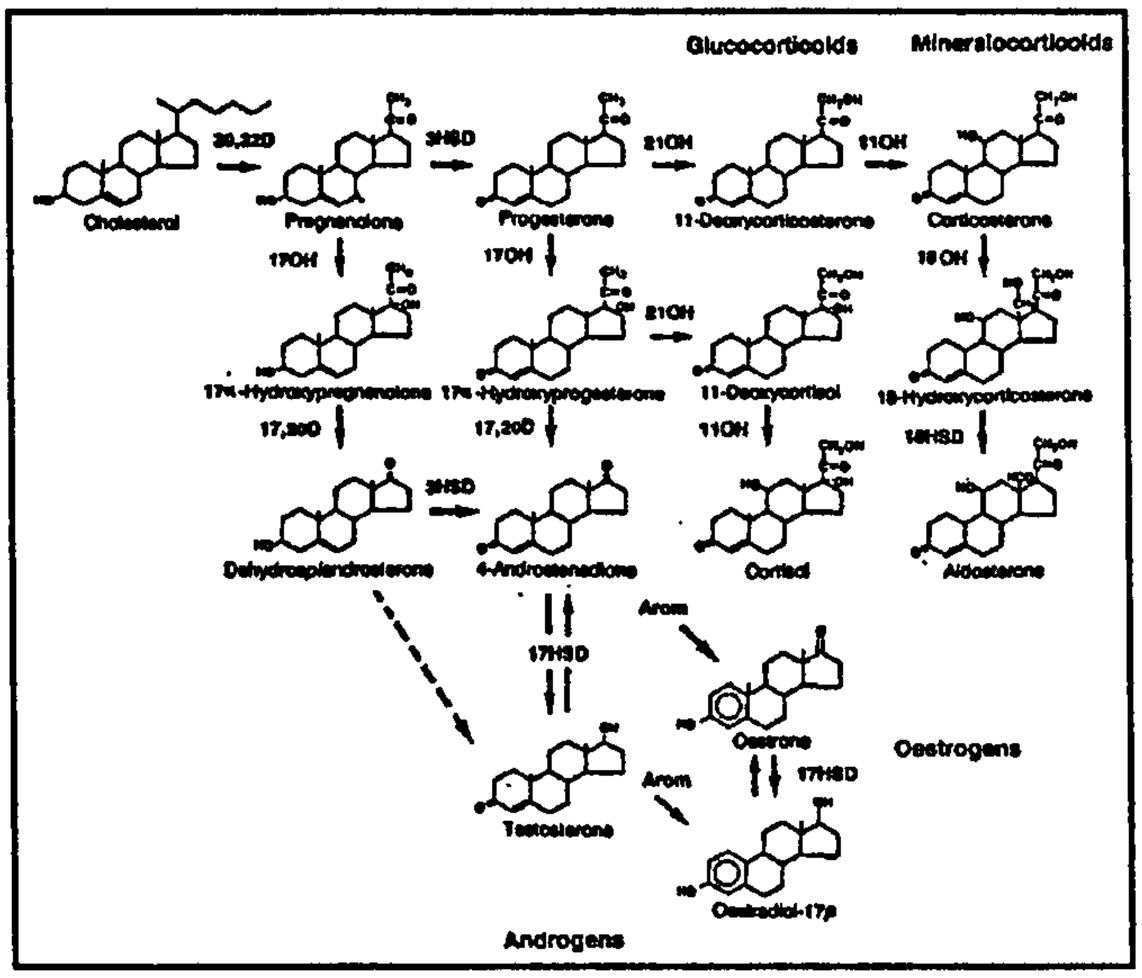


Figure 1.2: Steroid Metabolism

The critical step in the formation of steroids is the conversion of cholesterol to pregnenolone, which is under control of adrenocorticotrophin hormone (ACTH) in the adrenal and luteinising hormone in the gonads. Pregnenolone can either be converted to progesterone and via this intermediate to glucocorticoids, androgens or oestrogens. Alternatively 17-hydroxyprogesterone is formed from pregnenolone. This constitutes an alternative pathway formation of androgens or oestrogens (Norman *et al.* 1997).

The estrogens are 18-carbon steroids produced in the ovaries and fetal-placental unit. Oestrone and oestradiol are also produced from androstenedione and testosterone respectively in adipose tissue, muscle and breast tumours by the cytochrome p450 aromatase enzymes.

The adrenal cortex is divided into three zones that produce the different classes of the adrenal steroids. These are the *zona glomerulosa* (mineralocorticoids), *zona fasciculata* (glucocorticoids) and *zona reticularis* (androgens).

The androgens are all steroids with 19 carbons. The major naturally occurring steroids with androgenic activity are 5α –dihydrotestosterone (5α-DHT), testosterone, 5-androstene-3β,17β-diol (Adiol), androstenedione, dehydroepiandrosterone sulphate (DHEAS) and dehydroepiandrosterone (DHEA) .

1.2 Androgen receptor structure and functional domains

The androgen receptor is a member of the nuclear receptor superfamily of transcription factors. These proteins act as transcription factors in many different species and can be classified according to the type of ligand they bind: steroids (glucocorticoids, progestins, mineralocorticoids, androgens and oestrogens), steroid derivatives (vitamin D3) or non-steroids (thyroid hormones, retinoids, prostoglandins) (Tenbaum *et al.* 1997).

Nuclear receptors are related in structure and organization of their functional domains. They contain a highly conserved DNA binding region, a variable amino (N-) terminal and conserved carboxy (C-) terminal, suggesting that nuclear receptors are derived from a common ancestral form (Parker 1993).

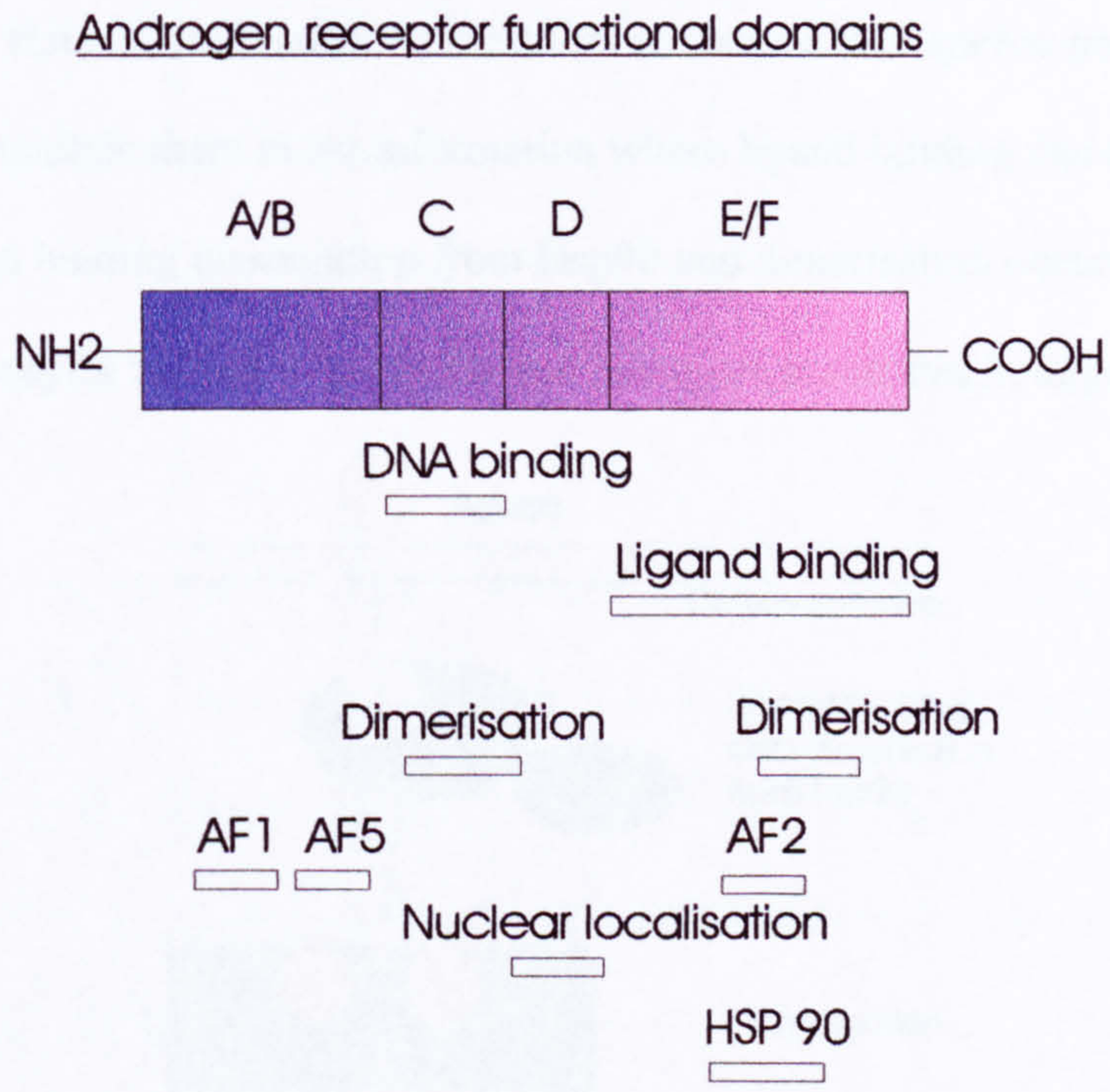


Figure 1.3: Androgen receptor functional domains

The highly conserved DNA binding domain is rich in cysteine residues and contains the zinc-finger motif required for binding to hormone response elements in target genes (Evans *et al.* 1988). This region also contains sequences responsible for dimerisation.

The C-terminus contains the hormone binding domain and amino acid residues responsible for interaction with the 90kD heat shock protein Hsp90 (Pratt *et al.* 1988) and a region involved in hormone dependent nuclear translocation of the androgen receptor (Picard *et al.* 1987).

In their inactive state, steroid hormone receptors are bound to chaperon proteins such as Hsp90, which maintain them in a conformation where ligand binding can occur. Following ligand binding dissociation from Hsp90 and dimerisation occurs allowing the ligand-bound receptor to bind to promoter and enhancer sequences in target genes.

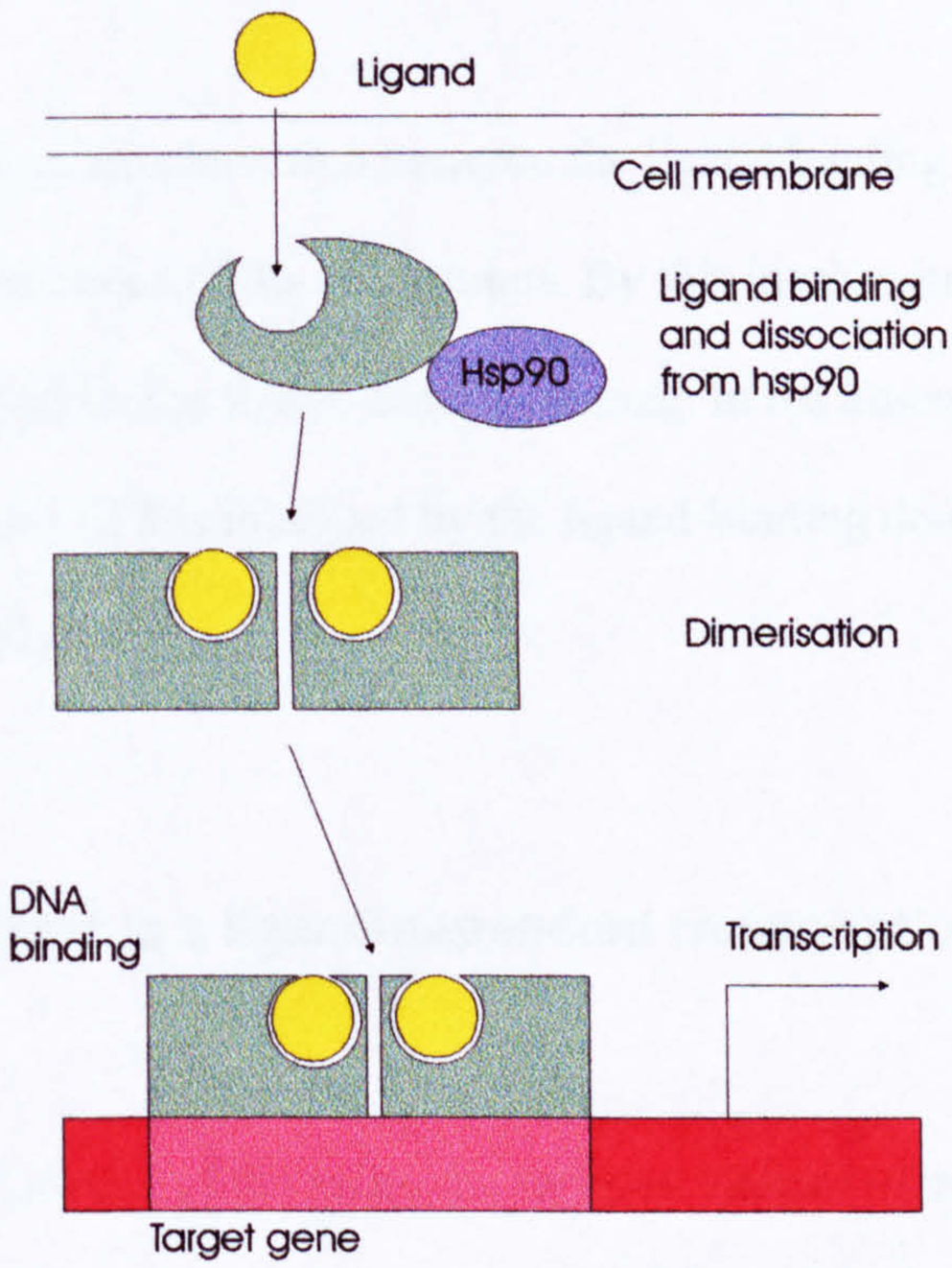


Figure 1.4: Ligand dependent activation of the androgen receptor

The involvement of additional trans-activation domains located on the C-terminus and N-terminus are required for full transcriptional activity of the steroid receptor. The trans-

activation function 2 (AF2) located on the C-terminus is activated on binding of ligand (Kumar *et al.* 1987). Point mutations in the AF2 domain have no effect on the binding of ligand, dimerisation or DNA-binding but abolish transcriptional activation.

Another trans-activation function AF1 is located on the N-terminus and is essential for transactivation activity in the full-length receptor (Jenster *et al.* 1991). More recently a third trans-activation function AF5, which is involved in the transactivation capacity of the constitutively active C-terminus truncated androgen receptor has been identified on the N-terminus (Jenster *et al.* 1995).

Recent evidence suggests an interaction between the ligand binding domain of the C-terminus and the AF functions of the N-terminus. By this mechanism the trans-activation function AF1 is inhibited by the ligand-binding domain in the absence of ligand and the trans-activation function AF5 is inhibited by the ligand-binding domain in the presence of ligand (Brinkman 1999).

1.3 Androgen receptor is a ligand-dependent transcription factor

The trans-activation domains of the androgen receptor interact with components of the basal transcription complex to initiate transcription. The basal transcription complex is composed of RNA polymerase II and various transcriptional factors such as TFIIB and TFIID, which assemble at the gene promoter and are essential for transcription to occur (Latchman 1997). It has become apparent, more recently, that another layer of proteins

called **co-activators** and **co-repressors** regulate transcriptional activity of hormone receptors by interacting with the basal transcriptional complex and the trans-activation function AF2 on the C-terminus of the hormone receptor.

The observations that steroid receptors could interfere with each other's function (Meyer *et al.* 1989) and possess a conserved region required for ligand-dependent transcriptional activity provided initial evidence of the existence of coactivator proteins that mediate AF2 function.

A large number of co-activator proteins have now been identified. Two families of co-activators are paramount in terms of ability to potentiate transcriptional activity of the nuclear receptor: the p160 family of co-activators including steroid receptor co-activator 1/ nuclear receptor co-activator 1 (SRC1/N-CoA1), p300/CBP co-integrator associate protein/ amplified in breast cancer 1 (p/CIF/AIB1) and transcriptional intermediary factor 2/ glucocorticoid receptor interacting protein 1 (TIF2/GRIP1) and the CREB binding protein/p300 (CBP/p300) family (Glass *et al.* 1997).

Studies demonstrated that overexpression of SRC1/N-CoA1 stimulated the transcriptional activities of steroid hormone receptors in response to their ligands (Onate *et al.* 1995). Subsequently, further proteins termed TIF2/GRIP1 and p/CIF/AIB1, highly homologous to SRC1/N-CoA1 were identified with a similar ability to potentiate the transcriptional activity of steroid hormone receptors (Voegel *et al.* 1996) (Anzick *et al.* 1997).

Overexpression of CBP/p300 also potentiates ligand-dependent transcriptional responses of nuclear receptors (Kamei *et al.* 1996). The CBP/p300 family of co-activators has been shown to act as essential co-activators for a large number of transcription factors including steroid receptors. It has been postulated that CBP/p300 may therefore act as an integrator of multiple signal transduction pathways, and competition for CBP/p300 may serve as a mechanism for antagonistic interactions between signaling pathways (Glass *et al.* 1997).

Shang *et al* have proposed the formation of an androgen receptor transcription complex in which agonist-bound androgen receptors, bound to promoter and enhancer DNA sequences, are linked by a **coactivator complex** containing p160 proteins, CBP/p300 and p300/CBP associated factor (pCAF) to the basal transcription machinery (Shang *et al.* 2002). PCAF is a coactivator known to possess separate sites for interaction with nuclear receptors, p160 proteins and CBP/p300 (Blanco *et al.* 1998). By this mechanism enhancer and promoter sequences are brought in close proximity, and communication occurs between enhancer and promoter via the coactivator complex.

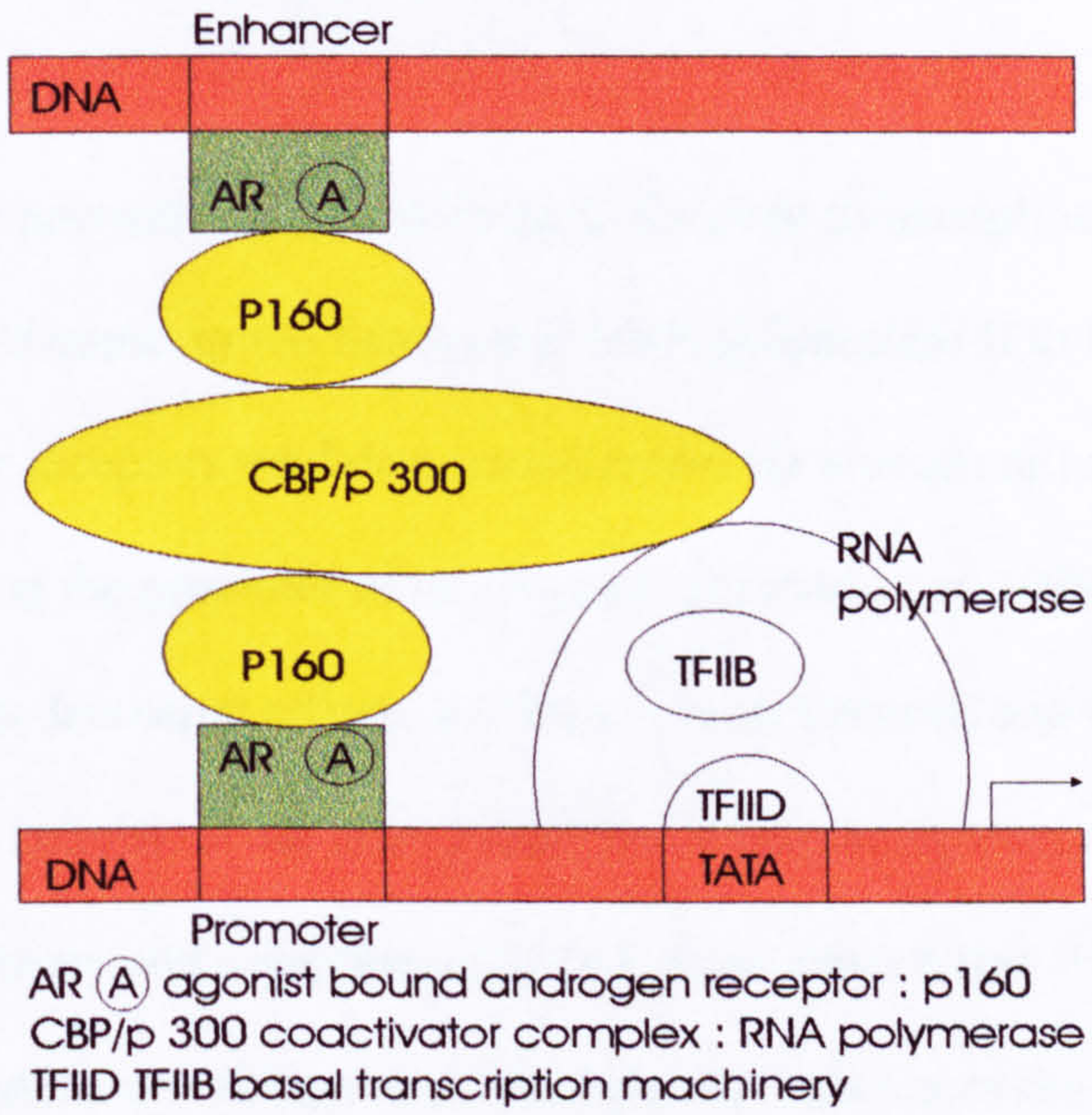


Figure 1.5: Coactivator Complex

A large number of nuclear hormone receptor coactivators that do not form part of this p160/p300/CBP/pCAF complex have now been identified including BRCA1 (Park *et al.* 2000), ARA70 (Yeh *et al.* 1996) and TIP60 (Brady *et al.* 1999). The roles of these proteins are unknown. It has been postulated that they may be specifically required for different target genes; they may be restricted to certain tissues or they may be selectively involved in some but not all, nuclear receptors.

In addition to proteins, which interact with nuclear receptors to enhance transcription, proteins, termed corepressors, have been discovered which repress transcription. Nuclear receptor corepressor (NcoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT) were originally identified to interact with retinoic acid and thyroid

hormone receptors in the inactive state (Chen *et al.* 1995). Subsequently it has been found that they are recruited by antagonist bound oestrogen receptors (Lavinsky *et al.* 1998).

Chromatin structure presents a physical obstacle for gene transcription, presumably by limiting the access of transcription factors and RNA polymerase II to the target DNA (Struhl 1998). Nuclear receptors modulate transcription via alterations in the state of chromatin structure at the promoter of target genes (Lemon *et al.* 1999). Histone acetylation results in decreased affinity between histone subunits and DNA and correlates with transcriptional activity. It has been found that coactivator proteins have histone acetylase (HAT) activity and corepressors have histone deacetylase (HDAC) activity. The balance between histone acetylation and deacetylation determines the outcome of ligand binding to nuclear receptor. Ligands act as switches to recruit HATs with the dismissal of HDACs (Xu *et al.* 1999).

A mechanism of ligand-dependent transcriptional activation can therefore be proposed. In the absence of ligand, steroid hormone receptors exist as inactive complexes with chaperone proteins such as Hsp90. Following hormone binding the complex dissociates, dimerisation occurs and the hormone receptor acts as a transcription factor by binding to DNA in the vicinity of target genes. Transcription is mediated by interaction of the trans-activation functions with the basal transcription complex. This process is modulated co-activator and co-repressor proteins, which act by adding or removing acetyl groups to chromatin, thereby altering the accessibility of target DNA to transcriptional factors and RNA polymerase.

1.4 Androgen receptor expression in breast cancer

Although measurement of oestrogen receptor is well established as prognostic and predictive factors in the management of breast cancer (Hawkins *et al.* 1980) the role of androgen receptors is less well defined.

Early studies measuring androgen receptor expression in breast cancer used ligand-binding assays. Androgen receptor expression was reported as being 35% (Miller *et al.* 1985), 84% (Lea *et al.* 1989) and 91.1% (Soreide *et al.* 1992). However, ligand-binding assays had technical limitations and did not provide any information about cellular distribution of the receptor.

More recently, following development of a monoclonal antibody to AR, immunohistochemical studies have been described and androgen receptors were reported to be present in 76% (Kuenen-Boumeester *et al.* 1996) (Kuenen-Boumeester *et al.* 1992) and 79% (Isola 1993). Indeed androgen receptor is reported as the most frequently expressed steroid receptor in breast cancer (Lea *et al.* 1989).

Kuenen-Boulmeester *et al* reported that 53% of breast cancers expressed AR, ER and PR (Kuenen-Boumeester *et al.* 1992). Several other groups have also found a significant association between AR and ER, PR expression in breast cancer (Lea *et al.* 1989) (Isola 1993) (Soreide *et al.* 1992). Only one study did not find any correlation between androgen receptor and oestrogen receptor expression, though they did report a correlation

between androgen receptor and progesterone receptor levels in breast cancer (Langer *et al.* 1990).

Whereas several studies have investigated the level of androgen receptor expression in breast cancer, none have hitherto looked at the variability of androgen receptor expression within individual tumours. However, androgen receptor, expressed as the sole hormone receptor, has been reported to occur 9% of breast cancers (Kuenen-Boumeester *et al.* 1992) and is better preserved during the process of metastasis than either oestrogen or progesterone receptor expression. Lea *et al* found that androgen receptors were present in 76% of metastatic breast cancer compared to 86% of primary breast tumours and that 25% of metastatic breast cancers have the AR+ER-PR- phenotype (Lea *et al.* 1989). In a multi-step model for the progression of breast cancer from a hormone-sensitive ER+PR+AR+ phenotype to a hormone-insensitive ER-PR-AR- phenotype, androgen receptor appears to be the last receptor to be lost.

In a study of 1026 patients with primary and recurrent breast cancer Lea *et al* found that androgen receptor concentration increased with age of the patient (Lea *et al.* 1989). Oestrogen receptor expression in breast cancer similarly increases with age of the patient. This is presumably an adaptation by up-regulation of the receptor apparatus to declining supply of circulating sex steroids found in postmenopausal women.

Several lines of evidence suggest that androgen receptor expression is a marker of good prognosis in breast cancer. In a large study of 1371 women with primary and recurrent

breast cancer, Bryan *et al* reported that patients with androgen receptor negative tumours had a significantly poorer response to hormonal therapy and a significant trend towards shorter overall survival, than those with androgen receptor-positive tumors (Bryan *et al.* 1984). Furthermore, androgen receptor expression was an independent prognostic factor for response to hormonal therapy and survival. Other groups have reported a positive correlation between androgen receptor expression, overall survival (Langer *et al.* 1990) and disease-free survival (Kuenen-Boumeester *et al.* 1996).

Studies investigating the relationship between androgen receptor and tumour grade have shown that androgen receptor expression is inversely correlated to histological grade (Kuenen-Boumeester *et al.* 1996) (Isola 1993) and S-phase fraction (Isola 1993).

In keeping with the observation that androgen receptor expression correlated with markers of good prognosis, it would have been expected that androgen receptor expression correlated inversely with lymph node metastases in breast cancer. However, the data on androgen receptor expression and lymph node status has been conflicting. Kuenen-Boumeester *et al* failed to show any significant association between immunohistochemically detected androgen receptor status and lymph node status (Kuenen-Boumeester *et al.* 1996). Indeed one group found a positive correlation between androgen receptor expression and the presence of axillary metastasis with multivariate analysis showing that androgen receptor content was an independent predictor of the likelihood of axillary metastases (Soreide *et al.* 1992).

There has only been one published report investigating a relationship between the levels of androgen receptor expression in breast cancer and circulating androgen levels. Langer *et al* in a study of 61 women with operable breast cancer found no significant correlation between serum testosterone, dehydroepiandrosterone sulphate (DHEAS) and tumour androgen receptor expression. However, they did find a significant positive association between serum androstenedione levels and androgen receptor expression (Langer *et al.* 1990).

1.5 Serum androgens levels in breast cancer

Several epidemiological studies have reported elevated adrenal androgen levels in the serum of postmenopausal women who subsequently developed breast cancer compared to controls (Cauley 1999) (Dorgan 1997) (Gordan 1990) (Zeleniuch-Jacqotte 1997). In a study comparing 97 postmenopausal women and 244 controls Cauley *et al* found significantly elevated serum androstenedione, DHEAS, and testosterone levels in women who subsequently developed breast cancer. However, in a statistical model that included bioavailable testosterone and oestradiol only bioavailable oestradiol was significantly related to breast cancer risk. This suggested that DHEAS and androstenedione contribute to the risk of breast cancer by acting as precursors to oestrogens (Cauley 1999).

Dorgan *et al* reported significantly elevated levels of DHEA, DHEAS and androstenediol in 71 postmenopausal women who subsequently developed breast cancer (Dorgan 1997). Gordon *et al* in a study of 30 postmenopausal women who developed breast cancer and

59 controls reported a significant association between DHEA and breast cancer risk and a non-significant association between DHEAS levels and breast cancer risk (Gordan 1990).

Zeleniuch-Jacquotte *et al* likewise reported that DHEAS were non-significantly elevated in 85 postmenopausal women who developed breast cancer compared to 163 controls (Zeleniuch-Jacqotte 1997). However, Barrett-Connor *et al* found no difference in DHEAS levels between women who developed breast cancer and controls (Barrett-Connor 1990).

Fewer studies have measured serum androgens in women with confirmed breast cancer. Secreto *et al* found that the risk of breast cancer was positively associated with serum levels of testosterone, DHEAS, androstenedione, and dihydrotestosterone in 75 women with breast cancer compared to 150 controls (Secreto 1991). Likewise, Zumoff *et al* reported supra-normal levels of DHEA and DHEA in postmenopausal women with primary operable breast cancer (Zumoff 1981). In this study, DHEA and DHEAS levels were found to be age invariant in women with breast cancer in contrast to a marked decline in levels of these androgens with age in controls.

Studies of adrenal androgen levels in premenopausal women who develop breast cancer have been less numerous. Several have reported subnormal levels of adrenal androgens in women with breast cancer compared to controls (Helzlsouer 1992) (Bulbrook 1986) (Zumoff 1981).

Helzlsouer *et al* measured serum DHEA and DHEAS in 15 premenopausal women who subsequently developed breast cancer. They reported that serum DHEA levels were lower in women who developed breast cancer, though there was no difference in DHEAS levels between cases and controls (Helzlsouer 1992). Zumoff *et al* reported subnormal levels of both DHEA and DHEAS in premenopausal women with breast cancer (Zumoff 1981). In a study of premenopausal women on the island of Guernsey, Bulbrook measured urinary metabolites of DHEAS (etiocholanolone and androsterone) in 110 women who developed breast cancer. Women who developed the disease in premenopausal years had significantly lower levels of these metabolites than age-matched controls (Bulbrook 1986).

In a review of the association between abnormal androgen production and breast cancer, Secreto and Zumoff summarize the evidence implicating excessive androgen production of ovarian origin in the aetiology of breast cancer (Secreto 1994). Polycystic ovarian syndrome, a condition characterised by anovulatory hyperplasia of the endometrium and elevated testosterone secretion is a risk factor for breast cancer (Coulam 1983). In addition elevated testosterone levels are found in women with abdominal obesity; such women are also at increased risk for breast cancer (Schapira *et al.* 1990).

Several investigators have found circulating levels of testosterone to be elevated in women with breast cancer (Malarkey 1977) (Zeleniuch-Jacqotte 1997) (Secreto 1991) (Sauter 2002) (Hill 1985) (Thomas 1997) (McFayden 1976) (Cauley 1999).

Five studies have found elevated circulating testosterone levels in postmenopausal women with breast cancer (Zeleniuch-Jacqotte 1997) (Cauley 1999) (Hill 1985) (Secreto 1991) (Thomas 1997). Only two measured serum testosterone in premenopausal women with breast cancer and found levels to be elevated compared to controls (Malarkey 1977) (Sauter 2002).

Whether circulating testosterone levels are independently associated with breast cancer risk is not established. Two studies have reported that the association of circulating testosterone levels to breast cancer risk is not independent of bioavailable oestrogen (Thomas 1997) (Zeleniuch-Jacqotte 1997). Whereas, only one has found that testosterone levels are an independent risk factor for breast cancer (Cauley 1999).

Several mechanisms have been proposed by which androgens may increase the risk of breast cancer, either directly or indirectly. We discuss in the next section mechanisms by which androgens may stimulate the proliferation of breast cancer cell lines via the oestrogen receptor, and these are well documented in the literature (Rochefort *et al.* 1984). On the other hand, androgens have been shown to inhibit oestrogen-induced proliferation of breast cancer cell lines by an androgen receptor-mediated mechanism (Poulin 1988) (MacIndoe 1981) (Rochefort *et al.* 1984).

Androgens may indirectly affect the growth of breast cancer by two mechanisms. They may be converted to oestrogens by the aromatization of testosterone and androstenedione (Longcope 1978). In addition, both oestradiol and testosterone are both bound in the

circulation to sex hormone-binding globulin (SHBG), however testosterone binds sex hormone-binding globulin with greater affinity than oestradiol (Brooks 1984). Elevated testosterone levels therefore indirectly increase bioavailable oestrogens by displacing oestradiol from sex hormone-binding globulin (Siiteri *et al.* 1982).

Recently, in a review of the role of adrenal androgens in breast cancer, Adams proposed that women who develop breast cancer in premenopausal years tend to have subnormal serum levels of adrenal androgens. Women who develop the disease in postmenopausal years have supranormal levels of these hormones. This he argues is due to androgens, acting via the androgen receptor, opposing oestrogen-stimulated growth in premenopausal years and stimulating growth via the oestrogen receptor in postmenopausal women (Adams 1998).

In contrast to adrenal androgens testosterone has been reported to be elevated in both pre and postmenopausal women with breast cancer (Secreto 1994). The reason for this is unknown. The major metabolite of testosterone, 5α-dihydrotestosterone, appears to inhibit the growth of breast cancer cell lines (Poulin 1988), though some groups have found that it can stimulate or inhibit the proliferation of breast cancer cell lines (Birrell 1995).

Testosterone may affect the proliferation of breast cancer by one of the mechanisms described above. In addition, Secreto and Zumoff have proposed that testosterone may stimulate the secretion of peptide growth factors such as epidermal growth factor which

act in a autocrine or paracrine loop to stimulate proliferation of breast cancer (Secreto 1994).

1.6 Growth actions of androgens on breast cancer cell lines

Birrell *et al* showed that androgens can both stimulate and inhibit growth of breast cancer cell lines (Birrell 1995). This effect appeared to be independent of the level androgen receptor expression by the breast cancer cell line. However, androgen receptor expression was necessary for the growth regulatory effects of androgens to be observed, as the growth of the two androgen receptor negative cell lines BT-20 and MDA-MB-231 was unaffected by androgens.

On investigating the effect of the naturally occurring androgen 5α-dihydrotestosterone (5α-DHT) and the synthetic, non-metabolized androgen mibolerone on six breast cancer cell lines, Birrell *et al* found that T47-D and ZR-75-1 were growth inhibited by androgens, while the growth of MCF-7 and MDA-MB-453 was stimulated by the presence of androgens. Co-incubation of 5α-DHT with the anti-androgen hydroxyflutamide resulted in reversal of inhibitory and stimulatory effects of 5α-DHT on T47-D, MCF-7 and MDA-MB-453 cell proliferation suggesting an androgen receptor mediated mechanism.

Birrell *et al* reported that stimulatory or inhibitory effects on growth were only observed after 6 days incubation with 5α-DHT, an observation that has been confirmed by other groups (Poulin *et al.* 1988). This is considerably longer than the time required by 17β-

oestradiol to exert its effects on cell proliferation, again suggesting different pathways for the action of androgens and estrogens on cell proliferation.

Two breast cancer cell lines MDA-MB-453 (Hall *et al.* 1994) and MFM-223 (Hackenberg *et al.* 1991) are unique in that they express high levels of androgen receptors in the absence of estrogen and progesterone receptors. However, although they have similar steroid receptor profiles they differ markedly in their response to androgens, MDA-MB-453 being growth stimulated and MFM-223 growth inhibited.

Why 5α-dihydrotestosterone can stimulate or inhibit the growth of cell lines with similar receptor profiles is unclear. Metabolism to oestrogenic substrates, which interact with the oestrogen receptor, is one possibility. This may explain the stimulatory action of 5α-dihydrotestosterone on MCF-7 cell line. MCF-7 cells possess glucuronyltransferase activity enabling 5α-DHT to be metabolized to compounds such as androstanediol (Roy 1992). Furthermore, androstanediol has been shown to stimulate the proliferation of MCF7 cells via interaction with the oestrogen receptor (Hackenberg 1993).

However, both MFM-223 and MDA-MB-453 cell lines do not express oestrogen receptors, and the growth effects of 5α-dihydrotestosterone can be inhibited by antiandrogens, suggesting an androgen receptor mediated mechanism. Clearly, there is a need for a more detailed understanding of the mechanisms of androgenic growth control in human breast cancer.

In a 12-day incubation of physiological doses of 5α-DHT with the ZR-75-1 breast cancer cell line, Poulin *et al* found that 5α-DHT inhibited cell proliferation and that the mitogenic effect of 17β-oestradiol on ZR-75-1 cells was completely inhibited by increasing concentrations of androgens (Poulin 1988). This anti-proliferative action was reversed by addition of the antiandrogen hydroxyflutamide suggesting that the effect was mediated by the androgen receptor and not by competition for binding with the oestrogen receptor.

In a study of the MCF7 breast cancer cell line, MacIndoe demonstrated that 5α-DHT inhibited oestrogenic induction and ongoing stimulation of progesterone receptor synthesis. Again this inhibitory activity of 5α-DHT was blocked by the addition of several anti-androgens, supporting the concept that the anti-oestrogenic effect of androgens is mediated by an androgen receptor mechanism (MacIndoe 1981).

In 1976 Lippman showed that 5α-DHT stimulated the growth of MCF7 breast cancer cell line. However, the concentration of 5α-DHT required to saturate the androgen receptor *in vitro*, was 1000 times lower than that which maximally stimulated MCF7 proliferation (Lippman 1976). If the mechanism of androgen action on the MCF7 cell line was due to androgens binding to the androgen receptor, then maximal stimulation would have occurred at 5α-DHT concentration sufficient to saturate the androgen receptor i.e. $1/1000^{th}$ of that observed.

There are two explanations of this anomaly. Firstly, 5α-dihydrotestosterone was metabolized to androstanediol by MCF7 cells, which stimulates proliferation via the oestrogen receptor, as discussed above, or 5α-dihydrotestosterone interacted directly with the oestrogen receptor.

While Zava *et al* also showed that 5α-DHT stimulated the growth of MCF7 cells at pharmacological doses (10^{-6}M) they found no effect on cell growth at physiological doses (10^{-8}M), despite 5α-DHT translocating the androgen receptor to the cell nucleus at this lower concentration. In contrast to Lippman's group, Zava *et al* found that 10^{-6}M 5α-DHT competed with oestradiol for binding to the oestrogen receptor, translocated oestrogen receptor to the cell nucleus and stimulated progesterone synthesis.

These findings suggested that the growth effects of 10^{-6}M 5α-DHT on MCF7 cells were mediated by direct interaction of 5α-DHT with the oestrogen receptor. The effects of 10^{-6}M 5α-DHT on cell growth, receptor depletion and progesterone synthesis was almost comparable to 10^{-8}M 17β-oestradiol and the affinity of 5α-DHT for the oestrogen receptor was estimated as approximately 1000-fold less than oestradiol (Zava 1978; Zava 1978). The above discussion has been restricted to 5α-dihydrotestosterone, an androgen with a high affinity for the androgen receptor. However, as discussed elsewhere, there are a large number of metabolically related androgens with a variety of androgenic and oestrogenic activities (Rochefort *et al.* 1984). The metabolism of androgens and interactions with the androgen and oestrogen receptors are illustrated below.

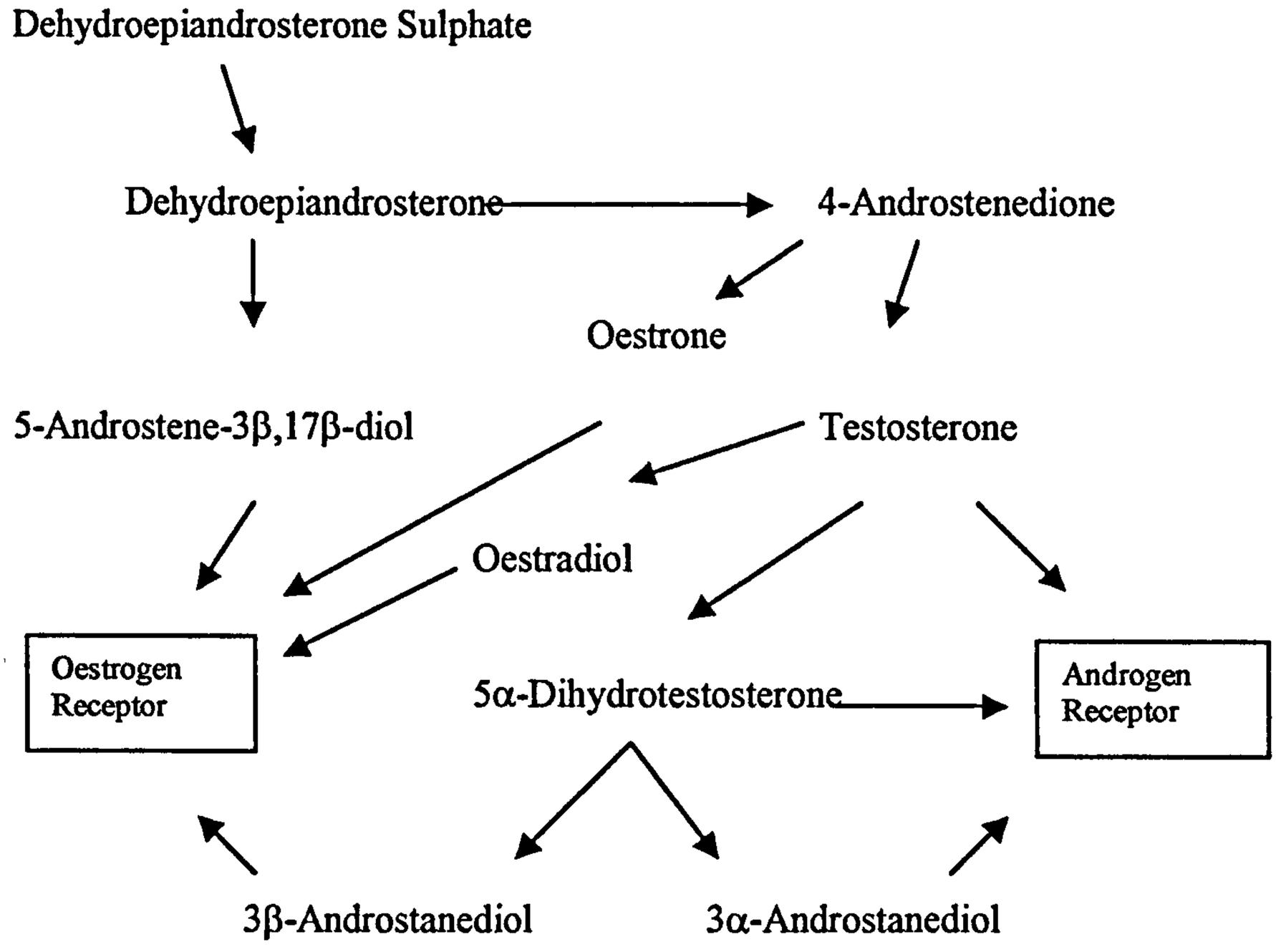


Figure 1.6: Androgen Metabolism in peripheral tissues

Rochefort and Garcia (Rochefort *et al.* 1984) divide androgens into three groups:

1) Androgens, such as 5α-dihydrotestosterone and testosterone, with a high affinity for the androgen receptor
2) Androgens, such androstenedione and testosterone which are converted to oestrogens by aromatization
3) Androgens of adrenal origin such as dihydroepiandrosterone sulphate (DHEAS), androstenediol and androstanediol which have some affinity for the oestrogen receptor

While 5α-dihydrotestosterone stimulates proliferation and induces progesterone receptor expression at pharmacological concentrations (Zava 1978), adrenal androgens produce similar effects at physiological concentrations.

5-androstene-3β,17β-diol a metabolite of DHEA has been shown to stimulate the proliferation of hormone dependent breast cancer cell lines at physiological concentrations via interaction with the oestrogen receptor (Adams *et al.* 1981) (Poulin *et al.* 1986) (Hackenberg 1993).

Similarly, DHEAS has oestrogen-like actions on MCF-7 and T47D breast cancer cell lines at physiological concentrations (LeBail *et al.* 1998) (Poulin *et al.* 1986) and DHEA stimulates MCF-7 breast cancer cell lines by a similar mechanism (Boccuzzi *et al.* 1992) (Poulin *et al.* 1986).

In a recent study of the oestrogenic activity of androgens LeBail *et al* found that DHEA, DHEAS, androstenediol, androstanediol, androstenedione, 5alpha dihydrotestosterone and testosterone were all able to elicit oestrogen-like actions on MCF-7 and T47D breast cancer cell. However, only DHEAS demonstrated oestrogenic activity at physiological concentrations (LeBail *et al.* 1998). This is in disagreement with other groups who have found that androstenediol not DHEAS has oestrogenic activity at physiological concentrations (Poulin *et al.* 1986).

Adrenal androgens have been shown to inhibit the growth of the MFM-223 breast cancer cell line, by an androgen receptor mediated mechanism (Hackenberg 1993). 5-Androstene-3β,17β-diol and DHEA have likewise been shown to inhibit oestrogen-stimulated growth of MCF7 cells by an androgen receptor mediated mechanism (Boccuzzi *et al.* 1993) (Boccuzzi *et al.* 1994).

The action of adrenal androgens depends on the endocrine environment: adrenal androgens stimulate the growth of hormone dependent breast cancer in the absence of oestrogens, and inhibit growth in the presence of oestrogens. These actions occur by the oestrogen and androgen receptor respectively and may underlie the findings of several studies that prediagnostic levels of adrenal androgens are elevated in postmenopausal women with breast cancer and reduced in premenopausal women with breast cancer (Adams 1998).

1.7 Insulin-like growth factor system in breast cancer

Insulin growth factor receptor 1 (IGFR1) is a member of the tyrosine kinase receptor superfamily and shares 70% homology with the insulin receptor (IR) (Ullrich *et al.* 1986). It is ubiquitously expressed in human tissues and has been implicated in mitogenesis, cellular transformation, survival and differentiation (Rubin *et al.* 1995).

IGFR1 expression is elevated in breast cancer compared with non-malignant tumours or normal breast epithelium (Peyrat *et al.* 1992) and the ligands for IGFR1 (IGFI and IGFII)

are potent mitogens for human breast cancer cell lines (Furlanetto *et al.* 1984). A substantial body of evidence implicates the IGF system as a key growth regulatory pathway in human breast cancer, which may be a therapeutic target for novel breast cancer therapy (Lee *et al.* 1998).

Oestradiol has been shown to upregulate the expression of IGFR1 mRNA in MCF7 breast cancer cell line (Stewart *et al.* 1990) as well as downstream molecules in the IGF pathway such as insulin receptor substrate-1 (IRS-1) (Lee *et al.* 1999). These results suggest that an important mechanism, by which oestrogens stimulate the proliferation of hormone-dependent breast cancer involve sensitization to the proliferative effects of IGFs. Oestadiol and IGFs have been shown to act synergistically to stimulate the proliferation of breast cancer cell lines (Stewart *et al.* 1992).

Activation of IGFR1 results in oligomerization, autophosphorylation and activation of tyrosine kinase (Ullrich *et al.* 1986). IGFR1 is coupled to downstream signaling molecules insulin receptor substrate –1 (IRS-1) and Src homology 2 containing sequence, which are phosphorylated by activated tyrosine kinases and interact with multiple signal pathways including the MAPkinase pathway (Myers *et al.* 1994) (Pelicci *et al.* 1992). Thus providing a pathway for the interaction between IGFR1 and steroid hormone receptors, to be discussed later.

Several large studies have reported no significant associations between IGFR1 expression and a variety of prognostic parameters including tumour grade, lymph node status, and

disease-free and overall survival (Surmacz *et al.* 1998). However, it is established that IGFR1 is co-expressed with oestrogen receptor in human breast cancer (Peyrat *et al.* 1988) (Pekonen 1988). Despite ongoing investigations, the exact role of the IGF system in the progression of human breast cancer remains obscure.

1.8 Epidermal growth factor system in breast cancer

Epidermal growth factor receptor (EGFR) is a cell surface receptor with tyrosine kinase activity, which has shown to be the cellular homologue of the v-erbB2 oncogene (Downward *et al.* 1984). Like the IGF system an interaction between the EGF system and steroid hormone receptors occurs. EGF activates the oestrogen receptor via the MAPKinase pathway resulting in phosphorylation of the ser^{118} in the transactivation domain AF1 located in the N-terminus (Bunone *et al.* 1996).

EGFR is important in the growth regulation of normal breast and can be detected in all samples of normal breast (Dittardi *et al.* 1993). However, it can be identified only in approximately one third of human breast carcinomas, where it is associated with high tumour grade (Walker *et al.* 1999), poor response to endocrine therapy (Nicholson *et al.* 1989), and is inversely associated with oestrogen receptor (Sainsbury *et al.* 1985) (Pekonen 1988) (Walker *et al.* 1999).

EGFR has been implicated in the progression of human breast cancer to hormone independence. Oestrogen has been shown to have a biphasic effect on EGFR expression

in breast cancer cell lines. In the presence of oestrogen, there is a rapid and transient up-regulation of EGFR expression, followed by a down-regulation to basal levels, which is dependent on protein synthesis. This suggests that hormone-dependent breast cancer cells possess active mechanisms to maintain low levels of EGFR (Yarden *et al.* 1996).

WalkerRA *et al* have proposed that in a proportion of patients lacking oestrogen receptors this regulation is lost, leading to an upregulation of EGFR. Overexpression of EGFR is therefore a result, not a cause of hormone independence (Walker *et al.* 1999).

Chrysogelos *et al* proposed a multistep model for the progression of breast cancer from a hormone dependent ER+/EGFR- phenotype to a hormone independent ER-/EGFR+ phenotype (Chrysogelos *et al.* 1994).

1.9 Cross-talk between steroid hormone and growth factor signal transduction pathways in cancer

The evidence from a large number of studies has established that growth factor signal transduction and steroid hormone pathways interact. This interaction is termed **"crosstalk"**. Power *et al* first showed that dopamine, an extracellular ligand was able to stimulate transcriptional enhancement of oestrogen receptor (Power *et al.* 1991). Subsequently it was shown that epidermal growth factor (EGF) induced oetrogen-like effects on mouse reproductive tract, which were attenuated by administration of an oestrogen receptor antagonist (Ignar-Trowbridge *et al.* 1992).

These results suggested that EGF induced these effects by an interaction between the EGF signaling pathway and the oestrogen receptor. More recently it has been confirmed that the oestrogen-like effects of EGF in the mouse uterus require the oestrogen receptor. Curtis *et al* showed that EGF treatment induced DNA synthesis and progesterone receptor mRNA in wildtype but not oestrogen receptor knockout (ERKO) mice (Curtis *et al.* 1996).

EGF was also shown to modulate the transcriptional activity of oestrogen responsive elements (EREs) in endometrial and ovarian carcinoma cells. In this study, it was demonstrated by the use of truncated steroid hormone receptors, that EGF interacted with the N-terminus of the oestrogen receptor (Ignar-Trowbridge *et al.* 1993).

Crosstalk between peptide growth factors and oestrogen receptor is not restricted to EGF. Insulin-like growth factor-1 (IGF-1) has also been shown to mediate oestrogen receptor-mediated transcription in the rat uterus (Aronica *et al.* 1993) and the MCF7 human breast cancer cell line (Katzenellenbogen *et al.* 1990).

In addition agents, which increase cyclic adenosine monophosphate (cAMP), are able to stimulate oestrogen receptor-mediated transactivation in the rat uterus (Aronica *et al.* 1993) and protein kinase activators act synergistically with oestradiol to increase oestrogen-receptor mediated transcription (Cho *et al.* 1993).

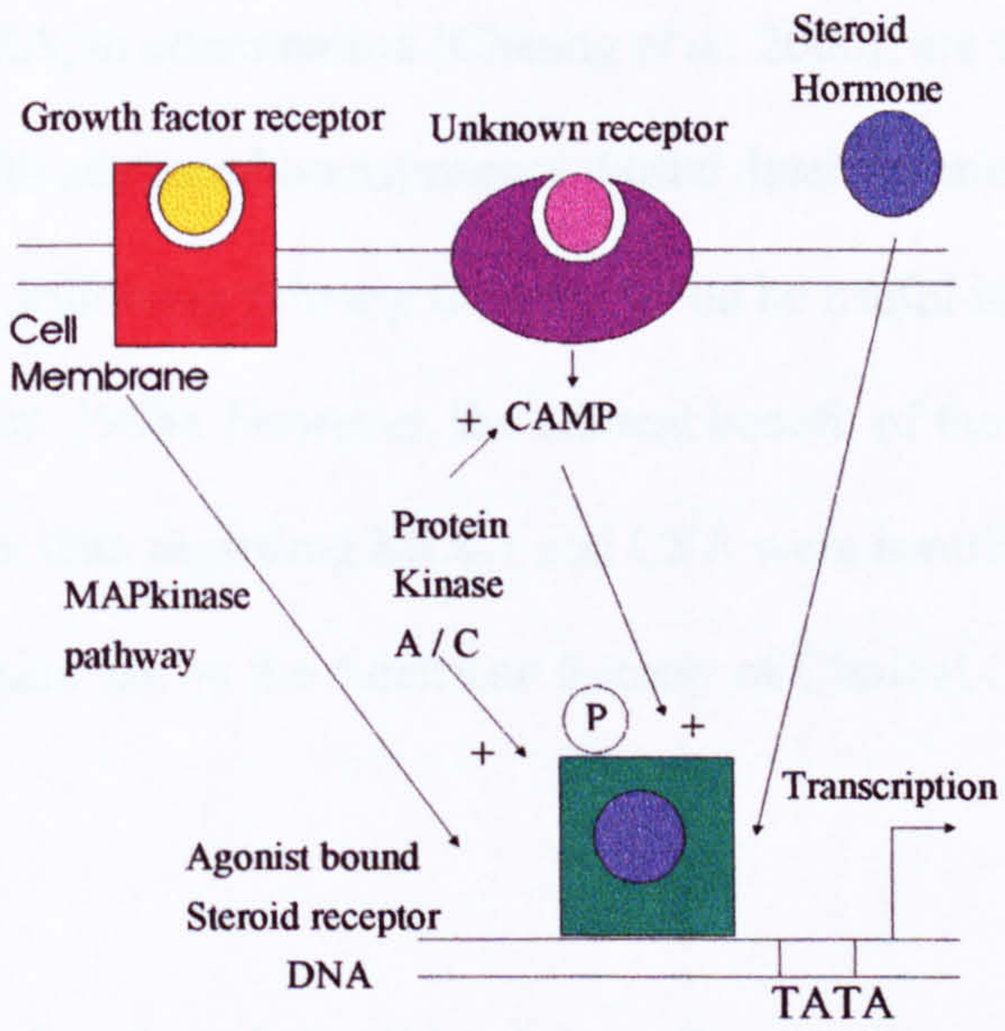
Synergy exists between growth factor and oestrogen-mediated proliferation of breast cancer cells. Oestradiol was shown to increase the proliferative effects of a number of growth factors in MCF7 cells (Stewart *et al.* 1992). IGF-binding protein-1 (IGF-BP-1) an inhibitor of IGF-1 action, inhibits not only IGF-1 mediated activation of oestrogen receptor, but also oestradiol mediated action in MCF7 cells (Lee 1997). Similarly, anti-oestrogens partially inhibited IGF-1 mediated growth of MCF7 cells in addition to completely inhibiting oestradiol mediated growth (Wakeling *et al.* 1988). Several reports have provided evidence that oestrogen can affect EGF and IGF action *in vitro* and *in vivo* (Dickson *et al.* 1987).

The growth factor and oestrogen-mediated growth factor pathways are interrelated in breast cancer. The mechanism of crosstalk appears to be bi-directional, with each signaling pathway positively influencing the other, resulting in reinforcement of both signaling pathways. The insulin-like growth factor pathway is required for maximal oestrogen-mediated stimulation of growth and *vice versa* (Yee *et al.* 2000).

In prostate cancer, there is evidence supporting a similar interaction of growth factor and androgen receptor pathways in the control of prostate cancer growth. IGF-1, EGF and keratinocyte growth factor (KGF) have been shown to activate androgen receptor-mediated transcription in the absence of androgens in the prostate cancer cell line DU-.145 transfected with androgen receptor (Culig 1994). Again, a mitogen activated protein kinase (MAPK) pathway has been identified which phosphorlates ser^{513} on the N-terminus of the androgen receptor activating androgen receptor mediated transcription in

response to peptide growth factors (Abreu-Martin *et al.* 1999). Like the oestrogen receptor the androgen receptor can also be activated by agents, which increase intracellular cAMP levels, in the absence of androgens. This effect was inhibited by protein kinase A inhibitor and anti-androgen, suggesting that activation of androgen receptor may occur through a protein kinase A signaling pathway in prostate carcinoma (Nazareth *et al.* 1996)

Further studies were undertaken to investigate the mechanism of signal transduction pathway responsible for ligand-independent activation of the oestrogen receptor. These showed that IGF-1 elicited only weak transcriptional activation in the presence of a deletion mutant that lacked the N-terminus. This suggested that ligand-independent activation of oestrogen receptor transcription by peptide growth factors is mediated through AF1 by a mechanism distinct from oestradiol and protein kinase C (Ignar-Trowbridge *et al.* 1996). It has subsequently been shown that transcriptional activation of the oestrogen receptor by EGF and IGF is mediated through the membrane- associated receptor tyrosine kinase-Ras-Raf-MAPK cascade, which enhances the activity of oestrogen receptor AF1 by phosphorylating Ser^{118} residue located in the N-terminus (Kato *et al.* 1995).



P = Phosphorylation by MAPkinase pathway

Figure 1.7: Ligand independent activation of the androgen receptor

1.10 Tumour Markers in Breast Cancer

The term "**tumour marker**" embraces a spectrum of molecules of widely divergent characteristics, but sharing an association with malignancy that facilitate their application in the clinical detection (diagnosis, screening) and management (monitoring, prognosis) of cancer patients (Sturgeon *et al.* 1999).

A large number of tumour markers exist for breast cancer, including the glycoprotein encoded by the MUC1 gene; carcinoembryonic antigen (CEA); tissue polypeptide antigen (TPA) and tissue polypeptide specific (TPS) antigen, which are soluble markers of cytokeratin 18; and oncoproteins such as HER-2.

MUC1 antigen and CEA, in combination (Cheung *et al.* 2000), are the most useful serum markers in patients with advanced breast cancer. Serial determinations of these markers may detect recurrence following primary treatment, and be useful in monitoring response to therapy (Stearns *et al.* 1998). However, the clinical benefit of their use is not established and present data regarding MUC1 and CEA were insufficient for them to be recommended for routine use by the American Society of Clinical Oncology (Bast *et al.* 1996).

Although a tumour marker that distinguishes benign from malignant breast disease would be very useful, no tumour marker is sufficiently sensitive to be recommended for the diagnosis and screening of breast cancer (Sturgeon *et al.* 1999). The detection of elevated serum HER-2 is unlikely to be useful as a tool for the diagnosis of breast cancer as it is only elevated in a subgroup of patients who over-express HER-2 at the tissue level (Breuer *et al.* 1993).

Serum HER-2 may be useful for the monitoring of tumour recurrence in patients with HER-2 over-expressing primary tumours (Isola *et al.* 1994) and predicting resistance to hormonal therapy (Harris *et al.* 1999). However, the most promising use of serum HER-2 would appear to be in monitoring response to **herceptin** therapy, the recombinant humanized monoclonal antibody against HER-2. Though further studies are needed to fully evaluate the role of serum HER-2 for this purpose (Cheung *et al.* 2000).

1.11 Retroviruses, proto-oncogenes and oncogenes

One common feature of all types of cancer, regardless of tissue of origin, is a loss of normal growth control mechanisms. This results in unchecked proliferation of neoplastic cells, which contributes to tumour formation. Rous first hypothesized the existence of an infectious agent, which could cause tumour formation in chickens this led to the discovery of the Rous sarcoma virus, a transforming retrovirus (McKenzie 1991).

During the life cycle of a retrovirus genetic material from the host is incorporated into its genome, in some cases modifying the gene and creating an **oncogene**. Retroviruses that can induce tumours in infected animals carry sequences responsible for their oncogenesis. These nucleotide sequences are called oncogenes and are derived from normal cellular genes called **proto-oncogenes.** These precursors to oncogenes, termed proto-oncogenes, are intimately involved in the control of cellular proliferation and differentiation and encode growth factors, growth factor receptors, signal transducers or nuclear receptors (McKenzie 1991).

Genetic mutations acquired en route from proto-oncogene to oncogene, which release the gene product from its normal cellular control mechanisms maybe responsible for the transforming activity of oncogenes (Bishop 1987). **Avian erythroblastosis virus** a retrovirus that induces erythroid leukaemia and sarcomas in infected chickens contains the **v-erbB oncogene** responsible for its transforming activity (Graf *et al.* 1978).

1.12 HER-2 proto-oncogene and protein in breast cancer

Comparison of the structure of the AEV transforming gene v-erbB with the human epidermal growth factor receptor revealed close sequence homology (Yamamoto *et al.* 1983). The predicted v-erbB transforming protein is a truncated form of the epidermal growth factor receptor, which contains the transmembrane and cytoplasmic domains, but lacks the extracellular EGF binding domain. It is postulated that the absence of the EGF binding domain might remove the control generated by ligand binding and results in the continuous generation of a signal (or constitutive activation) equivalent to that produced by EGF, causing the cells to proliferate rapidly (Downward *et al.* 1984).

Another proto-oncogene, termed ***neu***, identified originally in ethinylnitrosurea-induced rats (Shih *et al.* 1981) is also related to v-erbB. Using v-erbB as a screening probe (Coussens *et al.* 1985) a gene on q21 of chromosome 17 has been isolated which encodes a cell surface receptor with tyrosine kinase activity, now known to be the human counterpart of *neu*, termed **HER-2** (human epidermal growth factor receptor 2) or **cerbB-2** (cellular erbB2).

HER-2 encodes a transmembrane receptor of 185kDa with intrinsic tyrosine kinase activity termed **p185** (Stern *et al.* 1986), which is highly homologous but distinct from the EGFR (Bargmann *et al.* 1986) (Yamamoto *et al.* 1986). We now know that HER-2 belongs to a family of closely related growth factor receptors including EGFR, HER-3 and HER-4, identified through their homology with the transforming gene v-erbB. This

family encodes receptor tyrosine kinases possessing a cysteine-rich extracellular domain, a transmembrane domain and an intracellular domain possessing tyrosine kinase activity (Sahin 2000).

A point mutation in the transmembrane domain of rat *neu* gene has been identified which results in activation of the gene. This results in an amino acid substitution, which presumably alters the protein confirmation such that the tyrosine kinase becomes constitutively active (Bargmann *et al.* 1986). No such mutation has been reported in its human counterpart HER-2.

Activated HER-2, as well as interacting with other members of the epidermal growth factor family to form homodimers or heterodimers (Dougall *et al.* 1994), interacts with a variety of downstream substrates. These downstream molecules include mitogen-activated protein (MAP) kinase, Src and PI 3-kinase. MAP kinase is a pathway involved in the growth and transformation of many cell systems, which is overexpressed in malignant breast tissue: Src a nonreceptor tyrosine kinase which has also been implicated in mammary tumorigenesis; and PI 3-kinase a pathway that regulates cell death machinery (Hung *et al.* 1999).

One of the mechanisms implicated in the multistep process of tumorigenesis involves the amplification of one or more proto-oncogenes and the concomitant over-expression of its protein product (Weinberg 1989). Amplification of a normal HER-2 gene rather than

mutation to an abnormal gene appears to activate HER-2 in human breast cancer (Slamon *et al.* 1989).

King *et al* first identified HER-2 amplification in a breast cancer cell line (King *et al.* 1985). Subsequently, Slamon *et al* reported that HER-2 was amplified in 25-30% of human breast cancers. Amplification of HER-2 was found have prognostic significance for disease-free and overall survival in the lymph node positive group in this study (Slamon *et al.* 1987). Review of data from studies published to date, suggest that HER-2 is amplified or over-expressed in 10-34% of human breast cancers (Ross *et al.* 1999).

Following reports of the amplification of HER-2, several groups investigated the significance of HER-2 protein product expression in breast cancer. Amplification of HER-2 and over-expression of the HER-2 protein-product are significantly correlated in breast cancer (Venter *et al.* 1987). However some tumours, which contain only a single copy of the HER-2 gene were found to express high levels of the HER-2 protein product, suggesting that mechanisms other than gene amplification may lead to enhanced gene expression (Tandon *et al.* 1989).

HER-2 over-expression in breast cancer has been found to be an important independent prognostic factor in breast cancer, only lymph node status being a greater predictor of disease-free and overall survival (Paik *et al.* 1990) (Wright *et al.* 1989). Like HER-2 amplification, a large number of studies have reported a significant correlation between HER-2 over-expression and reduced disease-free and overall survival in lymph node

positive patients (Sahin 2000). However, although some have reported similar findings in lymph node negative patients (Guterson *et al.* 1992), the most studies have found no correlation between HER-2 status and outcome in this group (Slamon *et al.* 1989; Tandon *et al.* 1989; Clark *et al.* 1991).

In studies of an association with biomarkers of poor differentiation HER-2 over-expression has been shown to correlate with lack of oestrogen and progesterone receptors and high nuclear grade in human breast cancer (Berger *et al.* 1988) (Tandon *et al.* 1989) (Paik *et al.* 1990) (Wright *et al.* 1989).

Review of studies implicating HER-2 as a predictive marker of response to breast cancer therapy suggest that HER-2 over-expression may be linked to resistance to CMF-based (cytoxan, methotrexate, 5-fluorouracil) therapy and an enhanced response to high-dose doxorubicin regimens. More studies are required to confirm these findings before clinical decisions based on HER-2 status can be made regarding adjuvant chemotherapy (Pegram *et al.* 1998).

In contrast, several studies have shown that HER-2 over-expression predicts poor response to hormonal therapy (*i.e.* tamoxifen) (Pegram *et al.* 1998). The mechanism of hormone resistance in HER-2 over-expressing breast cancer has been the subject of intense laboratory investigation. Data from these studies suggest that overexpression or ligand activation of HER-2 results in functional signaling between HER-2 receptor

tyrosine kinase and oestrogen receptor (Pietras *et al.* 1995). However, to date no ligands that directly bind to HER-2 protein have been clearly identified (Hung *et al.* 1999).

HER-2 receptor protein is an ideal therapeutic target as it is expressed in primary and metastatic tumours, and its expression is low in normal tissues (Press *et al.* 1990). The anti-HER2 antibody, which is directed against the extracellular domain of HER-2 was humanised by inserting its murine antigen-binding region into the framework of a human immunoglobulin molecule (Carter *et al.* 1992).

The recombinant humanised monoclonal antibody, called **trastuzumab** or **herceptin,** inhibits growth in breast cancer cell lines that over-express HER-2 (Hudziak *et al.* 1989) and increases the response rate when given alone (Cobleigh *et al.* 1998) or in combination with chemotherapy (Slamon *et al.* 1998) in HER-2 positive advanced breast cancer.

1.13 Serum cerbB-2

Zabrecky *et al* detected, using two monoclonal antibodies specific for the extra-cellular domain of the HER-2 protein product, a protein of molecular mass 105kDa in conditioned media from cultures of SK-BR-3 cells (a breast cancer cell line known to express HER-2). They proposed that this molecule was the extra-cellular domain of HER-2 and SK-BR-3 cells release this portion of the receptor into its growth medium, and its detection could prove useful as a tumour marker (Zabrecky *et al.* 1991).

Growth signal transduction by HER-2 appears to involve receptor dimerisation and increased tyrosine kinase activity (Dougall *et al.* 1994). This is accompanied by release of extra-cellular domain of HER-2 and its accumulation in the extra-cellular environment. Thus accumulation of the extra-cellular domain of HER-2 in the extra-cellular environment represents a potential marker of uncontrolled growth signal transduction at the cellular level (Brandt-Rauf 1995). The mechanism of release of the soluble extra-cellular domain of HER-2 is believed to involve proteolytic cleavage from the surface of tumour cells. The release of the extra-cellular domain generates a truncated membrane receptor, which is constitutively activated (Pupa *et al.* 1993).

It was subsequently shown that a similar antigen is shed into the serum of nude mice bearing tumours that over-express HER-2. In addition, increasing antigen levels correlated with over-expression of HER-2 and increasing tumour volume. The utility of such an assay to detect HER-2 for the diagnosis and monitoring of patients with breast cancer was therefore proposed (Langton *et al.* 1991).

Studies of serum HER-2 levels in human breast cancer have shown that they are elevated in 23% of patients with locally advanced or metastatic disease (Leitzel *et al.* 1992). Other groups have confirmed that serum HER-2 levels are higher among patients with recurrent or metastatic breast cancer who have an increased tumour burden (Yasasever *et al.* 2000) (Isola *et al.* 1994). Though results from other groups have been conflicting (Brandt-Rauf 1995) suggesting that there is no association between tumour stage and serum HER-2 (Harris *et al.* 1999).

Elevated serum HER-2 levels were found almost exclusively among patients whose tumours expressed HER-2 (Isola *et al.* 1994). In addition serum HER-2 levels were found to return to normal values following removal of the tumour (Breuer *et al.* 1993), strongly suggesting that the source of the increased serum HER-2 was the tumour. It was therefore suggested that serum HER-2 might prove useful as a tumour marker in the monitoring of patients with HER-2 over-expressing primary tumours for recurrence (Isola *et al.* 1994) (Harris *et al.* 1999).

Commercially available kits are now available to measure serum HER-2. They are enzyme linked immunosorbent assays (ELISAs) using the two-site double antibody sandwich principle, i.e. they employ an antibody to the extracellular domain of HER-2 to capture the molecule from the serum and another antibody, directed to a different epitope on the ECD of HER-2, as a marker. This second antibody is usually conjugated to an enzyme, such as horseradish peroxidase which catalyses a colour change in a substrate. Chiron® and Oncogene Science® manufacture such kits.

In the present study an assay, which measures glycoconjugates present on the surface of the cerbB-2 glycoprotein present in the serum of women with breast cancer has been described. This assay used the lectin enzyme-linked immunoassay (ELISA) format. This employed an antibody against cerbB-2 in the solid phase to capture antigen and a protein, which binds carbohydrate, termed a lectin, in the liquid phase, to detect glycoconjugate groups expressed by the cerbB-2 antigen. The rational for using the lectin ELISA format was that glycoconjugates are altered in malignancy. Measuring altered glycoforms of the

cerbB-2 glycoprotein present in the serum of women with breast cancer might therefore form the basis of a more specific test for breast cancer.

In the following sections the classification of glycoproteins and the changes in glycoconjugate strusture that accompany malignancy are described. The properties of lectins and the results of histochemical studies investigating lectin binding in breast cancer sections have then been summarized.

1.14 Glycoproteins

Glycoproteins are proteins that have oligosaccharide or **glycan** chains attached to their polypeptide backbone. Almost all plasma proteins, with the exception of albumin are glycoproteins. **Glycoconjugate** and **complex carbohydrate** are equivalent terms to denote molecules containing one or more carbohydrate chains covalently linked to either protein or lipid. Glycoproteins, glycosylphosphatidylinositol anchors and glycolipids are all glycoconjugates. In addition **proteoglycans** (formerly called **mucopolysaccharide)** are a particular type of glycoprotein consisting of a complex of glycosaminoglycans covalently attached to protein. Glycoproteins can be divided into two major classes based on the nature of the linkage between the polypeptide and oligosacharride chains. Those containing a serine or threonine *O*-acetylgalactosamine linkage are termed ***O*-linked glycoproteins** and those containing asparagine *N*-acetylglucosamine linkage ***N*-linked glycoproteins** (Murray 1993).

Although about two hundred monosaccharides are found in nature, only about seven are commonly found in the oligosaccharide chains of glycoproteins. Each *N*-linked glycoprotein contains a common pentasaccharide core consisting of mannose (Man), N-acetylglucosamine (GlcNac) and glucose (Glc) but differs in its outer branches to form three classes of glycoconjugate **complex**, **hybrid** and **high-mannose**. These terminal elaborations consist of sugars associated with adhesion and recognition functions such as *N*-acetylneuraminic acid (NeuAc), galactose (Gal) and *N*-acetylgalactosamine (Gal-Nac) (Drickamer *et al.* 1998).

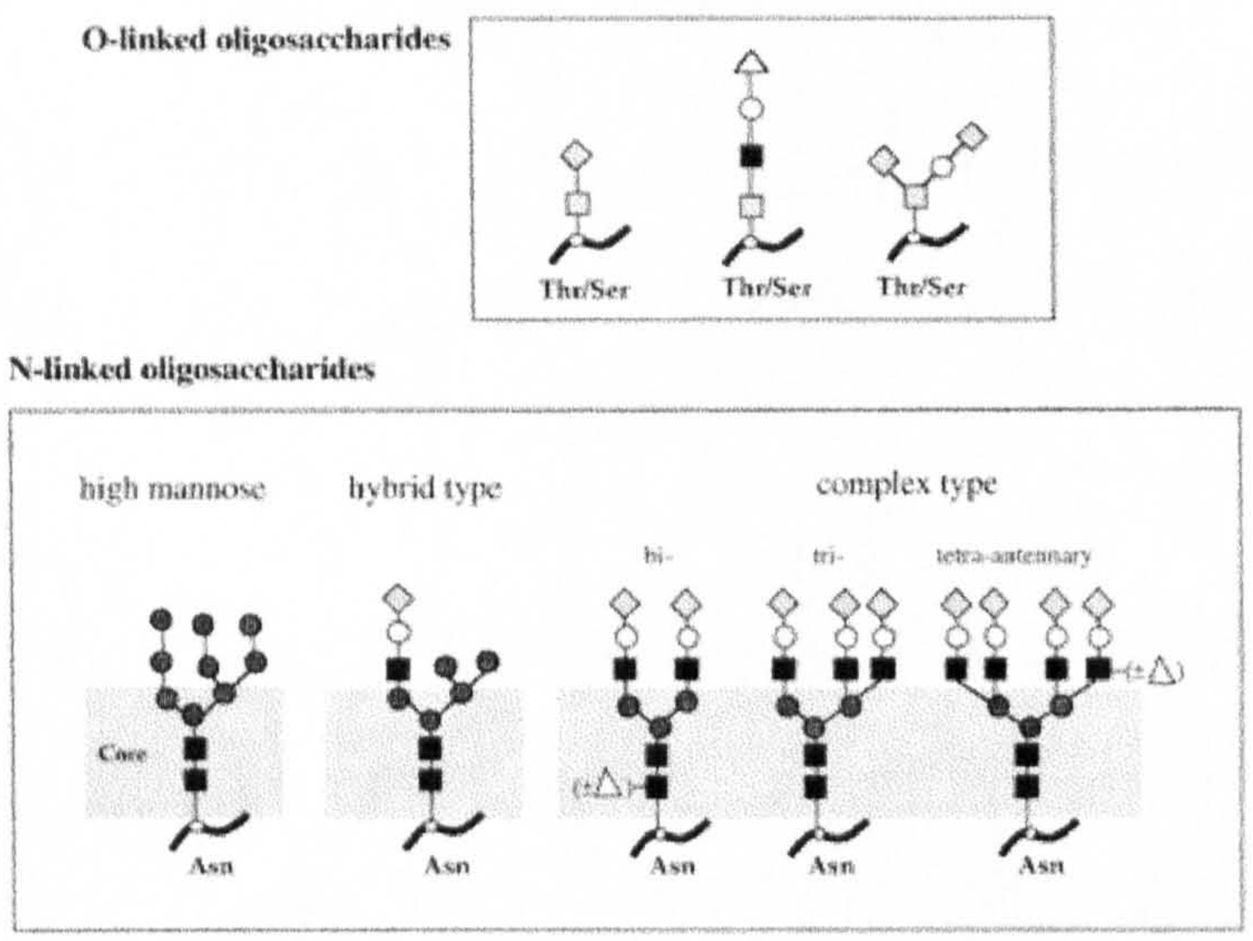


Figure 1.8: *N*-linked and *O*-linked glycoproteins ▩ GalNAc; ■ GlcNAc; ○ galactose; • mannose; △ fucose; ◇ SA; ~ polypeptide chain (Durand, 2000)

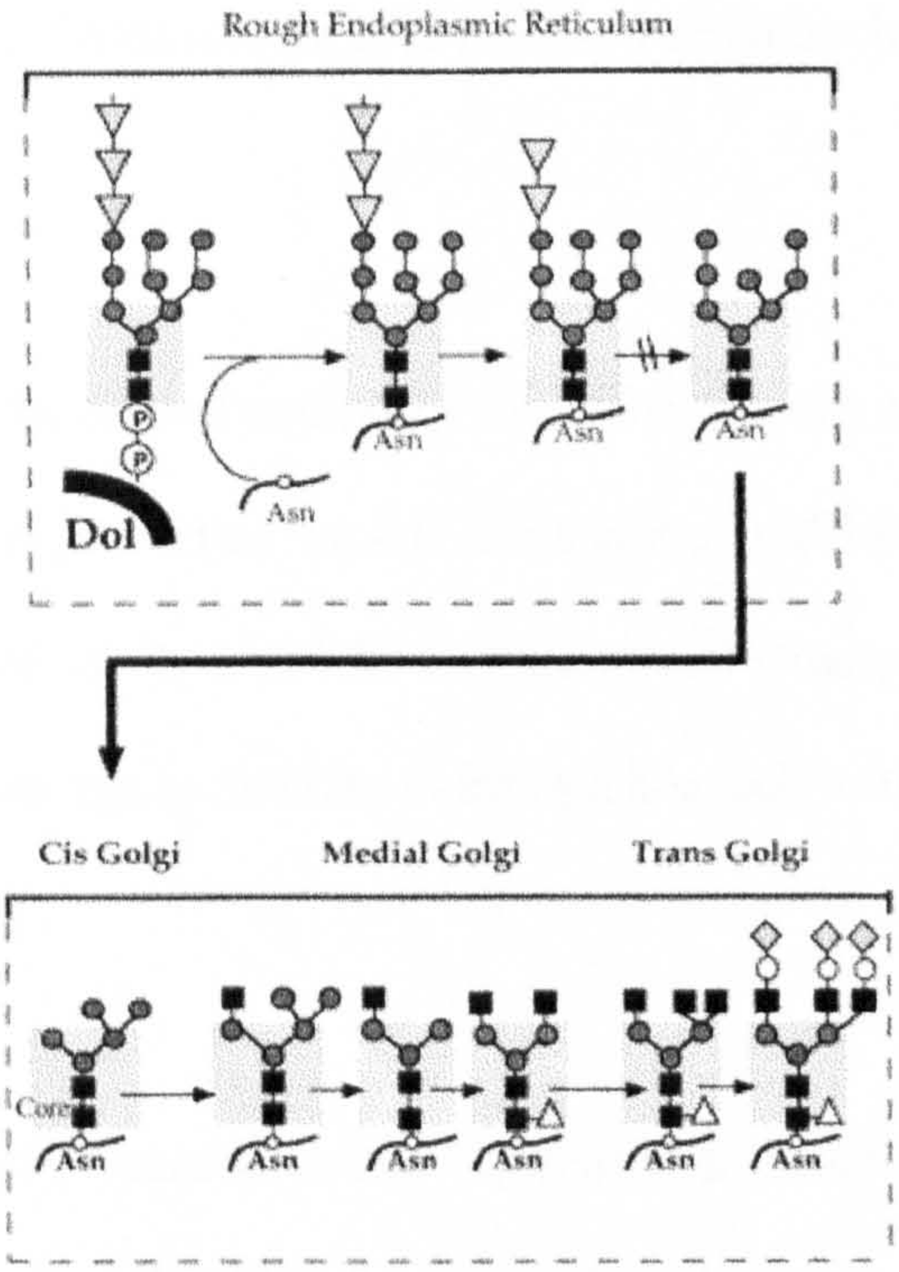


Figure 1.9: Processing of *N*-linked glycoproteins ▩ GalNAc; ■ GlcNAc; ○ galactose; • mannose; △ fucose; ◇ SA; ~ polypeptide chain, Dol Dolichol, P phosphate (Durand, 2000)

Biosynthesis of *N*-linked oligosaccharide chains begins in the endoplasmic reticulum (ER) with a large precursor molecule containing 14 sugar residues. This precursor is linked to dolichol pyrophosphate, which acts as a carrier molecule, and transferred *en bloc* to an asparagine residue on the growing polypeptide chain. Transfer to the Golgi complex follows and the action of glycosidases and glycosyltransferases, which remove and add sugar molecules, completes the maturation process. During this pathway the inner five residues, which constitute the common pentasaccharide core are conserved. High-mannose and hybrid oligosaccharides appear as intermediates along this pathway,

while the complex type is the mature form of *N*-linked oligosaccharide (Drickamer *et al.* 1998).

Glycan structure affects the overall conformation of the glycoprotein and as a consequence its molecular properties such as localisation, half-life and biological activity. In addition the cell surface location and three-dimensional structure allow the glycan to be recognised by receptors and to mediate events such as cell trafficking and cell adhesion (Murray 1993).

The structural diversity of glycans is dictated by tissue-specific regulation of glycosyltransferase genes, availability of sugar nucleotides in the Golgi and competition between enzymes for acceptor intermediates during glycan elongation. Wild-type glycoproteins consist of mixtures of glycosylated variants known as **glycoforms**, in which the same peptide sequence is associated with different glycans at the same glycosylation site. This phenomenon is termed **micoheterogeneity** (Hakomori 1989).

The potential structural diversity of glycans attached to proteins is enormous given the number of monosaccharides, linkages, branches and variable lengths of glycan chains, and has presented a considerable obstacle to the analysis of glycan structure. Analytical techniques of glycoconjugates such as sequential removal of oligosaccharides followed by mass spectometry or nuclear magnetic resonance analysis are time-consuming and expensive (Dwek *et al.* 1993). This has led to many laboratories using lectin-based

methods for the analysis of glycosylation, though they have their limitations. These lectin-based methods will be discussed in a later section.

The lysosome, which contains an array of glycosidases and proteases, constitutes the major catabolic organelle for glycoproteins. A variety of inherited diseases result from defects in glycoconjugate synthesis or catabolism and are reviewed by Durand *et al* (Durand *et al.* 2000). In addition glycan structure is altered in both benign and malignant acquired disease. The latter will be discussed in more detail in the following section.

1.15 Altered Glycosylation in Cancer Cells

Malignant transformation is associated with changes in glycosylation of cell surface proteins (Hakomori 1989) (Kim *et al.* 1997) (Fukuda 1996) (Dennis *et al.* 1999).

Interest in the pathophysiological role of glycosylation in cancer arose from findings that malignant transformation was associated with an increase in the size of membrane glycoproteins. This is known as the "Warren-Glick" phenomenon and it is now known that this is due to an increase in tri- and tetra-antennary branched chains in malignancy (Kobata 1998).

The cellular location of cell surface glycoconjugates suggests that they are involved in cell social functions such as cell to cell and cell to matrix interactions. Whereas the early stages of tumour formation are dependent on aberrant cell housekeeping functions,

regulated mainly by phosphorylation, the later stages of invasion and metastasis are due to aberrant cell social function, which are regulated by cell surface glycosylation (Hakomori 1996).

A number of different aberrations of glycosylation have been identified which are associated with malignancy. These can be due to increased branching of glycans or changes in terminal oligosaccharides. Several lines of evidence have implicated ***N*-acetylglucosaminyltransferase V (GlcNAc-TV).** This enzyme initiates a specific β1,6 branch to the trimannosyl core of the glycan during cancer progression (Dennis *et al.* 1999).

In a histochemical study of sections of breast and colon tissue Fernandes *et al* found a significant increase in binding of the lectin *Phaseolus vulgaris* leukoagglutinin (L-PHA), a plant lectin which specifically binds β1,6 branched structures, in sections of carcinoma compared to benign breast tissue (Fernandes *et al.* 1991). Demetriou *et al* showed that expression of GlcNAc-TV in an immortalised lung epithelial cell line MvLu, results in loss of contact inhibition of growth and increased cell motility (Demetriou *et al.* 1995). Futhermore Lu *et al* showed that NIH3T3 cells transfected with a H-*ras* oncogene with reduced GlcNAc-TV expression showed reduced ability to from lung metastases after tail vein injection in nude mice (Lu *et al.* 1994).

These and other studies suggest that increased GlcNAc-TV expression is a major factor in metastasis formation and is responsible for the increased glycan branching observed by

Warren and Glick (Kobata 1998). Of interest, it has been shown that NIH3T3 cells transfected with HER-2 oncogene showed increased GLcNAc-TV expression and β1,6 branching (Chen *et al.* 1998). This suggests a mechanism by which HER-2 a proto-oncogene associated with aggressive tumour behaviour may form metastasis.

In addition, changes in expression of terminal oligosacchides have been observed to be associated with malignancy. These have been identified by the use of monoclonal antibodies and are reviewed by Hakamori (Hakomori 1989). They include an increased expression of **Lewis antigens**. These are antigens carried on glycan chains, which normally express the blood group ABH determinants. Lewis^{x}, sialyl Lewis^{x}, Lewis^{y} and sialyl Lewis^{a} are over-expressed in human carcinomas (Itzkowitz *et al.* 1986) (Dennis *et al.* 1999) and may be important in the later stages of metastasis, as they have been shown to mediate attachment tumour cells to **selectins** *in vitro* (Mannori *et al.* 1995).

Selectins are endogenous adhesion molecules, which appear at the onset of inflammation and bind to neutrophils and monocytes slowing their movement and aiding extravasation through capillary endothelium and recruitment to the sites of inflammation. It has been hypothesized that as tumour cells express sialyl Lewis^{x} they use the same selectin-carbohydrate interaction as neutrophils in inflammation during the process of metastasis (Fukuda 1996) (Kim *et al.* 1997).

An increase in terminal **sialic acid** (NeuAc) has been associated with the transformed phenotype and metastatic potential. In addition, Santer *et al* showed that in NIH3T3

fibroblasts transfected with the H-*ras* oncogene, terminal α-Gal residues of *N*-linked oligosaccharides were replaced by NeuNAc suggesting that sialic acid was necessary for the complete expression of the transformed phenotype (Santer *et al.* 1989).

Other changes in glycosylation which have been reported to be associated with malignancy, and which will only be mentioned in passing are expression of *O*-linked oligosaccharides T, Tn and sialyl Tn by mucins and loss of normal ABH blood group expression, reviewed by Hakamori (Hakomori 1989).

1.16 Altered glycosylation of serum glycoproteins in cancer

Altered glycosylation of serum glycoproteins in serum of patients with cancer and other disorders such as liver and inflammatory disorders has been reported in several studies, and has been proposed as the basis for more discriminative tumour markers for the diagnosis and monitoring of disease (Turner 1992).

Cancer sera have been investigated by crossed affino-immunoelectrophoresis with *Concanavalin* A by a number of groups. However, results have been conflicting (Turner 1992). Another area, which has received particular attention, is the fucosylation and sialylation of serum glycoproteins in sera of cancer patients.

Measurements of serum concentrations of fucose and sialic acid have been reported to be elevated in patients with cancer. The concentrations of both fucose and sialic acid were

correlated with a number of acute phase proteins including **α1-anti trypsin, α1-acid glycoprotein** and **haptoglobin** in cancer sera. The reported sialic acid content of these proteins suggesting that the elevated levels of this sugar are due to the acute phase response (Turner *et al.* 1985).

Subsequent work has identified an abnormally fucosylated haptoglobin in sera of patients with ovarian (Thompson *et al.* 1987) and breast cancer (Thompson *et al.* 1991), levels of which correlated with tumour burden. An abnormally fucosylated form of α1-anti trypsin has been identified in patients with ovarian cancer that predicted unresponsiveness to chemotherapy (Thompson *et al.* 1988). In addition, the sialic acid content of another acute phase protein α1-acid glycoprotein was found to be elevated in cancer sera compared to sera from healthy controls and patients with other diseases (Moule *et al.* 1987). The source of both fucosylated acute phase proteins was suggested to be the liver, rather than the tumour.

Pekelharing was first to modify the enzyme-linked immunosorbent assay (ELISA) to study protein glycosylation. By replacing the enzyme-linked antibody of the conventional ELISA with a lectin he developed the lectin ELISA system. The sandwich, which consists of two monoclonal antibodies in the conventional ELISA, consists of one monoclonal antibody and one lectin in the lectin ELISA. Only glycoprotein meeting two conditions is therefore measured, it must be the right protein to bind the antibody and have the right glycan structures to bind the lectin. The specificity achieved is therefore greater than that by simply measuring the protein (Pekelharing *et al.* 1987).

Since then several groups have applied the lectin ELISA to the serum of cancer patients. Madiyalakan *et al* compared a lectin ELISA for the determination of CA125 antigen in ovarian cancer patients with conventional ELISA and found that it could detect 88% of ovarian cancer patients without compromising the specificity compared to 60-80% with the conventional assay (Madiyalakan *et al.* 1996).

Parker *et al* applied a lectin ELISA for CA19-9 to the sera of 79 patients with pancreatic cancer and reported a specificity of 76% and sensitivity of 78% for the diagnosis of pancreatic cancer (Parker *et al.* 1992).

1.17 Lectins

Lectins are proteins and glycoproteins extracted predominantly from plants, which have the capacity to bind sugars specifically (Walker 1989). Although extracted from plants, in which they are primarily found in seeds, lectins are present ubiquitously in animals and microorganisms. The biological role of lectins in plants is not clear, though it has been proposed that they act as mediators of symbiosis between plants and microorganisms, and protect plants against phytopathogens. Bacterial lectins are involved in the initiation of infection by mediating bacterial adherence to epithelial cells such as the urinary and gastrointestinal tract (Lis *et al.* 1986).

Lectins are isolated by their ability to agglutinate erythrocytes (**haemagglutination**) and are purified by affinity chromatography on immobilised carbohydrates. They are

classified according to the monosaccharide, (*e.g.* Man, Gal, GlcNAc, GalNAc, L-Fuc or NeuAc) which most effectively inhibits their haemagglutination, or by other techniques such as affinity chromatography or electrophoresis. Usually a particular plant source contains (a) lectin(s) belonging to a single specificity group. However, in some cases two or more lectins that differ in specificity are found in the same plant: for example, *Ulex Europeus* I has specificity for L-Fuc and *Ulex Europeus* II for GlcNAc (Lis *et al.* 1986).

Lectin	*Source*	*Sugar Specificify*
Concanavalin A (Con A)	Jackbean	Mannose/Glucose
Lens Culinaris (LCA)	Lentil	Mannose/Glucose
Ulex Europeus (UEA 1)	Gorse	Fucose
Triticum Vulgaris (WGA)	Wheat germ	N-acetyl glucosamine / sialic acid
Helix Pomatia (HPA)	Snail	N-acetyl galactosamine
Peanut agglutinin (PNA)	Peanut	Galactose / N-acetyl galactosamine
Ricinus Communis (RCA)	Rice	Galactose
Maakia Amurensis (MA)		Sialic acid

Table 1.1: Lectin carbohydrate specificity

Lectins have specificity for oligosaccharides defined by inhibition of haemagglutination.

Some *e.g. Arachis hypogaea* react only with terminal non-reducing sugars, others e.g.

Concanavalin A bind to monosaccharides which are external or internal constituents of glycan chains (Gallagher 1984). Some lectins bind to several different monosaccharides e.g. wheat germ agglutinin binds to both NeuAc and GlcNAc. Some lectins react with oligosaccharides rather than monosaccharides *e.g.Phaseolus vulgaris* leucagglutinin a lectin associated with metastatic potential (Fernandes *et al.* 1991) recogniz

es internally situated β1,6 branched structures.

It would therefore be apparent that the use of lectins for the analysis of glycan structures has limitations. In addition, Debray *et al* reports that the binding of lectins to their monosaccharide is influenced by neighbouring saccharides on the glycan chain. This may result in differences in binding of lectins with identical sugar specificities between samples. He concludes that the results of studies with lectins on cell surface carbohydrates should therefore be interpreted with caution (Debray *et al.* 1981).

The carbohydrate specificities of lectins have led to their utilisation as reagents for the study of glycoconjugates in solution and on cell surfaces, and the identification and separation of cells. Lectins were discovered due to their ability to agglutinate cells bearing appropriate carbohydrate groups; hence they frequently carry the term "agglutinin" as their common name. This ability is due to the fact that lectins are polyvalent and they have multiple carbohydrate binding sites. This property has enables lectins to be used routinely for the determination of ABO, and other blood group expression (McCoy 1987).

Some lectins such as *Phaseolus vulgaris* leucagglutinin have the ability to stimulate blast formation in lymphocytes by a process known as mitogenic stimulation. This is used clinically to assess the integrity of the immune system in patients with suspected immune deficiency (McCoy 1987).

Lectins have been used to investigate glycoprotein glycosylation by incorporating them into existing technologies: affinity chromatography, electrophoresis, ELISA, Western Blotting and histochemistry. **Crossed affino-immunoelectrophoresis** is a technique which involves separation of serum proteins on an agarose gel containing lectin, followed by separation at 90° in a second agarose gel containing antibody and a sugar, which displaces the lectin from the protein and allows protein to react with antibody. **Lectin blotting** is an extension of Western blotting, in which proteins separated by electrophoresis are transferred to a nitrocellulose membrane and a lectin is used to detect glycans on the bound glycoproteins (Turner 1992). **Affinity chromatography** is a technique, which involves passing a specimen down a column containing lectin coupled to Sepharose beads, the bound glycoproteins are collected by eluting with a sugar that competes with the glycoprotein for the lectin-binding sites (Cummings 1994).

Lectins have been used extensively as probes in histochemistry (Walker 1989) and immunoassays for serum glycoproteins (Pekelharing *et al.* 1987). These latter two applications are discussed in more detail in the following sections.

1.18 Lectin binding in histochemical sections of breast tumours

Franklin undertook a systematic examination of the lectin binding characteristics of twenty benign and malignant breast lesions. He reported that the pattern of staining differed between benign and malignant breast tumours. Wheat germ agglutinin gave the strongest and most consistent staining in malignant tissue, suggesting that a large proportion of glycans consist of GlcNAc and NeuAc in malignant breast tumours. *Ricin communis* agglutinin and *Arachis hypogaea* (peanut agglutinin) also showed strong staining suggesting a high proportion of galactosyl residues in malignant tumours (Franklin 1983). However, benign breast tissue also stained positively with wheat germ agglutinin, *Ricin communis* agglutinin and peanut agglutinin.

Walker in a study of the effect of fixation of tissue sections on lectin binding also reported that wheat germ agglutinin and peanut agglutinin consistently stained benign breast tissue. In addition this study showed that wheat germ agglutinin was binding to GlcNAc rather than NeuAc in these sections (Walker 1984). It would appear therefore that wheat germ agglutinin would have limited use in differentiating benign from malignant breast tissue.

In a study of the binding of two fucose specific lectins *Ulex europeus* I and *Lotus tetrogonolobus* in eighty frozen breast tissue sections, Walker reported a variable loss of binding of *Lotus tetrogonolobus* within carcinomas compared to benign breast tissue. This suggested breast malignancy is accompanied by alteration of fucosylation of

glycoconjugates (Walker 1984). In this study *Ulex europeus* I showed inconsistent binding to both benign and malignant breast tissue. However, when these sections were re-assessed to evaluate the use of *Ulex europeus* I to determine vascular invasion, it was found that *Ulex europeus* I binding could be useful in differentiating vascular from lymphatic invasion (Walker 1985).

A lot of attention has surrounded *Helix pomatia* agglutinin, a lectin extracted from snails, which binds terminal non-reducing GalNAc residues on glycoconjugates (Gallagher 1984). *Helix pomatia* agglutinin has been shown to be of prognostic value in adenocarcinomas of the breast, colon and stomach and is the subject of a review by Mitchell and Schumacher (Mitchell *et al.* 1999).

It was demonstrated that *Helix pomatia* agglutinin binds to both benign and malignant breast tissue, however, in primary breast cancer Brooks and Leathem first demonstrated a strong association between *Helix pomatia* agglutinin staining and the presence of lymph node metastasis (Brooks *et al.* 1991). Similar findings have been reported by other studies (Fenlon *et al.* 1987) (Thomas *et al.* 1993). Both of these studies found that *Helix pomatia* agglutinin staining correlated with lymph node stage, disease-free and overall survival. In addition Thomas *et al* showed that *Helix pomatia* agglutinin staining correlated with HER-2 status in primary breast cancer (Thomas *et al.* 1993).

Other groups were unable to reproduce these results and have disputed the prognostic value of *Helix pomatia* agglutinin staining in breast cancer (Mitchell *et al.* 1999).

However, Brooks subsequently showed that methodological differences could account for the discrepancy in findings, as considerable differences in lectin binding patterns were found between direct and indirect lectin histochemical techniques (Brooks *et al.* 1996).

The relationship between *Helix pomatia* agglutinin staining and metastasis in human breast cancer are supported by experimental work. Kjonniksen *et al* have shown that *Helix pomatia* agglutinin binds preferentially to melanoma and sarcoma cell lines in a manner that correlated with their ability to form pulmonary metastases in nude mice. In this study other lectins including wheat germ agglutinin, *Concanavalin* A, peanut agglutinin and *Ulex europeus* I failed to show a similar correlation with pulmonary mestatases (Kjonniksen *et al.* 1994).

The association of two lectins *Phaseolus vulgaris* leucagglutinin and *Helix pomatia* agglutinin with the formation of metastases led one group to investigate whether these lectins were recognising different oligosaccharides on a common metastasis-related glycoconjugate. However, the difference in the staining patterns observed with these two lectins in sections of breast cancer and breast cancer cell lines suggests that these lectins recognise oligosaccharides on different glycocojugates (Mitchell *et al.* 1998).

In order to investigate whether enhanced *Helix pomatia* agglutinin binding in breast cancer was due to over-expression of a single or group of glycoproteins, Schumacher *et al* investigated *Helix pomatia* agglutinin binding in seven metastatic breast cancer cell lines. Analysis of the isolated membrane glycoproteins from these cell lines on Western

blots suggested that, in fact several different membrane glycoproteins bound *Helix pomatia* agglutinin (Schumacher *et al.* 1995). However, one 55kDa glycoprotein, identified by protein sequencing to be a human immunoglobulin subclass A has been identified which binds *Helix pomatia* agglutinin in cancer but not benign tissue (Streets *et al.* 1996).

2.1 Central Hypothesis

In this study, serum and tumour samples were collected from women with primary operable breast cancer and controls. Serum samples were used to measure androgen levels (dehydroepiandrostene sulphate, androstenedione and testosterone levels) using conventional ELISA techniques. As "crosstalk" occurs between steroid hormone and growth factor pathways in breast (and prostate) cancer, it was proposed to measure cerbB-2 levels using a lectin ELISA format. This technique was chosen as a lectin ELISA for cerbB-2 had previously been developed in our laboratory, which showed encouraging results as a diagnostic test for breast cancer.

Elevated levels of serum androgens in postmenopausal women and reduced levels of serum androgens in premenopausal women with breast cancer have been reported by several recent studies though there is disagreement on the significance of levels of different androgens. On the basis of these observations and the results of cell line studies it has been proposed that androgens may have a role in the aetiology of breast cancer by stimulating growth of hormone-dependent breast cancer in postmenopausal women and inhibiting the growth of hormone-dependent breast cancer in premenopausal women (Adams 1998).

In order to test this hypothesis, in the present study, the levels of three androgens dehyroepiandrosterone sulphate, androstenedione and testosterone were measured in a sample of women with primary operable breast cancer and controls. Two further relationships, between serum androgen levels, body mass index and age have been investigated.

Cell line studies have been performed to examine the effect of two androgens, 5α-dihydrotestosterone and 5-androstene-3β,17β-diol, on the proliferation of hormone-dependent breast cancer cell lines MCF7 and T47D and the hormone-independent cell breast cancer cell line MDAMB231. 5α-Dihydrotestosterone and 5-androstene-3β,17β-diol have been chosen as they are true androgen metabolites, which act at the tissue level *in vivo* (Rochefort *et al.* 1984).

A flow cytometric technique has been used to investigate the effect of these androgens on DNA cell cycle analysis in 48-hour incubations and cell numbers in 9-day incubations. Cell line models have been set up to mimic the hormonal environment in pre and postmenopausal women in order to investigate mechanisms by which androgens may stimulate the proliferation of hormone-dependent breast cancer in postmenopausal and inhibit the proliferation of hormone-dependent breast cancer in premenopausal women.

A positive correlation between breast cancer incidence throughout the world and nutritional status has been demonstrated (DeWaard 1969). Nutritional status has been reported to affect androgen production and metabolism and therefore it has been

proposed that androgens are implicated in the development of "Western-environmental" type breast cancer (Adams 1977). In order to investigate this hypothesis a correlation between serum androgen levels and body mass index has been sought. Secondly, a correlation between serum androgen levels and age has been investigated, as it has been proposed that although serum androgen levels fall with age in healthy women they are independent of age in women with breast cancer (Adams 1998).

Flow cytometry is an established method for the determination of cell surface (Loken *et al.* 2000), cytoplasmic and nuclear antigens (Larson 2000). Multiparameter flow cytometry enables the simultaneous determination of expression of several antigens on tumour specimens. Flow cytometric methods for the determination of oestrogen receptor alpha (Brotherick *et al.* 1995), oestrogen receptor beta (Girdler *et al.* 2001) and epidermal growth factor receptor expression (Brotherick *et al.* 1994) have been established in our laboratory. In the present study a flow cytometric method for the determination of androgen receptor expression in breast cancer has been described.

A correlation between androgen receptor expression, determined by flow cytometry and a more established technique, immunohistochemistry, has been investigated. In order to investigate the clinical significance of androgen receptor expression in breast cancer, androgen receptor expression has been correlated with existing prognostic markers Bloom Richardson grade (Bloom *et al.* 1957) and lymph node status (Fisher *et al.* 1978).

Chapter 2 Central Hypothesis

Oestrogen receptor expression in breast cancer has been found to increase with age (Elwood *et al.* 1980). Expression of steroid hormone receptors is regulated by their ligands, by a mechanism known as "negative autoregulation". In order to determine whether negative autoregulation determines androgen receptor expression in breast cancer *in vivo* a correlation between androgen receptor expression and serum dehydroepiandrosterone, androstenedione and testosterone levels have been investigated. In addition, an association between androgen receptor expression and age has been investigated.

In the present study, in addition to androgen receptor expression, oestrogen receptor alpha and epidermal growth factor receptor expression has been determined. Flow cytometric methods for the determination of oestrogen receptor alpha (Brotherick *et al.* 1995) and epidermal growth factor receptor (Brotherick *et al.* 1994) in breast cancer have previously been established in our laboratory. The relationship between androgen receptor, oestrogen receptor and epidermal growth factor receptor expression has been examined.

The rational for investigating an association between androgen receptor and epidermal growth factor expression was that crosstalk occurs between steroid receptor and growth factor receptor pathways in breast (and prostate) cancer (Nicholson *et al.* 1999). An inverse correlation has been reported between oestrogen receptor expression and epidermal growth factor expression in breast cancer (Walker *et al.* 1999), though a relationship between androgen receptor and epidermal growth factor expression has not hitherto been investigated.

Materials and Methods

The role of androgens in breast cancer

In the following study Western Blotting was performed by Susan Cook in the Department of Surgery, immunohistochemistry by staff in the Department of Pathology. Flow cytometry was done mostly by the author under supervision of Susan Stamp and Dr Brian Shenton in the Department of Surgery and ELISA under the supervision of Dr David Cook in the Department of Clinical Biochemistry.

3.1 Principles of Flow Cytometry

Flow cytometry is a technique for making rapid measurements on particles or cells as they flow in a fluid stream one by one through a sensing point. A flow cytometer consists of a fluidic system, which delivers particles in suspension to a point intersected by a laser beam. This incident light is scattered by cells passing through the detection point as well as exciting fluorescent dyes bound to the cells. This scattered and fluorescent light is collected by photodetectors, which convert the photon pulses to electrical signals.

On passing through the laser beam several physical processes take place diffraction, absorption, refraction and reflection of incident light. Diffraction is of significance close to the optical axis and is closely related to the size of the cell passing through the laser beam. Low-angle scattered light is focused onto a photoelectric diode. This signal is

referred to as **forward angle light scatter (FALS).** At higher angles, refraction and reflection become increasingly important. These processes result from structural features of the cell such as granularity and surface convulsions and are referred to as **side scatter (SS)** (Carter *et al.* 2000). These processes are illustrated in the following figure.

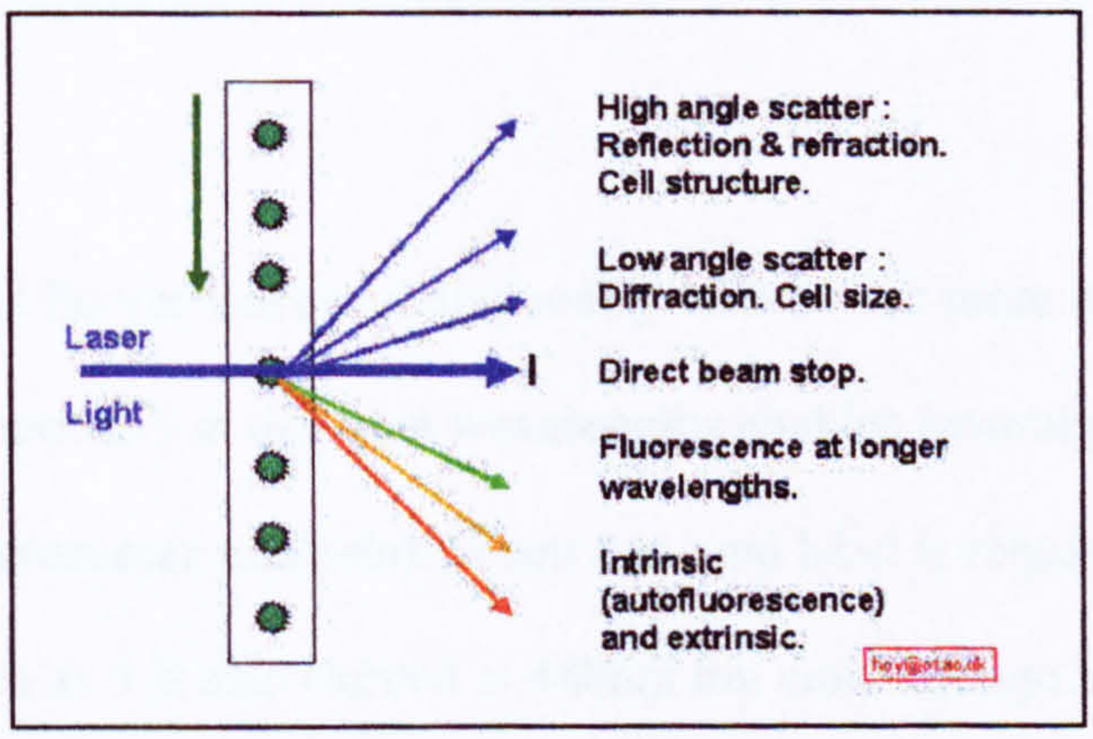


Figure 3.1: Diffraction, absorption, refraction and reflection of incident laser light by particles passing through a fluidic system (from hoy@cf.ac.uk)

3.2 Use of fluorescent probes in flow cytometry

The name laser is an acronym for "light amplification by stimulated emission of radiation". Laser light produces coherent radiation at discreet wavelengths. Fluorescence occurs when a molecule excited by light of one wavelength, returns to the unexcited state.

Together with light scatter, fluorescence is the major parameter measured by flow cytometry. Fluorochromes such as fluorescein and phytoerythrin covalently linked to

antibodies can be used to measure antigen expression in cell populations. Propidium iodide, which binds DNA, can be used to gain information on cell cycle.

There is a range of fluorescent molecules used in flow cytometry. The most commonly used is fluorescein isothiocyanate (FITC), which emits green light on excitation at 488nm. The isothiocyanate group links the fluorescein molecule to lysine residue on a protein molecule.

The ability to detect fluorescence simultaneously with two or more compounds fluorescing simultaneously at different wavelengths enables several parameters to be measured (**multiparameter analysis**). When a second label is required phycoerythrin (PE) is often chosen as it is also excited at 488nm but emits orange light, which can easily be distinguished from the green fluorescence of fluorescein (Ormerod 2000).

3.3 Optics

The optical configuration for the simultaneous measurement of light scatter and fluorescence is illustrated below. The forward collection lens gathers light from approximately 1 to 20 degrees off the laser beam light. Separation of the mixture of scattered and fluorescent light emitted from cells is achieved with an arrangement of dichroic mirrors and band pass filters. Bandpass filters transmit light of the desired wavelength over a narrow band.

The dichroic mirrors are placed at 45 degrees to the incident beam. The first mirror reflects light below a given wavelength (500nm) towards the side scatter detector. Longer wavelengths pass on to the second mirror, which again reflects light below a given wavelength (560nm) via a band pass filter to a detector. In this way light emitted from different fluorochromes is detected by a series of photomultiplier tubes (Carter *et al.* 2000).

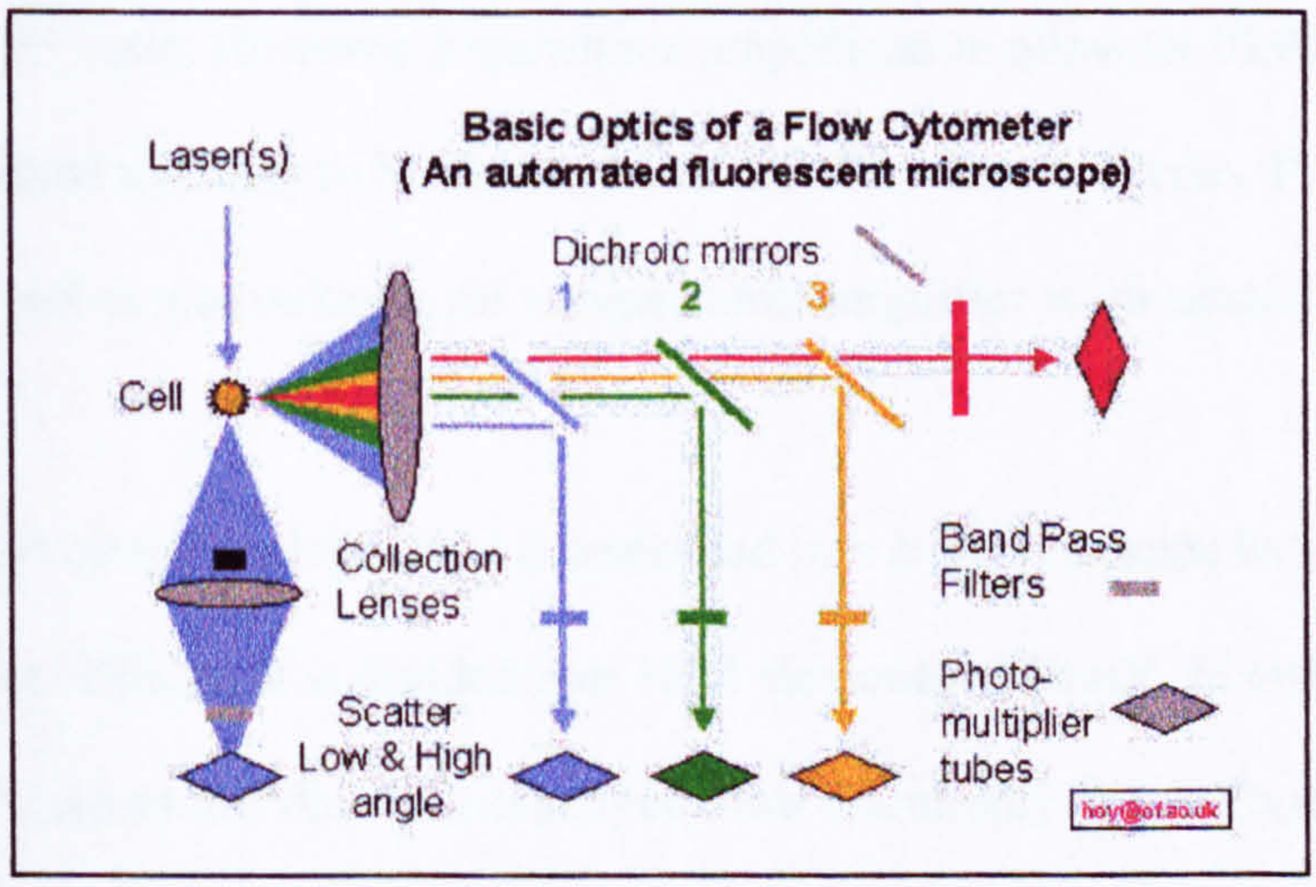


Figure 3.2: Optics of flow cytometer (from hoy@cf.ac.uk)

3.4 Interpretation of the signal: the dot-plot and histogram

A current is generated in photomultiplier tubes by incident light. The amplitude of which is proportional to the number of photons reaching the photomultiplier tube. The output signal from the photomultipier tube is then passed on to a pre-amplifier. After that logarithmic amplification may take place.

Logarithmic amplification amplifies weak signals, compresses strong signals and normalises data, which is skewed torwards lower values. The final step is conversion of the current pulse to a digital signal, which takes place in the analog-to-digital converter (ADC). These digital signals are displayed on a 1024-channel scale.

With linear amplification a 1000-fold difference in signal intensity can be displayed on the 1024-channel scale. However, logarithmic amplification allows a 10,000-fold difference in signal intensity to be displayed on the 1024-channel scale. The relationship between input and output voltages for a logarithmic amplifier is illustrated in figure 3.4.

The continuous voltage analog signal is translated into a discrete scale by the analog-to-digital converter. This scale is divided into 1024 elements of 10mV. In order to meaningfully interpret this data it is displayed most commonly in two forms a **frequency histogram** or **dot-plot**.

The frequency histogram is a direct graphical representation of the number of events occurring for each channel of the ADC. The dot-plot is a plot of the output from one ADC against the output from another. In this manner, populations of cells can be distinguished on dot-plots and statistical analyses carried out by setting boxes or polygons around regions of interest **(gating).** The antigen expression on a particular population of cells for instance, can be measured by limiting the analysis to the population within a certain gate (Carter *et al.* 2000). Examples of histograms and dot-plots are shown in figure 3.4.

3.5 Measurement of antigen expression by flow cytometry

Information provided by light scatter and immunofluorescence enables the identification of cell populations within a heterogeneous mixture of cells. Having identified the cell population of interest, fluoresecent probes conjugated to antibodies directed against cell surface, cytoplasmic or nuclear antigens are used to determine antigen expression on a particular population (Larson 2000) (Loken *et al.* 2000).

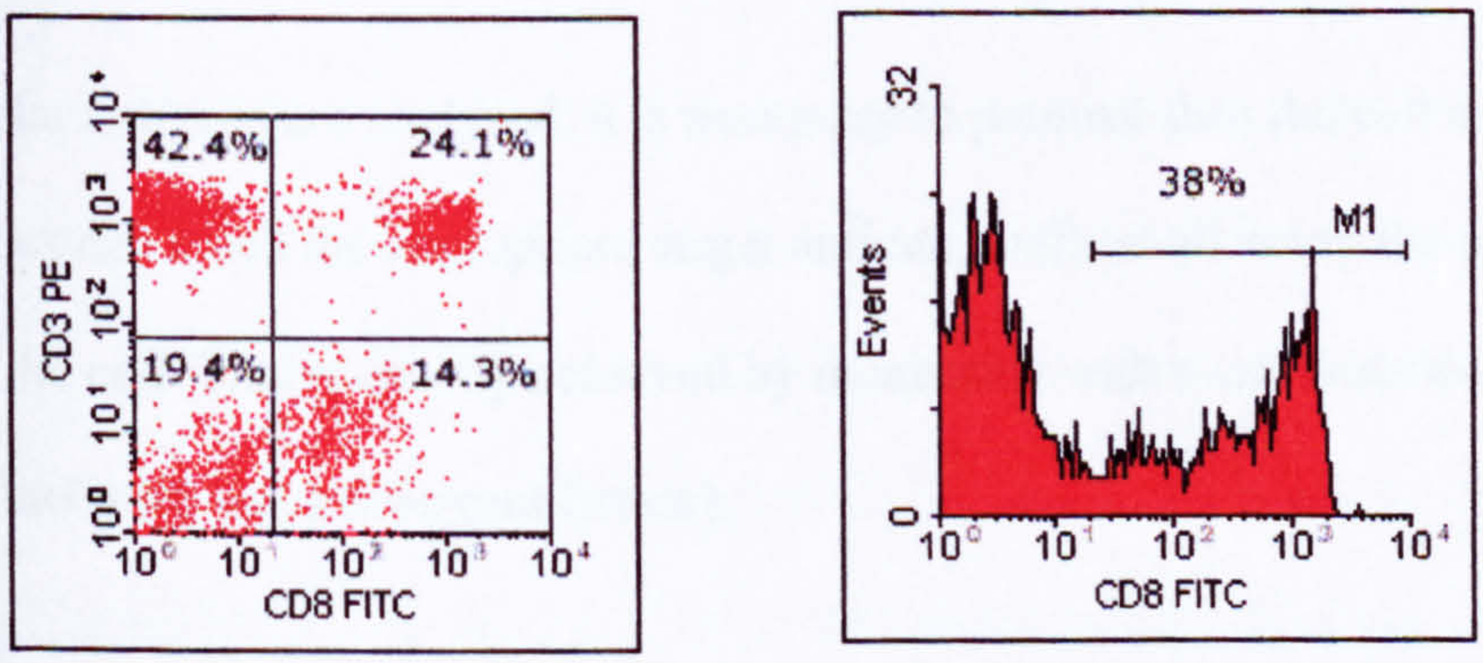


Figure 3.3: Histogram and dot plot of CD3 and CD8 expression on a population of lymphocytes (from hoy@cf.ac.uk)

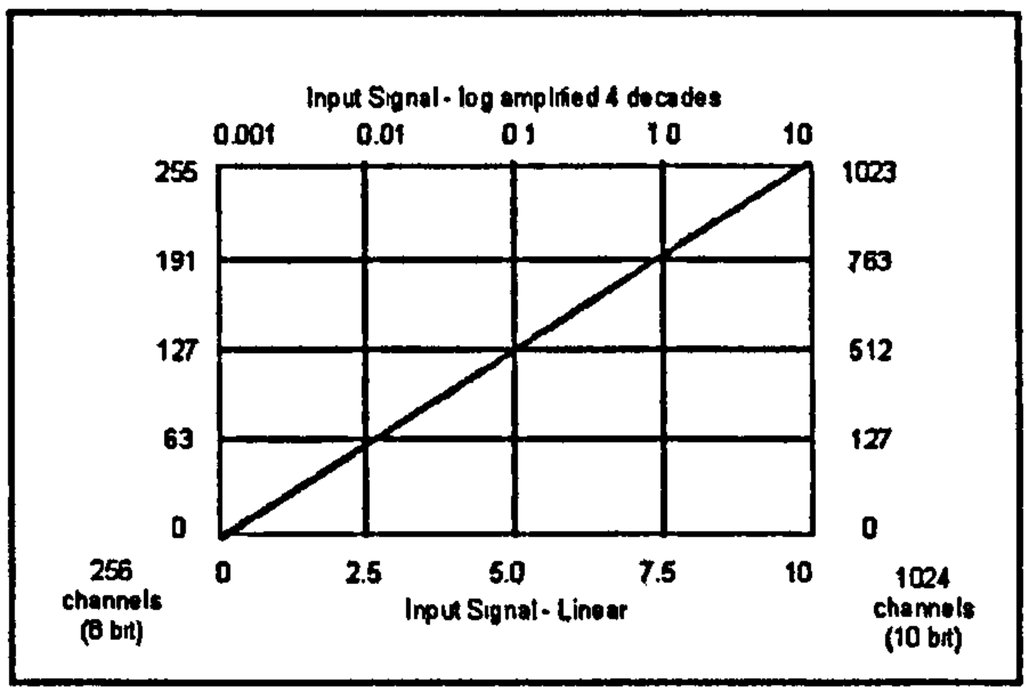


Figure 3.4: Channels/linear/log amplification (from hoy@cf.ac.uk)

If intracellular antigens are analysed, it is necessary to permeabilise the cell in order that the antibodies can reach the appropriate target antigen, without allowing the antigen to leak out of the cell. This is usually achieved by incubation with a combination of fixative (paraformaldehyde) and a detergent (triton).

3.6 Growth Medium

Full medium consisted of Dulbecco's modified Eagle medium (DMEM) with 1000mg/L glucose and without sodium pyruvate (GIBCO Life Technologies, Paisley, UK) was supplemented with 10% heat-inactivated fetal calf serum (Sigma, Poole, UK), 1% L-glutamine (GIBCO) and 0.5% Gentamycin (GIBCO). Stripped Medium consisted of DMEM with 1000mg/L glucose and without phenol red or sodium pyruvate (GIBCO) was supplemented with 10% charcoal/dextran treated foetal calf serum (HyClone, Perbio Science UK Ltd, Cheshire, UK), 1% L-glutamine (GIBCO) and 0.5% Gentamycin

(GIBCO). Phenol red was omitted from the stripped medium as it has been shown to have oestrogenic properties and produces a fluorescent signal, which interferes with the flow cytometric analysis.

3.7 Cell Lines

All cell lines were routinely cultured in DMEM full medium and grown in 75cm^3 flasks (Corning Ltd, High Wycombe, UK) at 37^{O}C in a humidified atmosphere containing 5% CO_2 in air. Cells were passaged every 3-4 days when they had grown to 90% confluence.

The breast cancer cell lines used were all epithelial cells, which grow as monolayers. They were the MCF7 cell line (European Collection of Animal Cell Cultures ECACC, Salisbury, Wiltshire, UK), which was established in 1970 from a pleural effusion obtained from a 69 year-old female (Soule *et al.* 1973): the T47D cell line (ECACC) which was established in 1974 from a 54 year old female with an infiltrating ductal carcinoma of the breast (Keydarl *et al.* 1979): the MDA-MB-231 cell line (ECACC) which was established in 1973 from the pleural effusion of a 51 year old female (Cailleau *et al.* 1974): and the SKBR3 cell line (American Type Tissue Culture Collection ATCC, Manassas, Virginia, USA) which was established from the pleural effusion of a 43 year old female.

To passage cell lines, culture medium was decanted from the flasks and the cells washed with 2ml of 1× trypsin-EDTA (GIBCO) at 37°C to remove any residual fetal calf serum (FCS). A further 8ml of 1× trypsin-EDTA was added to the flask and incubated for

approximately 5 minutes at 37°C until all the cells were detached. The trypsin-EDTA was inactivated by addition of 10ml full medium and decanted into a 30ml universal centrifuge tube (Bibby Sterilin Ltd, Staffordshire, UK). The recovered cells were pelleted by centrifugation at 1000rpm for 5 minutes. The cell pellets were either resuspended in full medium and split into flasks or retained in the pellet for cryopreservation (see below).

Cryopreservation of all cell lines was routinely carried out at an early stage of passage, freezing medium contained 80% full medium, 10% FCS (Sigma) and 10% dimethyl sulfoxide DMSO (Sigma). Approximately 5×10^6 cells were pelleted by centrifugation, resuspended in 1ml of freezing medium and transferred into cryogenic vials (Corning, USA). To control the rate of freezing cryogenic vials were initially frozen for 4 hours at –80°C before transfer to liquid nitrogen for long-term storage.

Reculture of cryopreserved cells was achieved by rapid thawing of the frozen cell suspension at 37°C and re-suspension in full medium. Cells were washed by centrifugation at 1000rpm for 5 minutes to remove the DMSO, resuspended in fresh full culture medium and transferred to culture flasks.

3.8 Clinical Material

Patients were recruited to this study from the Breast Unit at the Royal Victoria Infirmary, Newcastle upon Tyne over a 2-year period. Venous blood was taken from women with confirmed primary operable breast cancer on or preceding the day of operation. Venous blood was also collected from women in whom breast cancer had been excluded, as

controls who attended the diagnostic breast clinic. All venous blood samples were centrifuged at 2500rpm for 5 minutes the serum was removed and stored at -80°C until analysed.

Tumour samples were taken in the operating theatre immediately after excision of the breast cancer. A Bard Magnum™ (C.R.Bard Inc, Covington, USA) biopsy instrument was used to obtain core biopsies from palpable breast tumours. Core biopsies were snap frozen in liquid nitrogen and stored at –80°C until analysed. Ethical permission had been granted for this study, and all patients recruited had given their informed consent to tissue and serum collection.

3.9 Titration of Antibodies

All antibodies were titrated to determine their optimum concentration for use. For the titration of the biotinylated androgen receptor antibody (DAKO, Cambridge, UK) LNCap, a prostate cancer cell line known to express the androgen receptor (Horoszewicz *et al.* 1983) was used as a positive control and DU145, a prostate cancer cell line which does not express the androgen receptor (Stone *et al.* 1978) was used as a negative control. For the titration of phycoerythrin (PE) conjugated epidermal growth factor receptor EGFR monoclonal antibody (BD Pharminogen, San Diego, USA) the MCF7 cell line, which is known to express EGFR (Chrysogelos *et al.* 1994) was used as a positive control and lymphocytes were used as a negative control. The titration of biotinylated oestrogen receptor alpha antibody (DAKO, Cambridge, UK), which follows a similar method

outlined below, has previously been established in our laboratory and is described by Girdler (Girdler 2002).

Cultured cells were harvested from the flasks and pelleted by centrifugation. The cell pellet was resuspended to form a single cell suspension and incubated with 1% paraformaldehyde (BDH Chemicals, Poole, UK) for 30 minutes at 4°C. The cell suspension was centrifuged for 5 minutes at 2500rpm and resuspended in 0.025% triton (BDH Chemicals, Poole, UK) to give a final concentration of $1\%10^6$cells/ml. Increasing amounts of biotinylated androgen receptor antibody (1µl, 2µl, 3µl and 5µl) or PE conjugated EGFR (2.5µl, 5µl, 10µl, 20µl and 30µl) were incubated with 100µl aliquots of cell suspension for 30 minutes at 4°C. Cells were washed with 2ml of isoton (Becton Dickinson), centrifuged at 2500rpm for 5 minutes and resuspended in 100µl of isoton. To those cells labelled with biotinylated androgen receptor antibody 2µl of streptavidin phycoerythrin strep-PE (BD Biosciences, San Jose, USA) were added and incubated for 30 minutes at 4°C. The cell suspension was washed as above and resuspended in 250µl of isoton. Finally analysis was performed on a FACScan (Becton and Dickinson) using LYSYS II software with pre-stored settings. The medians of the FL2 histogram obtained were plotted against the different dilutions of antibody and the optimum dilution of antibody was selected as the point where the titration curve reached a plateau and the negative control remained at a low level.

3.10 Preparation and Staining of Tumour Samples and cell lines

Frozen tumour samples were allowed to thaw out before being dissected into small pieces and disaggregated by passing through a wire mesh of approximately 50µm diameter (Boots Pharmaceuticals Ltd, Nottingham, UK) to form a single cell suspension in isoton. To reduce clumping further, cells were then passed through a 53µm nylon mesh (Lockertex, Warrington, UK) and the resulting cell suspension centrifuged at 1000rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 2mls of 1% paraformaldehyde. Following incubation at 4°C for 20 minutes, this cell suspension was washed and centrifuged as above, and the cell pellet resuspended in 0.025% triton

100µl of the resulting fixed and permeabilised cells were aliquoted into FACS tubes (BD Labware, New Jersey, USA). To these aliquots primary antibody was added at the optimal quantity determined by titration, mixed thoroughly on a vortex mixer (Fisons Scientific, Loughborough, Leicestershire) and incubated for 30 minutes at 4°C. The primary antibodies used were biotinylated androgen receptor antibody, biotinylated oestrogen receptor alpha antibody, and phycoerythrin conjugated epidermal growth factor receptor antibody.

Following incubation with primary antibody, the cell suspension was washed and centrifuged as above, and resuspended in 100µl of isoton. At this stage, 2µl of streptavidin phycoerythrin was added to the cells labeled with biotinylated antibodies and

5µl of fluorescein isothiocyanate conjugated cytokeratin antibody CK-FITC (Novocastra, Newcastle upon Tyne, UK), an antibody which is directed towards cytokeratins 8 and 18 (Ferrero *et al.* 1990), was incubated with each aliquot for 30 minutes at 4°C. Following washing with 2ml isoton and centrifugation as above, the fixed, permeabilised and stained cells were resuspended in 250µl of isoton and were now ready for analysis.

The following controls were also performed for each sample; unlabelled cells, cells plus 2µl of streptavidin phycoerythrin, cells plus 5µl of fluorescein isothiocyanate conjugated cytokeratin antibody. Controls ensured that compensation was correctly adjusted to minimize interference between FITC and PE signals. Analysis was performed on a Becton Dickinson FACScan using LYSYS II software with pre-stored settings.

The preparation and staining of cell lines for determination of antigen expression follows a similar method to that described above for the titration of antibodies. Cultured cells were harvested from the flasks and pelleted by centrifugation. The cell pellet was resuspended to form a single cell suspension and incubated with 1% paraformaldehyde (BDH Chemicals, Poole, UK) for 30 minutes at 4°C. The cell suspension was centrifuged for 5 minutes at 2500rpm and resuspended in 0.025% triton (BDH Chemicals, Poole, UK) to give a final concentration of 1×10^6cells/ml.

Primary antibody, biotinylated androgen receptor or biotinylated oestrogen receptor alpha, were then added at the optimum quantity determined by titration to 100µl aliquots of this cell suspension. Cytokeratin antibody was not required when labeling cell lines, as

they are a homogenous population of epithelial cells. Following incubation for 30 minutes with primary antibody, the cell suspension was washed with isoton and centrifuged at 1000rpm. The cell pellet was then resuspended in 100µl of isoton, 2µl of streptavidin phycoerythrin was added and the cell suspension incubated for 30minutes at 4°C. Following further washing and centrifugation the fixed, permeabilised and stained cells were resuspended in 250µl of isoton and analysed on a Becton Dickinson FACScan using LYSYS II software with pre-stored settings.

3.11 Data Analysis & Quantification of Fluorescence

Data analysis on samples on the FACScan was performed using LYSYS II software. Events were gated on a CK-FITC (fluorescence-1) against PE (fluorescence-2) dot plot to distinguish cytokeratin positive cells from non-epithelial cells. Cytokeratin positive cells were gated R1.

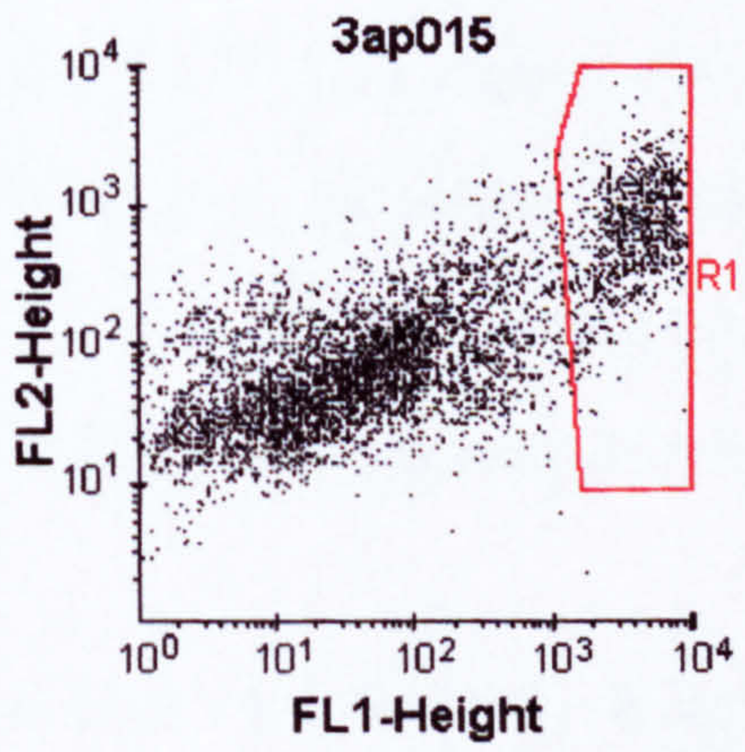


Figure 3.5: Dot plot of fluorescence-1 against fluorescence-2 to distinguish cytokeratin positive (*i.e.*epithelial) from non-epithelial cells

Fluorescence-2 (*i.e.* PE) histograms for streptavidin-PE, unlabeled cells and androgen receptor, oestrogen receptor alpha and EGFR labelled cells were generated for events falling within the R1 gate. The median fluorescence intensity was determined from these fluorescence-2 histograms.

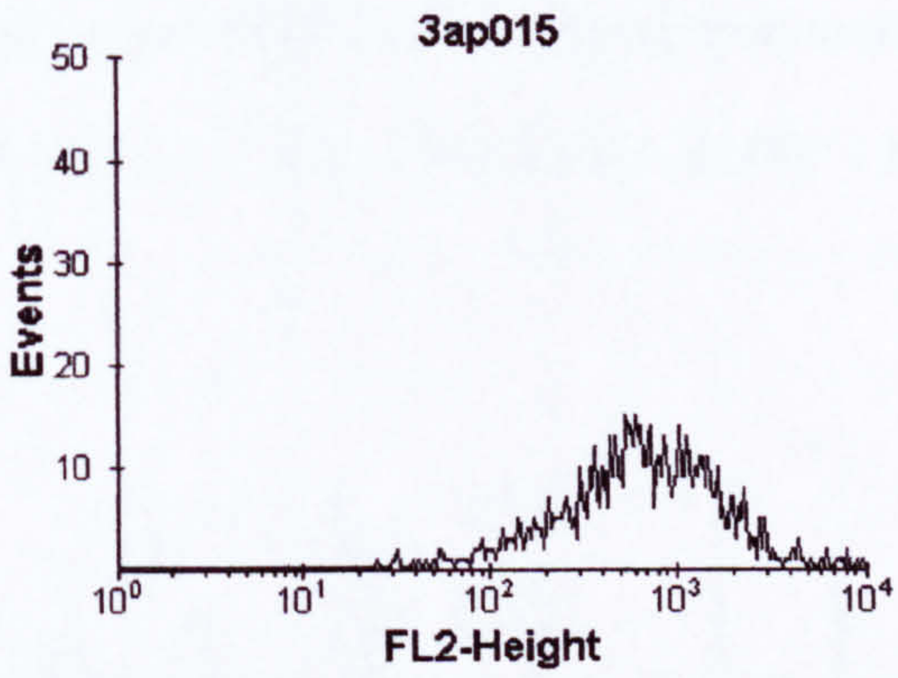


Figure 3.6: Fluorescence-2 histogram of cytokeratin positive cells

Analysis of antigen expression on tumour samples and cell lines by the above methods generates a fluorescence-2 histogram with a median measured in the arbitrary units of **median fluorescent intensity**. This fluorescence can be quantified into absolute units of **molecules of equivalent fluorochrome (MEF)** using fluorescent microbead standards.

Spherotec RCP50 beads were used to standardise the arbitrary units of median fluorescence intensity obtained on the cytometer into absolute units of molecules of equivalent fluorochrome. The beads were run using the same instrument settings

determined for cell line or tumour samples on FACScan and analysed using LYSYS II software.

Beads were gated as singlets on a dot plot of FSC against SSC and fluorescence-2 histograms were generated from the gated events. The number of molecules of equivalent fluorochrome of the beads was obtained by the manufacturer spectrophotometrically, with reference to solutions containing known phycoerythrin molecules of fluorescence. These values were as follows 425, 1131, 3765, 11628, 34589, 128918 and 294760.

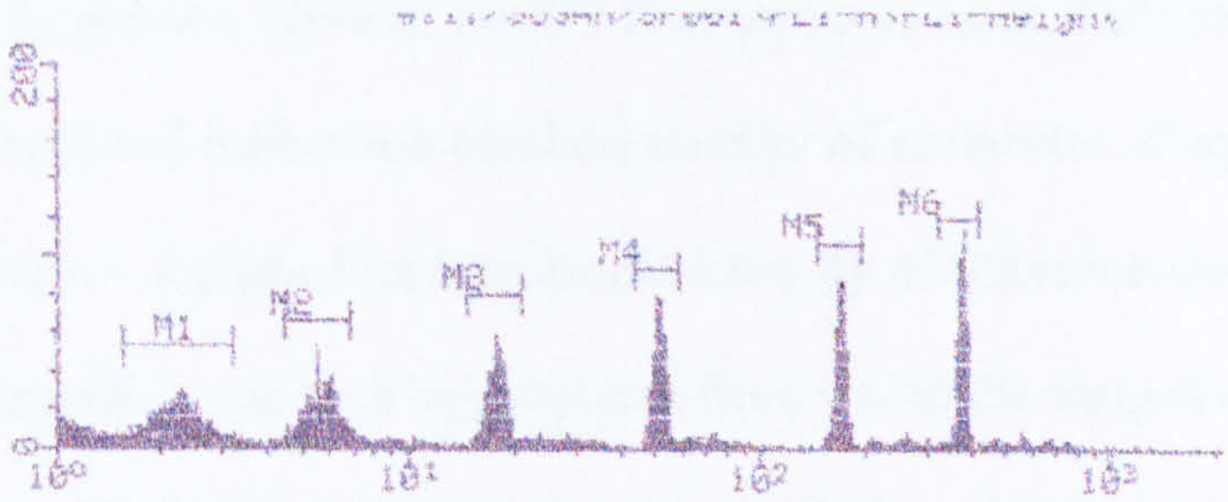


Figure 3.7: Fluorescence-2 histogram of Spherotec RCP50 beads

A regression line of median fluorescent intensity against molecules of equivalent fluorocrome (*i.e.*phycoerythrin) was generated using Tallycal software (DAKO, Cambridge, UK) for the bead populations. From the regression equation the number of molecules of equivalent fluorochrome for the phycoerythrin labelled cell lines was extrapolated.

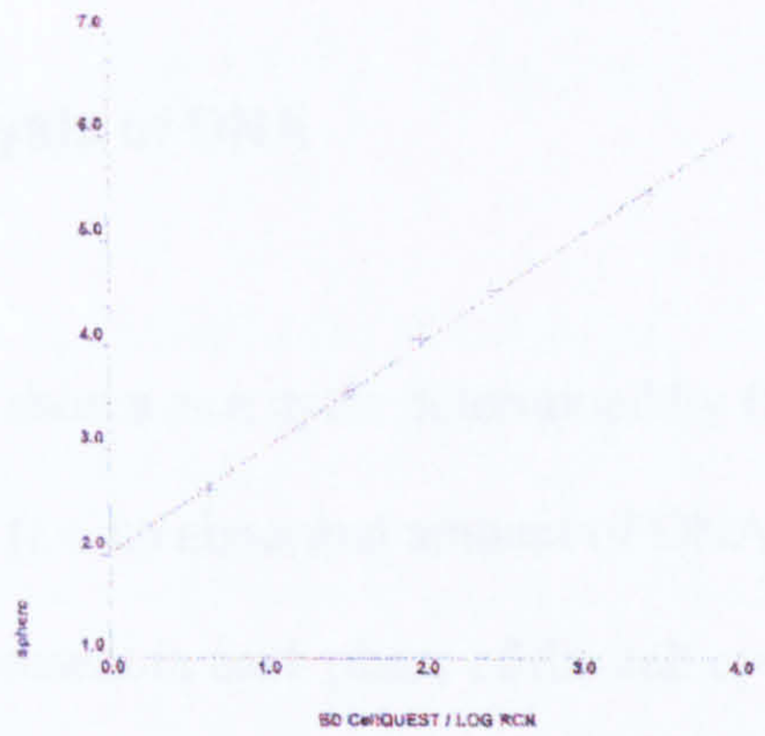


Figure 3.8: Regression curve of DAKO fluorospheres using Tallycal software:

X-axis = log [bead calibration number] number of molecules of equivalent

fluorochrome*Y*-axis = log [median fluorescent intensity of fluorescence-2 histogram].

The following equation can thus be generated from which the antigen expression of

tumour samples or cell lines can be quantified $y = 4.06\ x + 104.06$

Unlabeled cells and cells incubated with streptavidin-PE have a background fluorescence which does not reflect antibody binding to antigens expressed by the tumour cells. The number of molecules of equivalent fluorochrome was therefore determined for unlabelled cells and cells labelled with streptavidin-PE. In order to obtain the antigen expression the number of molecules of equivalent fluorochrome for cells incubated with streptavidin-PE was subtracted from the value obtained from cells stained with biotinylated androgen or oestrogen receptor antibody and streptavidin-PE. Likewise, the number of molecules of equivalent fluorochrome was determined for unlabeled cells and subtracted from the number of molecules of equivalent fluorocrome obtained for cells labeled with phycoerythrin conjugated EGFR antibody.

3.12 Staining and Analysis of DNA

The DNA content of a cell shown as a cycle determined by flow cytometry provides information on aneuploidy (*i.e.* an abnormal amount of DNA) and gives a static estimate of the percentage of cells present in each phase of the cell cycle (*i.e.* $G_0/_1$, S, and G_2/M). Cell lines were used as an *in vitro* model to assess the effects of 5α-DHT, androstenediol and 17β-oestradiol on cell cycle and cell proliferation. The study of synchronous populations of cells allowed steroid induced changes in the cell cycle to be quantified. To achieve synchronous cell populations, cells were cultured in full medium and harvested when approximately 80% confluent. The cells were then pelleted by centrifugation and resuspended in stripped medium. Cells were seeded in 12-well culture plates (Corning) for cell cycle studies and 6-well plates (Corning) for proliferation studies. The cells were allowed to adhere to the wells for 24 hours. At this point, cell cycle analysis was determined by processing the cells from wells, outlined below. The stripped medium was aspirated from the remaining wells and replaced with stripped medium supplemented with steroids.

The following method was used to prepare and stain cell lines for DNA analysis. Cultured cells were harvested with 1×trypsin/EDTA and pelleted by centrifugation at 2500rpm for 5 minutes. The cell pellet was resuspended in 350µl of full medium and incubated for 5 minutes at 37°C. 100µl of 0.25mg/ml propidium iodide PI (Sigma, Poole, UK) containing 5% triton X-100 (BDH Chemicals, Poole, UK) and 50µl of 1mg/ml

RNAse (Sigma, Poole, UK) was added to the cell suspension. Analysis was performed on a Becton Dickinson FACScan using LYSYS II software with pre-stored settings.

Permeabilisation of the cell membrane with the detergent triton X-100 was required to allow the dye propidium iodide access to the DNA. RNAse was required to remove RNA to which the propidium iodide would otherwise bind. Propidium iodide is a nucleic acid dye, which intercalates between the bases in double stranded nucleic acids. When excited by a 488nm laser, propidium iodide fluoresces at 620nm, which can be detected by the fluorescence-2 or fluorescence-3 photomultiplier tubes.

Data files were converted from HP to PC format using HP disk (Applied Cytometry Systems, South Yorkshire, UK). DNA data analysis was then performed using Multicycle (Phoenix Flow Systems, San Diego, USA). This software used algorithms to deconvolute the DNA histogram and fit a cell cycle model. The DNA was identified on a dot plot of fluorescence-2 area against fluorescence-2 width and gated to exclude doublet and triplet molecules.

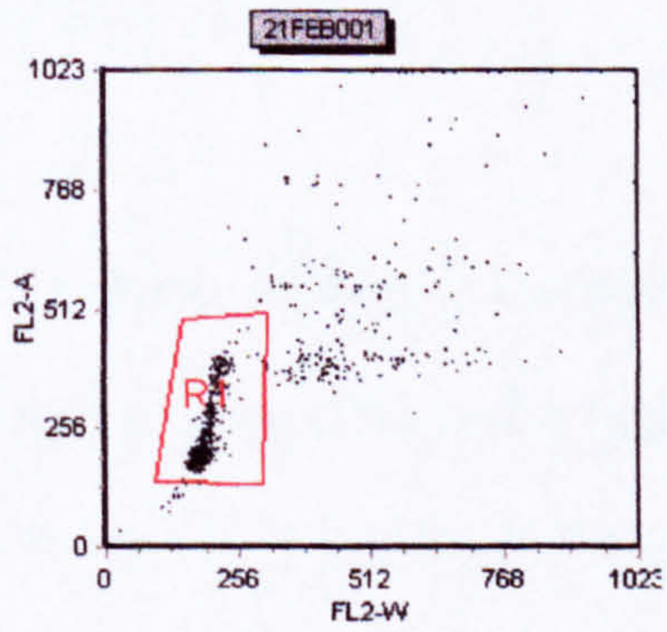


Figure 3.9: Dot plot of fluorescence-2 area against fluorescence-2 width. DNA from cell lines is gated to exclude doublet, triplet *etc.* populations.

A fluorescence-2 histogram of these gated events displays the DNA profile, and Multicycle was then used to fit the three phases of the cell cycle and calculate the percentage of cells within each phase. A DNA histogram exhibiting more or less than one $G_0/_1$ peak is classified as aneuploid (Ormerod 2000).

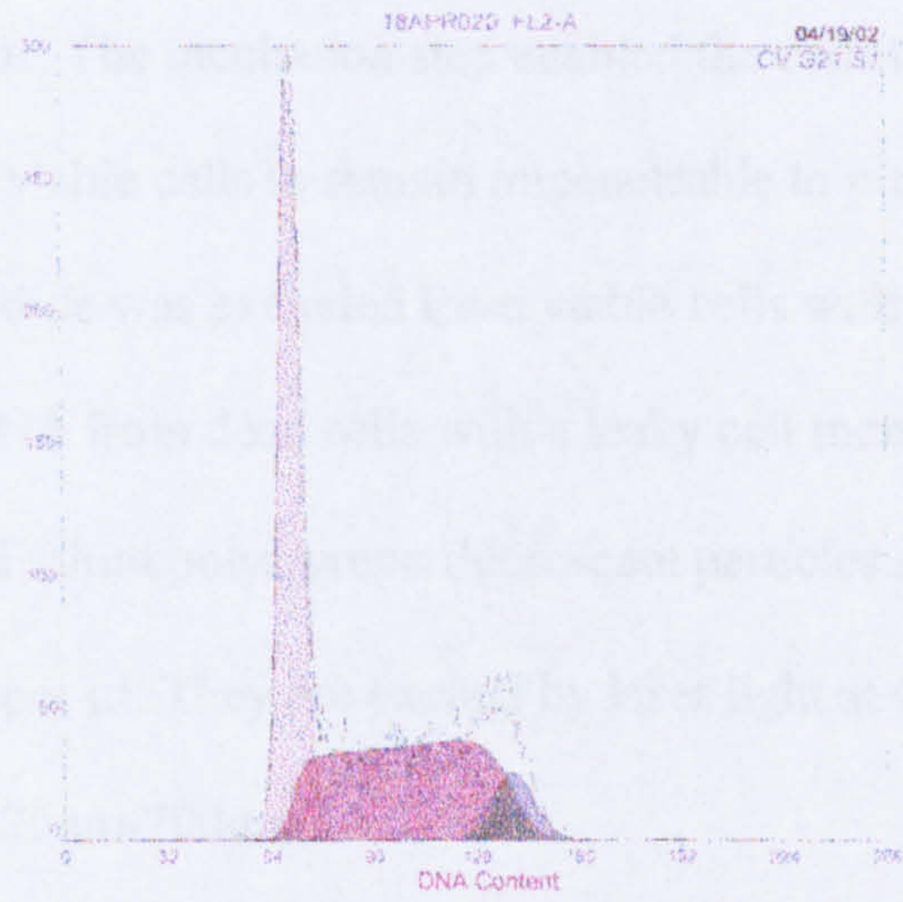


Figure 3.10: Fluorecence-2 histogram of gated events from the above dot plot. Multicycle software is used to determine the percentage of cells in each stage of the cell cycle from this histogram.

3.13 Cell proliferation studies

To assess the effect of steroids on the proliferation of cultured cells, the following method was followed. Cultured cells were harvested from 6 well plates by 1×trypsin/EDTA and pelleted by centrifugation at 2500rpm for 5 minutes in falcon tubes. The cells were then resuspended in 350µl of stripped medium and incubated for 5 minutes at 37°C. 40µl of 1mg/ml propidium iodide (Sigma, Poole, UK) and 10µl of Flow Count Fluorospheres

(Coulter, Bedfordshire, UK) were added. Analysis was performed on a Becton Dickinson FACScan using LYSYS II software on pre-stored settings.

The incubation step prior to adding propidium iodide was necessary, as harvesting with 1×trypsin/EDTA damages the cell membrane rendering the cell leaky to small molecules such as propidium iodide. The incubation step enabled the cells to repair the cell membrane allowing all viable cells to remain impenetrable to propidium iodide. Therefore propidium iodide was excluded from viable cells with an intact cell membrane, but intercalated with DNA from dead cells with a leaky cell membrane. Flow count fluorospheres consist of 10μm polystyrene fluorescent particles in an aqueous solution at a known concentration per μl. They are excited by laser light at 488nm and have a broad emission spectrum of 525nm-700nm.

Cells were analysed on a dot plot of FSC against SSC and a R1 gate set around cells to exclude debris and an R2 gate set on the fluorospheres. A dot plot of fluorescence-1 against fluorescence-2 was constructed for all events within the R1 and R2 regions. Dead cells took up propidium iodide staining with higher fluorescence-2 intensity, and therefore could be easily identified. Quadrants were set so that the upper left area contained the dead cells, the lower left area the live cells and the upper right area the fluorospheres.

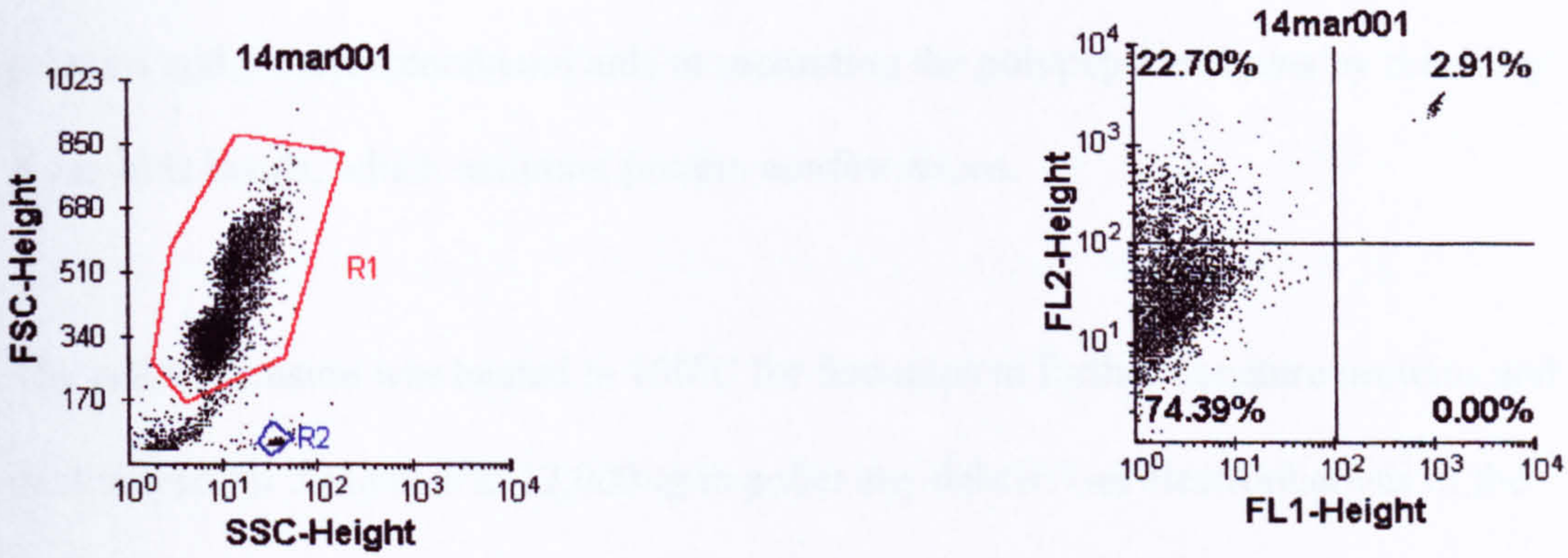


Figure 3.11: Dot plot of forward scatter (FSC) against side scatter (SSC) of cell lines and fluorospheres (gated R1 and R2 respectively). A dot plot of fluorescence-1 against fluorescence-2 is illustrated for the cells gated R1 and R2. Quadrants have been set to distinguish dead from live cells and fluorospheres. The number of live cells = number of events in lower left quadrant / number of events in upper right quadrant × fluorospheres per µl × volume of fluorospheres added to sample (µl)

3.14 Western Blotting and sodium dodecyl sulphate polyacrylamide gel electrophoresis SDSPAGE

Western blotting was used to confirm the specificity of the androgen receptor antibody. Cultured LNCap cells were harvested and pelleted by centrifugation at 180×*g* for 5minutes. Approximately 1×10^6 cells were lysed with 0.5ml of lysis buffer containing 0.125M Tris-HCl (BDH Chemicals, Poole, UK) at pH 6.8, 2% sodium dodecyl sulphate SDS (BDH Chemicals, Poole, UK), 10% glycerol (Sigma-Aldrich, Poole, UK), 0.001% bromophenol blue (BioRad, Hemel Hempstead, UK) and 10% β-mercaptoethanol (Sigma-Aldrich, Poole, UK). Sodium dodecyl sulphate is a detergent, which denatures

proteins and β-mercaptoethanol aids in separating the polypeptide chains by breaking disulphide bonds, which maintain protein confirmations.

The cell suspension was heated to 100°C for 5minutes to further denature proteins and centrifuged for 5 minutes at 12,000×*g* to pellet any debris. Gel electrophoresis of the proteins was performed using SDS-polyacrylamide gels to separate proteins by molecular weight. Polyacrylamide gels were set up using a running gel consisting of 10% acrylamide: bis-acrylamide 29:1 mix (Kramel Biotech, Cramlington, UK), 375mM Tris-HCl (BDH Chemicals, Poole, UK) at pH8.8, 0.1% sodium dodecyl sulphate (BDH Chemicals, Poole, UK), 1% ammonium persulphate (Sigma-Aldrich, Poole, UK) and 1% tetramethylethylenediamine TEMED (Sigma-Aldrich, Poole, UK). TEMED catalyzed the formation of free radicals from ammonium persulphate, which caused the acrylamide and bis-acrylamide to polymerize to form a gel matrix, which could be used for sieving macromolecules.

Having allowed the running gel to set, it was overlaid with a stacking gel (5% acrylamide: bis-acrylamide mix, 125mM Tris-HCl at pH6.8, 0.1% SDS, 1% ammonium persulphate and 1% TEMED). Gels were assembled in a pouring frame and having set were transferred to a running frame (Mini Protean II, Bio-Rad, Hemel Hempstead, UK) containing electrophoresis buffer containing 25mM Tris, 190mM glycine (BDH Chemicals, Poole, UK) and 0.1% SDS. 20µl of cell lysates and 3µl of Seeblue standards (Novex, Frankfurt, Germany) were loaded into preformed wells in the stacking gels.

Electrophoresis was carried out at 200V for 45-60 minutes in electrophoresis buffer. The negatively charged proteins migrating towards the anode and separating on the basis of polypeptide chain size. Following electrophoresis, the proteins were transferred to a Hybond-C nitrocellulose membrane (Amersham Life Sciences Ltd, Amersham, UK) by electrophoresis at 30V for 16 hours in transfer buffer containing 25mM Tris HCl pH 8.3, 150mM glycine and 10% methanol (BDH Chemicals, Poole, UK).

Nitrocellulose membranes have a high binding capacity for proteins. Therefore it was important to block any non-specific protein binding sites before incubating the membrane with antibody. Membranes were immersed in blocking solution for 1 hour at room temperature containing 5% non-fat milk powder (Marvel, Merseyside, UK), 20mM Tris-HCl at pH 7.5, and 500mM NaCl (BDH Chemicals, Poole, UK) and washed with Tris-Tween solution (0.1% Tween-20, 20mM Tris-HCl, 500mM NaCl).

Membranes were then transferred to primary antibody solution containing androgen receptor mouse anti-human monoclonal antibody (Dako, Cambridge, UK), 0.05% skimmed milk protein and Tris-Tween solution, wrapped in cellophane, heat sealed and incubated for 1 hour at room temperature on a rotating wheel. To remove any unbound primary antibody, the membrane was washed twice for 5 minutes with Tris-Tween solution and incubated with secondary antibody solution containing 1 in 500 horse radish peroxidase conjugated mouse immunoglobulin HRP-IgG (Dako, Cambridge, UK) in Tris-Tween solution containing 0.05% non-fat milk powder for 1 hour with agitation.

Unbound secondary antibody was removed by two washes for 5 minutes in Tris-Tween solution, followed by a wash for 10 minutes in Tris buffer solution. A chemiluminescence system (ECL Plus, Amersham Life Sciences Ltd) was used to HRP conjugated secondary antibody and signals were visualised by exposure to X-ray film.

3.15 Immunohistochemistry of LNCap cells with androgen receptor antibody (AR441)

Immunohistochemistry of LNCap cells was performed to confirm that the androgen receptor antibody AR441 bound to antigen on sections of a cell line known to express androgen receptors (Horoszewicz *et al.* 1983). Preparation of a cellblock from the cultured LNCap cells was as follows. Cells were harvested from flasks with 3mM ethylenediaminetetraacetic acid EDTA (Sigma-Aldrich, Poole, UK), pelleted into 15ml Falcon tubes at 1700×g for 5 minutes. The supernatant was decanted the pellet fixed by incubation with 4% formalin (Sigma-Aldrich, Poole, UK) in phosphate buffer solution at pH 7.0 for 1 hour at room temperature. All but 1ml of the fixative was decanted, the block loosened with a gentle tap on the bench and removed with forceps.

This cellblock was then processed by hand by sequential 30 minute incubations with increasing concentrations of ethanol (70%, 95%, 100% and 100%), followed by incubation with xylene for 30 minutes and 60 minutes and finally wax twice for 60 minutes, before embedding the block.

Sections were cut from the cellblock and pre-treated with 0.5% hydrogen peroxide solution (BDH Chemicals, Poole, UK) in methanol (BDH Chemicals, Poole, UK) for 10minutes and rinsed with running tap water prior to pressure-cooking sections for antigen retrieval. Pressure-cooking was performed in 1.5L of citrate buffer containing 21g citric acid monohydrate powder, 140mM of 2MNaOH (BDH Chemicals, Poole, UK), 10L of distilled water at 100°C for 2 minutes.

Sections were then rinsed in cold water, transferred to 0.005M Tris buffer solution TBS (BDH Chemicals, Poole, UK) for 5 minutes, and covered with normal rabbit solution NRS (Sigma-Aldrich, Poole, UK) for 10 minutes. Incubation with primary antibody solution containing 1 in 75 mouse anti-human androgen receptor monoclonal antibody AR441 (Dako, Cambridge, UK) was then undertaken for 30 minutes. Following incubation with primary antibody, the sections were rinsed twice with TBS and incubated for 30 minutes with secondary antibody- biotinylated rabbit anti-mouse antibody RAM (DAKO, Cambridge, UK) diluted 1 in 500 in normal rabbit serum.

Sections were again rinsed twice for 5 minutes in TBS, excess removed, before incubation with 1 in 100 tertiary antibody, streptavidin-biotin-peroxidase (DAKO, Cambridge, UK) in normal rabbit serum for 30 minutes. Sections were rinsed twice in TBS for 5 minutes; peroxidase activity was then developed, by incubating the sections for 5 minutes in diaminobenzidine tetrahydrochloride DAB solution (Sigma-Aldrich, Poole, UK). DAB solution was prepared by mixing 0.2ml of a solution containing a 10mg DAB tablet (Sigma-Aldrich, Poole, UK) in 10mls TBS at pH 7.6 to 0.2mls of a solution

containing 30vol hydrogen peroxide (BDH Chemicals, Poole, UK) in 5.8mls of distilled water.

Following incubation with DAB, sections were rinsed under running tap water for 10 minutes, counterstained with haemotoxylin, mounted in DPX (CellPath) and visualised under a microscope.

3.16 Immunohistochemistry of paraffin sections of breast tumours with androgen receptor antibody AR441

Sections were pre-treated with 0.5% solution of hydrogen peroxide in methanol for 10 minutes, washed in running tap water and rinsed in 0.005M tris buffered solution (TBS) pH 7.6 for 5 minutes. Sections were then incubated with normal rabbit serum diluted 1 in 10 with tris buffered solution for 10 minutes, excess serum was removed and replaced with primary antisera containing a 1 in 200 dilution of the androgen receptor monoclonal antibody AR441 (DAKO) in normal serum for 30 minutes. After two further 5 minute rinses with tris buffered solution, sections were then incubated with secondary antiserum containing a 1 in 500 dilution of biotinylated rabbit anti-mouse antibody in normal rabbit serum for 30 minutes. Sections were rinsed again with tris buffered solution, as above, prior to incubation for 30 minutes with tertiary antiserum, which contained 1µl streptavidin, 1µl biotinylated horseradish peroxidase and 100µl of normal rabbit serum. After two further 5 minute rinses with tris buffered solution peroxidase activity was developed by a 5-minute incubation with diaminobenzidine tetrahydrochloride solution

(DAB). Sections were then rinsed in running tap water, conterstained with haematoxylin, mounted in DPX and visulaised under a microscope.

3.17 Measurement of serum androstenedione, DHEAS and testosterone

Serum was collected pre-operatively from women presenting with primary operable breast cancer on or preceding the day of surgery. Controls were taken from the diagnostic breast clinic from women presenting with breast symptoms, in whom the diagnosis of breast cancer had been excluded. Patients on exogenous oestrogens were excluded from the study. Blood samples were spun at 2,500 rpm for 5 minutes and the serum decanted and frozen at –70°C until analyzed. Serum DHEAS, androstenedione and cortisol were measured using ELISA kits (DRG Diagnostic©). The limit of detection defined as the steroid concentration corresponding to the mean absorbance of 20 replicates of the zero standard minus 2 standard deviations, and the intraassay and interassay coefficients of variation for these kits are summarized in the following table.

	Lowest detectable limit	Intra assay coefficient of variation %	Inter assay coefficient of variation %
DHEAS	0.02microg/ml	2.13-5.07	2.96-8.67
Androstenedione	0.043ng/ml	2.79-6.30	5.95-8.09
Cortisol	2.5ng/ml	3.98-4.70	4.96-9.59

Table 3.1: Specifications of serum androgen kits

Materials and Methods

A lectin ELISA for cerbB-2/HER-2

4.1 Lectin ELISAs

The lectin-based methods summarised above are not suitable for the investigation of a large number of clinical specimens. Lectin-based assays using the familiar sandwich ELISA technology in multiwell plates have been developed for this purpose. Pekelharing *et al* first described a lectin-enzyme immunoassay for the measurement of transferrin sialovariants (Pekelharing *et al.* 1987).

By replacing the immobilized or enzyme-linked antibody with a lectin a heterologous lectin-enzyme immunoassay to study the glycosylation of glycoproteins is constructed. The sandwich consists of a lectin and an antibody. Only glycoprotein that meets two conditions is measured. It must be the right protein to be bound by the antibody and have the relevant glycan structure to bind the lectin (Pekelharing *et al.* 1987).

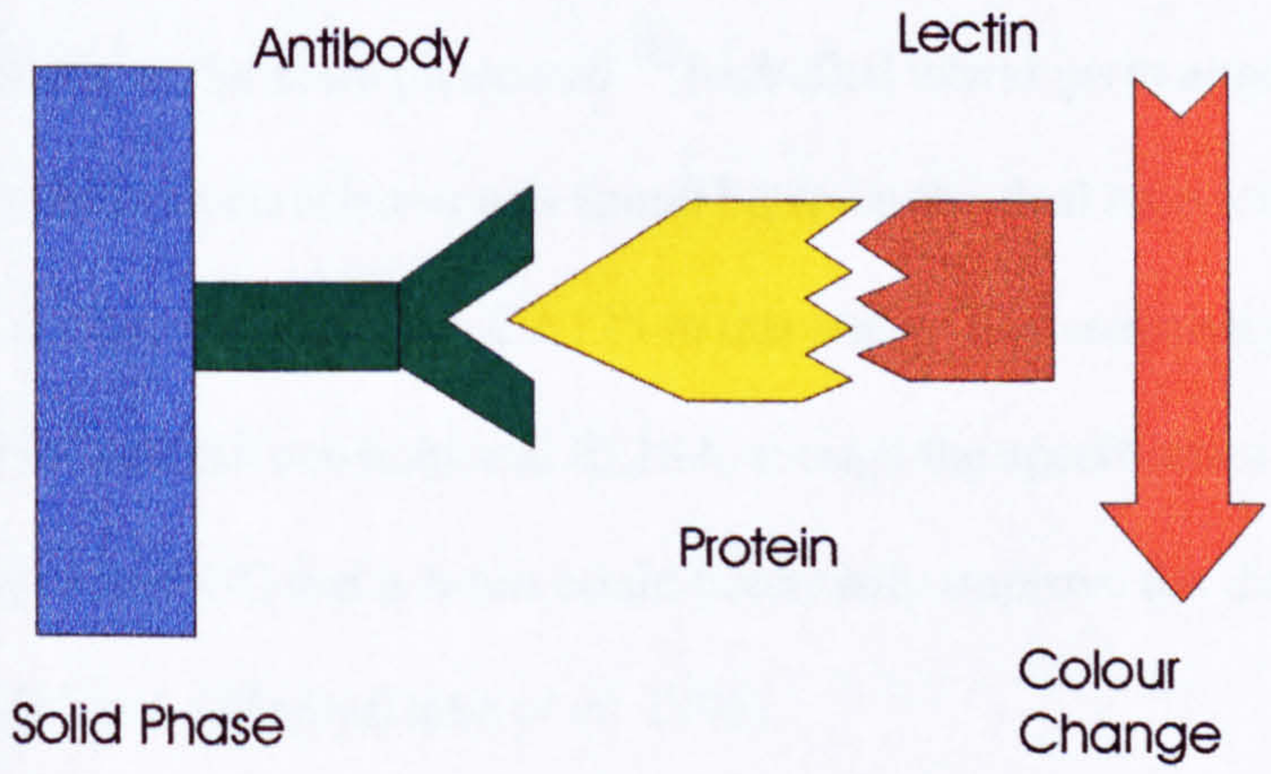


Figure 4.1: Lectin ELISA

Two configurations are possible, both of which have drawbacks. If the lectin is immobilised, it may bind glycans of other glycoproteins in the sample, which compete with the molecule of interest for the available binding sites. This may strongly influence the results and reduce the sensitivity of the method. On the other hand, when an antibody is used as a capture molecule, care must be taken to ensure that the antibody is not binding to the same determinant as that reacting with the lectin (Turner 1992).

Both types of assay bear the risk of binding of the lectin to the carbohydrate determinants of the immunoglobin used as an antibody: IgG contains 5% carbohydrate, IgM 12% and IgA 7.5%. This may cause elevated binding in the absence of sample (Pekelharing *et al.* 1987).

Lectin ELISAs have subsequently been reported for the investigation of serum glycoproteins in a variety of diseases. Madiyalakan *et al* reported a lectin ELISA for the determination of CA125 antigen in ovarian cancer patients using an anti-CA125 monoclonal antibody in the solid phase and ^{125}I-labelled wheat germ agglutinin in the liquid phase. A moderate correlation was found between the dual monoclonal antibody sandwich assay and lectin ELISA for CA125 in this study. The sensitivity of the lectin ELISA was higher than the conventional ELISA, though the specificities were comparable. This suggested that a lectin could potentially improve the diagnostic utility to detect ovarian cancer (Madiyalakan *et al.* 1996).

Parker *et al* developed a lectin ELISA for the diagnosis of pancreatic cancer, in which a lectin wheat germ agglutinin was bound to the solid phase and an anti-mucin antibody CAM17.1 and peroxidase-tagged antibody in the liquid phase were used as a detection system. The sensitivity and specificity of this assay compared with conventional radioimmunoassay for another mucin antigen CA19-9 (Parker *et al.* 1992).

4.2 Biotinylation of synthetic p185 peptide

A synthetic p185 peptide (Cambridge Research Biochemicals Ltd, Cheshire, UK), which consists of the following sequence of amino acids, Pro-Glu-Ser-Phe-Asp-Gly-Asp-Pro-Ala-Sec-Asn-Thr-Ala-Pro-Leu-Glu-Pro-Cys was biotinylated by the following method. This sequence of amino acids forms part of the extracellular domain of HER-2. This synthetic peptide was used as the antigen against which the monoclonal antibody OM-11-954 (Genosys Biotechnologies) was raised.

150µg of p185 peptide was dissolved in 0.1ml of 0.1M phosphate buffer solution 0.1M NaPO4 (BDH Chemicals, Poole, UK) to give a p185 solution of 1500µg/ml.

Molecular weight p185 peptide = 2150

Therefore the concentration of p185 solution = 1500/2150 = 0.7 µmol/L

2ml of EZ-link™ NHS-LC biotin (Perbio Science, Cheshire, UK) was dissolved in 12µl of dimethyl sulfoxide DMSO (Sigma-Aldrich, Poole, UK) and 188µl of deionised water to give an EZ-link™ solution of 10mg/ml.

Molecular weight of EZ-link™ = 556.8

Therefore the concentration of EZ-link™ solution = 10/556.8 = 17.9 μmol/L

To give a 10:1 ratio of EZ-link™: p185 solution

$X = 10 \times 0.7/18 = 0.39$ mls of EZ-link™ need to be added per ml of p185 protein

or, 19.5 μl EZ-link™ to 50μl of p185 solution.

Therefore, 50μl of p185 solution and 19.5μl of EZ-link™ solution were vortexed and incubated for 4 hours at 4°C. The biotinylated p185 peptide was separated from the excess biotin molecules by eluting the mixture through a 6×1 cm column containing sephadex G-25 (Sigma-Aldrich, Poole, UK) with 0.01M phosphate buffer solution. 1ml fractions were collected following elution with this buffer.

4.3 Capture of biotinylated synthetic p185 by cerbB2 antibody OM-11-954

Carbonate buffer: 0.1M $NaCO_3/NaHCO_3$ (BDH Chemicals, Poole, UK), pH 9.6

PBS-Tween solution: 0.1M NaPO4 (BDH Chemicals, Poole, UK), 0.1%Tween-20 (Pharmacia, Uppsala, Sweden), pH 7.4

Tris-Tween solution: 0.1M TrisHCl (BDH Chemicals, Poole, UK), 0.1% Tween-20, pH 7.6

Immulon 4 microtiter plates (Dynex Technologies, Chantilly, USA) were incubated overnight with 100µl of 1 in 1000 OM 11-954 (Genosys Biotechnologies) in carbonate buffer at 4°C, washed ×3 with PBS-Tween solution and slapped dry.

100µl dilutions of biotin p185 in 1% bovine serum albumin BSA (Sigma-Aldrich, Poole, UK) PBS-Tween solution were added per well and incubated overnight at 4°C, washed ×6 with Tris-Tween solution and slapped dry.

100µl of 1 in 2000 streptavidin alkaline phosphatase (Sigma-Aldrich, Poole, UK) in 1% BSA Tris-Tween solution was added per well and incubated for 2 hours at room temperature, then washed ×6 with Tris-Tween solution and slapped dry.

100µl of BCIP substrate (KP Laboratories, Maryland, USA) was then added per well, and the absorbance was read on a *vmax* kinetic microplate reader (Molecular Devices) at 620nm.

4.4 Competition between unconjugated syntheic p185 and biotinylated synthetic p185 for binding to cerbB2 antibody (OM-11-954)

100µl of 1 in 1000 OM-11-954 in carbonate buffer was added per well and incubated overnight at 4°C. Wells were washed × 3 with PBS Tween solution and slapped dry. Dilutions of unconjugated synthetic p185 in 1% BSA PBS Tween were prepared. 50µl of biotinylated synthetic p185 containing 800ng of protein in 1% BSA PBS Tween and 50µl of unconjugated synthetic p185 containing 0-1500ng of protein were added per well and incubated overnight at 4°C. Wells were washed × 6 with TrisTween solution and 100µl of 1 in 1000 streptavidin alkaline phosphatase in 1% BSA Tris Tween solution was

added per well and incubated for 2 hours at room temperature. Wells were then washed ×6 with Tris-Tween solution and slapped dry. 100µl of BCIP substrate (KP Laboratories, Maryland, USA) was then added per well, and the absorbance was read on a *vmax* kinetic microplate reader (Molecular Devices) at 620nm.

4.5 Preparation of Zabrecky Lysate of SKBR3 cells

Lysis Buffer: 10mM NaPO4 (BDH Chemicals, Poole, UK), 140mM NaCl (BDH Chemicals, Poole, UK), 1% Triton X-100 (BDH Chemicals, Poole, UK), 2.5ml Trasylol (Bayer), 0.1% sodium dodecyl sulphate (BDH Chemicals, Poole, UK), 1% sodium deoxycholate (BDH Chemicals, Poole, UK) and 1mM phenylmethylsulfonylfluoride (Sigma-Aldrich, Poole, UK)

Preparation of a SKBR3 cell lysate follows the method described by Zabrecky (Zabrecky *et al.* 1991) and is as follows. The SKBR3 cell line was chosen as it has been shown to express high levels of HER-2 protein (Leitzel *et al.* 1992).

500µl of SKBR3 cells were removed from storage in liquid nitrogen, thawed and 250µl aliquots were transferred to 1ml Eppendorf tubes. The cells were then centrifuged at 13,000rpm for 15minutes at 4°C to from a cell pellet. The supernatant was decanted and the pellet resuspended by vortexing in lysis buffer (described above). This cell suspension was incubated for 30minutes at 4°C, centrifuged for 20minutes at 13,000rpm and the supernatant decanted into 1ml Eppendorf tubes. This supernatant was re-centrifuged for

10minutes at 13,000rpm and 25µl aliquots added to 100µl of buffer containing 50mM HEPES (Sigma-Aldrich, Poole, UK), 140mM NaCl (BDH Chemicals, Poole, UK), 0.1% Triton X-100 (BDH Chemicals, Poole, UK), at pH 7.5. These 125µl aliquots of Zabrecky lysate were stored at –80°C.

4.6 Binding of OM11-954 with Zabrecky Lysate of SKBR3 cells

This experiment was undertaken to demonstrate that the OM11-954 antibody, used as the capture antibody in the lectinELISA, bound to p185 protein coated to wells incubated overnight with Zabrecky lysate of SKBR3 cells. Medium from the SKBR3 cells, obtained after centrifuging the cell suspension in the first step of the preparation of Zabrecky lysate was also investigated to establish whether this contained any shed p185 protein. The HER-2 protein content of the Zabrecky lysate of SKBR3 cells was also measured using cerbB-2 kits (Oncogene Science).

Immulon 4 microtitre plates were incubated overnight at 4°C with 100µl of Zabrecky lysate or medium of SKBR3 cells diluted in carbonate buffer, washed 3× with PBS-Tween solution and slapped dry. 100µl of 1 in 1000 OM11-954 in 1% BSA PBS-Tween solution was added per well, incubated overnight at 4°C, washed ×6 with Tris-Tween solution and slapped dry. 100µl of 1 in 1000 alkaline phosphatase conjugated anti-mouse IgG (Sigma-Aldrich, Poole, UK) in 1% BSA Tris-Tween solution was added per well and incubated overnight at 4°C. After washing ×6 with Tris-Tween solution and slapping the

wells dry, 100µl of BCIP was added and absorbance was read at timed intervals on a *vmax* kinetic microplate reader (Molecular Devices) at 620nm.

4.7 Binding of wheat germ agglutinin to complex carbohydrates

Fetuin, a heavily glycosylated glycoprotein was used to check that the lectin used in the assay, wheat germ agglutinin, binds to glycoconjugate. 3mg/ml fetuin in phosphate buffer solution was diluted to a final concentration of 30µg/ml. Immulon 4 plates were coated overnight at 4°C with 100µl dilutions of 30µg/ml fetuin in carbonate buffer, washed ×6 with PBS-Tween solution and slapped dry. 100µl of 1 in 1000 biotin conjugated wheat germ agglutinin b-WGA (Vector) in 1% PBS-Tween was added per well, incubated overnight at 4°C, washed ×6 with Tris-Tween solution and again slapped dry. Finally, wells were incubated with 100µl of 1 in 1000 streptavidin alkaline phosphatase (Vector) in 1% BSA Tris-Tween solution for 2hours at room temperature, washed ×6 with Tris-Tween solution, slapped dry and 100µl of BCIP added per well. Absorbance was read at timed intervals on a *vmax* kinetic microplate reader (Molecular Devices) at 620nm.

4.8 The HER-2/cerbB-2 lectin ELISA

Having established by the above methods that the capture antibody OM11-954 binds p185 the extracellular domain of HER-2, the Zabrecky lysate of SKBR3 cells contains

the HER-2 protein and that OM11-954 binds to this protein, and the wheat germ agglutinin binds to complex carbohydrates, the next step was to construct the HER-2 lectin ELISA.

Immulon 4 microtitre plates were incubated overnight at 4°C with 100µl of 1µg/ml OM11-954 in carbonate buffer, then washed ×3 with PBS-Tween and slapped dry. 100µl of SKBR3 Zabrecky lysate diluted in 1% BSA PBS-Tween was added per well, incubated overnight at 4°C, washed ×6 with PBS-Tween and slapped dry. 100µl of 1 in 1000 biotinylated wheat germ agglutinin in 1% BSA PBS-Tween was added per well, incubated overnight at 4°C, washed ×6 with Tris-Tween solution and slapped dry. Finally, after 2 hours incubation at room temperature with 100µl of 1 in 1000 streptavidin alkaline phosphatase in 1% BSA Tris-Tween, followed by further washing in Tris-Tween solution and slapping dry, 100µl of BCIP was added and absorbance was read at 620nm at timed intervals, as above.

Results and discussion

Serum androgens levels in breast cancer

All patients were recruited to this study between August 2000 and July 2002 from the Breast Unit at the Royal Victoria Infirmary, Newcastle upon Tyne. Cases had confirmed primary operable screen-detected or symptomatic breast cancer. Controls were recruited from the diagnostic breast clinic and had a variety of benign breast disorders. The case and control groups had a similar mean, median and range of ages. This data is summarized in the following table.

	Number	Age Range	Median Age	Mean Age
Premenopausal case	23	33-51	46	44.7
Premenopausal control	25	34-52	46	44.8
Postmenopausal case	58	48-80	63	63.1
Postmenopausal control	37	48-86	62	63.0

Table 5.1: Sample demographics DHEAS and androstenedione

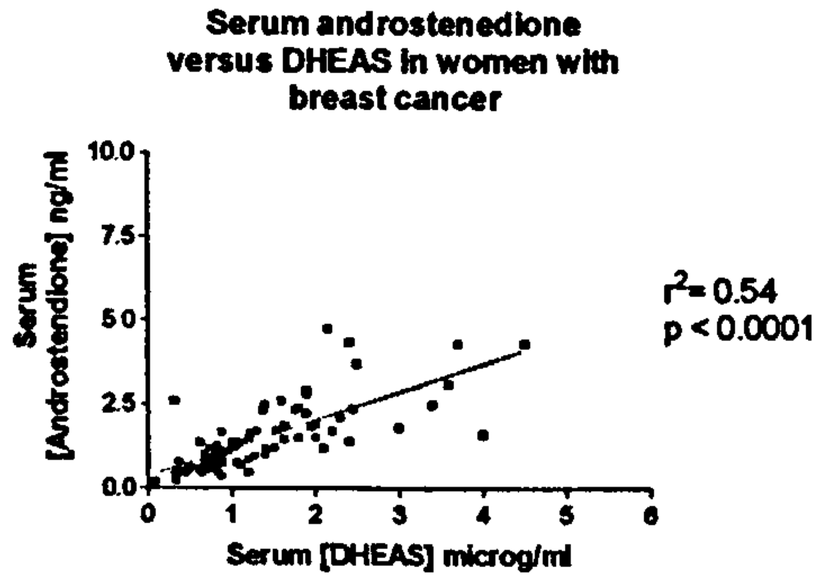
	Number	Age range	Mean age	Median age
Premenopausal case	21	33-51	45.0	47.5
Premenopausal control	22	34-52	45.0	46
Postmenopausal case	51	46-86	63.0	63
Postmenopausal control	41	48-88	63.0	64

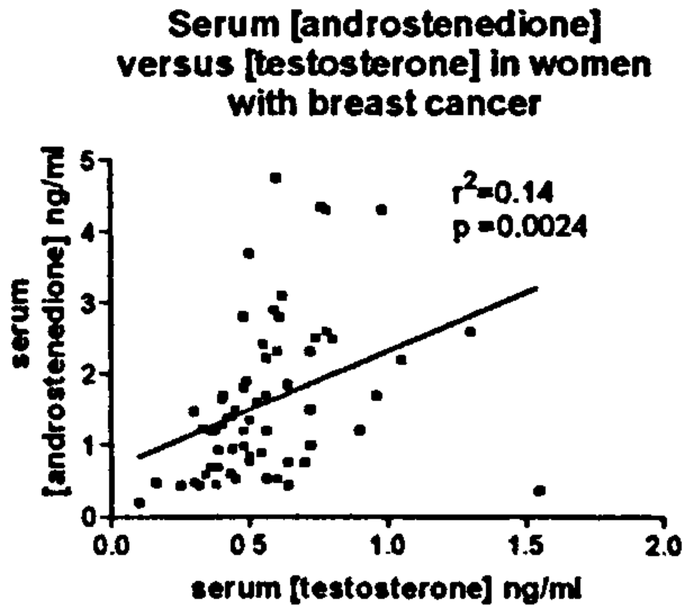
Table 5.2: Sample demographics serum testosterone

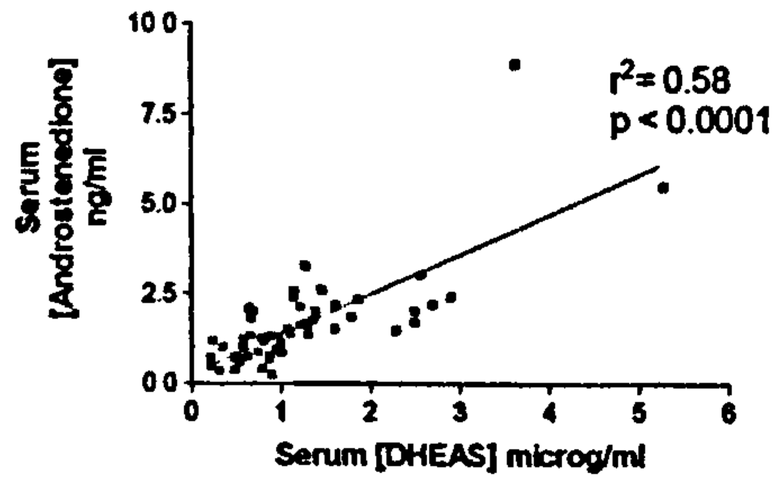
Results

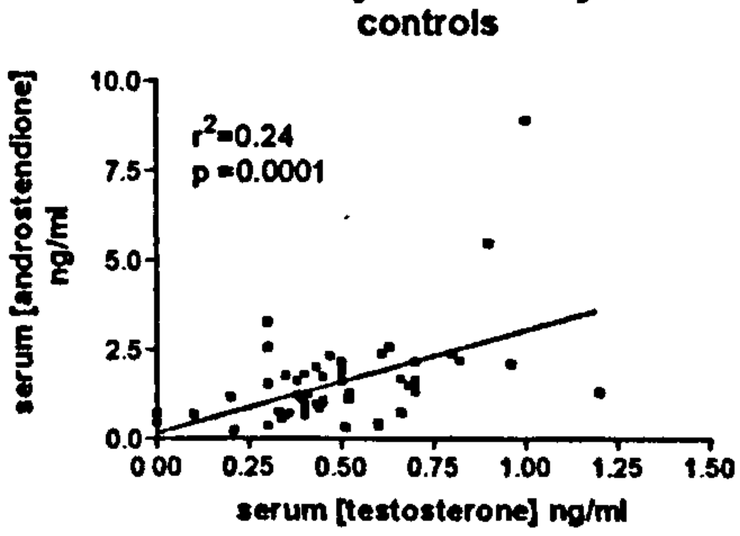
5.1 Relationship between serum androstenedione, DHEAS and testosterone levels in women with breast cancer and controls

In this study serum androstenedione levels were significantly correlated with DHEAS in women with breast cancer ($p<0.0001$, $r^2=0.54$) and controls ($p<0.0001$, $r^2=0.58$). Serum testosterone levels were correlated with androstenedione in women with breast cancer ($p=0.0024$, $r^2=0.14$) and controls ($p=0.0001$, $r^2=0.24$) and DHEAS in women with breast cancer ($p=0.02$, $r^2=0.07$) and controls ($p<0.0001$, $r^2=0.30$). This data is illustrated in the following graphs and tables.









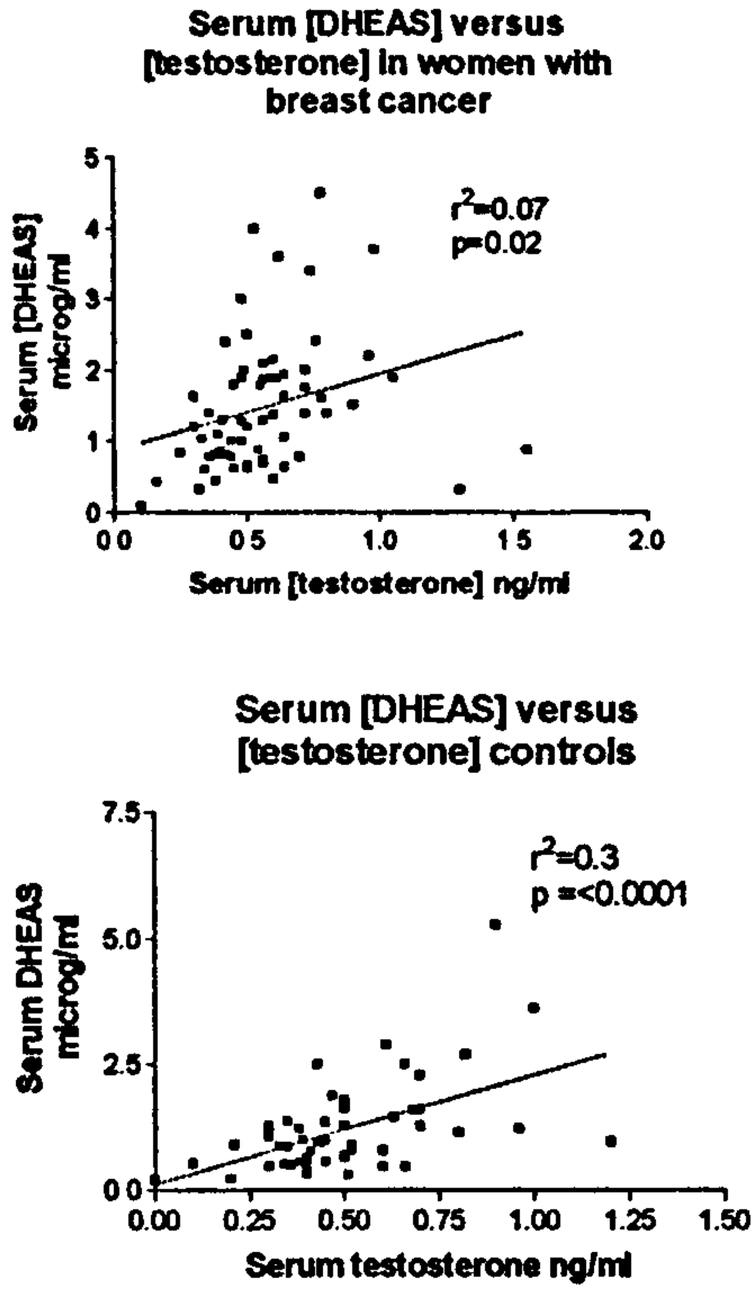


Figure 5.1: Serum testosterone versus DHEAS and androstenedione

	Testosterone	
	Case	**Control**
DHEAS	p=0.02	p<0.0001
	r^2=0.07	r^2=0.30
Androstenedione	p=0.0024	p=0.0001
	r^2=0.14	r^2=0.24

Table 5.3: Results of linear regression analysis of serum testosterone against DHEAS and androstenedione for women with breast cancer and controls

Androstenedione		
	Case	**Control**
DHEAS	$p<0.0001$	$p<0.0001$
	$r^2=0.54$	$r^2=0.56$

Table 5.4: results of linear regression analysis of serum DHEAS against androstenedione for women with breast cancer and controls

5.2 Relationship between serum androstenedione, DHEAS and testosterone levels and age in women with breast cancer and controls

In the control group of this study, we observed a decline in serum DHEAS levels with increasing age. The association between serum DHEAS and age in the control group was significant (linear regression $p=0.006$) though the correlation was low ($r^2=0.09$). Likewise serum testosterone levels were found to decline with age, the association between serum testosterone and age was significant (linear regression $p=0.006$) but again the correlation coefficient was low ($r^2=0.09$). Serum androstenedione levels also fell with age in the control group, though the association between serum androstenedione and age was not significant (linear regression $p=0.08$).

The association between age and serum adrenal androgen levels in women with breast cancer was significant (linear regression $p=0.003$) but again the correlation was low ($r^2=0.1$). Serum testosterone levels also fell with age, though the association between age and serum testosterone levels in women with breast cancer failed to reach significance

(linear regression p=0.07). The relationship between serum adrenal androgen levels and age are demonstrated in the following graphs.

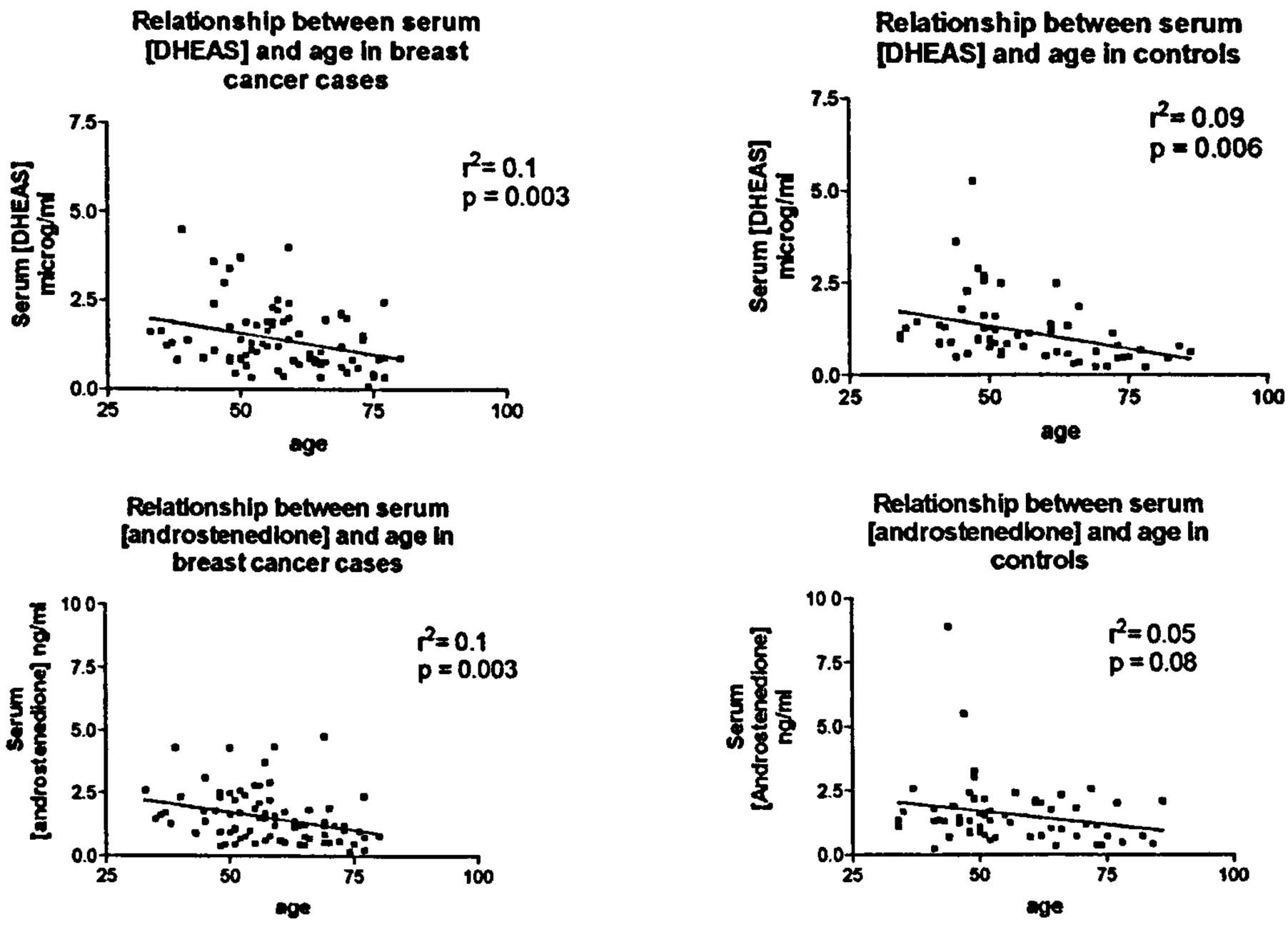
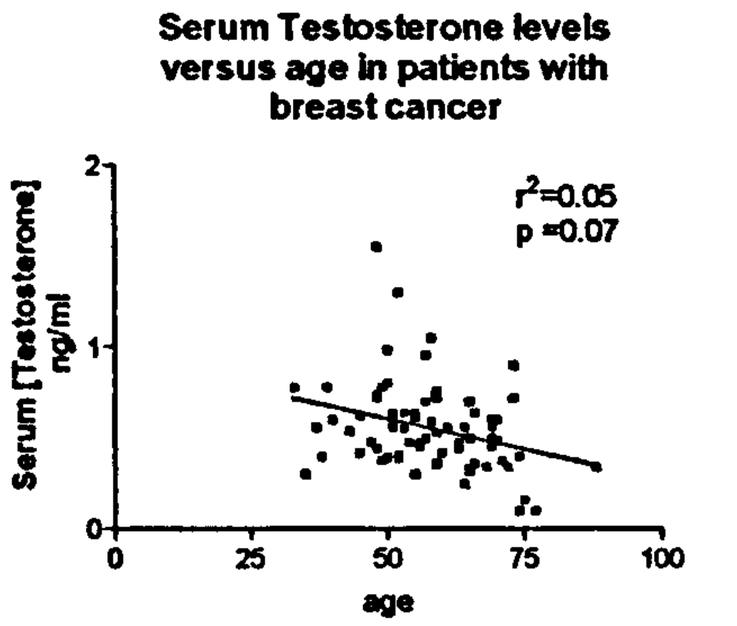


Figure 5.2: Relationship between serum androstenedione, DHEAS and age in women with breast cancer and controls



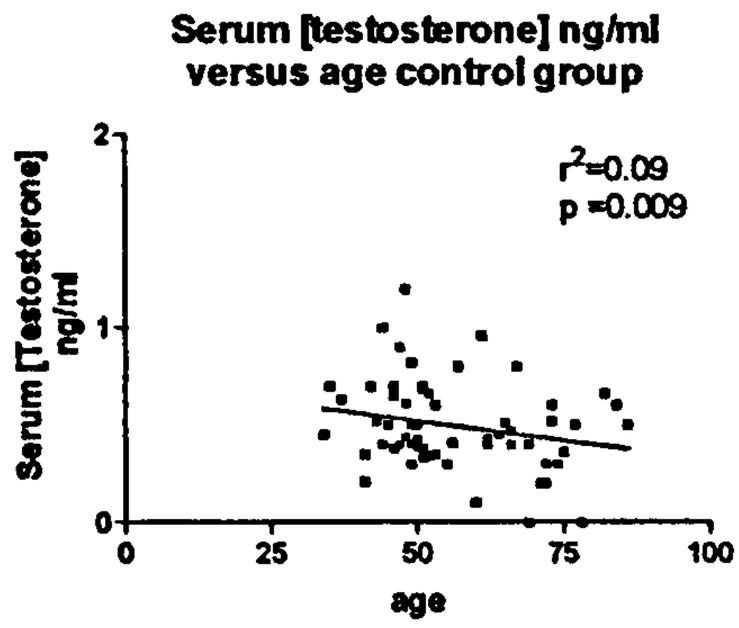


Figure 5.3: Relationship between serum testosterone levels and age in women with breast cancer and controls

		Testosterone	DHEAS	Androstenedione
Case	R^2	0.05	0.1	0.09
	P value	0.07	0.003	0.006
Control	R^2	0.09	0.1	0.05
	P value	0.009	0.003	0.08

Table 5.5: Relationship between serum testosterone, DHEAS and androstenedione levels and age in women with breast cancer and controls

5.3 Serum androstenedione, DHEAS and testosterone levels in women with breast cancer and controls

Next the serum levels of androstenedione, DHEAS and total testosterone in pre and postmenopausal women with breast cancer and control groups were compared. Both mean and median serum DHEAS levels were elevated in postmenopausal women with breast cancer. However, although the difference in median serum DHEAS between postmenopausal women with breast cancer and controls was significant (Mann-Whitney test p=0.038), the difference between mean serum DHEAS just failed to reach significance (*t* test p=0.056). No difference was observed in serum DHEAS levels between premenopausal women with breast cancer and controls. Serum androstenedione levels were not found to differ between women with breast cancer and controls in either premenopausal or postmenopausal groups.

Although mean serum testosterone levels were higher in both pre and postmenopausal women with breast cancer than controls the difference between the means failed to reach significance in either group. Serum DHEAS, androstenedione and testosterone levels in premenopausal and postmenopausal women with breast cancer and controls are illustrated in the following table and graphs.

Serum [androstenedione]	Median ng/ml	Mean ng/ml	Standard error of mean	95% Confidence interval
Premenopausal case	1.47	1.73	0.22	1.30-2.19
Premenopausal control	1.36	1.89	0.34	1.19-2.59
Postmenopausal case	1.2	1.42	0.13	1.17-1.67
Postmenopausal control	1.16	1.37	0.12	1.12-1.62

Table 5.6: Androstenedione cases and controls

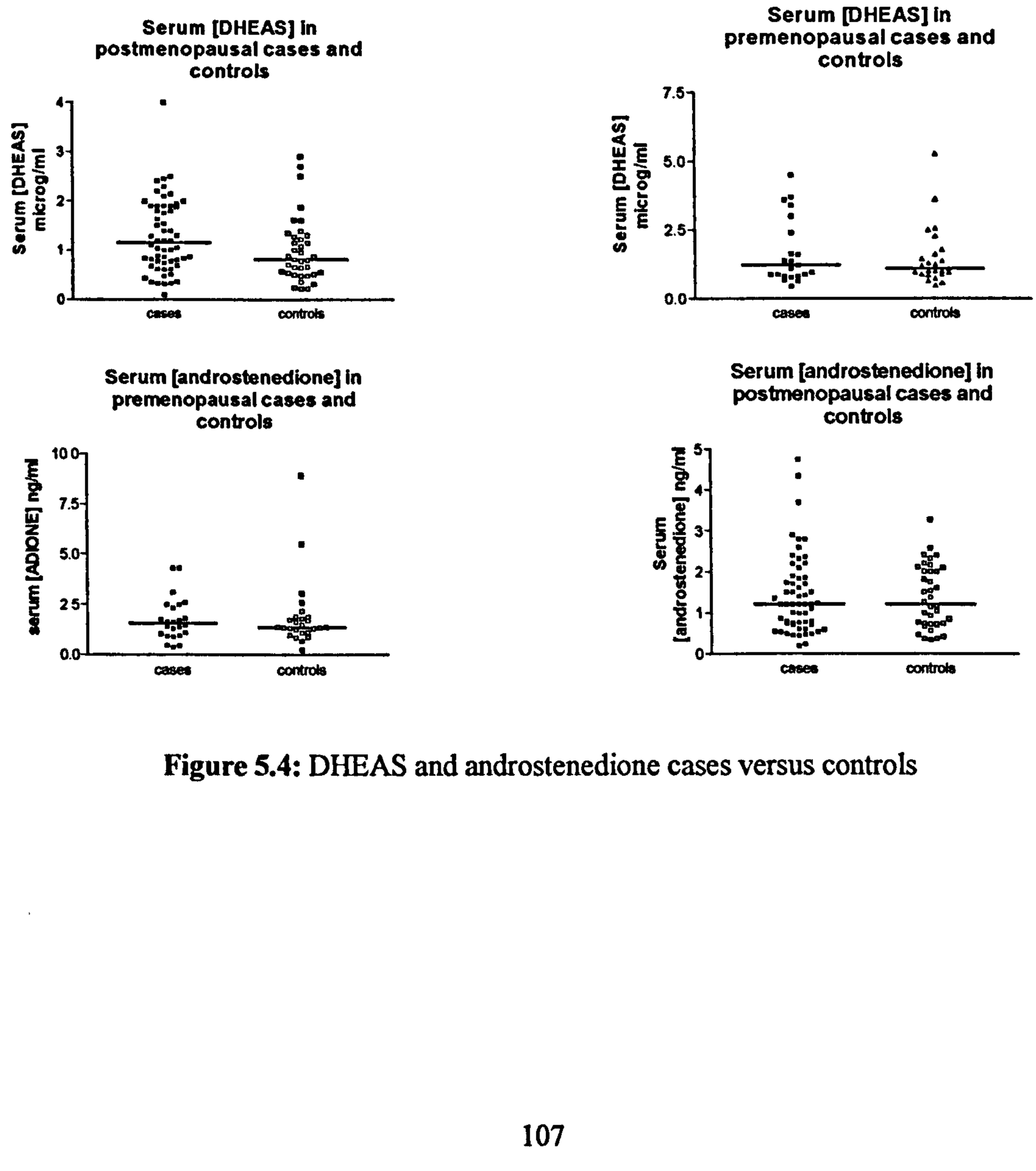
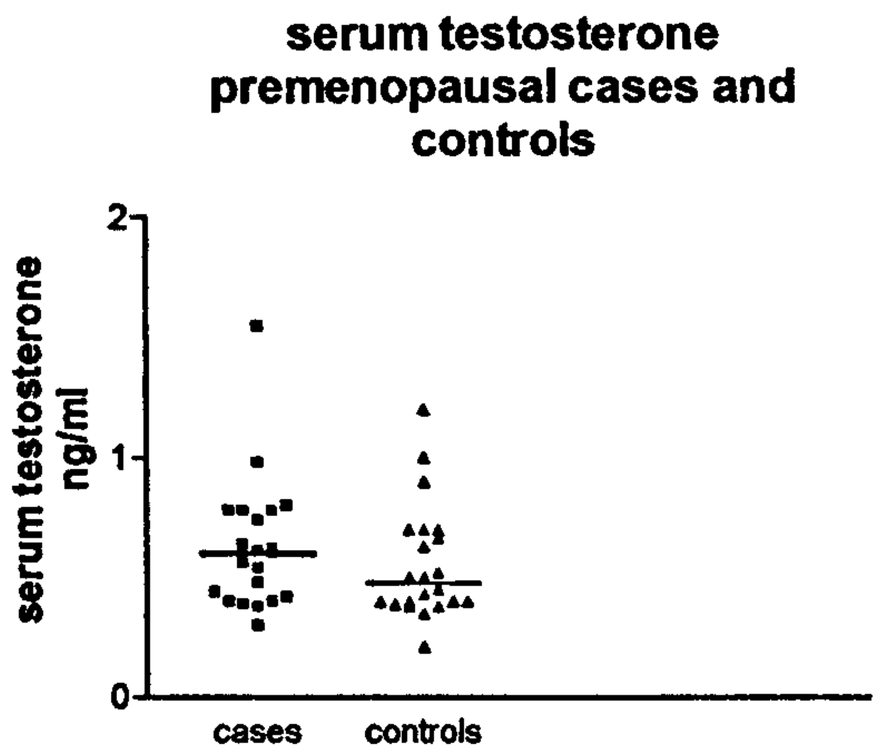


Figure 5.4: DHEAS and androstenedione cases versus controls

Serum [DHEAS]	Median microg/ml	Mean microg/ml	Standard error of mean	95% Confidence interval
Premenopausal case	1.22	1.65	0.24	1.14-2.16
Premenopausal control	1.1	1.50	0.21	1.06-1.94
Postmenopausal case	**1.15***	1.28	0.10	1.09-1.48
Postmenopausal control	**0.8***	0.99	0.11	0.77-1.21

Table 5.7: serum DHEAS cases and controls



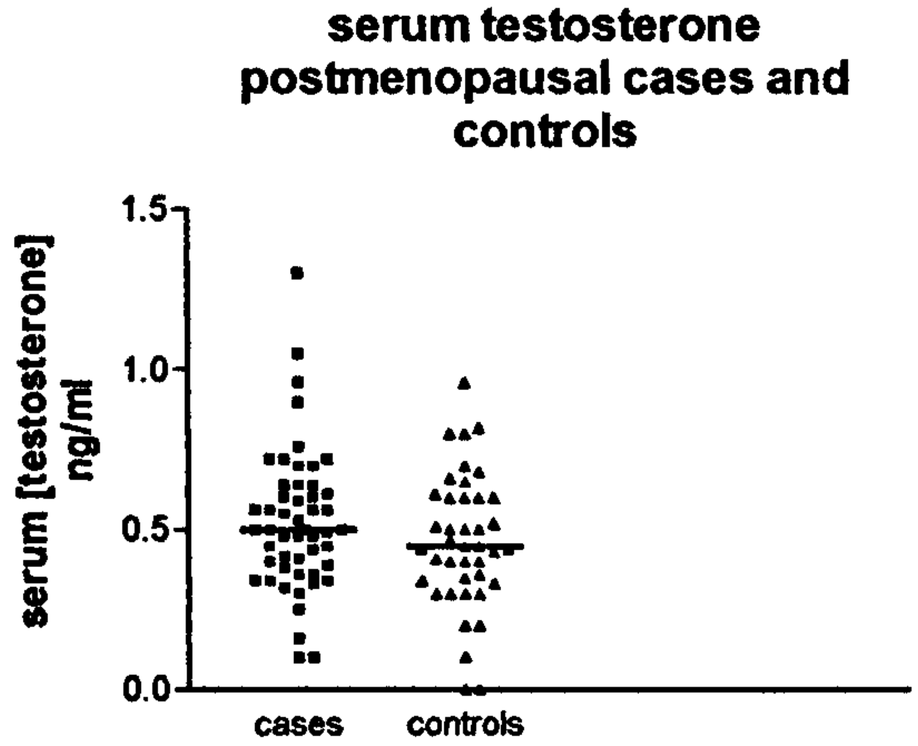


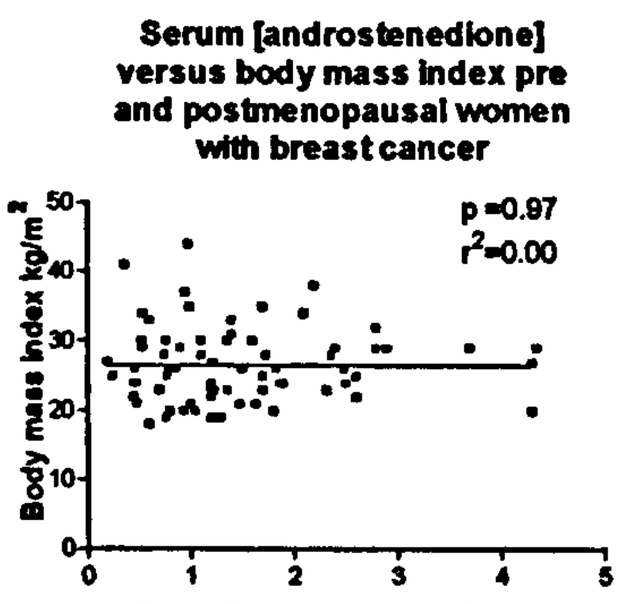
Figure 5.5: Serum testosterone cases and controls

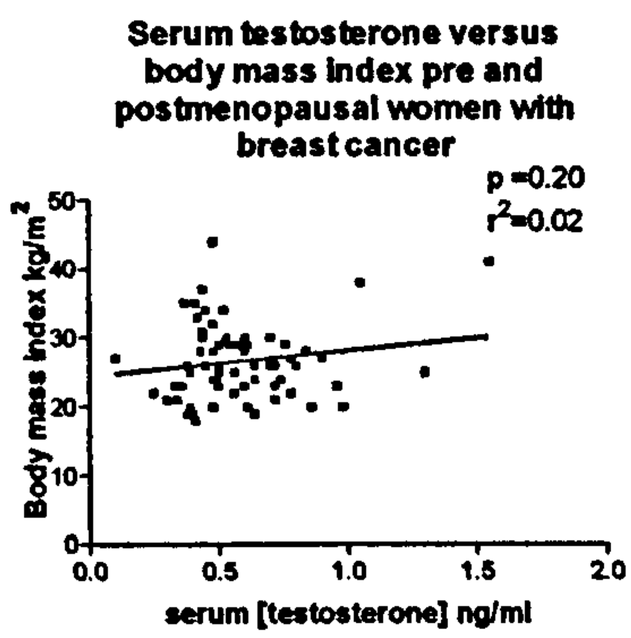
Serum [testosterone]	Median ng/ml	Mean ng/ml	Standard error of the mean	95% Confidence Intervals
Premenopausal case	0.60	0.63	0.06	0.50-0.75
Premenopausal control	0.47	0.55	0.05	0.45-0.66
Postmenopausal case	0.50	0.52	0.03	0.46-0.59
Postmenopausal control	0.45	0.46	0.03	0.39-0.52

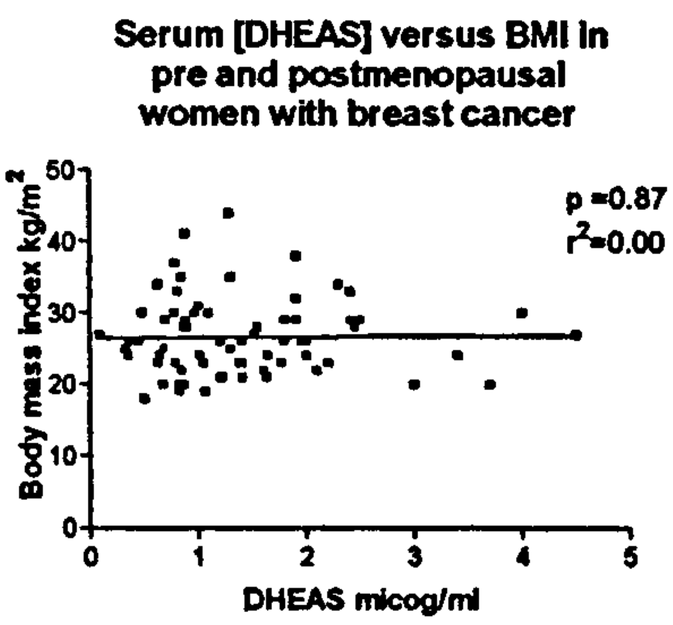
Table 5.8: serum testosterone cases and controls

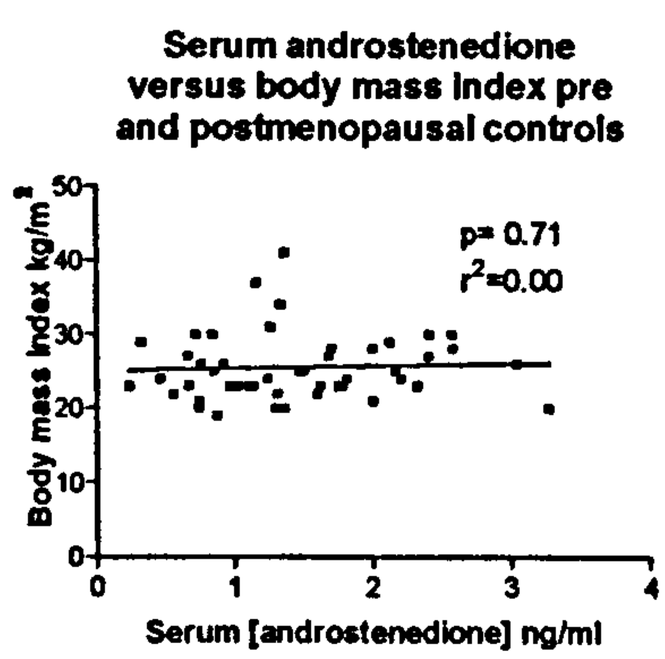
5.4 Relationship between serum androstenedione, DHEAS and testosterone levels and body mass index

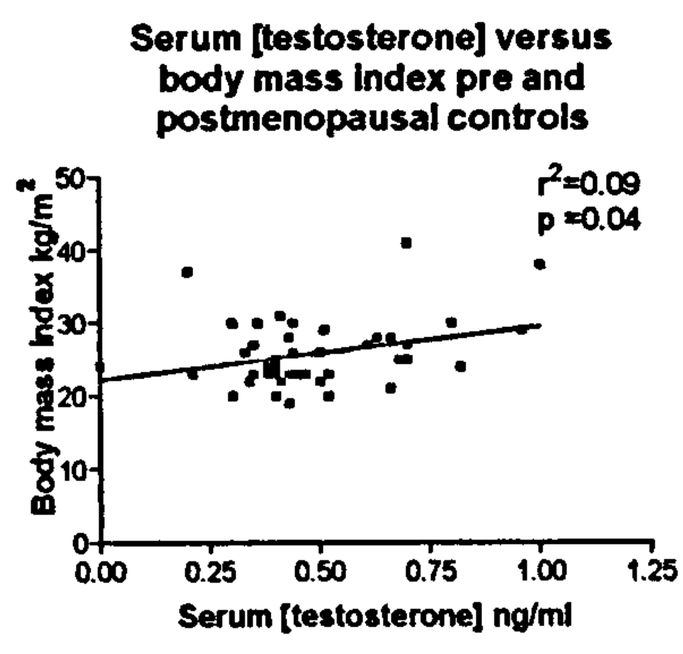
An association between serum adrenal androgen levels and body mass index was sought. No significant association was found between serum DHEAS and body mass index in women with breast cancer (linear regression $p=0.87$, $r^2=0.00$) or controls (linear regression $p=0.198$, $r^2=0.036$). Likewise serum androstenedione was not associated with body mass index in either women with breast cancer (linear regression $p=0.97$, $r^2=0.00$) or controls (linear regression $p=0.71$, $r^2=0.00$). These results suggest that serum adrenal androgen levels are independent of body mass index, and are illustrated in the following.











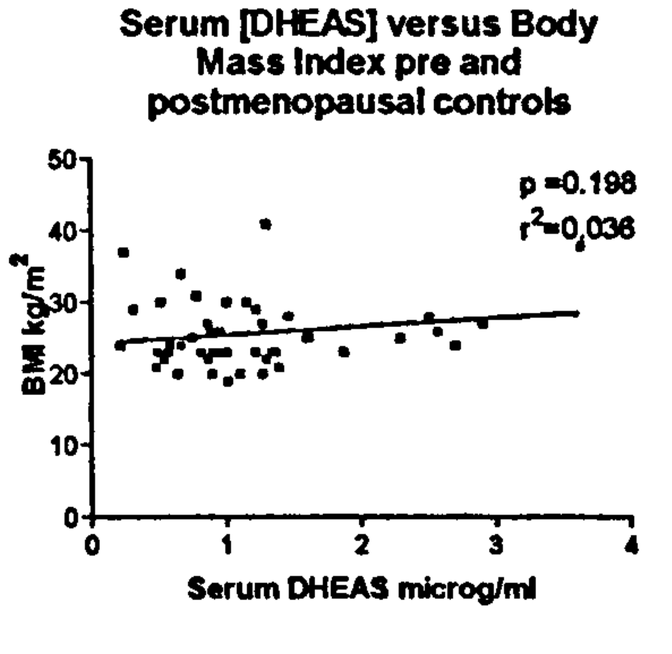


Figure 5.6: Relationship between serum androstenedione, DHEAS and testosterone levels and body mass index

		Testosterone	DHEAS	Androstenedione
Case	R^2	0.04	0.00	0.00
Control	P value	0.20	0.87	0.97
Case	R^2	0.09	0.036	0.71
Control	P value	0.04	0.198	0.00

Table 5.9: Relationship between serum androstenedione, DHEAS and testosterone levels and body mass index

5.5 Relationship between serum androgens and stress

Adrenal androgens are secreted in response to changes in ACTH as part of the pituitary-adrenal axis (Parker *et al.* 1980). As serum was taken from women with breast cancer on or preceding the day of surgery we investigated whether pre-operative anxiety may have contributed to the elevated adrenal androgen levels observed in women with breast cancer. Cortisol is a steroid, which is also secreted by the adrenal cortex in response to changes in ACTH levels accompanying trauma, infection, surgery and emotional disturbance (James 1984). Serum cortisol levels were therefore measured and plotted against serum DHEAS. The absence of an association between serum cortisol and DHEAS suggests that serum DHEAS levels are truly elevated in postmenopausal women with breast cancer and are not spuriously raised due to the stress response. The following graph illustrates that serum DHEAS were independent of serum cortisol.

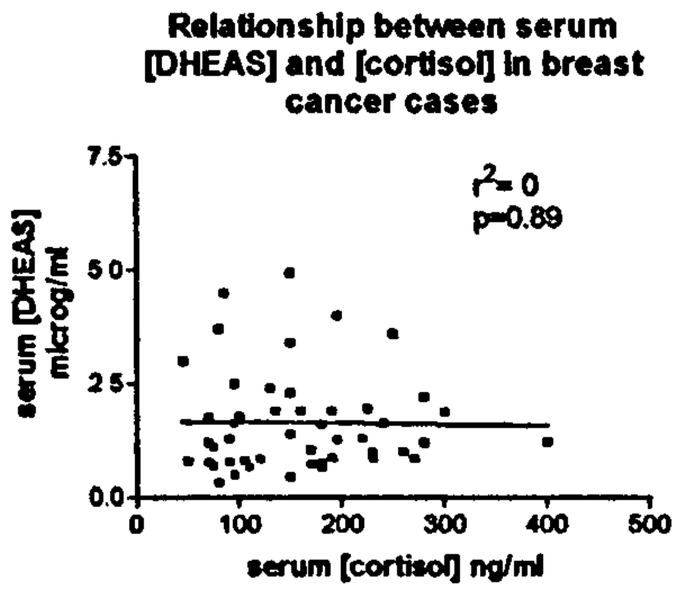


Figure 5.7: Relationship between serum DHEAS and cortisol levels

Discussion

5.6 Sources of androgens in women

We have mentioned previously that androgens are derived from the adrenal cortex and ovary. The adrenal secretes primarily DHEA, DHEAS and androstenedione, but also smaller amounts of testosterone (Longcope 1986). Adrenal androgens, in addition to cortisol and aldosterone are secreted by the adrenal cortex in response to changes in serum adrenocorticotrophin hormone (ACTH) concentrations. This mechanism is referred to as the pituitary adrenal axis (Parker *et al.* 1980). Because of the selective increase in adrenal androgen levels observed at the adrenarche and the apparent dissociation between adrenal androgen and cortisol levels in fasting, obesity, anorexia nervosa, surgical stress and aging, the existence of an adrenal androgen stimulating hormone (AASH) has been postulated (Parker *et al.* 1980). However, the evidence for the existence of a separate trophic hormone for adrenal androgens is not conclusive and it has been suggested that prolactin may fulfill this role (Adams 1985).

In premenopausal women the ovary secretes testosterone, androstenedione and DHEA under action of luteinising hormone (Longcope 1986). Ovarian adrenal androgen secretion varies during the menstrual cycle. Testosterone and androstenedione reach a peak at mid-cycle whereas ovarian DHEA secretion is constant throughout the menstrual cycle (Abraham 1974). In postmenopausal women the ovary continues to secrete androstenedione and testosterone (Longcope 1986).

When we consider the contribution each organ makes to the serum levels of androgens, the situation is more complex. Studies of androgen concentrations in ovarian and adrenal veins have shown that DHEAS is secreted only by the adrenal gland. DHEA is produced mainly in the adrenal cortex. However small amounts are secreted in the ovary in premenopausal women (Longcope 1986).

The ovary and adrenal cortex contribute equally to serum androstenedione levels except at the midcycle in premenopausal women when the ovarian contribution is twice that of the adrenal (Abraham 1974). In a recent study of postmenopausal women Dorgan *et al* have found that both the ovaries and adrenal cortex also contribute equally to the elevated serum androstenedione levels found with breast cancer (Dorgan *et al.* 2001).

The major source of testosterone is the peripheral conversion of androstenedione. However, despite androstenedione secretion varying with the menstrual cycle, serum testosterone levels remain stable. In addition to peripheral conversion of androstenedione the ovary and adrenal also contribute equally to serum testosterone levels (Longcope 1986).

On the other hand neither the adrenal nor the ovary contributes significantly to the production of androstenediol or 5alpha dihydrotestosterone, they can therefore be considered as end-points in the metabolism of androgens (Longcope 1986).

In practice, DHEAS, DHEA and androstenediol are often referred to as "adrenal" androgens, though this term only strictly applies to DHEAS. Testosterone is often referred to as ovarian androgen, though this term is strictly incorrect, as it arises from both the ovary and adrenal. Rochefort and Garcia (Rochefort *et al.* 1984) classify androgens into three groups: androgens with high affinity for the androgen receptor (5alpha-dihydrotestosterone and testosterone); androgens which are converted to oestrogens by the aromatase enzymes (androstenedione and testosterone); and those of adrenal origin (DHEAS, DHEA and androstenediol), which have affinity for the oestrogen receptor.

5.7 Relationship between serum androstenedione, DHEAS and testosterone levels in women with breast cancer and controls

The correlation we observed between serum androgen levels are in agreement with those of Phillips *et al* who showed that serum androstendione, DHEAS and testosterone were correlated in healthy women (Phillips 1996). As outlined above the adrenal cortex secretes androgens in response to ACTH and the ovary secretes androgens in response to luteinising hormone. These two systems are independent. Therefore a high correlation coefficient and level of significance between serum androgen levels suggests a common source of secretion.

Androstenedione and DHEAS are highly correlated in both women with breast cancer and controls. This suggests that androstenedione and DHEAS are predominantly secreted

from the adrenal cortex in women with breast cancer and controls. The differences observed between correlation coefficients for serum testosterone versus DHEAS and, to a lesser extent, androstenedione in women with breast cancer compared to controls suggests that testosterone and/or androstenedione are derived from a more heterogeneous source in women with breast cancer than controls.

5.8 Relationship between serum androstenedione, DHEAS and testosterone levels and age in women with breast cancer and controls

A significant negative correlation has been reported between serum adrenal androgen levels and age (Labrie *et al.* 1997) (Orentreich *et al.* 1984) (Sulcova *et al.* 1997). Serum levels of DHEA and DHEAS reach a peak in early adulthood and decline thereafter (Sulcova *et al.* 1997). A concomitant decrease in cortisol, another adrenal cortical steroid secreted in response to ACTH does not occur with aging (Bonney *et al.* 1984). The reason for the age-related decline in DHEA and DHEAS levels has been attributed to alterations in the *zona reticularis* of the adrenal gland, whereas the reduction in serum androstenedione with aging is probably due to reduced ovarian secretion after the menopause (Parker *et al.* 2000).

Few studies have commented on a relationship between serum adrenal androgens and age in women with breast cancer. In contrast to the age invariance of serum adrenal androgen levels described by Zumoff *et al* (Zumoff 1981), both serum DHEAS and androstenedione declined with age in women with breast cancer in this study.

Serum testosterone levels have also been shown to decrease with increasing age (Bancroft *et al.* 1996), in agreement with the findings of the present study. Serum total testosterone levels are unaffected during the menopausal transition (Burger *et al.* 2000). SHBG levels fall as a result of the menopause, and this leads to a rise in free testosterone levels in postmenopausal women (Burger *et al.* 2000).

5.9 Serum androstenedione, DHEAS and testosterone levels in women with breast cancer and controls

The finding of a raised serum DHEAS in postmenopausal women with breast cancer compared to controls is in agreement with the findings of other groups (Cauley 1999) (Dorgan 1997) (Gordan 1990) (Zeleniuch-Jacqotte 1997) (Secreto 1991) (Zumoff 1981). DHEAS is secreted almost exclusively by the adrenal cortex (Longcope 1986). Elevated levels of DHEAS measured in postmenopausal women with breast cancer suggest increased adrenal androgen secretion, and supports a role for adrenal androgens in the aetiology of breast cancer. It has been proposed that in postmenopausal women adrenal androgens stimulate the proliferation of hormone-responsive breast cancer by interaction of a product of DHEAS metabolism, androstenediol with the oestrogen receptor (Adams 1998). Furthermore, after the menopause almost all oestrogens are synthesized in peripheral tissues from precursor steroids of adrenal origin (Labrie 1991). The importance of DHEAS is suggested by the findings that among the androgens only

DHEAS had oestrogenic activity at physiological concentrations on breast cancer cell lines (LeBail *et al.* 1998).

No significant difference in serum androstenedione levels between postmenopausal women with breast cancer and controls was observed in this study, though other groups have found elevated androstenedione levels in postmenopausal women who developed breast cancer (Cauley 1999) (Secreto 1991) (Dorgan *et al.* 2001).

No significant differences were observed in serum DHEAS and androstenedione levels between premenopausal women with breast cancer and controls. This is in agreement with the findings of Helzlsouer *et al* (Helzlsouer 1992) for DHEAS but contradicts those of Zumoff and Bulbrook (Zumoff 1981) (Bulbrook 1986). Our findings do not therefore support a role for adrenal androgens in the development of premenopausal breast cancer. However, fewer numbers of premenopausal women in this study and failure to control for all potential variables discussed below may have affected our results.

The present study observed that although mean serum testosterone levels were elevated in premenopausal and postmenopausal women with breast cancer no significant difference was found between serum testosterone levels between women with breast cancer and controls. The results of other groups suggest that serum testosterone levels are elevated in premenopausal and postmenopausal women with breast cancer (Malarkey 1977) (Sauter 2002) (Zeleniuch-Jacqotte 1997) (Cauley 1999) (Hill 1985) (Secreto 1991) (Thomas 1997). The failure of this study to find significantly elevated serum testosterone levels in

women with breast cancer may be a reflection of the size of the group studied or failure to control for the variables outlined below.

One further factor might have to the failure to observe a difference in serum androgen levels between these groups. This was the observation that serum androgen levels have been reported to be elevated in women with benign breast disease (Parlati *et al.* 1992; Lee *et al.* 1999; Mady *et al.* 2000). As controls for this study were taken from the one-stop breast clinic, a high proportion would have had benign breast disease.

Several factors have been reported to affect androgen levels include cigarette smoking (Baron *et al.* 1995), diurnal variation (Nicolau *et al.* 1984), body mass index (Kirschner *et al.* 1982), menstrual cycle (Abraham 1974) and exogenous oestrogens (Kraemer *et al.* 2001).

5.10 Relationship between serum androstenedione, DHEAS and testosterone levels and body mass index

A strong correlation between the degree of obesity and serum oestrogen levels in postmenopausal women is established (Kirschner *et al.* 1982) (Cauley *et al.* 1989). This is consistent with the fact that aromatization of androgens occurs primarily in adipose tissue (Longcope 1986). However, the relationship between serum androgens and adiposity is less straightforward.

An increased production of adrenal androgens and excretion of 17-ketosteroids has been found in obese subjects (Glass *et al.* 1981) (Adams 1985). In addition obesity is associated with decreased sex hormone binding globulin levels and consequently an elevated free testosterone levels, which may explain the increased incidence of amenorrhoea and hirsutism reported in obese patients (Glass *et al.* 1981).

Studies investigating an association between serum adrenal androgen levels and obesity suggest that serum androstenedione (Kirschner *et al.* 1982) (Cauley *et al.* 1989) and DHEAS (Williams *et al.* 1993) (DePergola *et al.* 1991) (Barret-Connor *et al.* 1996) levels are independent of body mass index. These results are supported by the observations of the present study. However, an inverse association has been reported between serum DHEA levels and obesity (Field *et al.* 1994) (DePergola *et al.* 1991) (Barret-Connor *et al.* 1996) (Tchernof *et al.* 1995).

In premenopausal women, Kirschner *et al* found no significant difference between plasma androstenedione levels in obese women compared to controls; but reported that the production, metabolic clearance rate, and aromatization of androstenedione was significantly higher in obese women (Kirschner *et al.* 1982). Two other groups have failed to find a correlation between serum DHEAS levels and body mass index in premenopausal women (Williams *et al.* 1993) (DePergola *et al.* 1991). However serum DHEAS levels have been shown to be associated with truncal distribution of body fat in women (Williams *et al.* 1993) (Barret-Connor *et al.* 1996). Likewise, in men and

postmenopausal women no association between androstenedione levels (Cauley *et al.* 1989), DHEAS (Barret-Connor *et al.* 1996) and obesity has been reported.

However, an inverse association between serum DHEA and body mass index has been reported in premenopausal women (DePergola *et al.* 1991) and postmenopausal women (Barret-Connor *et al.* 1996). In addition, DHEA has been found to have anti-obesity properties in rodents (Williams 2000) and to be inversely associated with body mass index in men (Field *et al.* 1994) (Tchernof *et al.* 1995).

No clear association between serum total testosterone and obesity in women has been demonstrated (Kirschner *et al.* 1982). However, obesity is associated with decreased serum sex hormone binding globulin (SHBG) levels, and consequently elevated free testosterone levels (DePergola *et al.* 1991). This finding may explain the increase in menstrual disturbance and hirsutism reported in obese women (Kirschner *et al.* 1982).

The relationship between adrenal androgens and obesity may therefore be similar to that between cortisol and obesity. Normal plasma cortisol levels are maintained in obesity as the enhanced production is balanced by an accelerated metabolism of cortisol (Glass *et al.* 1981). In the same way serum adrenal androgen levels are normal in obesity, though production, metabolism and urinary excretion may be increased.

5.11 Relationship between serum androgens and stress

Surgical stress has been reported to induce a marked and sustained increase in adrenal androgen secretion (Batrinos 1999). In the present study no association between preoperative serum levels of DHEAS and cortisol in women undergoing surgery has been demonstrated. The lack of correlation between serum DHEAS and cortisol levels may be due to the relatively large pool of DHEAS in the serum, which masks an elevation in serum DHEAS due to episodic secretion (Rosenfeld *et al.* 1975). In addition these results suggests that the elevated DHEAS levels we have observed in postmenopausal women with breast cancer are not due to emotional stress in the pre-operative period.

5.12 Serum androgens and smoking

The anti-oestrogenic effect of cigarette smoking particularly evidence that smoking reduces the risk of endometrial cancer (Lesko *et al.* 1985) has stimulated interest in the possible effect of smoking on steroid hormones. Several studies have reported that smokers have elevated levels of androstenedione (Longcope *et al.* 1988) (Cauley *et al.* 1989) (Khaw *et al.* 1988) (Baron *et al.* 1995) (Field *et al.* 1994) and DHEAS (Khaw *et al.* 1988) (Baron *et al.* 1995) (Field *et al.* 1994). Other studies have reported that serum DHEAS levels did not differ between smokers and non-smokers (Key *et al.* 1991). The association between androgens and smoking appears to be restricted to adrenal androgens, as those studies which have measured serum testosterone levels have not found them to differ between smokers and non-smokers (Longcope *et al.* 1988) (Khaw *et*

al. 1988). The mechanism of the association between smoking and elevated adrenal androgen levels is unknown. However, Baron *et al* noted that smoking caused not only an elevation of adrenal androgen levels but a generalized disturbance in adrenal cortical hormone levels (Baron *et al.* 1995).

5.13 Diurnal variation in serum androgens

Adrenal androgens are secreted by the adrenal cortex, along with cortisol in response to changes in ACTH and are a component of the pituitary-adrenal axis (James 1984). Over a 24-hour period plasma DHEA and androstenedione levels vary synchronously and episodically with cortisol secretion in response to pulsatile changes in ACTH secretion (Rosenfeld *et al.* 1971). Although DHEAS is also secreted in response to ACTH, serum levels do not vary synchronously with other adrenocortical hormones. This may be due to the relatively long half-life of DHEAS and therefore the large pool of DHEAS in the serum, which masks an elevation in DHEAS levels, due to episodic secretion. Serum DHEAS levels are thus less labile than other adrenocortical hormone, however, a diurnal variation in DHEAS secretion has been described (Nicolau *et al.* 1984) with a maximum level during the day and a minimum at night (Jones *et al.* 1987). Though other studies have failed to find evidence for a diurnal variation in serum DHEAS levels over a 24-hour period (Garde *et al.* 2000). A diurnal variation in serum testosterone levels has also been demonstrated (Nicolau *et al.* 1984). Testosterone is also secreted episodically (West *et al.* 1973) though peaks do not occur synchronously with cortisol.

5.14 Summary

Controls in this study have been matched for age. Patients on exogenous oestrogens have been excluded. Controls have not been matched with respect to cigarette smoking, body mass index and time of venepuncture. Failure to control for these factors, for the reasons outlined above may have affected our findings. However, few of the studies investigating serum androgens in breast cancer have matched cases and controls for all of these factors, apart from age.

In conclusion, these findings show an elevated serum DHEAS level in postmenopausal women with breast cancer. No difference was observed in serum androstenedione levels between postmenopausal women with breast cancer and controls. Nor were levels of either adrenal androgen different between premenopausal women with breast cancer and controls. Mean testosterone levels were non-significantly raised in both premenopausal and postmenopausal women with breast cancer. These results, in conjunction with those reported by other groups (Gordan 1990; Dorgan 1997; Zeleniuch-Jacqotte 1997; Cauley 1999) suggest a role for DHEAS in the aetiology of postmenopausal breast cancer. However the failure to find any differences in androstenedione or testosterone levels in this group and a difference in DHEAS levels that was only just significant at the 5% level in postmenopausal women does not exclude the possibility that these results could have occurred by chance.

Mechanisms for an action for DHEAS are discussed in chapter 8: in an oestrogen-depleted environment DHEAS stimulates the proliferation of hormone-dependent breast cancer via its metabolite 5-androstene-3β,17β-diol or via aromatisation to 17β-oestradiol.

Serum adrenal androgens have been implicated in the aetiology of "western-environmental" breast cancer. It has been postulated that dietary factors or body mass index may account for differences in adrenal androgens observed in western women. This study has demonstrated that serum androgen levels are independent of body mass index in a sample of western women with breast cancer. This suggests that body mass index has no effect on serum androgen levels.

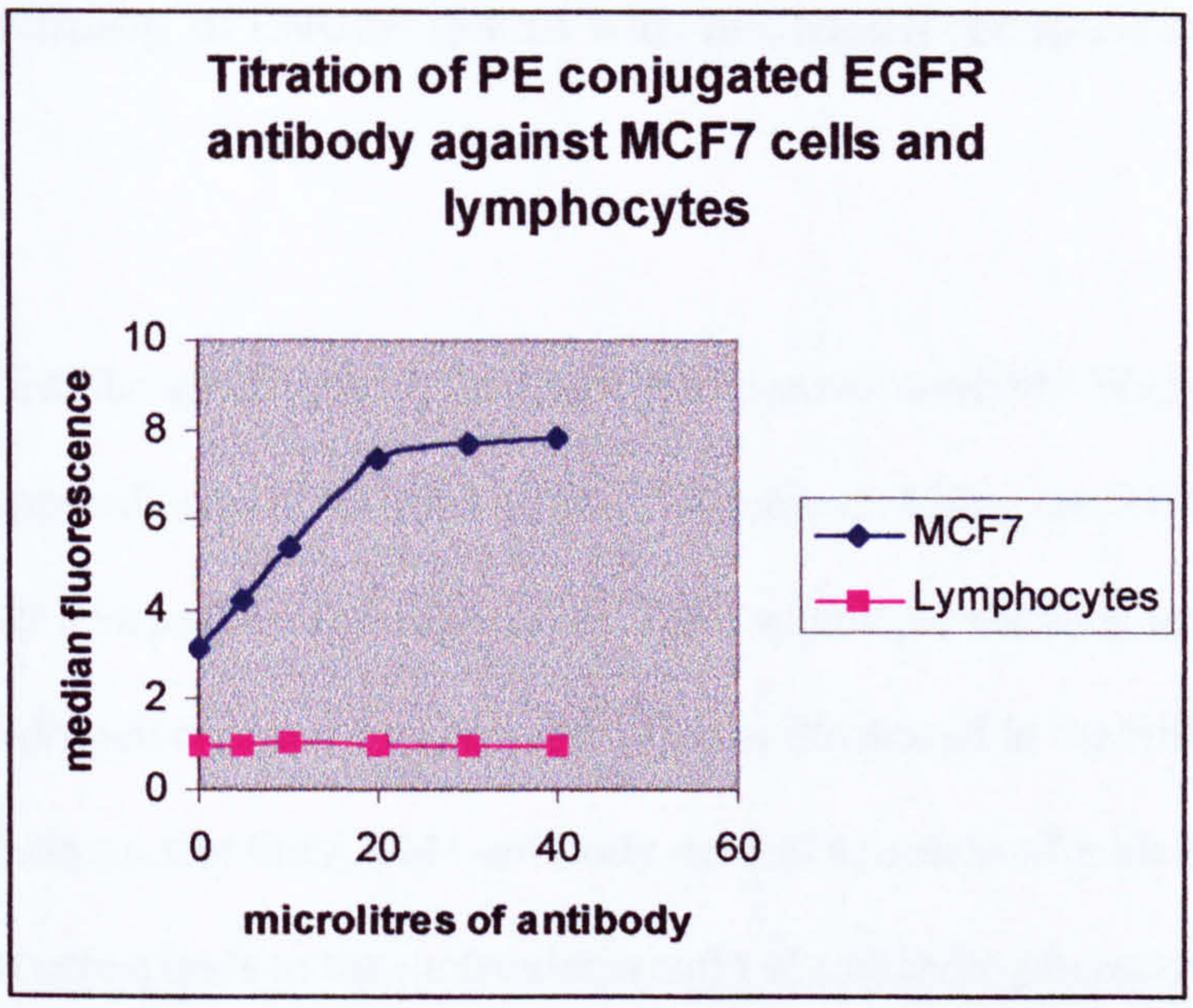
Finally this study has shown that serum androgen levels decline with increasing age in women with breast cancer, as they do in controls. This contradicts previous studies, and suggests there is no fundamental difference in the age-related decline in androgen production between women with breast cancer and healthy women.

Results and Discussion

Androgen receptor expression in primary operable breast cancer

6.1 Titration of androgen and epidermal growth factor receptor antibodies

Titration of the biotinylated mouse anti-human androgen receptor antibody (DAKO, Cambridge, UK) against the androgen receptor positive and negative prostate cancer cell lines LNCap and Du154 is illustrated in the following figure. This indicates that the titration curve reaches a plateau, while the control remained low, at a concentration of 3µl of androgen receptor antibody per 10^5 cells. Therefore 3µl of androgen receptor antibody was used in subsequent experiments to determine the androgen receptor expression in breast tumours. Likewise, the titration curve of the phycoerythin conjugated epidermal growth factor antibody against MCF7 cells and lymphocytes reaches a plateau at 20µl of antibody per 10^5 cells, indicating that this quantity of antibody was optimal.



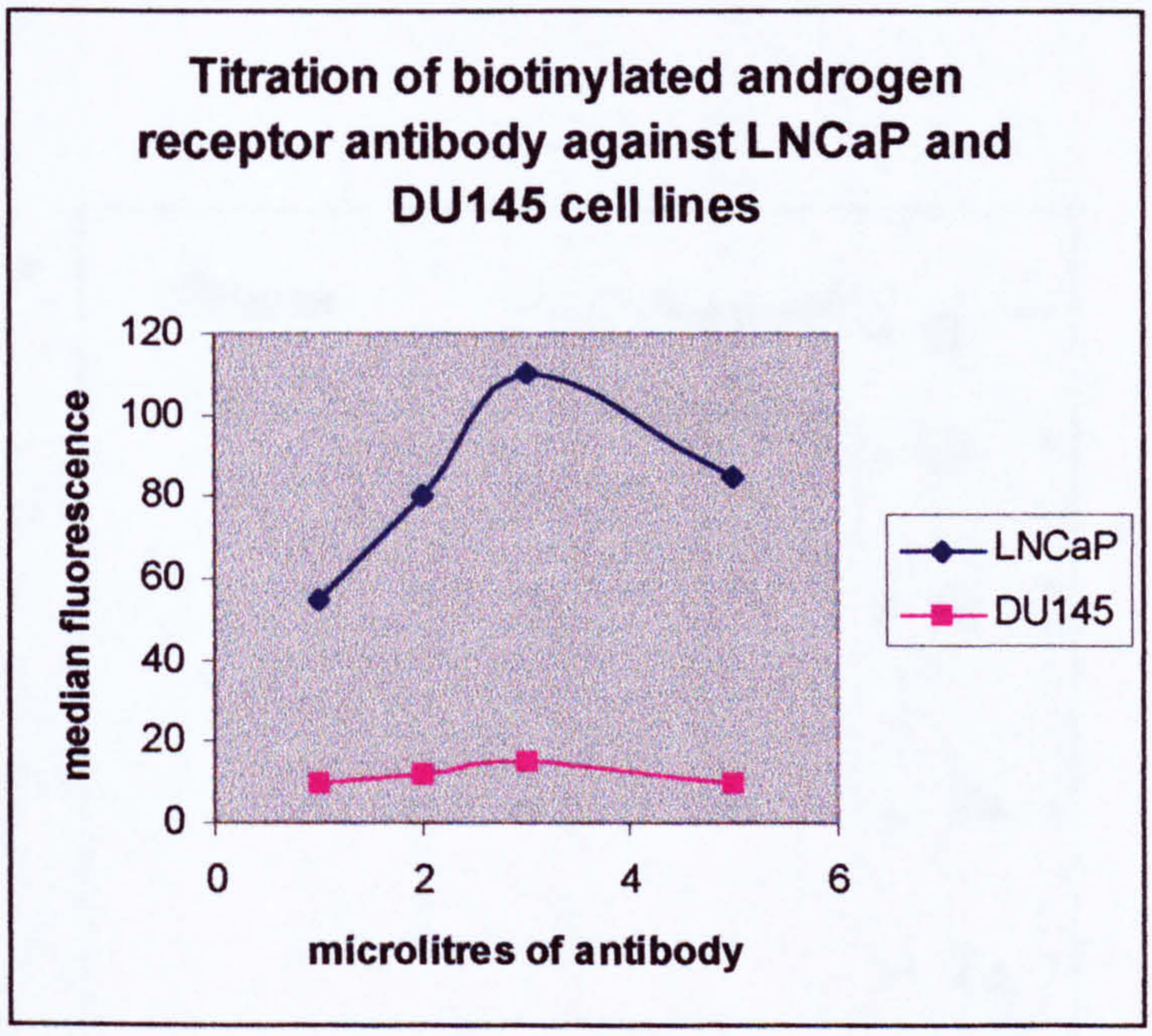


Figure 6.1: Androgen and epidermal growth factor receptor antibody titration curve

6.2 Western blotting of LNCaP lysate with androgen receptor antibody (AR441)

In order to confirm the specificity of the androgen receptor antibody Western blotting of a lysate of LNCap cells was performed as described above. Using the AR441 androgen receptor antibody as a probe a transcript at 110kDa, which corresponds to the molecular weight of the androgen receptor was detected. This is illustrated in the following figure. These results illustrate that the AR441 antibody detects a protein of molecular weight 110kDa, which corresponds to the molecular weight of the androgen receptor (van Laar *et al.* 1989).

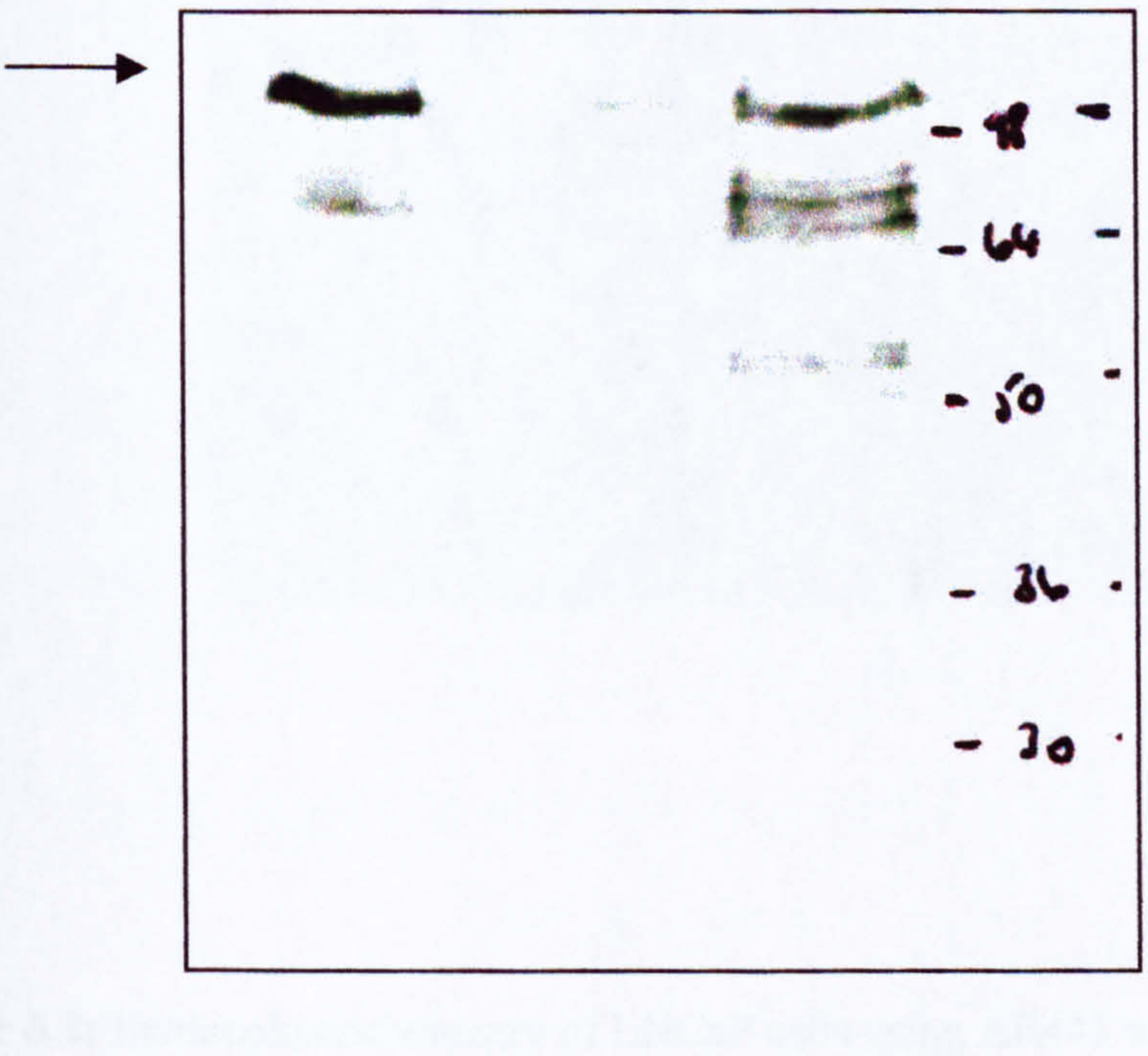


Figure 6.2: Western blot of LNCaP lysate with the AR441 androgen receptor antibody

6.3 Immunohistochemistry of LNCaP cells with androgen receptor antibody (AR441)

Immunohistochemistry demonstrated after counterstaining with haematoxylin that both LNCap cell nuclei and cytoplasm stained positively for androgen receptors. Cell nuclei stained more intensely than the cytoplasm. This suggests that the androgen receptor are localised predominantly to the cell nucleus. Immunopositive cell nuclei staining varied with some cells showing intense staining and others faint or no staining. The results of immunohistochemistry are demonstrated in the following figure.

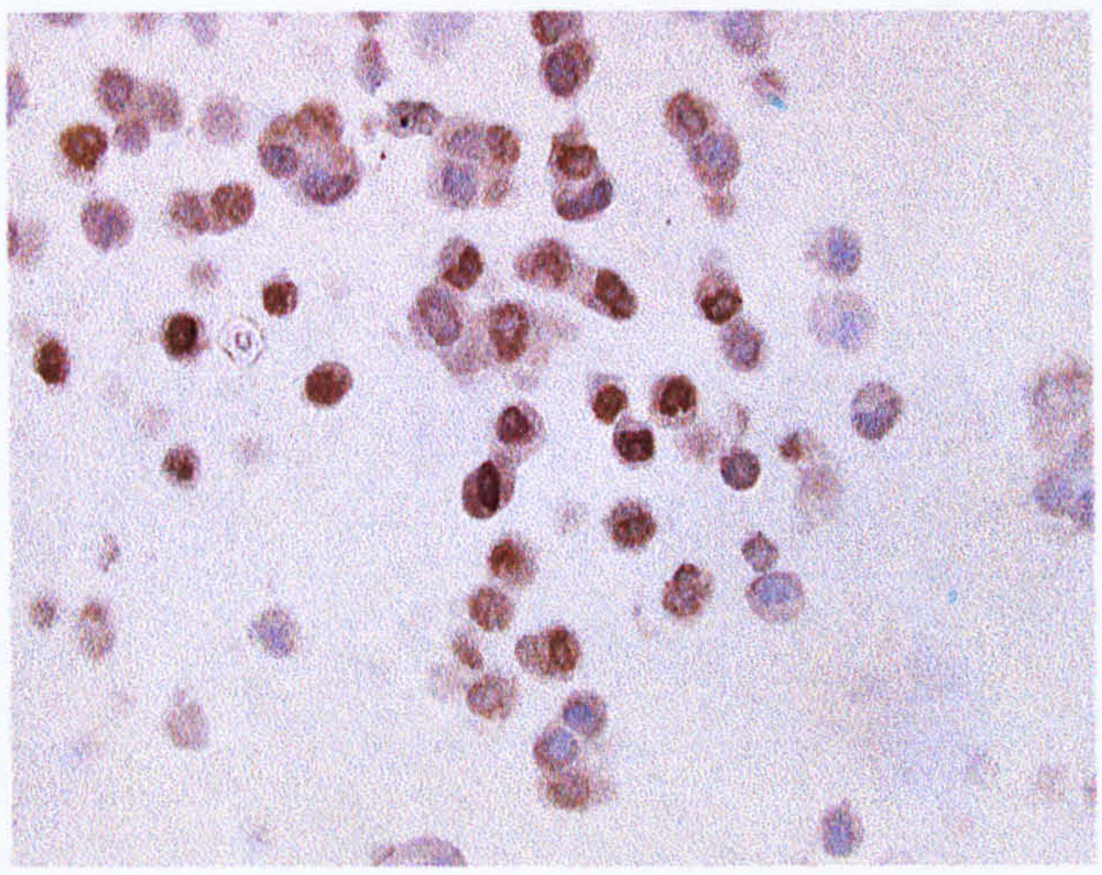


Figure 6.3: Immunohistochemistry of LNCaP cells using AR441 androgen receptor antibody

6.4 Immunohistochemistry of paraffin sections of breast tumours with androgen receptor antibody (AR441)

Immunohistochemistry of paraffin sections of breast tumours demonstrated variable levels of androgen receptor expression. Androgen receptors were predominantly located in the nucleus, but cytoplasmic staining was present. Figures 6.4-6 illustrate breast tumour sections, which stained strongly, moderately and weakly for androgen receptor expression.

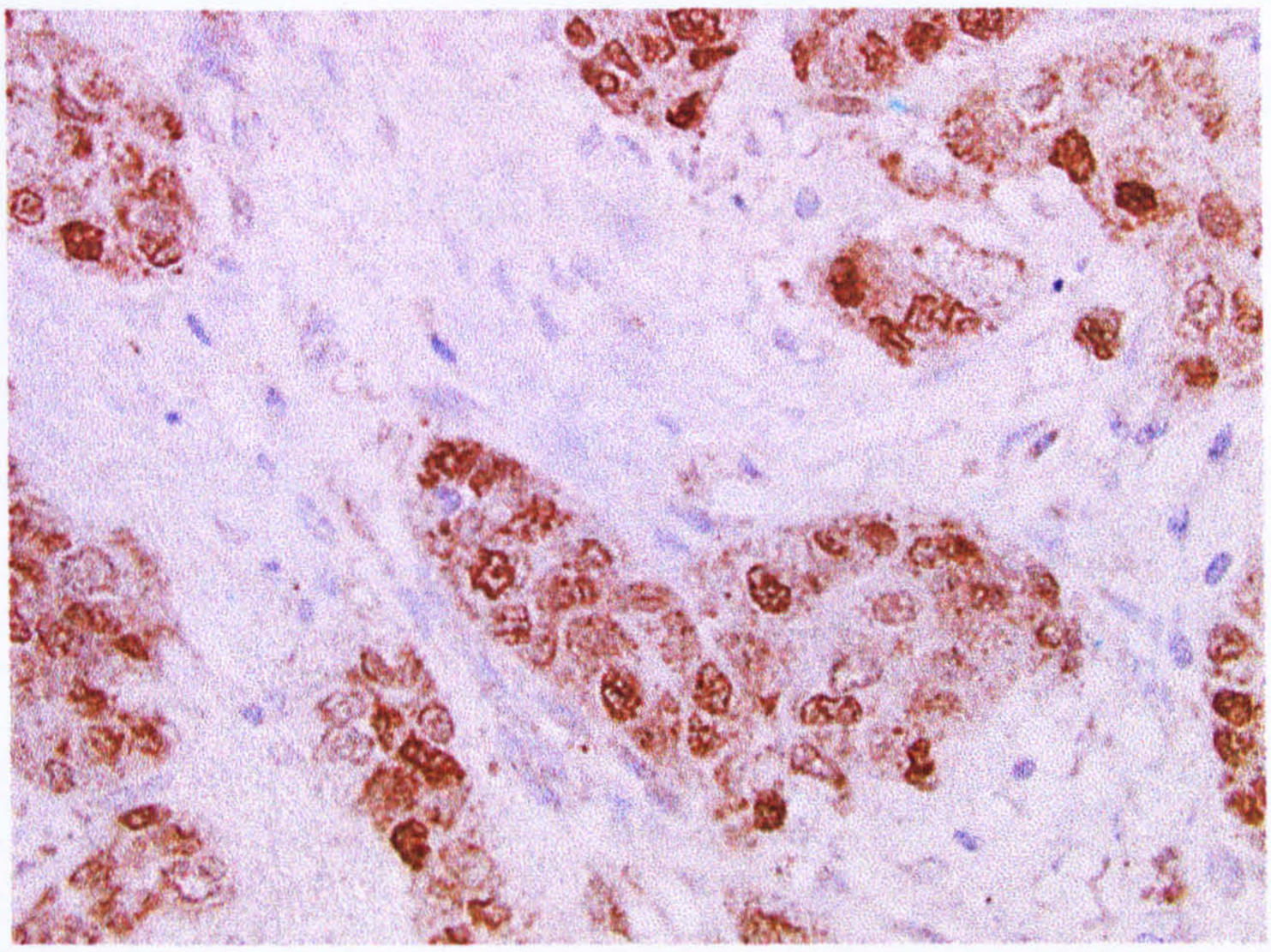


Figure 6.4: Paraffin section of breast tumour demonstrating strong staining for androgen receptor expression

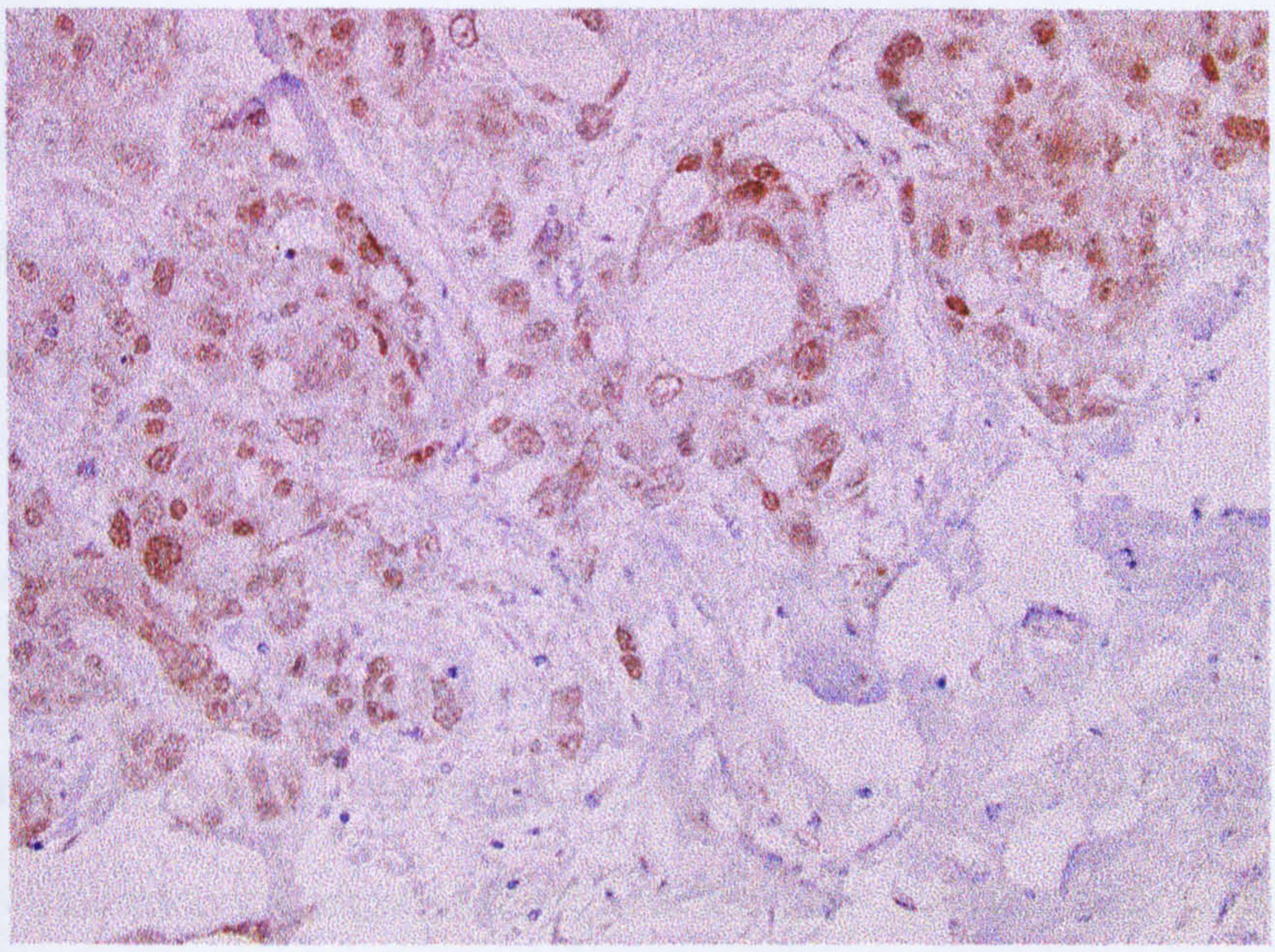


Figure 6.5: Paraffin section of breast tumour demonstrating moderate staining for androgen receptor expression

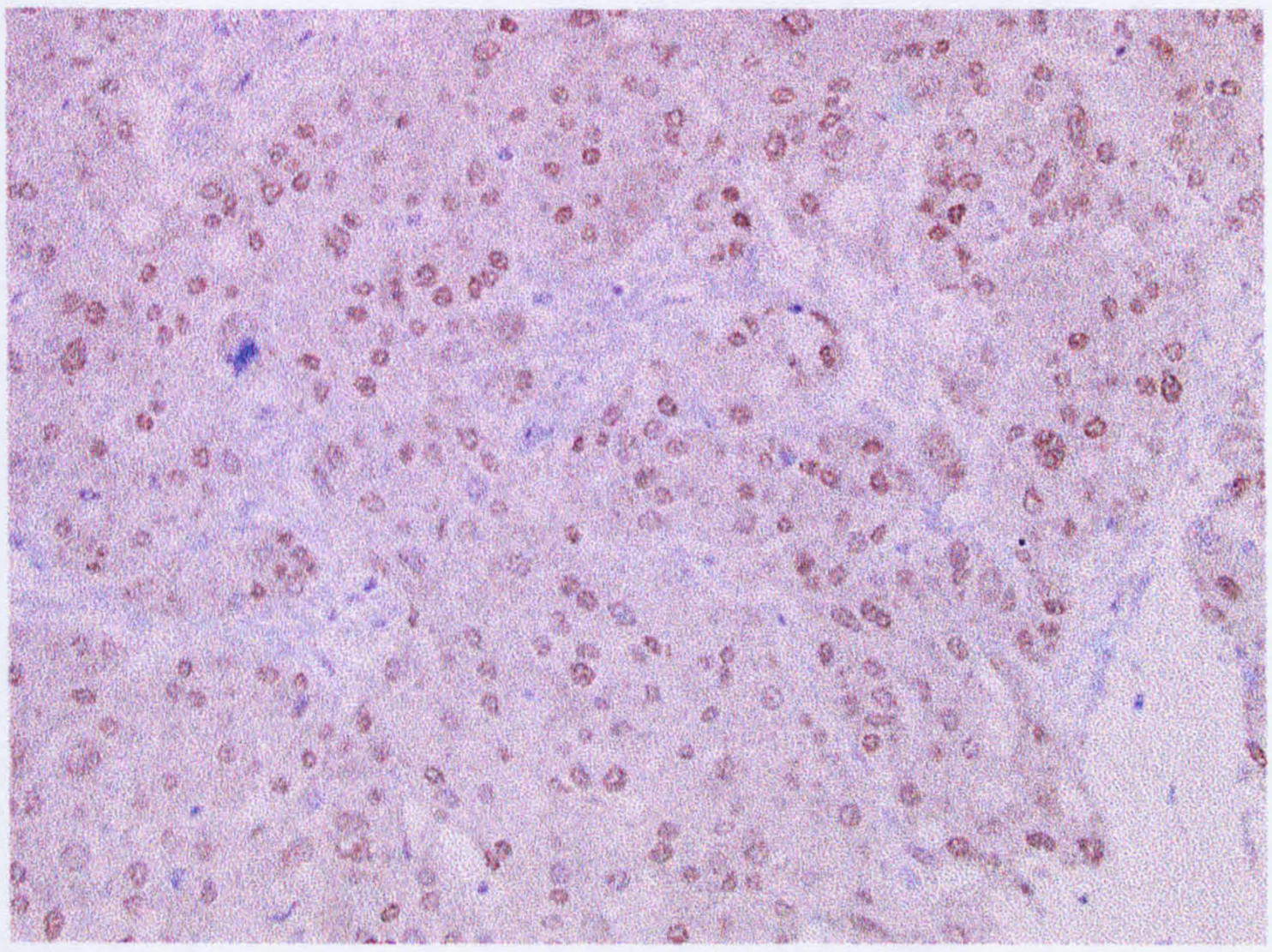
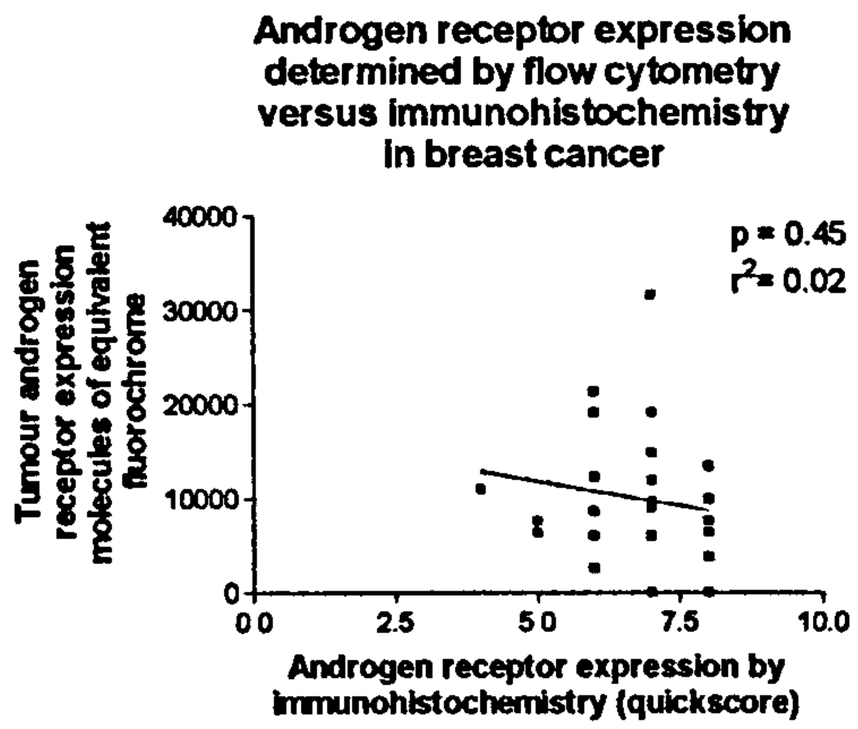


Figure 6.6: Paraffin section of breast tumour demonstrating weak staining for androgen receptor expression

Breast sections were graded for the intensity with which they stained for androgen receptor expression (1=weak, 2=moderate, 3=strong) and the percentage of positively staining cells. These figures were added to arrive at a number termed a "quickscore" (Detrie *et al.* 1995).

Androgen receptor expression determined by flow cytometry, which was expressed in units of molecules of equivalent fluorochrome, was plotted against the quickscore and intensity of androgen receptor staining determined by immunohistochemistry. In order to determine whether any correlation existed between these methods, linear regression analysis was performed. Figures 6.7 and 6.8 illustrate that no correlation occurred between immunohistochemical and flow cytometric methods for androgen receptor expression.



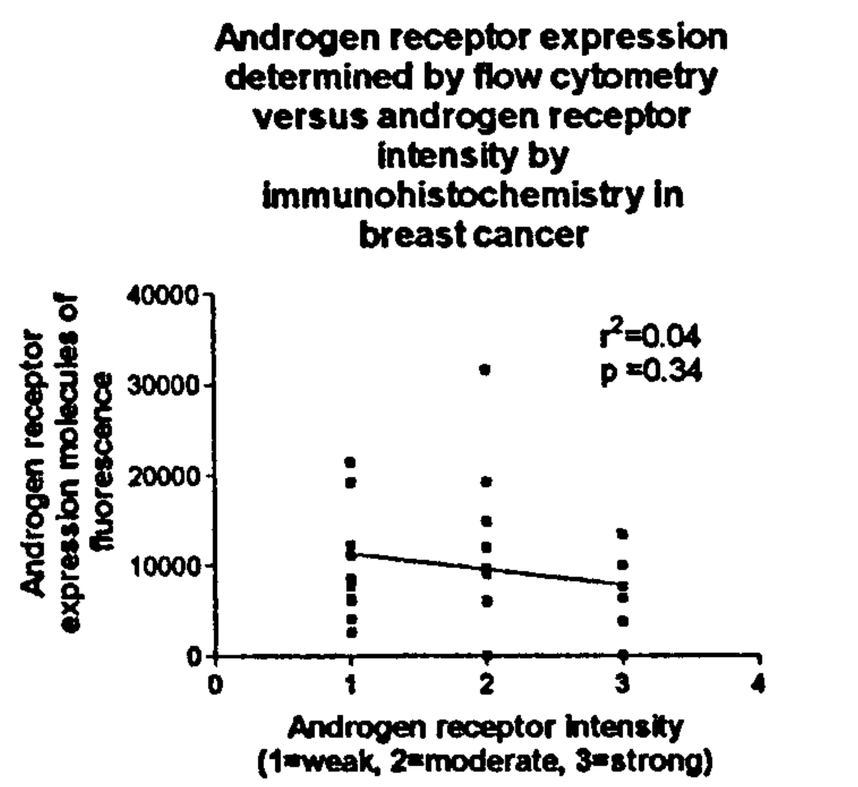


Figure 6.7: Androgen receptor expression determined by flow cytometry versus androgen receptor "quickscore" and intensity by immunohistochemistry. Note the tumours with a quickscore of "0" have been omitted from the graph and statistical analysis.

6.5 Androgen receptor expression of breast tumours

Eighty-six percent of breast tumours in this study were found by flow cytometry to express the androgen receptor. This level of androgen receptor expression in breast cancer is in agreement with reported studies of androgen receptor expression by ligand-

binding assay (Lea *et al.* 1989) (Soreide *et al.* 1992) and immunohistochemistry (Isola 1993) (Kuenen-Boumeester *et al.* 1992).

In addition the level of androgen expression observed was higher than the oestrogen receptor alpha expression, which was found to be 80%, and confirms previous reports that the androgen receptor is the most frequently expressed steroid receptor in breast cancer (Lea *et al.* 1989).

The level of expression of androgen receptor in the sixty-three breast tumours analyzed is illustrated in figure 6.8.

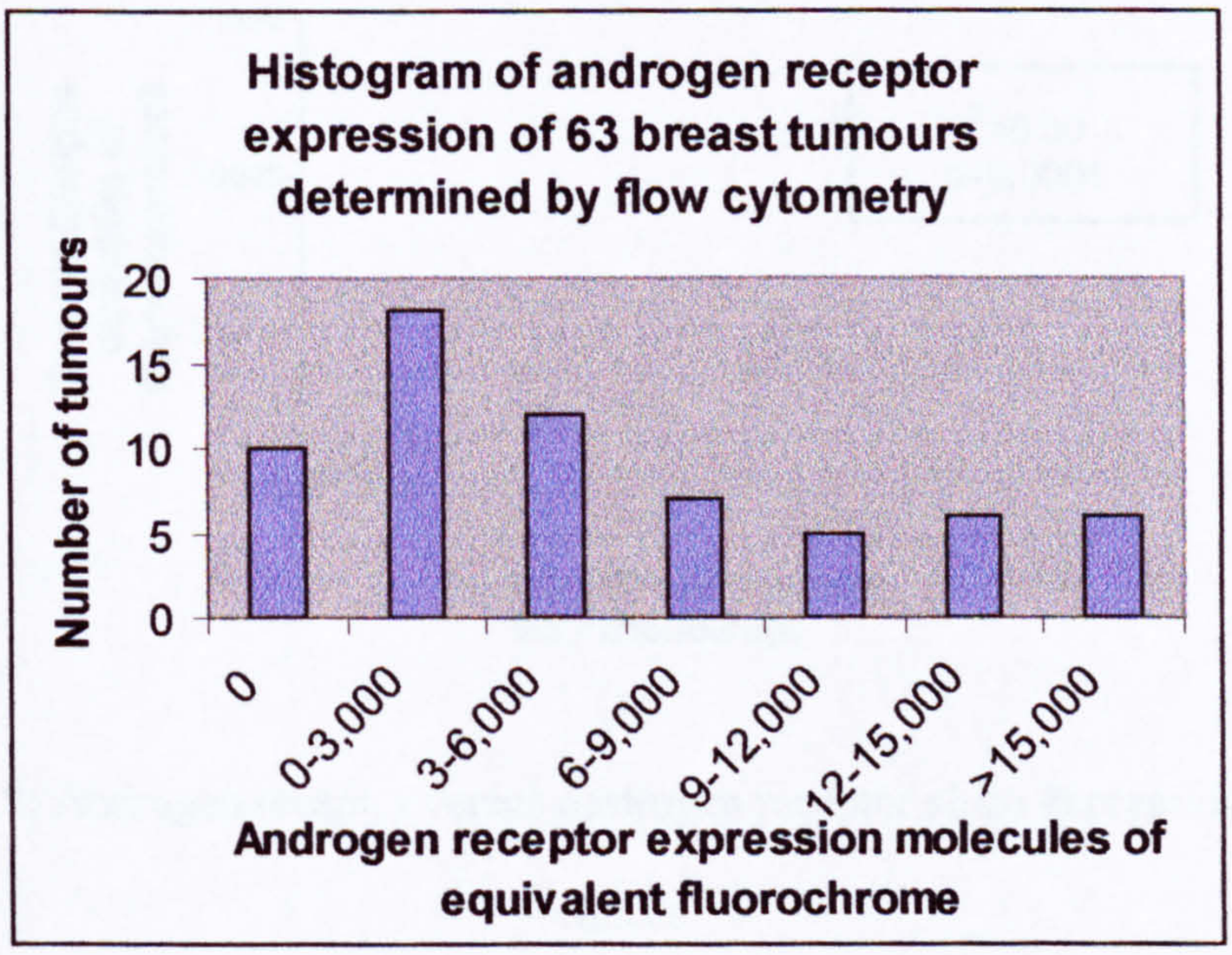


Figure 6.8: Androgen receptor expression in sixty-three primary operable breast cancers

6.6 Androgen receptor is co-expressed with oestrogen receptor alpha in breast cancer

A plot of androgen receptor expression against oestrogen receptor alpha in the 43 breast tumours analyzed demonstrates that there is a significant correlation between androgen receptor and oestrogen receptor alpha expression (linear regression r^2=0.30, p<0.0001). Previous studies have found a similar association between androgen and oestrogen receptor expression in breast cancer (Isola 1993) (Lea *et al.* 1989) (Soreide *et al.* 1992). Though the reason for co-expression of steroid receptors in breast cancer is unknown.

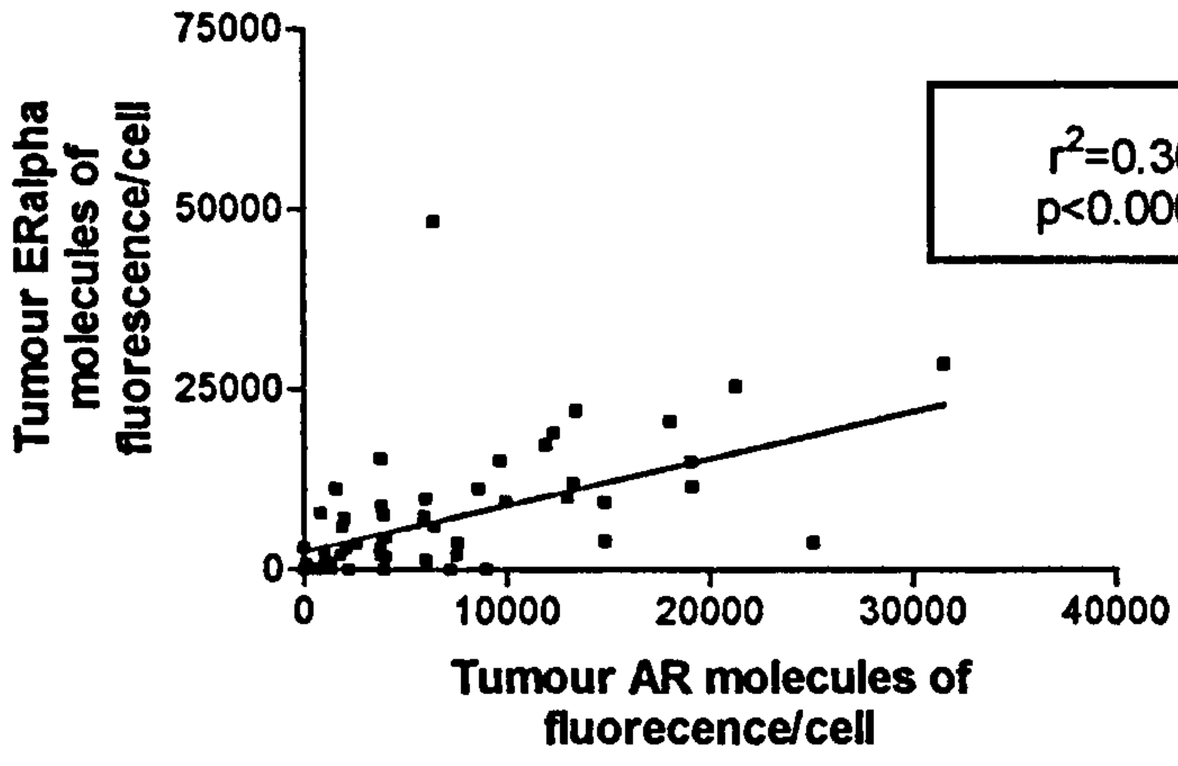


Figure 6.9: Androgen receptor versus oestrogen receptor alpha expression in breast cancer

6.7 Androgen receptor expression increases with age

Androgen receptor expression was observed to increase with increasing age. The association between breast tumour androgen receptor expression and age reached

significance but the correlation co-efficient was low (linear regression $p=0.02$, $r^2=0.08$). This is illustrated in the following figure.

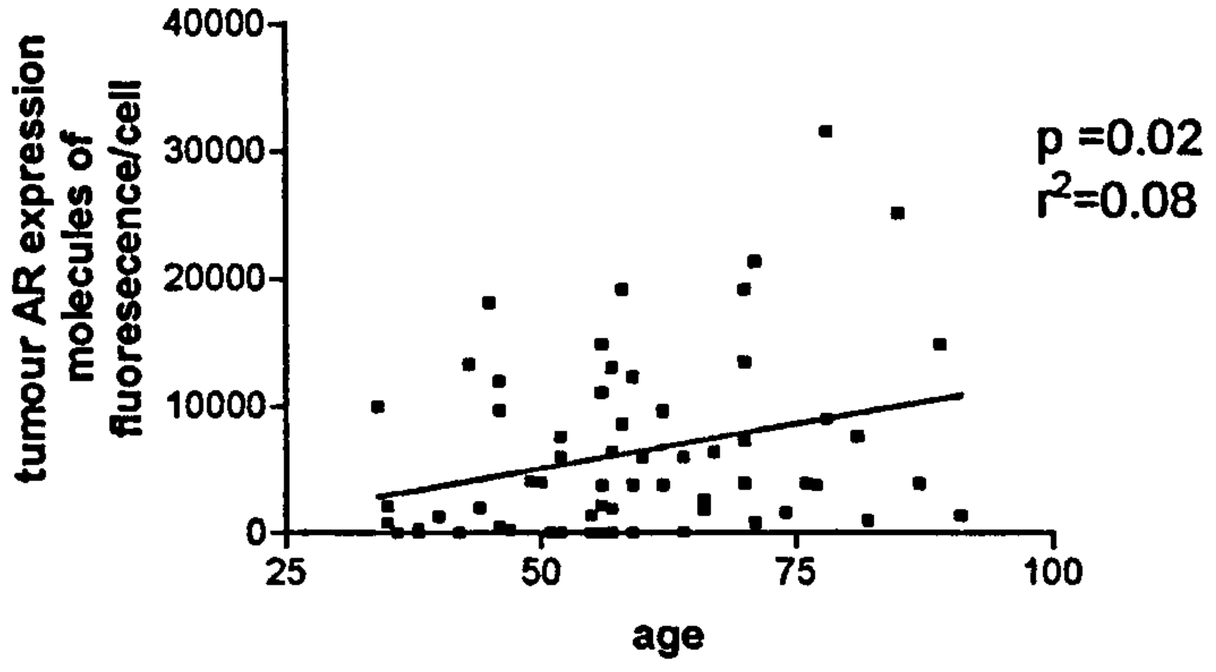


Figure 6.10: Androgen receptor expression versus age in breast cancer

Steroid receptor levels are regulated by their ligands. Adaption by up regulation of the receptor apparatus to compensate for the declining supply of sex steroids observed in advancing age has been proposed as an explanation for the increase in steroid hormone receptor expression observed in breast cancer (Lea *et al.* 1989). It has been demonstrated in chapter 5 that serum androgen levels decline in women with breast cancer. In order to further examine the hypothesis that negative autoregulation determines breast tumour androgen receptor expression *in vivo* we measured serum testosterone, dehydroepiandrosterone sulphate and androstenedione in these patients, and a correlation between tumour androgen receptor expression and serum androgen levels was sought.

Breast tumour androgen receptor expression increased with decreasing serum levels of DHEAS and androstenedione, and the association between tumour androgen receptor expression and serum DHEAS was approaching significance, though the correlation co-

efficient was low (linear regression, p=0.08, r^2=0.08). However, breast tumour androgen receptor expression appeared to be independent of serum testosterone levels (linear regression p=0.94, r^2=0.00). This data is illustrated in the following figures.

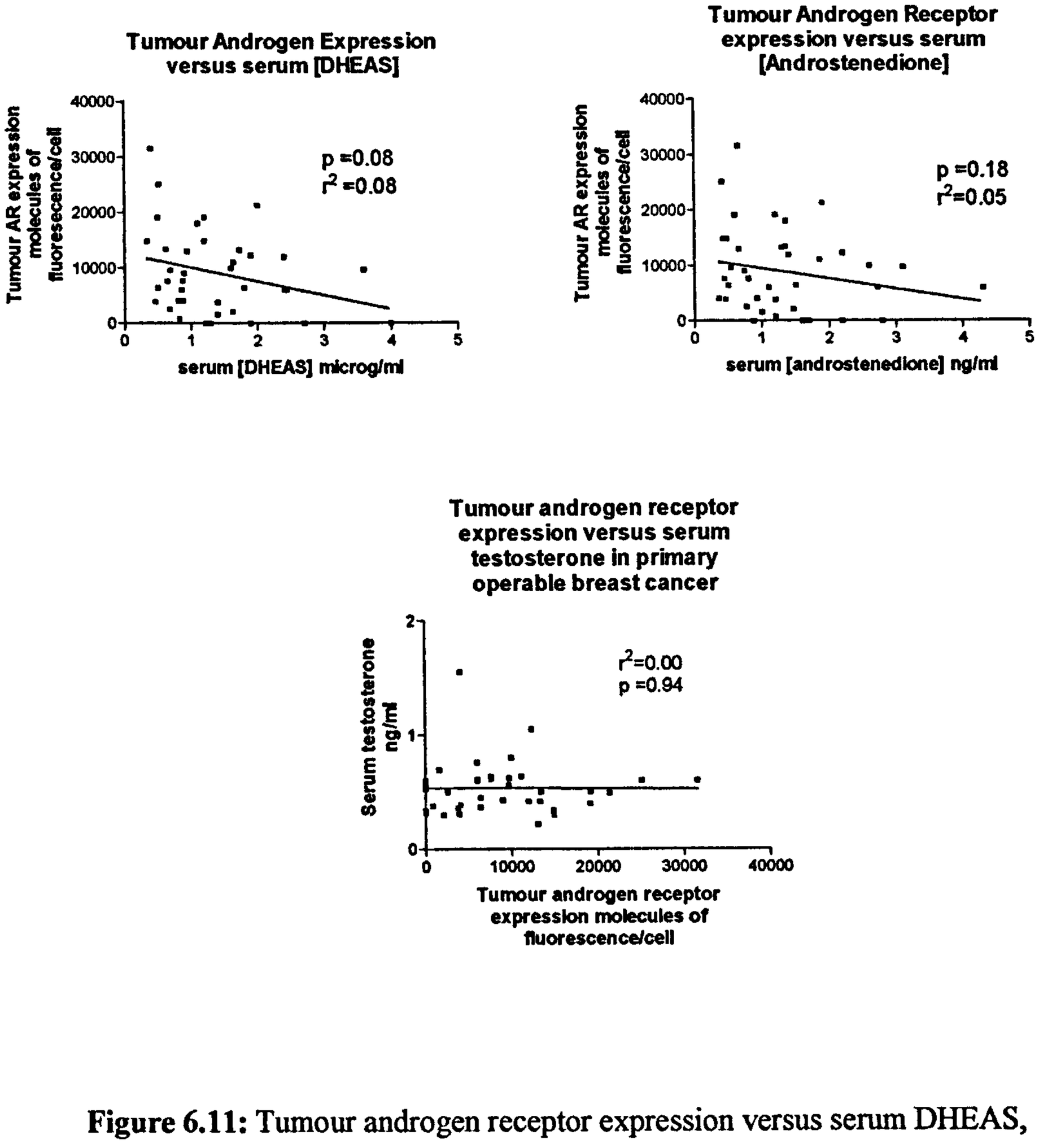


Figure 6.11: Tumour androgen receptor expression versus serum DHEAS, androstenedione and testosterone

6.8 Androgen receptor expression versus tumour grade, lymph node status and Nottingham Prognostic Index

Tumour androgen receptor expression was compared against the established prognostic markers, Bloom-Richardson grade (Bloom *et al.* 1957), lymph node status (Fisher *et al.* 1978) and Nottingham Prognostic Index. This data is presented in the following tables and figures.

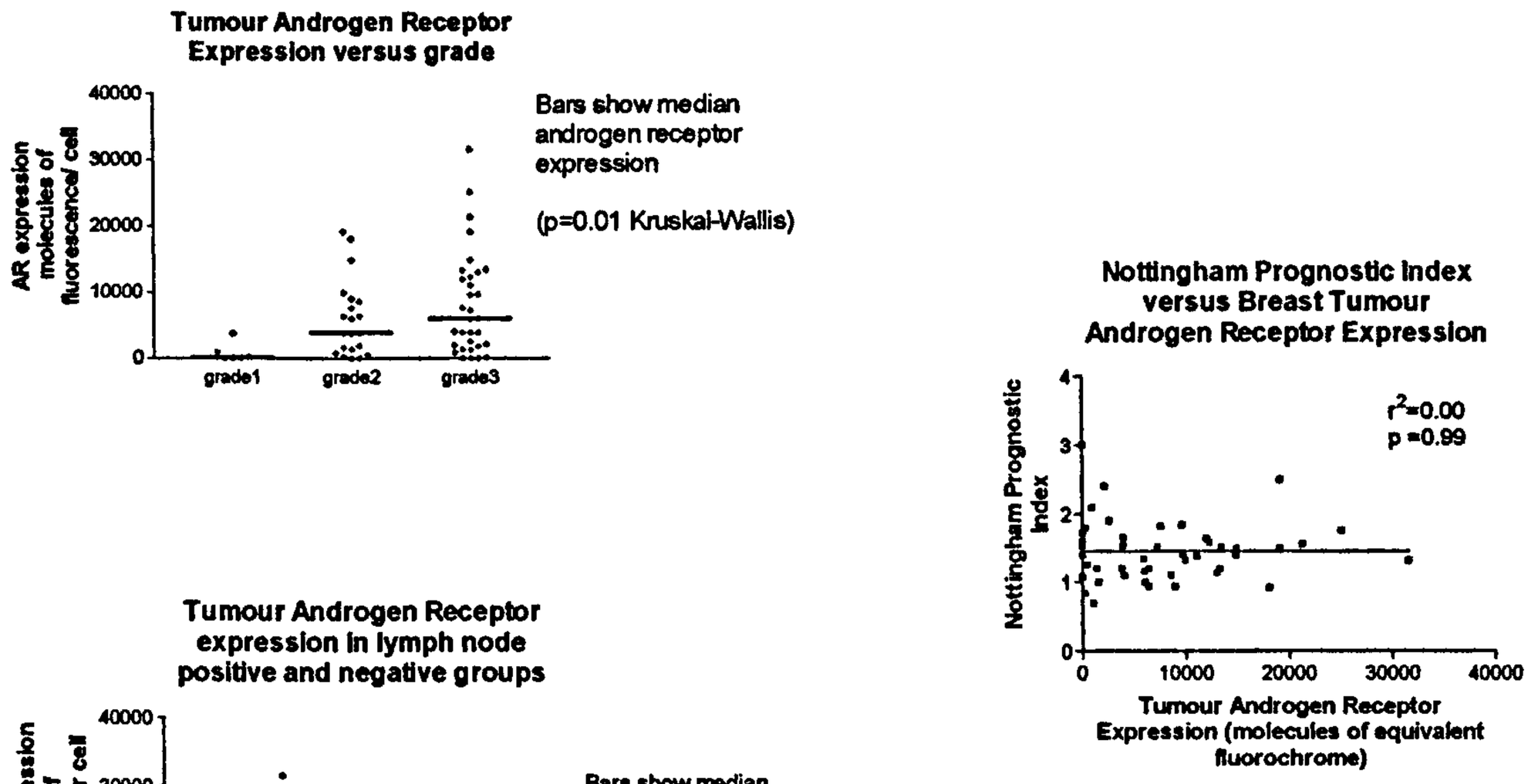


Figure 6.12: Androgen receptor status versus tumour grade, lymph node status and Nottingham Prognostic Index in breast cancer

Mean androgen receptor expression increased with tumour grade suggesting that androgen receptor expression is a marker of poor prognosis. A statistically significant difference was observed between these groups (Kruskal-Wallis p=0.01). No difference in mean androgen receptor expression was observed between axillary lymph node positive and negative groups (Mann-Whitney p=0.84). No association was observed between androgen receptor expression and Nottingham Prognostic Index.

6.9 Expression of androgen receptor and epidermal growth factor receptor are positively correlated in breast cancer

We investigated whether an association between epidermal growth factor receptor expression and androgen receptor expression occurs in breast cancer. A positive correlation between epidermal growth factor receptor and androgen receptor expression in breast cancer in the present study (linear regression, p=0.01 r^2=0.13) illustrated in the following figure suggests a functional interaction between androgen receptor and the epidermal growth factor receptor pathways in breast cancer.

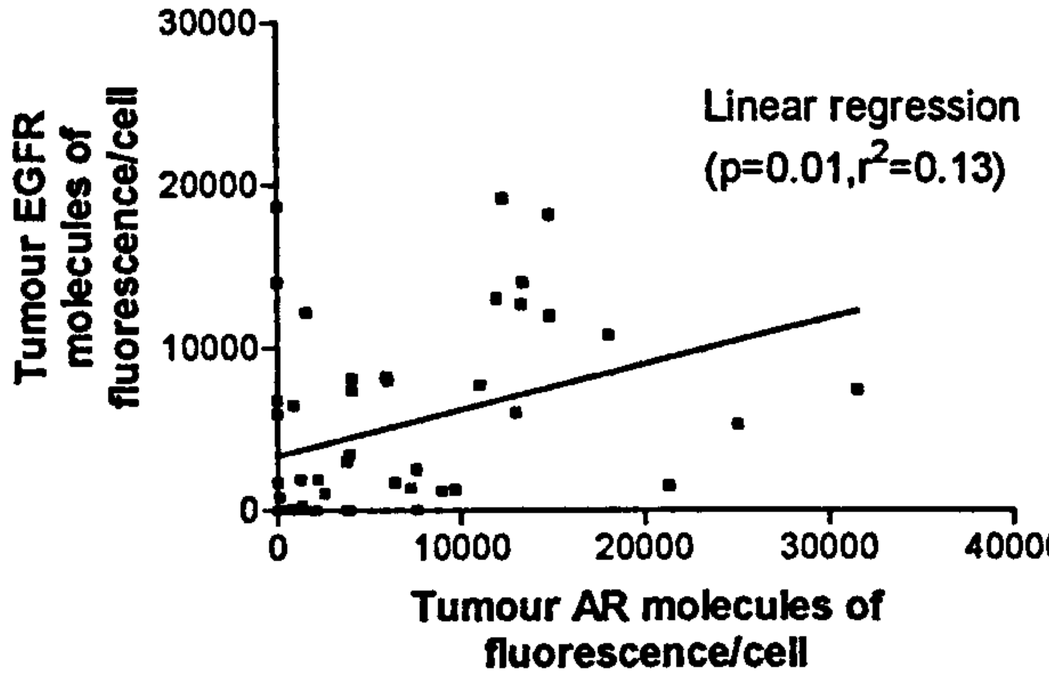


Figure 6.13: Androgen receptor versus epidermal growth factor receptor expression

Discussion

6.10

A flow cytometric method for the determination of androgen receptor expression in breast cancer

We report here a novel method for the determination of androgen receptor expression in primary operable breast cancer by flow cytometry. The observations that androgen receptor expression, determined by this method occurs in 86% of breast tumours and is co-expressed with oestrogen receptor alpha are in agreement with previous studies investigating androgen receptor status in breast cancer (Lea *et al.* 1989) (Soreide *et al.* 1992) (Isola 1993) (Kuenen-Boumeester *et al.* 1992) (Allegra *et al.* 1979).

Flow cytometry is an established method for the determination of surface, cytoplasmic and nuclear antigen expression (Loken *et al.* 2000) (Larson 2000). In our laboratory, flow cytometric methods for the determination of oestrogen receptor alpha (Brotherick *et al.* 1995), oestrogen receptor beta (Girdler *et al.* 2001) and epidermal growth factor receptor (Brotherick *et al.* 1994) in breast tumour specimens have been established.

An increasing number of prognostic and predictive factors in breast cancer have now been described (Slooten *et al.* 2001). In addition to providing information on DNA ploidy and S-phase fraction (Ormerod 2000) the use of flow cytometry to measure cell surface and cytoplasmic antigen expression provides a rapid, reliable and objective method for the simultaneous determination of multiple parameters in breast cancer.

There was however no correlation between immunohistochemical and flow cytometric methods for the determination of androgen receptor expression. Both methods have disadvantages. Immunohistchemistry has become established as a routine method for the determination of antigen expression on clinical specimens. Flow cytometry is not used in routine clinical practice to measure antigen expression, however its capability to simultaneously measure the expression of several antigens on clinical specimens suggests a clinical utility.

Immunohistochemistry used a subjective determination of the intensity of antigen expression plus the percentage of positive cells to arrive at a "quickscore". Whereas, flow cytometry determined the number of antigen molecules expressed per cell. This figure

was not arrived at entirely objectively, as the placing of gates around cytokeratin positive cells (see figure 3.5) on a dot plot of fluorescence-1 against fluorescence-2 was subjective. This "gating" did affect the final measurement, as antigen expression was determined on cells within the gate, and the cytokeratin population was not always so easily distinguished as those shown in figure 3.5.

Immunohistochemistry has the advantage of directly visualizing tumour cells. Flow cytometry measured antigen expression on cytokeratin-positive (*i.e.* epithelial) cells. This assumes that all the cytokeratin-positive cells in the sample were tumour cells. This may not be the case, particularly as core biopsies of palpable breast tumours were used in this study. Immunohistochemistry of paraffin-embedded sections of breast tumours demonstrated that epithelial cells in normal breast tissue also frequently stained positively for androgen receptor expression. The inability of flow cytometry to distinguish cytokeratin-positive tumour cells from normal epithelial cells may have influenced the results presented in this chapter and contributed to the lack of correlation with immunohistochemistry.

Interestingly, previous flow cytometric methods, developed in our laboratory, for epidermal growth factor receptor and oestrogen receptor alpha both correlated with ligand-binding assays (Brotherick *et al.* 1994) (Brotherick *et al.* 1995). Ligand-binding assays, which have been superseded by immunohistochemistry in routine clinical practice, also have the disadvantage of not showing the cellular distribution of antigen expression.

6.11 The association between tumour androgen receptor status, lymph node status, grade and Nottingham Prognostic Index

The present study has reported a significant association towards increasing androgen receptor expression with higher grade. This observation is at odds with previous studies, which have found androgen receptor expression to be associated with low grade and favorable prognosis in breast cancer (Isola 1993) (Kuenen-Boumeester *et al.* 1996) (Bryan *et al.* 1984) (Langer *et al.* 1990).

No significant difference in androgen receptor expression was observed between lymph node positive and negative breast cancers. Few studies have examined the relationship between androgen receptor expression and lymph node metastases. Kuenen-Boumeester *et al* reported no association between the presence of lymph node metastases and androgen receptor status (Kuenen-Boumeester *et al.* 1996), whereas Soreide *et al* found a positive correlation between androgen receptor expression and the presence of axillary metastases (Soreide *et al.* 1992).

Androgen receptor is co-expressed with oestrogen receptor alpha in breast cancer (Kuenen-Boumeester *et al.* 1992) (Isola 1993) (Lea *et al.* 1989) (Allegra *et al.* 1979). The reason why steroid hormone receptors should be co-expressed in breast cancer is not established. Oestrogen receptor expression in breast cancer is correlated with differentiated tumours, response to endocrine therapy and improved survival (Hawkins *et al.* 1980). As androgen receptors are co-expressed with oestrogen receptors in breast

cancer, one may expect androgen receptor expression to be also correlated with improved outcome.

However, Lea *et al* found that androgen receptors are expressed as the sole steroid receptor in 25% of breast cancer metastases (Lea *et al.* 1989). This suggests that during the de-differentiation of hormone receptor positive to hormone receptor negative tumours androgen receptor is the last steroid receptor to be lost. It is for this reason that we may expect androgen receptor expression to be associated with a more aggressive phenotype than oestrogen receptor alpha expression.

6.12 Breast tumour androgen receptor expression and serum androgen levels

Negative autoregulation of steroid receptor expression by their own ligand is a phenomenon common to the steroid receptor family of transcription factors and may reflect a homeostasis mechanism modulating hormone signals towards a steady state. It is a complex process, which is incompletely understood but is regulated at the level of transcription and by post-transcriptional processes.

Upregulation of ARmRNA *in vivo* has been reported on androgen withdrawal in rat ventral prostate, epididymis, seminal vesicle, kidney and brain and *in vitro* in the prostate cancer cell line LNCaP (Quarmby *et al.* 1990). However, regulation of ARmRNA differs between androgen responsive tissues, downregulation of ARmRNA occurs on treating

LNCaP and the breast cancer cell lines T47D, MFM223 and EFM19 with androgens (Wolf *et al.* 1993) (Krongrad *et al.* 1991) (Hackenberg *et al.* 1992). Whereas, levels of ARmRNA in genital skin fibroblasts are unaffected by androgens (Krongrad *et al.* 1991) and upregulation of ARmRNA occurs in the prostate cancer cell lines DU145 and PC3 (Dai *et al.* 1996) by incubation with androgens.

Regulation of AR protein expression by androgen has also been reported to vary in a tissue specific manner. Downregulation of AR mRNA has been reported to coincide with a rise in AR protein expression in LNCaP on treatment with androgen due to increased translational efficiency or stabilisation of the receptor protein (Krongrad *et al.* 1991). However, other groups did not observe an upregulation of AR protein expression under these conditions, but found prolonged incubation of the LNCaP with androgen led to a functional inactivation of the androgen receptor (Wolf *et al.* 1993). However, downregulation of ARmRNA is accompanied by a fall in AR protein expression on treating mammary cancer cell lines with androgen (Hackenberg *et al.* 1992).

The regulation of androgen receptor expression *in vivo* is complicated by the finding that oestrogens, and to a lesser extent progestins, may also downregulate AR protein expression in MCF7 cells by an oestrogen receptor mediated mechanisms (Stover *et al.* 1987). Autoregulation of oestrogen receptor expression in breast cancer has also been demonstrated and is reviewed by Martin *et al* (Martin *et al.* 1994). Autoregulation has also been described for glucocorticoid receptors (Okret *et al.* 1986) and the progesterone receptors (Read *et al.* 1988).

Evidence from *in vivo* studies of ER protein expression in human breast tumours has established that ER protein expression increases with age (Elwood *et al.* 1980) (McCarty *et al.* 1983). Studies, which have investigated an association between serum oestrogen levels and tumour ER protein expression, have reported a negative correlation (Nagai *et al.* 1979) (Theve *et al.* 1978). These results provide strong evidence for negative autoregulation of ER protein expression in breast tumours *in vivo*. Only one study has investigated an association between serum adrenal androgens and oestrogen receptor expression in breast cancer. This reported that serum DHEAS levels were non-significantly higher in patients with oestrogen receptor positive breast tumours (Skinner *et al.* 1984).

The observation that breast tumour androgen receptor expression increased with age is consistent with previous findings (Lea *et al.* 1989). In addition, it has been demonstrated in chapter 5 that serum androgen levels decline with patient age in women with breast cancer. Few studies have sought a correlation between serum androgen levels and androgen receptor expression. Langer *et al* found no correlation between serum DHEAS, serum testosterone levels and tumour androgen receptor expression and a positive correlation between serum androstenedione and tumour androgen receptor expression in 61 women with breast cancer, though he had difficulty interpreting these findings (Langer *et al.* 1990).

The results of the present study have not showed a significant correlation between serum androgen levels and tumour androgen receptor expression in women with primary operable breast cancer. However, a trend towards increasing tumour androgen receptor expression with decreasing serum DHEAS levels was observed.

DHEAS is quantitatively the most important steroid hormone in the circulation and is metabolized to androgenic metabolites such as testosterone and 5alpha-dihydrotestosterone, oestrogenic androgens such as androstenediol and via aromatase enzymes to oestrogens (Rochefort *et al.* 1984). DHEAS could therefore give rise to a variety of steroids, which could regulate the expression of androgen receptor expression in breast cancer cell lines *in vitro.*

The finding of an inverse correlation between serum DHEAS and androgen receptor expression, though not reaching significance, suggests DHEAS is a determinant of androgen receptor expression in breast cancer. The observation that serum DHEAS levels decline with age and breast tumour androgen receptor levels rise with age suggests that negative autoregulation is the mechanism underlying this observation.

In contrast, tumour androgen receptor expression appeared to be independent of serum total testosterone levels. This finding is surprising as testosterone and its metabolite 5alpha-dihydrotestosterone have high affinity for the androgen receptor, and have been shown to regulate androgen receptor expression in breast cancer cell lines *in vitro* (Hackenberg *et al.* 1992). However, testosterone is bound in the circulation to sex

hormone binding globulin and albumin, only the unbound fraction is biologically active (Brooks 1984). Serum total testosterone has been measured in this study. It would therefore be necessary to measure free testosterone levels in order to investigate further an association between serum testosterone levels and androgen receptor expression in breast tumours.

6.13 Androgen receptor and epidermal growth factor receptor expression in breast cancer

This is the first study, which has examined a relationship between androgen receptor expression and epidermal growth factor expression in human breast cancer. As outlined above crosstalk between oestrogen receptor and epidermal growth factor pathways has been demonstrated to occur in breast cancer (Nicholson *et al.* 1999). Likewise crosstalk between androgen receptor and epidermal growth factor pathways has been demonstrated in prostate cancer (Culig 1994). The androgen receptor clearly has a very different role in breast cancer than prostate cancer. Though it has been speculated that crosstalk occurs between androgen receptor and growth factor pathways in breast cancer (Brys 2000), there is no evidence to substantiate this at present.

The results of the present study suggest that a functional interaction between epidermal growth factor and androgen receptor. This implies that crosstalk between androgen receptor and epidermal growth factor receptor-pathways. However, these results have to be interpreted with caution. Firstly there are only small numbers in this study, secondly

we have used a novel technique i.e. flow cytometry to measure steroid and growth factor expression and thirdly an association between oestrogen receptor and epidermal growth factor was demonstrated, though previous results have demonstrated an inverse association between these antigens in breast cancer (Walker *et al.* 1999). Further investigation of this area will require *in vitro* studies to establish whether crosstalk occurs between growth factor and androgen receptor pathways in breast cancer. This should help us further understand the role of the androgen receptor in breast cancer.

6.14 Summary

The results of a flow cytometric method for the determination of androgen receptor expression in breast cancer are presented in this chapter. Western blotting demonstrates that the androgen receptor antibody AR441 detects a protein of molecular weight 110kDa corresponding to the androgen receptor. Immunohistochemistry of the androgen receptor positive prostate cancer cell line LNCap demonstrates positive nuclear staining for androgen receptors. Immunohistochemistry of parrafin sections of breast tumours show staining for androgen receptor expression. A clinical utility for the determination of antigen expression by flow cytometry is suggested by its capability to determine the expression of several antigens simultaneously. However, no correlation was observed between androgen receptor expression determined by immunohistochemistry and flow cytometry. The disadvantages of each method and possible causes for the failure to observe a correlation have been discussed. Androgen receptor expression increased with patient age and a trend towards increasing tumour androgen receptor expression with

decreasing serum androgen levels provides evidence for negative autoregulation of androgen receptor expression *in vivo*. In the present study androgen receptor expression was found to increase with increasing Bloom Richardson grade. This suggests that androgen receptor expression is a marker of poor prognosis. The lack of an association between androgen receptor expression and lymph node status suggests that androgen receptor is not important in the process of metastasis. Co-expression of androgen receptor and oestrogen receptor alpha confirms results of previous studies. A positive correlation between androgen receptor expression and epidermal growth factor expression suggests a functional interaction between these pathways in breast cancer and warrants further investigation.

Results and Discussion

The growth effects of 5α-dihydrotestosterone and 5-androstene-3β,17β-diol on MCF7, T47D and MDAMB231 breast cancer cell lines

Results

7.1 Analysis of steroid hormone receptor expression of breast cancer cell lines

The cell lines used in this study, namely MCF7, T47D and MDAMB231 were chosen as they express different levels of steroid receptors (Horwitz *et al.* 1978). MCF7 cells have been reported to express high levels of estrogen receptors and moderate levels of androgen receptors (Horwitz *et al.* 1975). Whereas T47D cells have been reported to express moderate levels of androgen and oestrogen receptors and MDAMB231 cells are steroid receptor negative (Horwitz *et al.* 1978).

The steroid receptor expression of the cell lines used in the present study has been determined using a flow cytometric technique. Flow cytometry is an established technique for the determination of cytoplasmic and nuclear antigens (Larson 2000). Methods for the determination of oestrogen receptor alpha (Brotherick *et al.* 1995) have been established in our laboratory. Methods for the determination of androgen receptor expression in breast cancer are described in the materials and methods section. The steroid receptor profile is illustrated in the following figure.

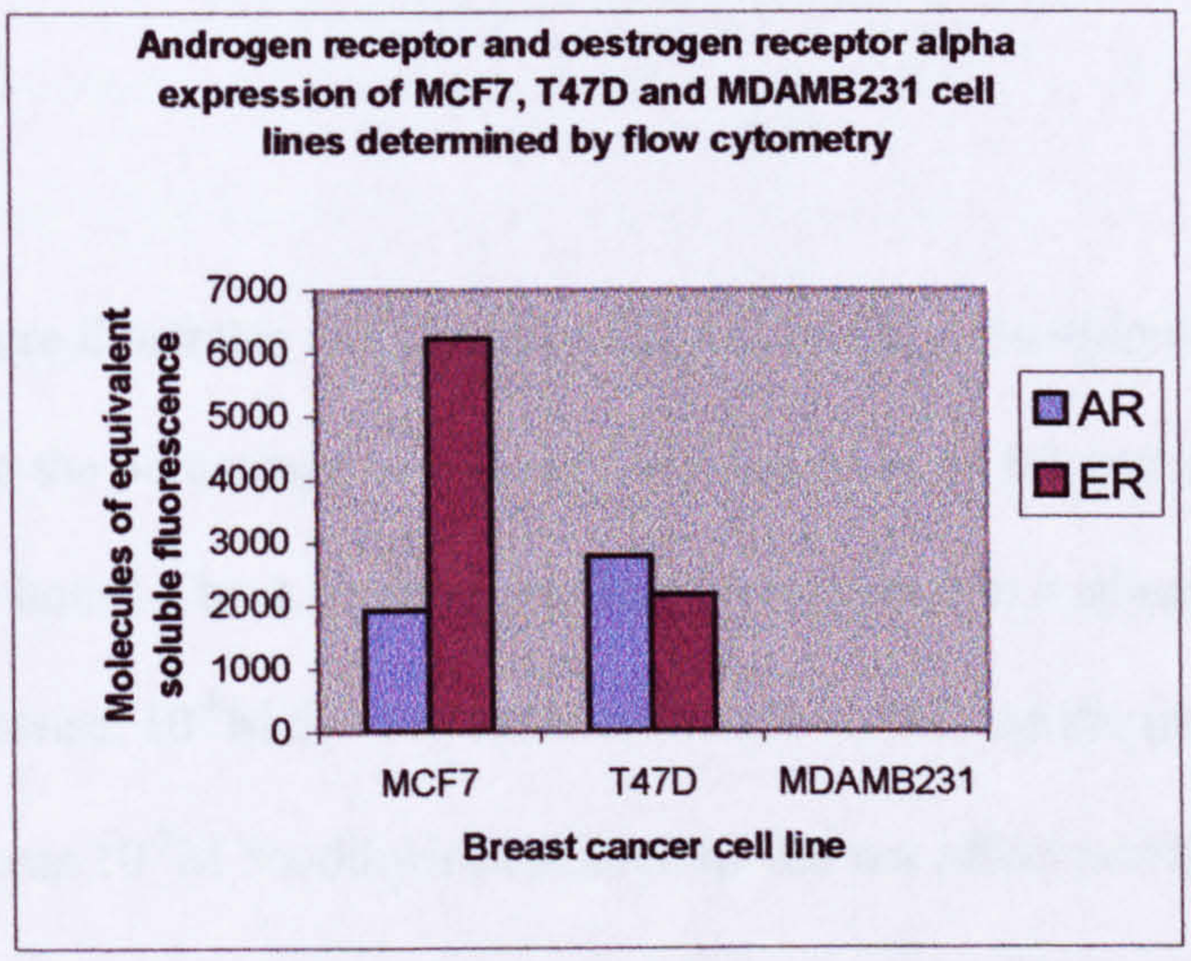


Figure 7.1: Androgen and oestrogen receptor alpha expression of MCF7, T47D and MDAMB231 cell lines

7.2 Analysis of DNA cell cycle during forty-eight hour incubation of MCF7, T47D, and MDA231 with 5α-dihydrotestosterone +/- faslodex

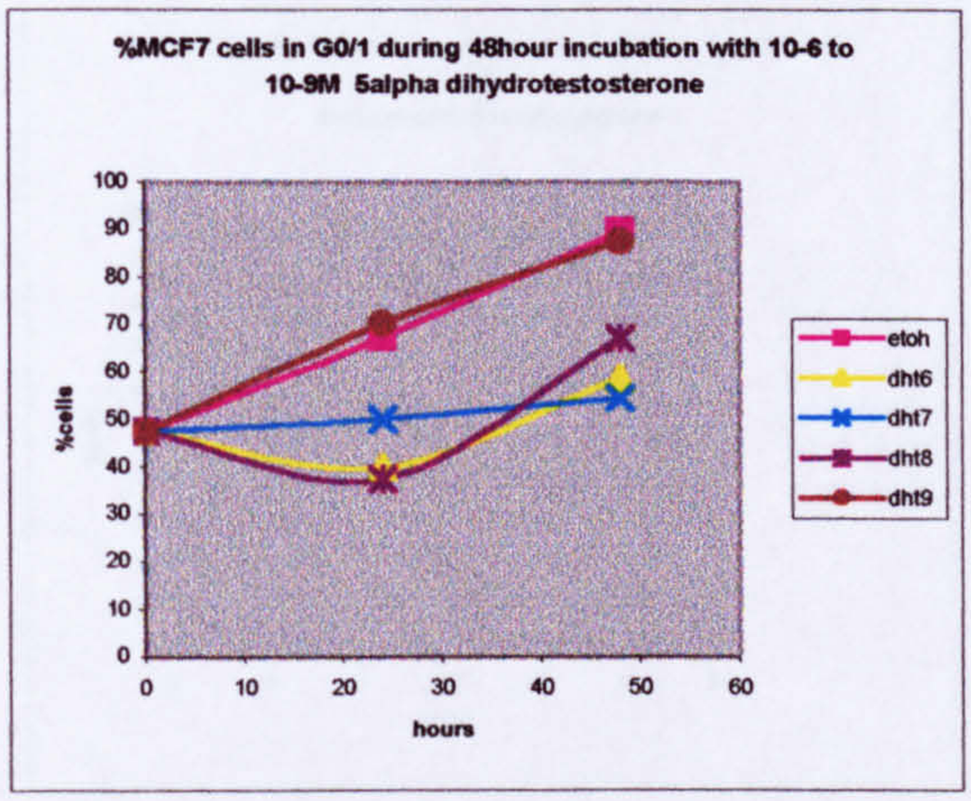
MCF7, T47D and MDA231 breast cancer cell lines were incubated over 48 hours with a range of concentrations of 5α-dihydrotestosterone. The percentage of cells in each phase of the cell cycle G0/1, S and G2/M was determined at 0, 24 and 48 hours as outlined in the materials and methods section above.

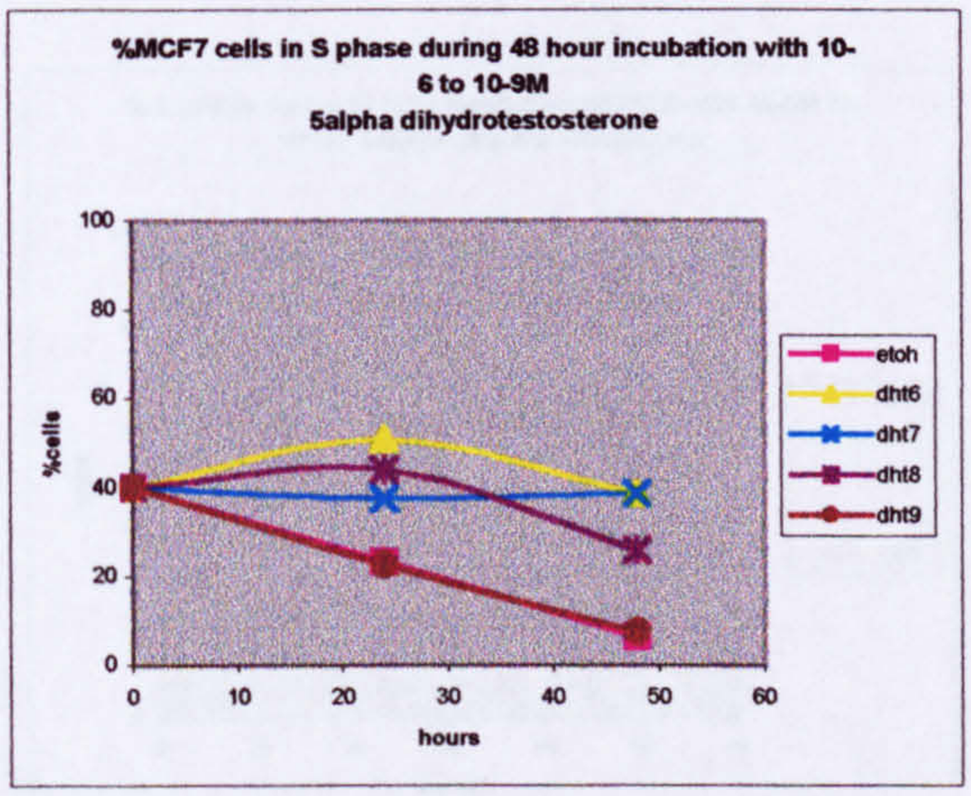
MCF7 cells were incubated with a range of concentrations of 5α-dihydrotestosterone from physiological (10^{-9}M to 10^{-8}M concentrations) to pharmacological levels (10^{-7}M to 10^{-6}M).

The following figure illustrates that pharmacological levels of 5α-dihydrotestosterone resulted in a fall in the percentage of cells in G0/1 and a rise in the percentage of cells in S phase within 24 hours. These findings would be interpreted as a stimulation of proliferation. Likewise, 10^{-8}M 5α-dihydrotestosterone stimulated the proliferation of MCF7 cells, whereas 10^{-9}M 5α-dihydrotestosterone did not affect proliferation over a 48 hour period. The effect on the progression of all breast cancer cells to G2/M was less consistent.

Similar results were obtained with T47D cells. Pharmacological doses of 5α-dihydrotestosterone stimulated the proliferation of T47D cells over 48 hours, however, physiological doses did not affect the proliferation of this cell line. Again the effect on progression of cells to G2/M was inconsistent. On incubating the MDA231 cell lines with 10^{-9}M to 10^{-6}M 5α-dihydrotestosterone and 5-androstene-3β,17β-diol which express low or zero amounts of oestrogen receptors, no significant effect on proliferation was observed. These results are illustrated below.

MCF7 and T47D cell lines were incubated with 5α-dihydrotestosterone and the anti-oestrogen 10^{-6}M faslodex. The percentage of cells in each phase of the cell cycle G0/1, S and G2/M was determined at 0, 24 and 48 hours, as previously. The following figures demonstrate that the proliferation of MCF7 cells by 5α-dihydrotestosterone was inhibited by faslodex confirming that 5α-dihydrotestosterone stimulates proliferation in the MCF7 cell line via interaction with the oestrogen receptor. However, faslodex failed to inhibit the proliferation of T47D cells, suggesting that 5alpha dihydrotestosterone stimulates proliferation in T47D cell line via a pathway other than the oestrogen receptor.





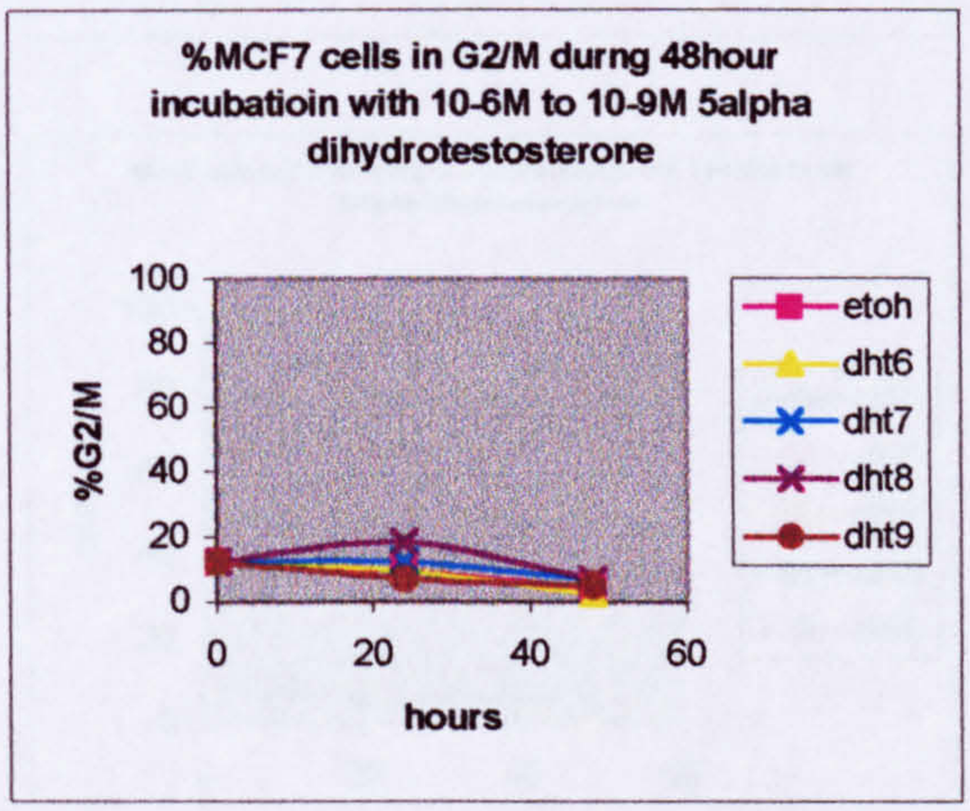
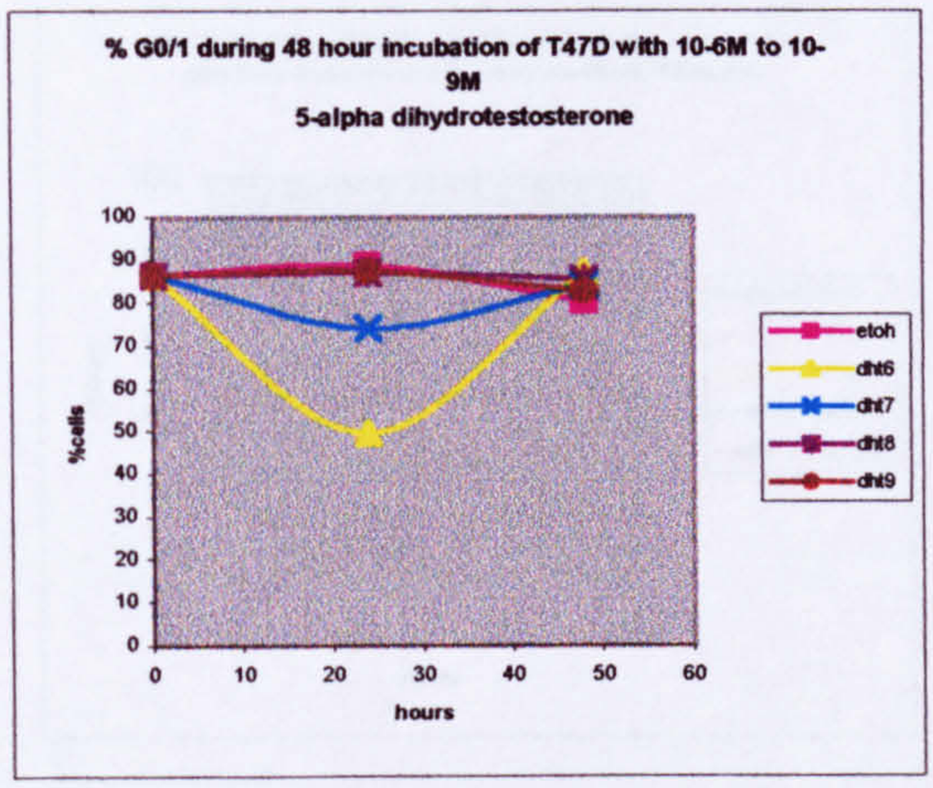
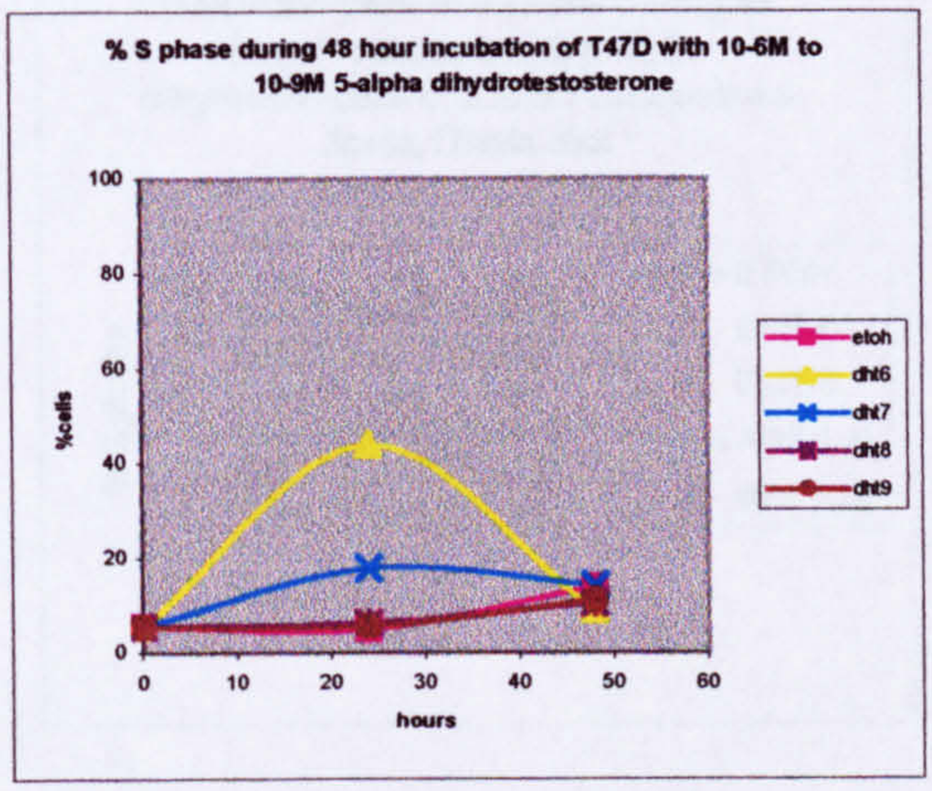


Figure 7.2: %MCF7 cells in G0/1,S and G2/M on incubation with 10^{-9}M to 10^{-6}M 5α-dihydrotestosterone (abbreviations: nDHT = 10^{-n}M 5α-dihydrotestosterone, ETOH = 0.01% ethanol)





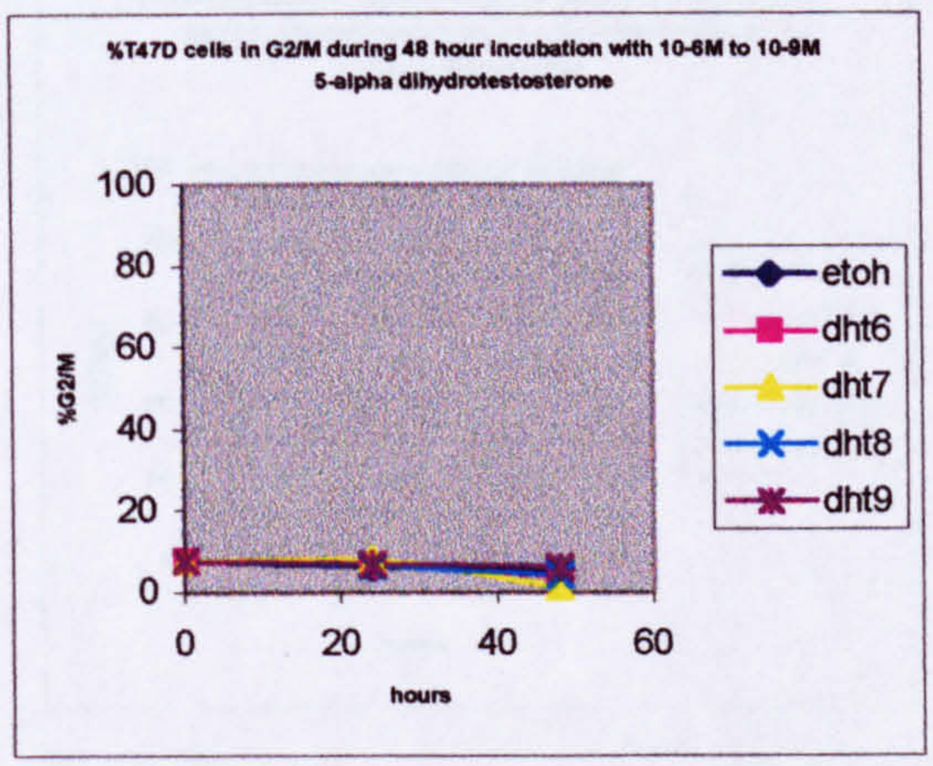
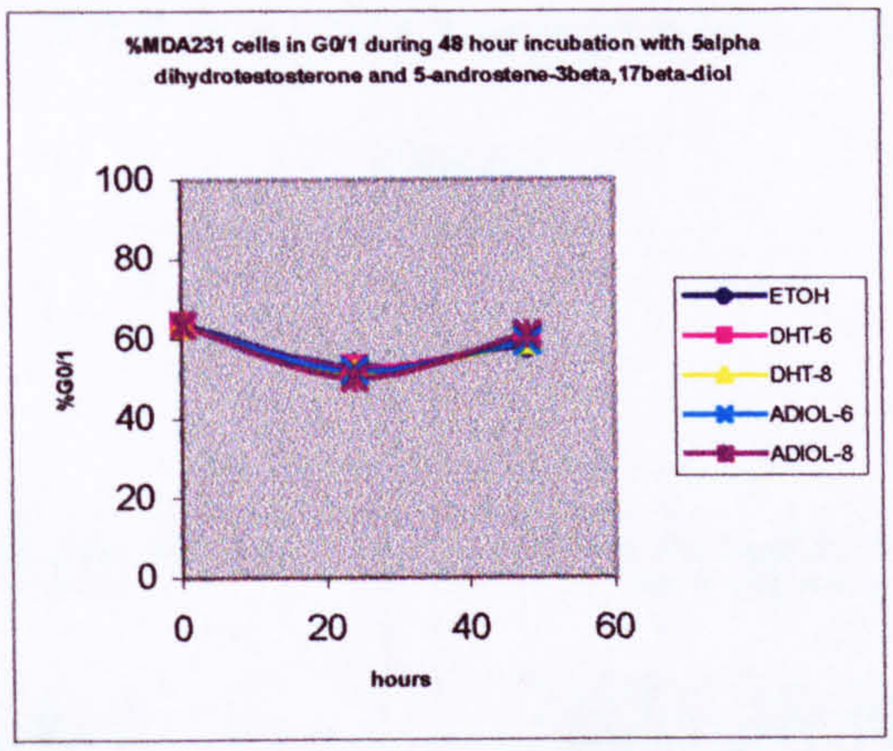
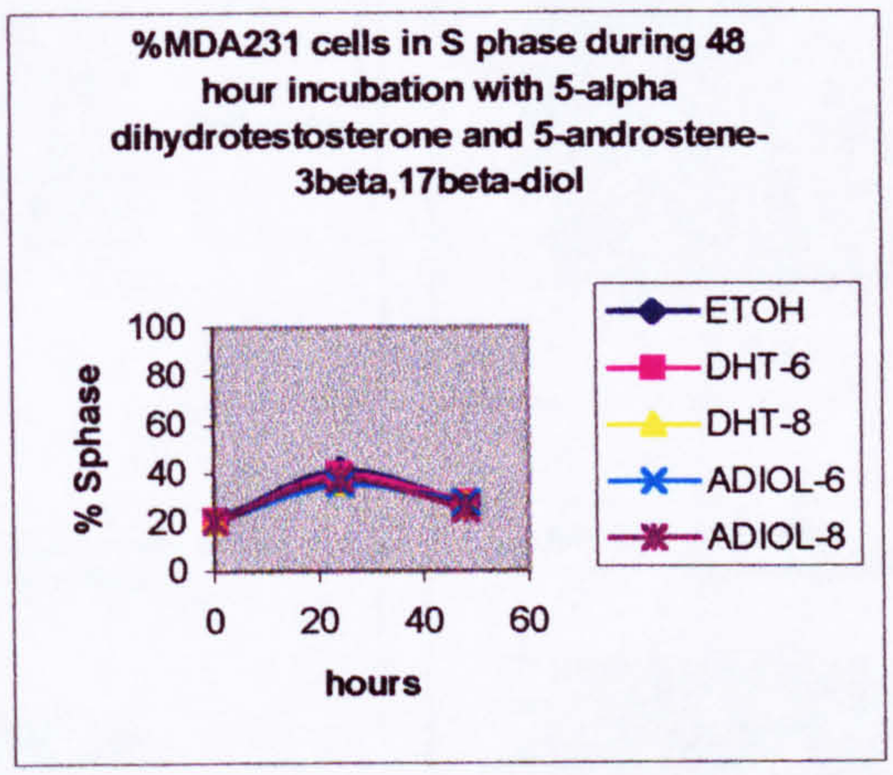


Figure 7.3: %T47D cells in G0/1,S and G2/M on incubation with 10^{-9}M to 10^{-6}M 5α-dihydrotestosterone (abbreviations: nDHT = 10^{-n}M 5α-dihydrotestosterone, ETOH = 0.01% ethanol)





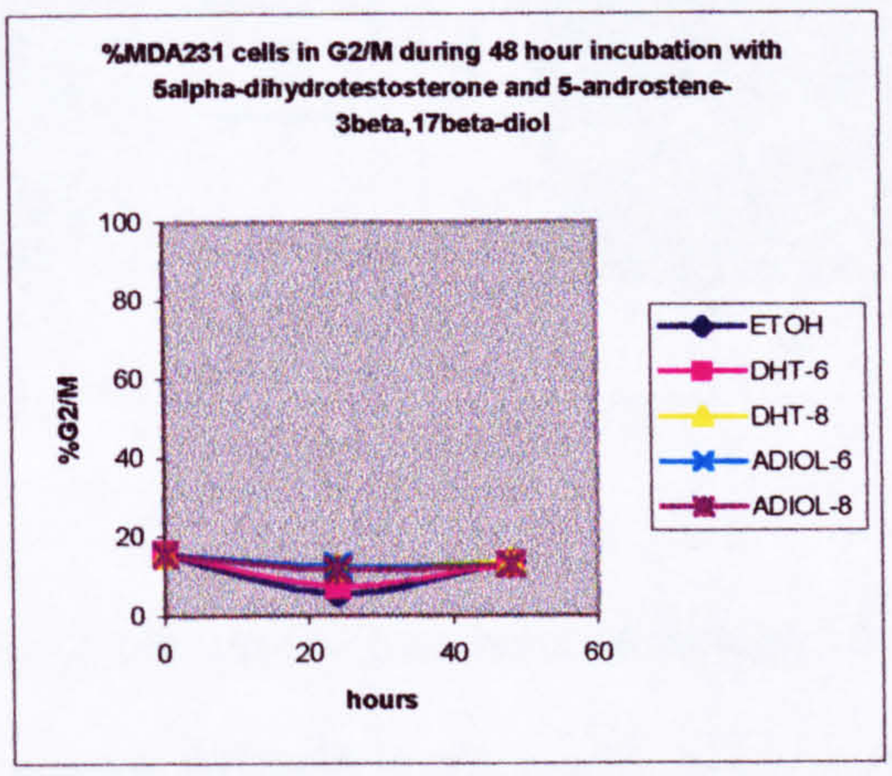


Figure 7.4: %MDA231 cell lines on incubation with 10^{-9}M to 10^{-6}M 5α-dihydrotestosterone and 5-androstene-3β,17β-diol (abbreviations: DHT-n = 10^{-n}M 5α-

dihydrotestosterone, ADIOL-n = 10^{-n}M 5-androstene-3β,17β-diol, ETOH = 0.01% ethanol)

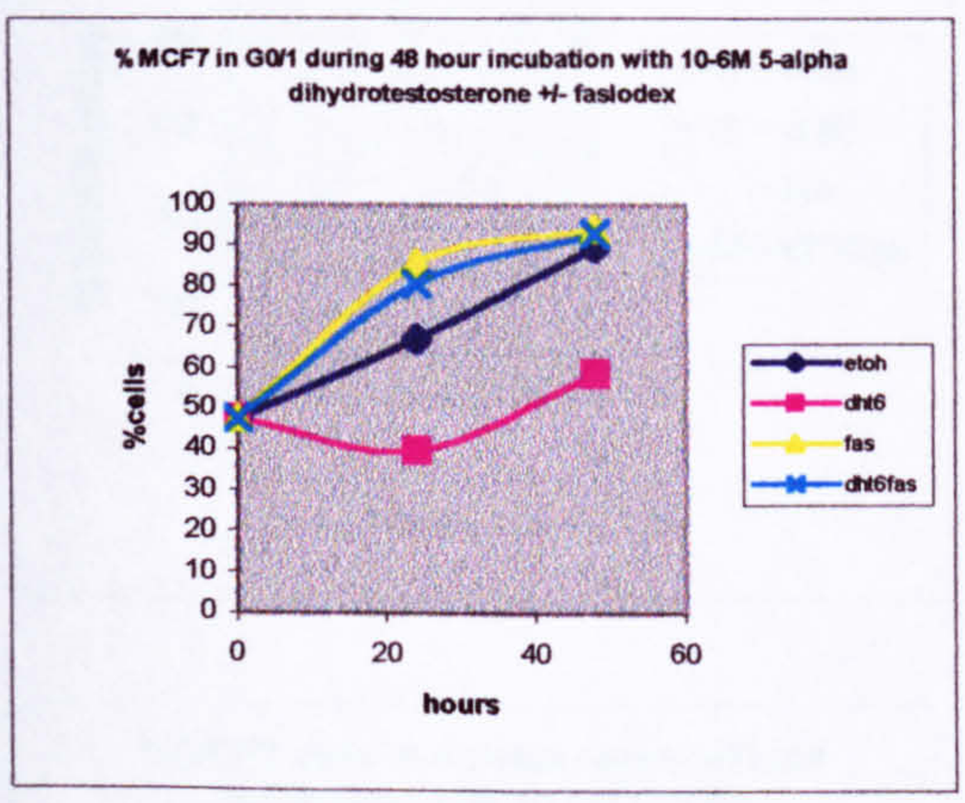


Figure 7.5: %MCF cells in G0/1 on incubation with 10^{-9}M to 10^{-6}M 5α-dihydrotestosterone and 10^{-6}M faslodex (abbreviations: nDHT = 10^{-n}M 5α-dihydrotestosterone, ETOH = 0.01% ethanol, FAS = 10^{-6}M Faslodex)

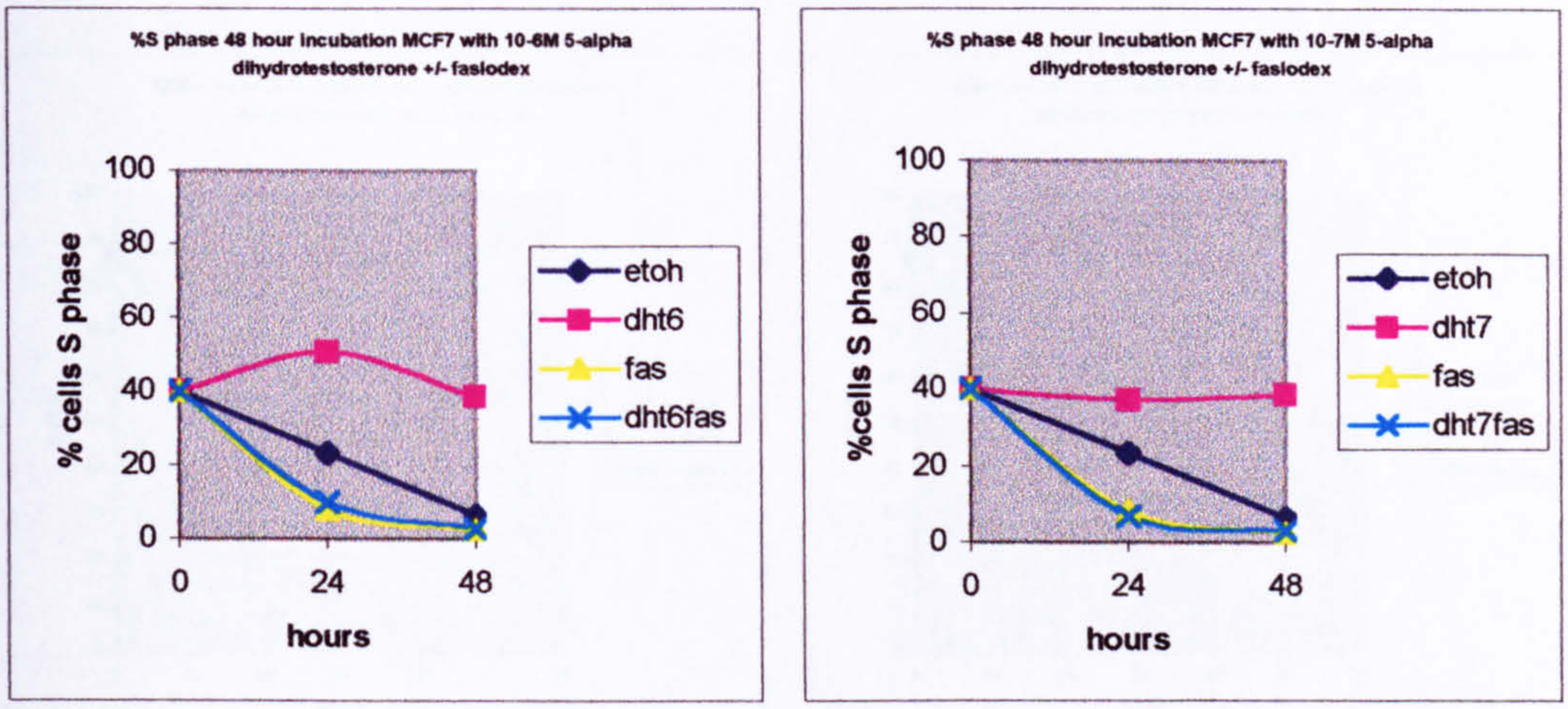


Figure 7.6: %MCF cells in S phase on incubation with 10^{-9}M to 10^{-6}M 5α-dihydrotestosterone and 10^{-6}M faslodex (abbreviations: nDHT = 10^{-n}M 5α-dihydrotestosterone, ETOH = 0.01% ethanol, FAS = 10^{-6}M Faslodex)

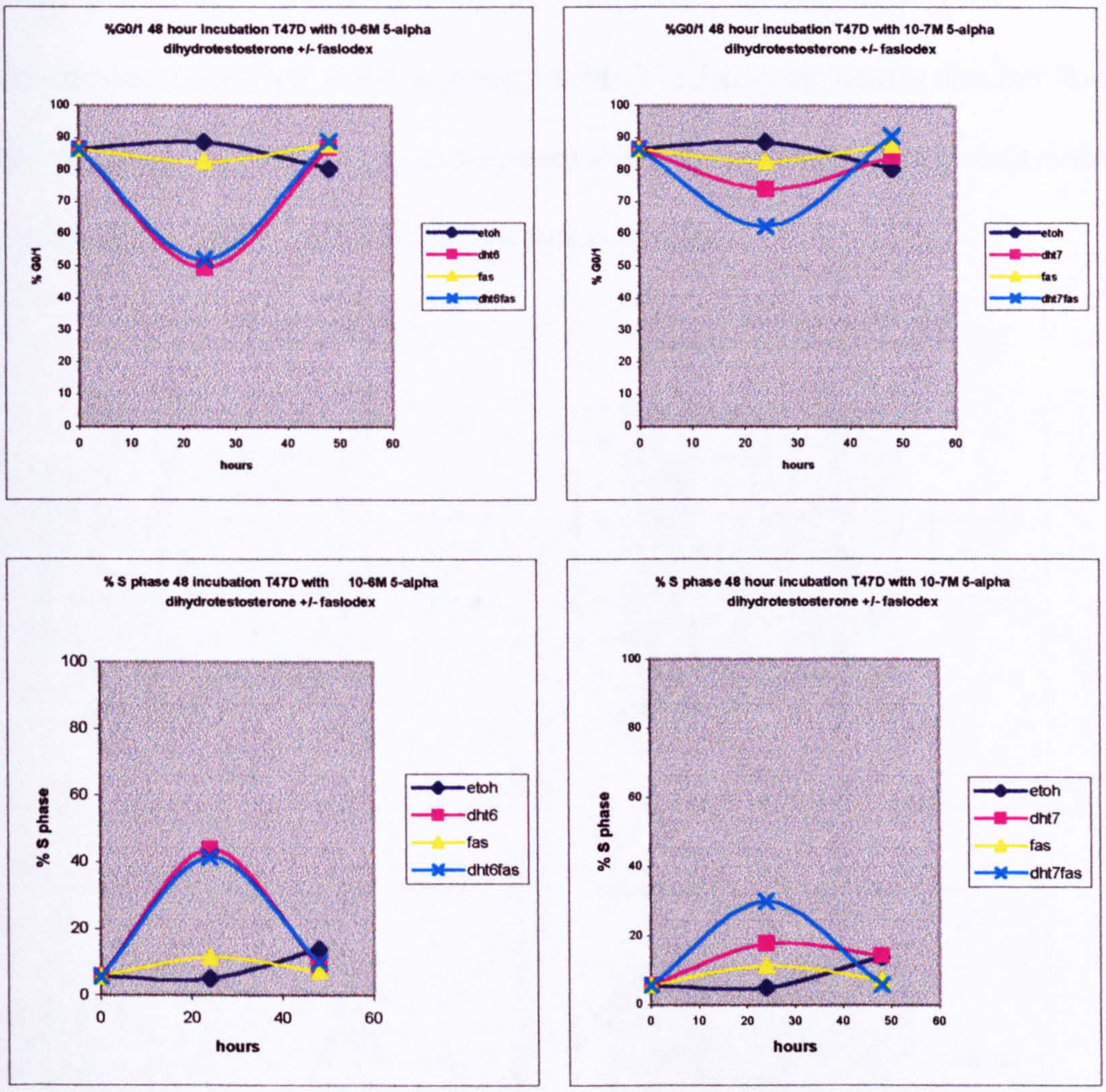


Figure 7.7: %T47D cells in G0/1 and S phase on incubation with 10^{-7}M to 10^{-6}M 5α-dihydrotestosterone and 10^{-6}M faslodex (abbreviations: nDHT = 10^{-n}M 5α-dihydrotestosterone, ETOH = 0.01% ethanol, FAS = 10^{-6}M Faslodex)

7.3 Cell proliferation of MCF7 and T47D cell lines during 9-day incubation with 5α-dihydrotestosterone

MCF7 and T47D cells were incubated for 9 days with physiological levels of 5α-dihydrotestosterone and 17β-oestradiol (10^{-9}M). The following figures illustrate that physiological levels of 5α-dihydrotestosterone inhibit the baseline and oestrogen-induced proliferation of MCF7 and T47D breast cancer cell lines.

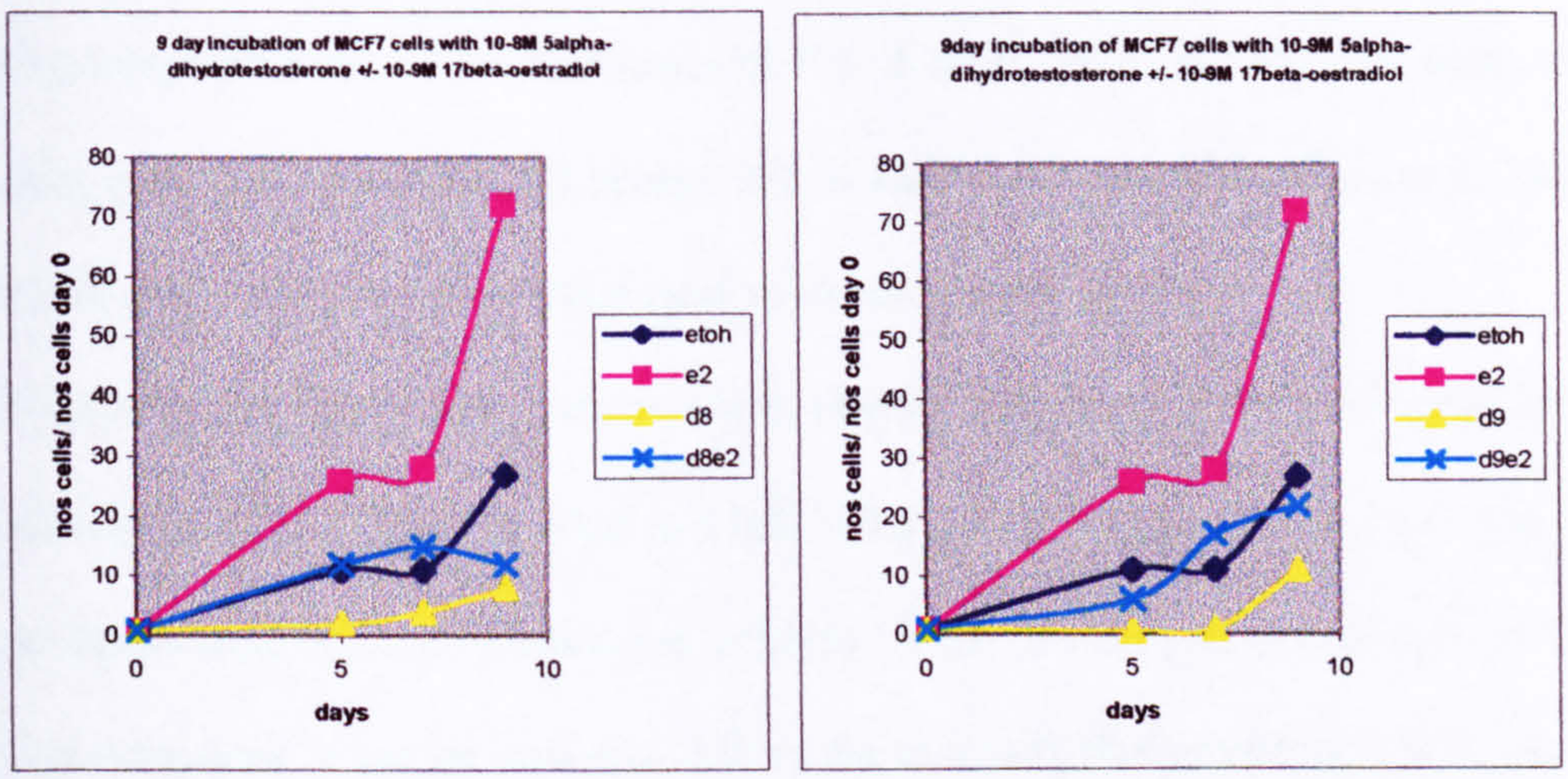


Figure 7.8: Proliferation of MCF7 and T47D breast cancer cell lines during 9-day incubation with physiological levels of 5α-dihydrotestosterone and 17β-oestradiol

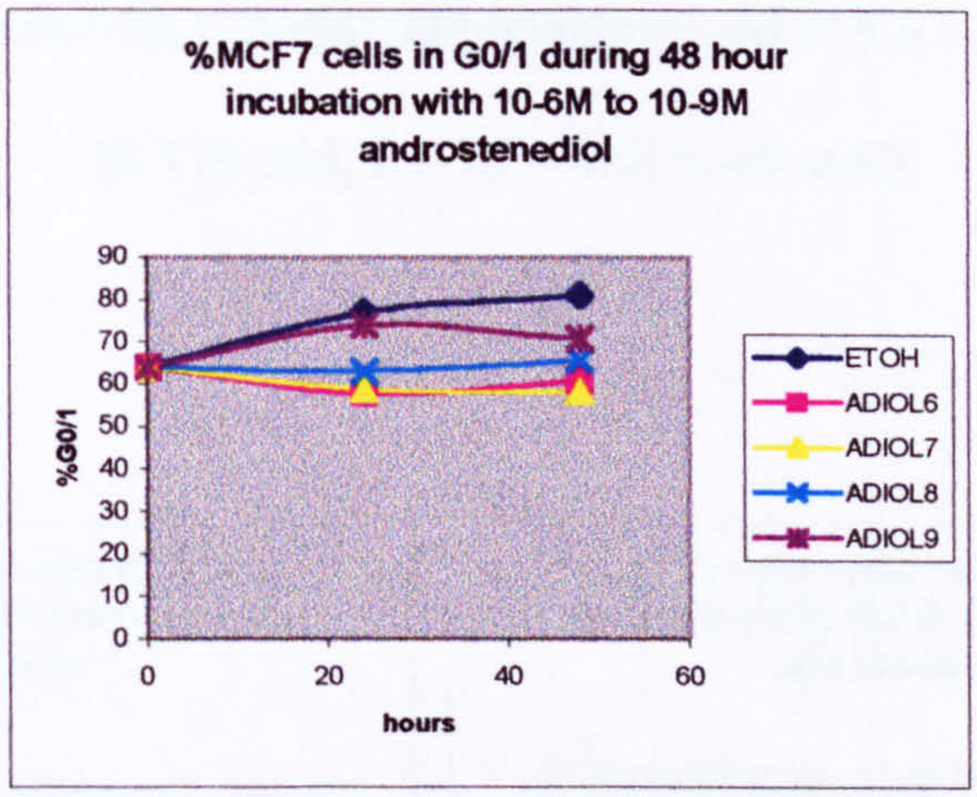
(abbreviations: nDHT = 10^{-n}M 5α-dihydrotestosterone, e2 = 17β-oestradiol, ETOH = 0.01% ethanol)

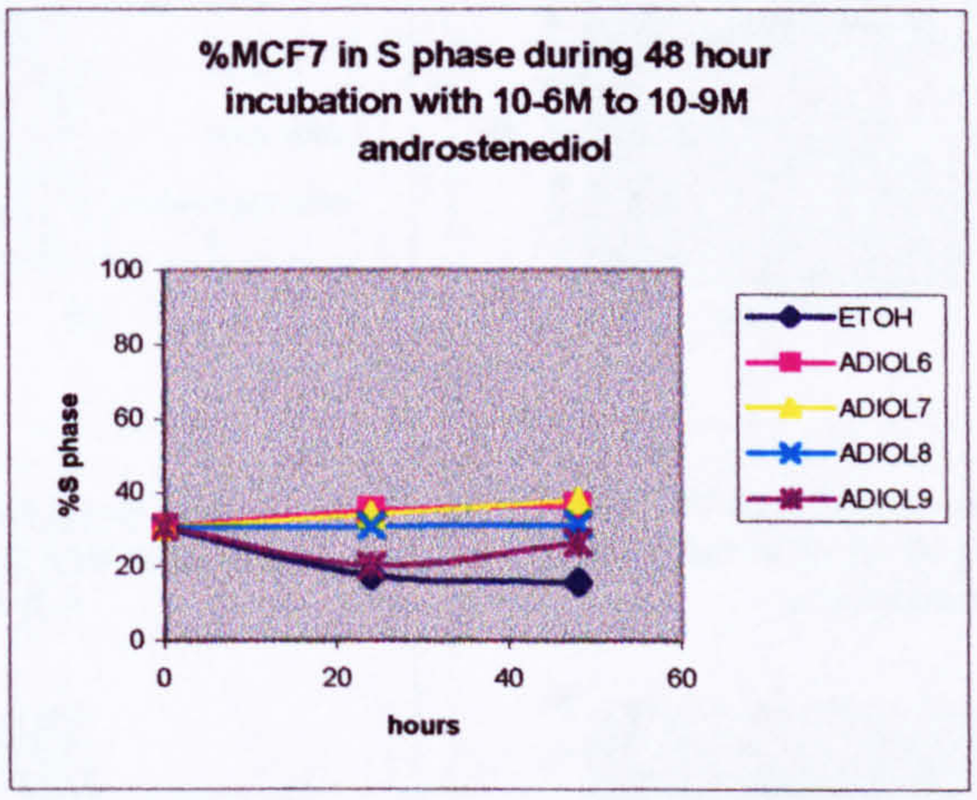
7.4 Analysis of DNA cell cycle during forty-eight hour incubation of MCF7 and T47D cell lines with 5-androstene-3β,17β-diol +/- faslodex

MCF7 and MDA231 breast cancer cell lines were incubated over a 48 hour period with physiological levels of 5-androstene-3β,17β-diol (10^{-9}M to 10^{-6}M). The percentage of cells in each phase of the cell cycle G0/1, S and G2/M was determined at 0, 24 and 48 hours as outlined in the materials and methods section above.

The following figures illustrate that both physiological and pharmacological levels of 5-androstene-3β,17β-diol resulted in a fall in the percentage of cells in G0/1 and a rise in the percentage of cells in Sphase at 24hours. Again the progression of cells to G2/M was less consistent. It can be interpreted from these results that physiological levels of 5-androstene-3β,17β-diol stimulate the proliferation of MCF7 cell line. In addition, stimulation increased up to a concentration of 100nM 5-androstene-3β,17β-diol, but no additional stimulation was observed at greater concentrations. No significant affect on proliferation of the MDA231 cell line was observed.

On co-incubating MCF7 cells with 5-androstene-3β,17β-diol and faslodex it can be seen from the following figures that faslodex completely inhibits the stimulatory affect of physiological doses of 5-androstene-3β,17β-diol and partially inhibits the stimulation of pharmacological doses of 5-androstene-3β,17β-diol.


%MCF7 cells in G0/1 during 48 hour incubation with 10-6M to 10-9M androstenediol
%G0/1
90
80
70
60
50
40
30
20
10
0
0
20
40
60
hours
ETOH
ADIOL6
ADIOL7
ADIOL8
ADIOL9


%MCF7 in S phase during 48 hour incubation with 10-6M to 10-9M androstenediol
%S phase
100
80
60
40
20
0
0
20
40
60
hours
ETOH
ADIOL6
ADIOL7
ADIOL8
ADIOL9

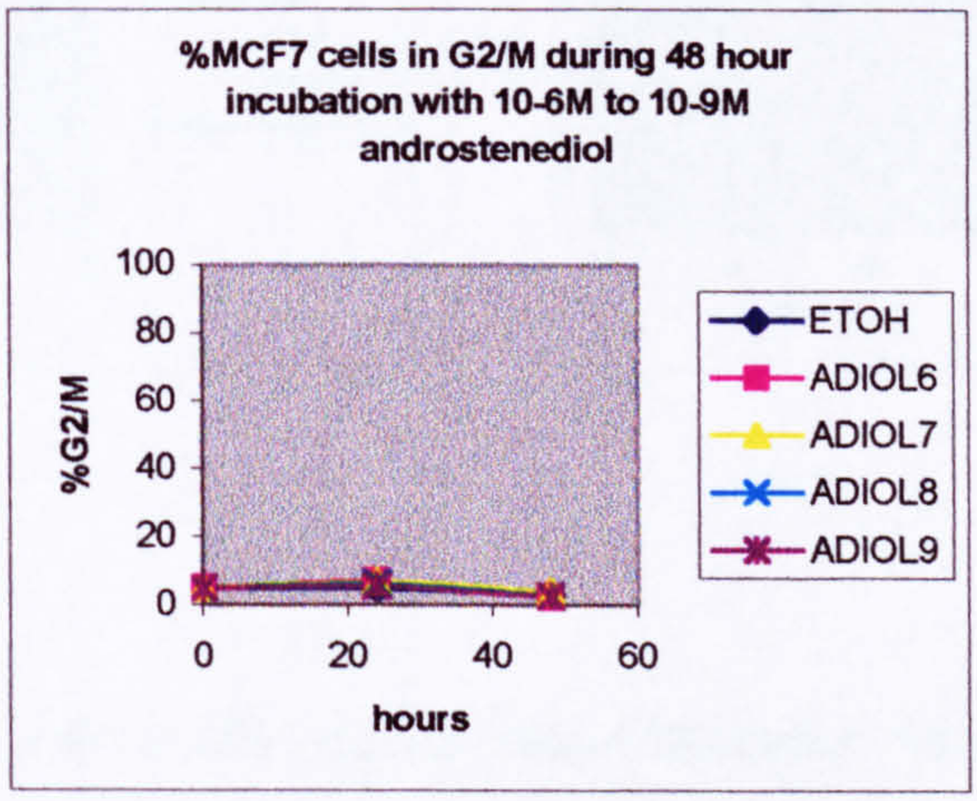
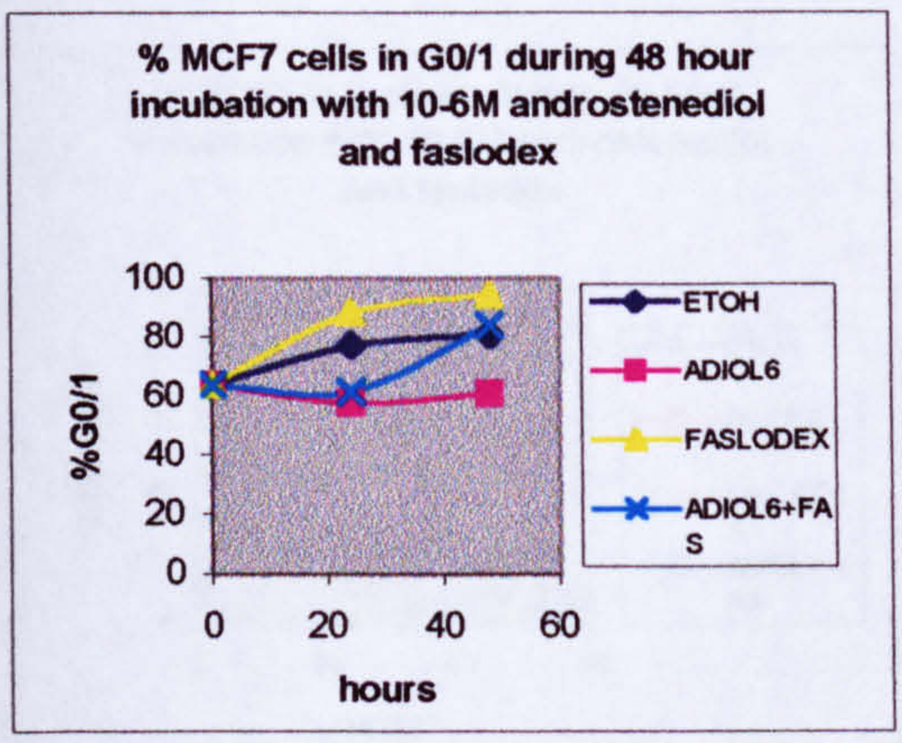
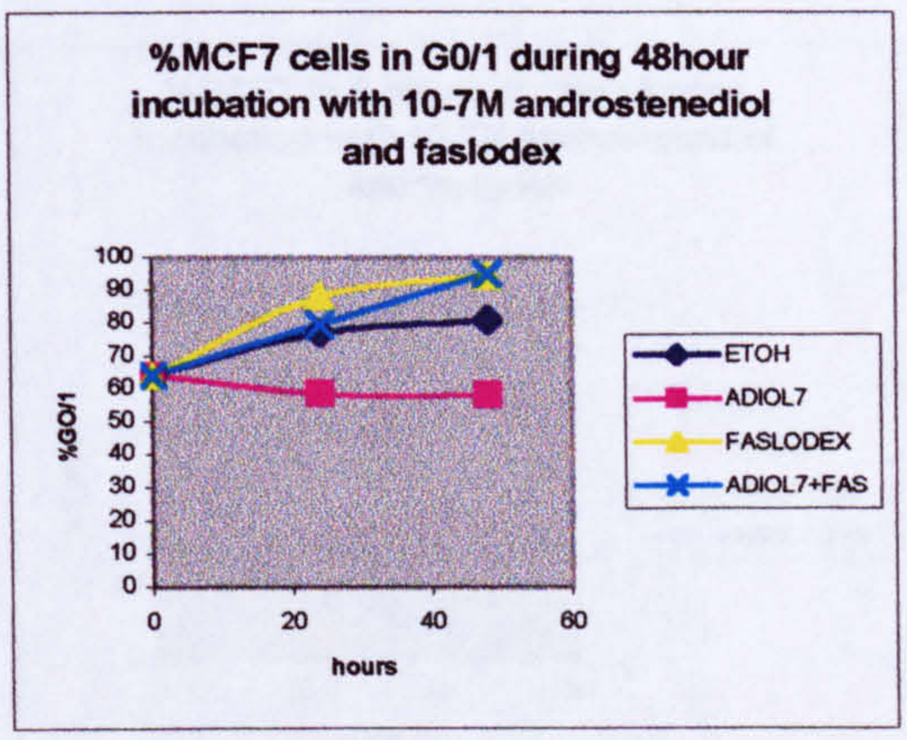
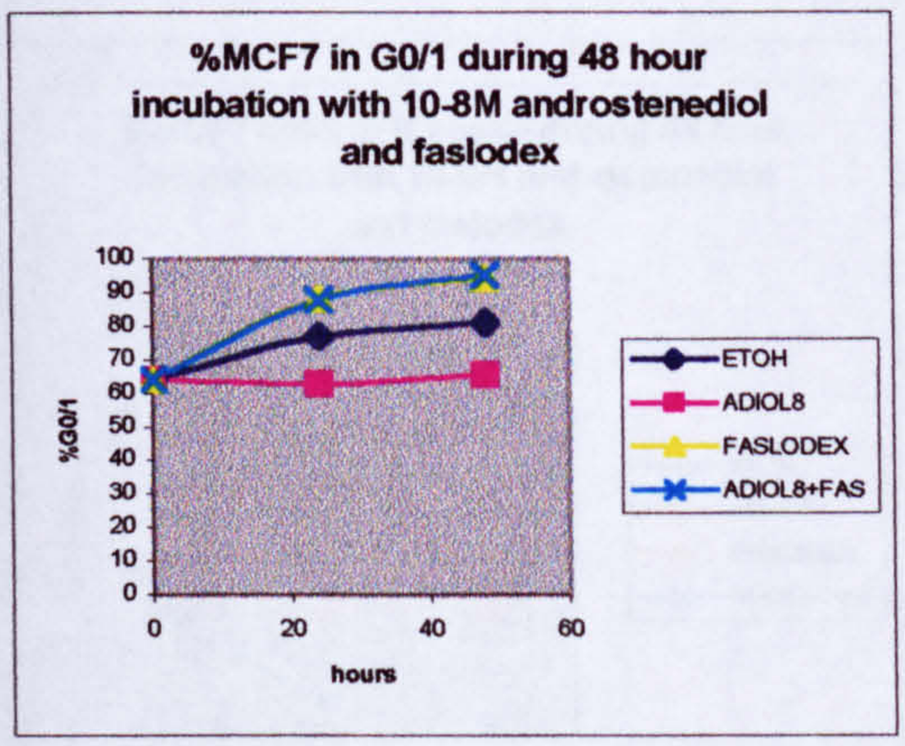

%MCF7 cells in G2/M during 48 hour incubation with 10-6M to 10-9M androstenediol
%G2/M
100
80
60
40
20
0
0
20
40
60
hours
ETOH
ADIOL6
ADIOL7
ADIOL8
ADIOL9

Figure 7.9: %MCF7 cells in G0/1, S phase and G2/M during 48hour incubation with 10-6 to 10-9M 5-androstene-3β,17β-diol (abbreviations: ADIOLn = 10^{-n}M 5-androstene-3β,17β-diol, ETOH = 0.01% ethanol)







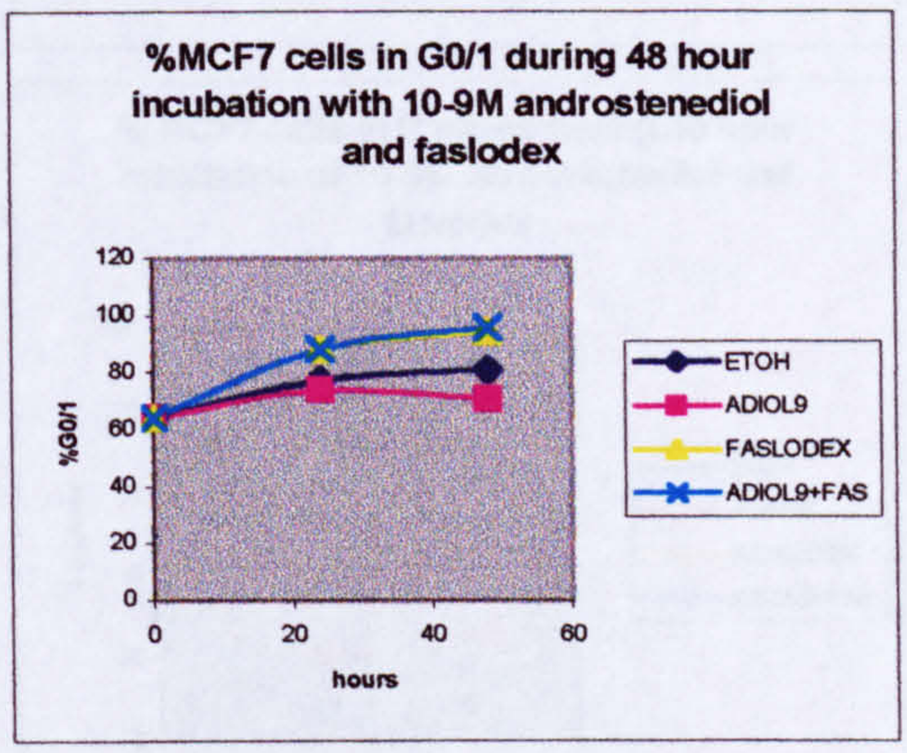
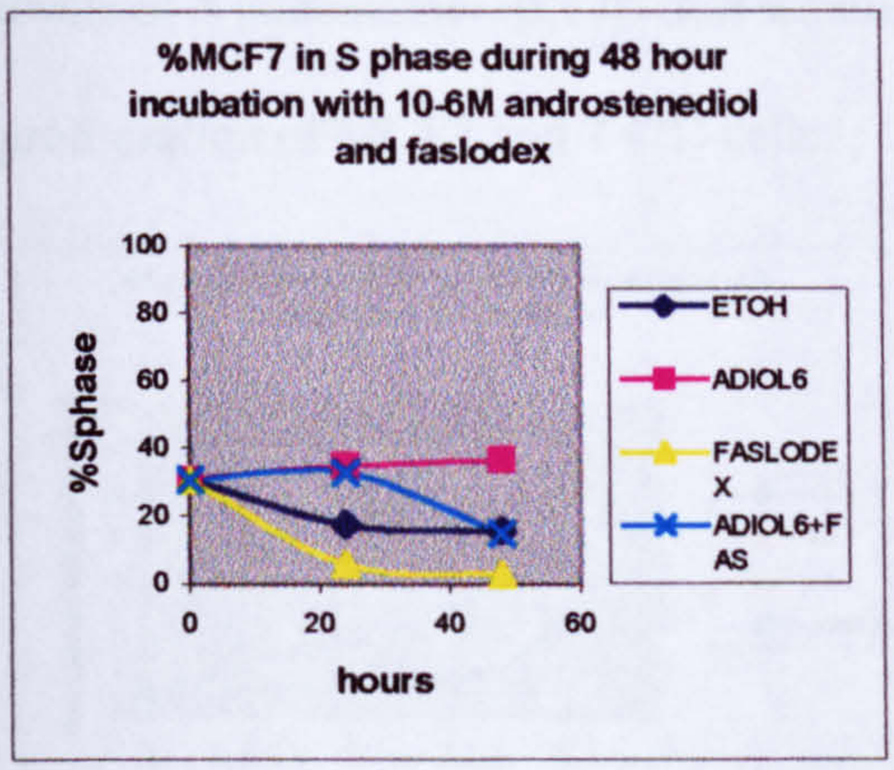
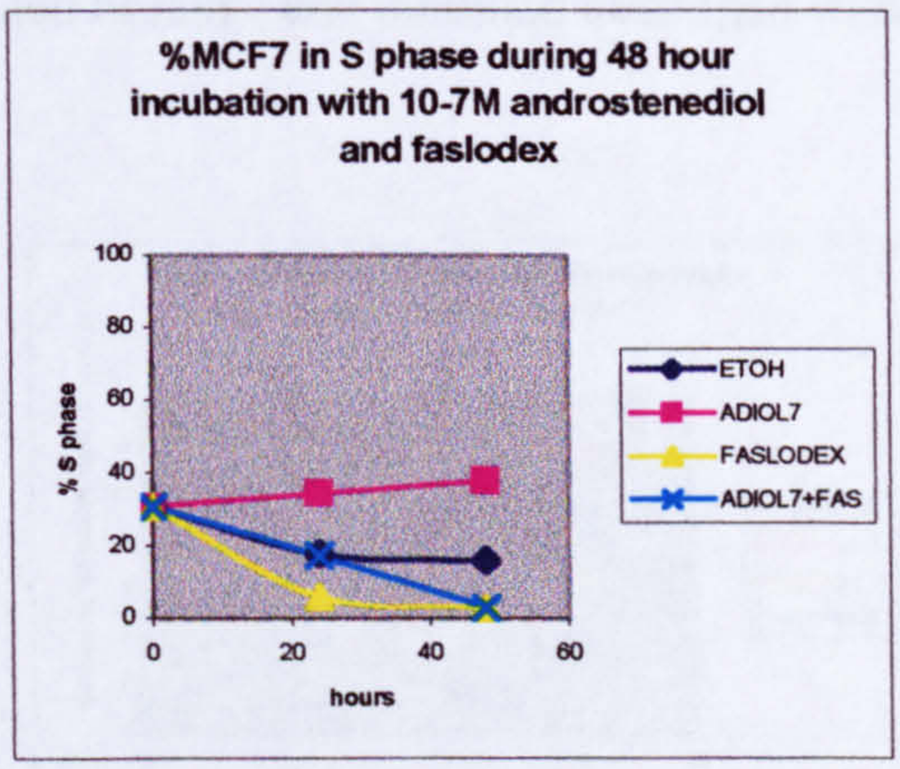
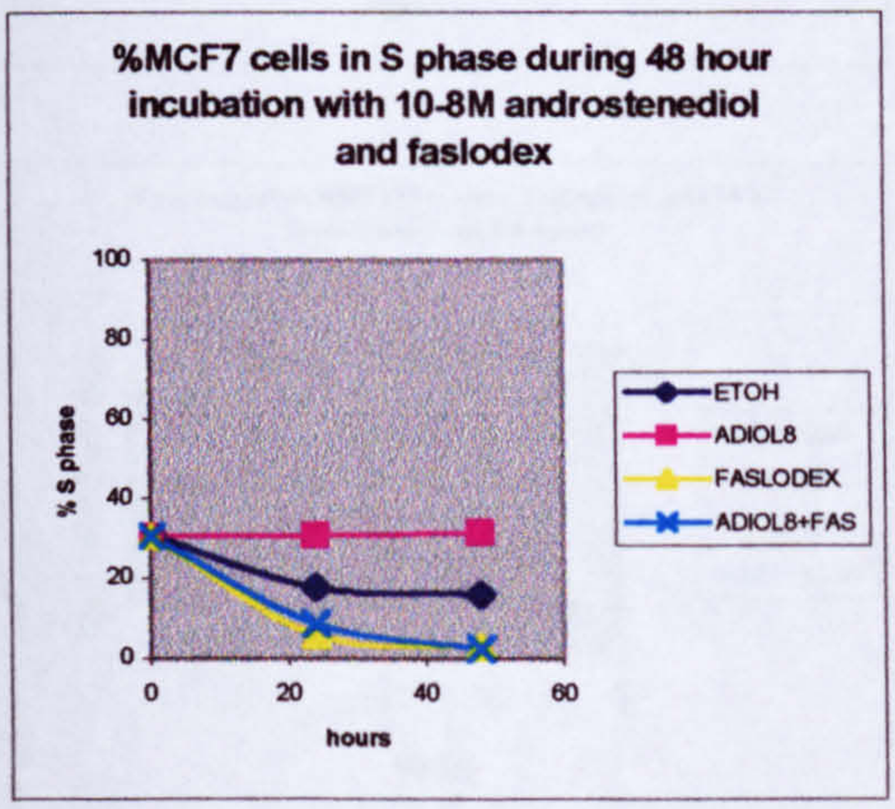


Figure 7.10: %MCF7 cells in G0/1 during 48hour incubation with 5-androstene-3β,17β-diol and faslodex (abbreviations: ADIOLn = 10^{-n}M 5-androstene-3β,17β-diol, ETOH = 0.01% ethanol, FAS = 10^{-6}M Faslodex)







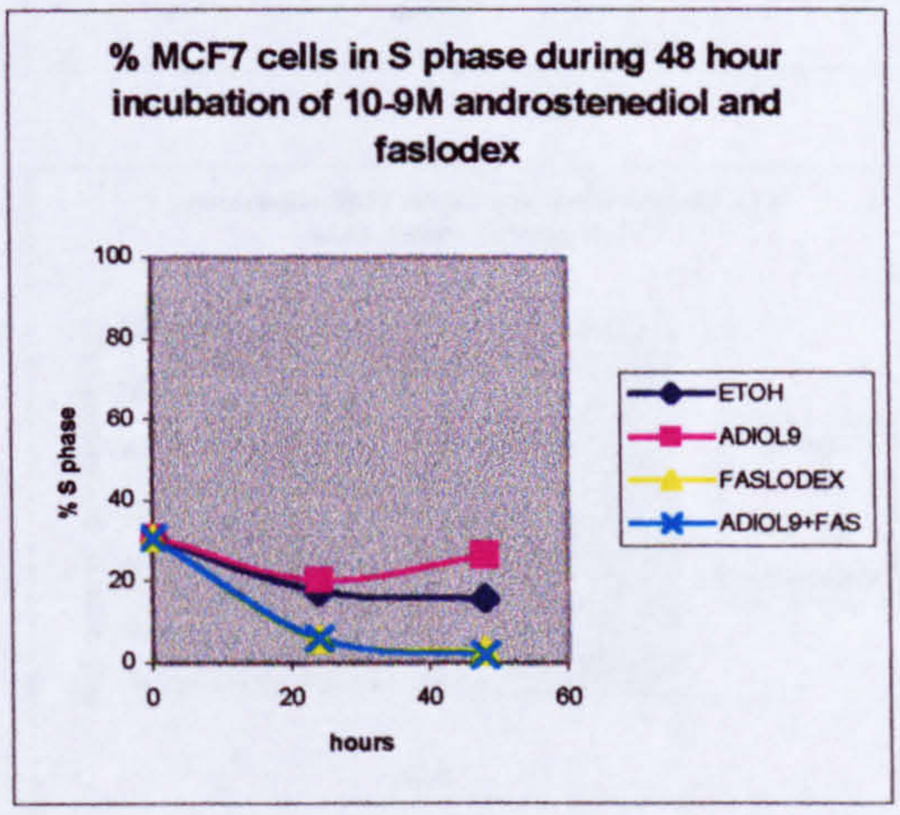


Figure 7.11: %MCF7 cells in G0/1 during 48hour incubation with 5-androstene-3β,17β-diol and faslodex (abbreviations: ADIOLn = 10^{-n}M 5-androstene-3β,17β-diol, ETOH = 0.01% ethanol, FAS = 10^{-6}M Faslodex)

7.5 Cell proliferation of MCF7 and T47D cell lines after 6 day incubation with physiological levels of 5-androstene-3β,17β-diol and 17β-oestradiol

MCF7 and T47D cell lines were incubated for 8 days with physiological levels of oestradiol and 5-androstene-3β,17β-diol. Cell numbers were determined after 6 and 8 days by the methods outlined above. As illustrated in the following figure, physiological levels of 5-androstene-3β,17β-diol stimulated baseline and inhibited oestrogen-induced proliferation of MCF7 and T47D cells.

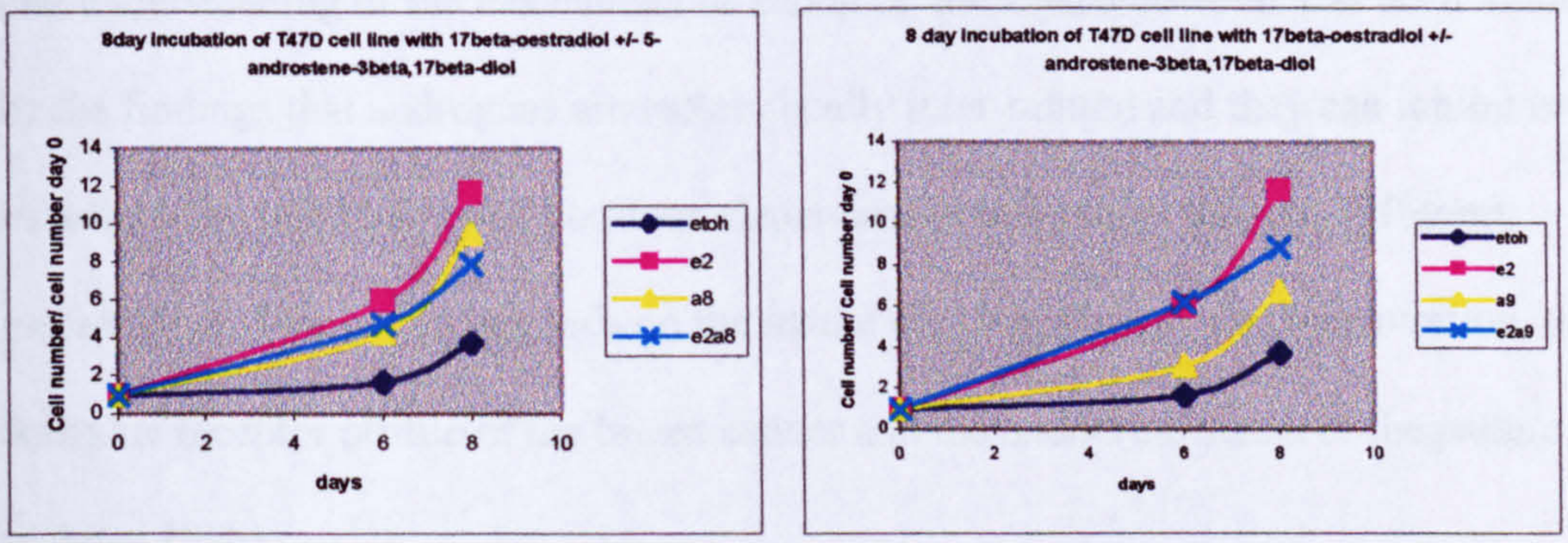


Figure 7.12: Proliferation of MCF7 and T47D cell lines after 8 days and 6 days incubation with physiological levels of 17β-oestradiol and 5-androstene-3β,17β-diol (abbreviations: ADIOLn = 10^{-n}M 5-androstene-3β,17β-diol, ETOH = 0.01% ethanol, e2 = 17β-oestradiol)

Discussion

As outlined in the introduction several studies have published results, which implicate androgens in the aetiology of breast cancer. Epidemiological studies have established that serum androgens are elevated in postmenopausal women who develop breast cancer (Cauley 1999) (Dorgan 1997) (Gordan 1990) (Zeleniuch-Jacqotte 1997) and subnormal in premenopausal women who develop breast cancer (Bulbrook 1986) (Helzlsouer 1992).

Our understanding of the mechanism of action of androgens however has been hindered by the findings that androgens are metabolically inter-related and they can inhibit or stimulate the proliferation of hormone-dependent breast cancer through different mechanisms. This action depends on the nature of the androgen, its concentration, the hormone receptor profile of the breast cancer and the endocrine status of the patient (Adams 1998).

The metabolism of androgens is illustrated below. Androgens are metabolised:

(a) By the sulfatase enzymes to 5-androstene-3β,17β-diol a metabolite with high affinity for the oestrogen receptor

(b) Via androstenedione and testosterone to 5α-dihydrotestosterone which has high affinity for the androgen receptor

(c) By the aromatase enzymes to oestrogens.

The ovary and the adrenal cortex both contribute to circulating levels of androgens. However neither produce significant quantities of either 5α-dihydrotestosterone or 5-androstene-3β,17β-diol, which may therefore be considered true metabolites, which act at tissue level (Longcope 1986).

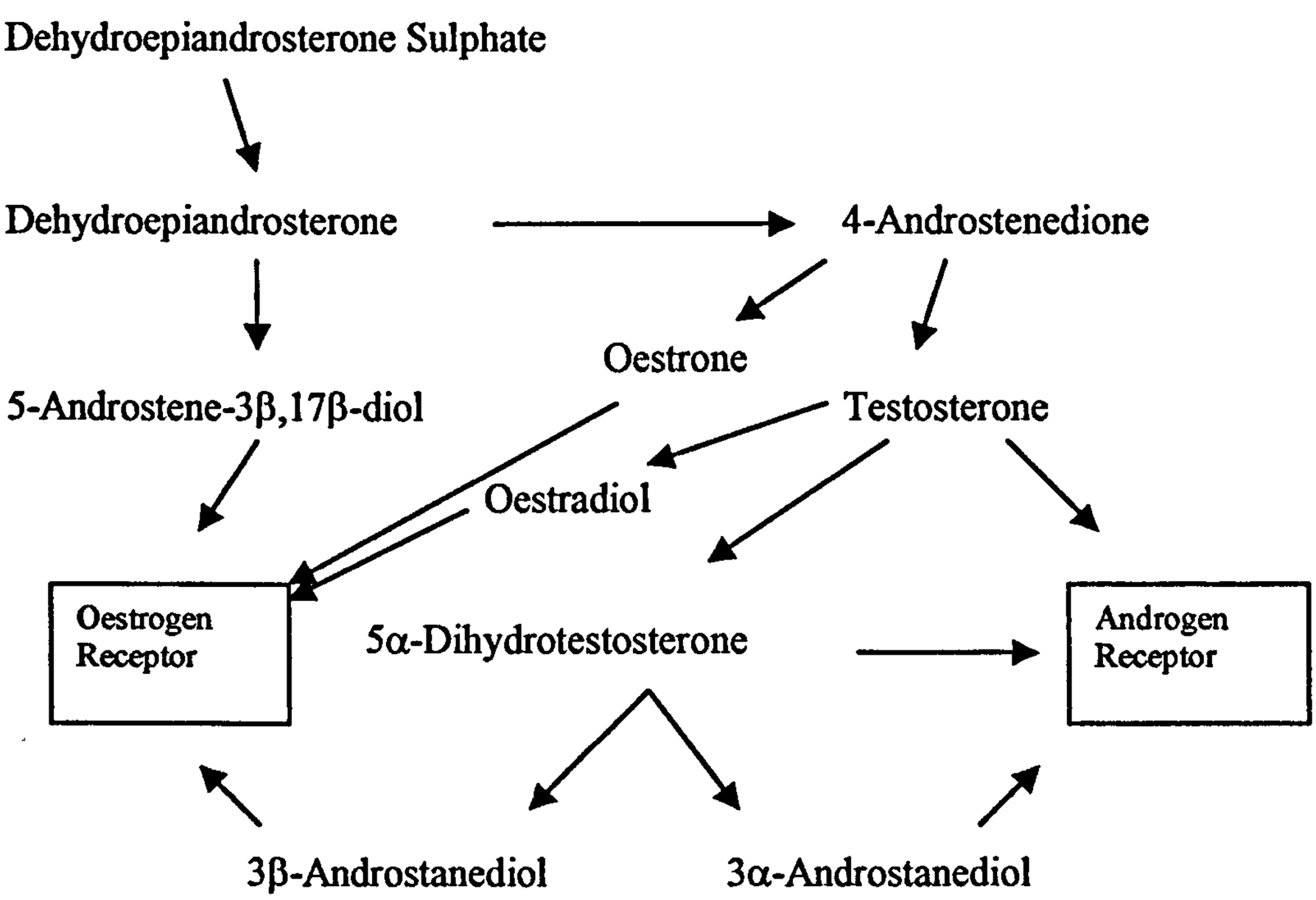


Figure 7.13: The Metabolism of Androgens (Rochefort *et al.* 1984)

In the present study we have therefore examined the actions of 5α- dihydrotestosterone and 5-androstene-3β,17β-diol on breast cancer cell lines. In general it appears that androgen receptor mediated mechanisms in breast cancer inhibit growth. The mechanism of this action is unknown. However, recently it has been suggested that this affect may be

mediated by the production of one or more proteins, which prevent entry of the target cell into the cell cycle. These proteins have been termed androcyclone II (Szelei *et al.* 1997). The aim of the present study was therefore to examine the affects of 5-androstene-3β,17β-diol and 5α-dihydrotestosterone on cell cycle and proliferation using a flow cytometric technique. The hormone-dependent cell lines MCF7 and T47D have been examined as well as the hormone-independent cell line MDA231.

7.6 Actions of 5α-dihydrotestosterone on proliferation of breast cancer cell lines

5α-Dihydrotestosterone, an androgen with high affinity for the androgen receptor and low affinity for the oestrogen receptor (Rochefort *et al.* 1984) has also been shown to have anti-oestrogenic activity at physiological levels in the MCF7 cell line (MacIndoe 1981). As outlined in the introduction, physiological levels of 5α-dihydrotestosterone inhibit the proliferation of T47D, ZR751 and MFM223 breast cancer cell lines via an interaction with the androgen receptor (Poulin *et al.* 1986) (Birrell 1995) (Hackenberg *et al.* 1991). Although 5α-dihydrotestosterone stimulates the proliferation of MCF7 and MDAMB453 cell lines at physiological levels this may be due to the activation of androgen receptor-independent pathways by active metabolites with oestrogenic activity (Hall *et al.* 1994) (Birrell 1995). At pharmacological levels, 5α-dihydrotestosterone has been shown to stimulate the proliferation of MCF7 cells via the oestrogen receptor (Zava 1978) (Lippman 1976).

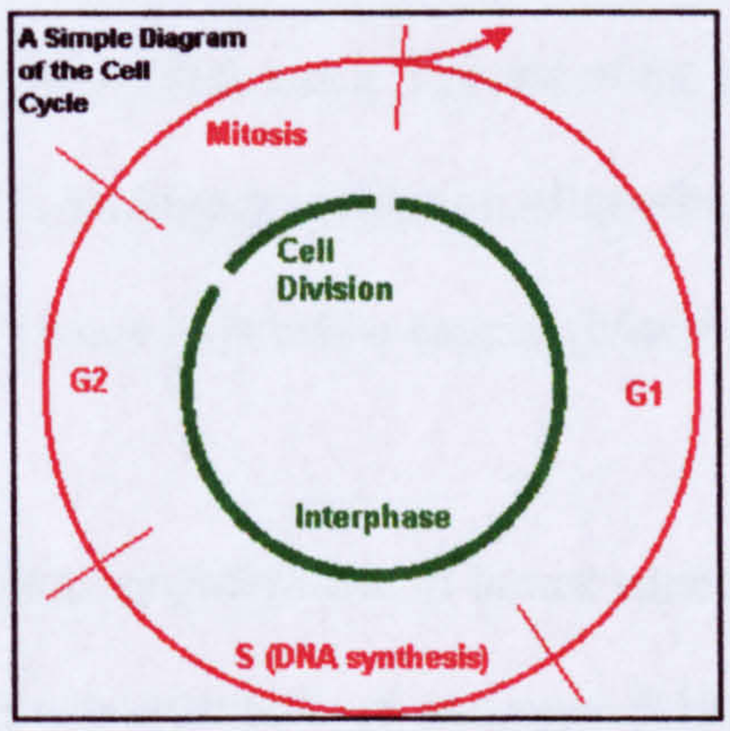
This study has observed that over 8-9 day incubations physiological concentrations of 5α-dihydrotestosterone inhibit the proliferation of MCF7 and T47D cell lines. Physiological levels of 5α-dihydrotestosterone have previously been reported to either stimulate (Birrell 1995) (Hackenberg *et al.* 1988) or not affect (Zava 1978) the proliferation of MCF7 cells and inhibit the proliferation of T47D breast cancer cells via an androgen receptor mediated mechanism (Birrell 1995).

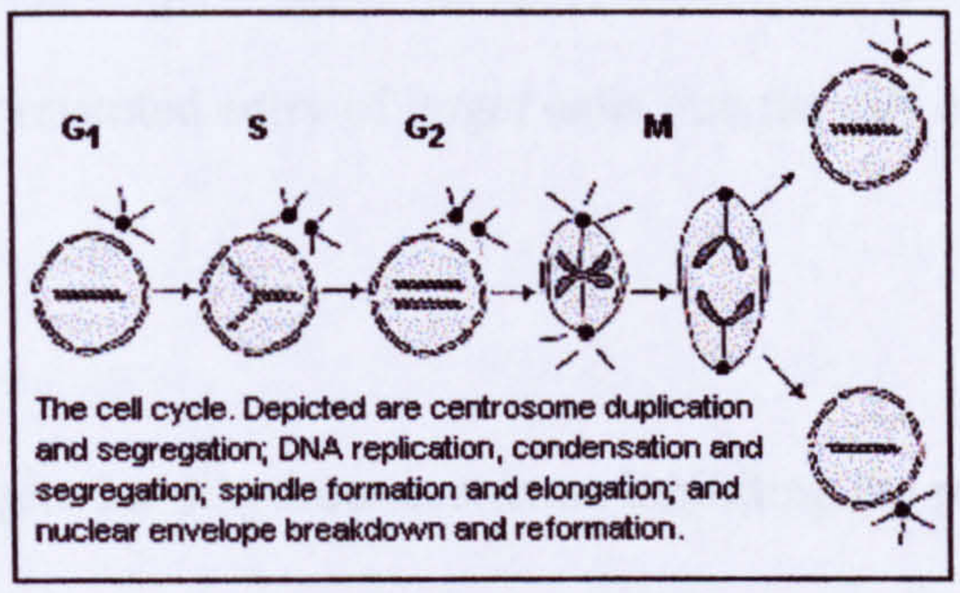
MCF7 cells have been shown metabolise 5α-dihydrotestosterone to androstane-3α,17β-diol, an androgen with oestrogenic properties (Roy 1992). This may account for the stimulatory affect of 5α-dihydrotestosterone observed in MCF7 cells, in contrast to the inhibitory action of 5α-dihydrotestosterone observed in other breast cancer cell lines (Roy 1992) (Birrell 1995). The MCF7 cell line used in this study has been passaged several hundred times. It is not known how this affects glucuronyltransferase activity. It may be that repeated passages result in downregulation or loss of glucuronyltransferase expression in MCF7 cells, which may account for the inhibitory affect of physiological concentrations of 5α-dihydrotestosterone observed in this study.

In addition to the inhibition of MCF7 and T47D cell lines demonstrated in this study, 5α-dihydrotestosterone has been observed to inhibit the oestrogen-induced proliferation of MCF7 and T47D cell lines. These findings are consistent with previous studies on MCF7 (MacIndoe 1981) and ZR751 cell lines (Poulin 1988) by a mechanism, which is mediated via the androgen receptor.

Over 48 hours 10^{-9}M 5α-dihydrotestosterone does not affect the progression of MCF7 or T47D cells through the cell cycle. Likewise 10^{-8}M 5α-dihydrotestosterone does not affect the proliferation of T47D cells during over 48 hour incubation, however, 10^{-8}M 5α-dihydrotestosterone stimulates the proliferation of MCF7 cells via an oestrogen receptor mediated mechanism.

The phases of the cell cycle are illustrated below. The quiescent cell is in the G0 phase of the cell cycle. On the initiation of division the cell moves into G1 phase and produces proteins required for DNA replication. Replication of DNA takes place in the S phase, by the end of which the DNA content of the cell has doubled. At this point the cell enters G2 phase followed by mitosis (M phase) during which the cell divides. Following cell division the cell either returns to the quiescsent state (G0 phase) or if cell division is to be sustained to G1.





The cell cycle. Depicted are centrosome duplication and segregation; DNA replication, condensation and segregation; spindle formation and elongation; and nuclear envelope breakdown and reformation.

Figure 7.14: Phases of the cell cycle

Cell cycle progression in breast cancer is regulated by a series of cyclin-dependent kinases and cyclin-dependent kinase inhibitors (Grana *et al.* 1995) (Hunter *et al.* 1994). These proteins control the transition through checkpoints between different cell cycle states occurring, for example, at the initiation of DNA replication (S phase) and cell division (mitosis). Androgens have been shown to upregulate expression of the cyclin-dependent kinases CDK2 and CDK4 and downregulate expression of the cyclin-dependent kinase inhibitor CKIp16 in the androgen-dependent prostate cancer cell line LNCaP. Thereby stimulating the cell to enter S phase of the cell cycle and enhancing proliferation (Lu *et al.* 1997). Androgen regulation of another cyclin-dependent kinase inhibitor p27 is also of importance in prostate cancer (Macri *et al.* 1999).

How androgens regulate cellular proliferation in breast cancer remains poorly understood. Szelei *et al* using a synthetic non-metabolised androgen R1881 and MCF7 cells transfected with androgen receptor observed these MCF7-AR1 cells were arrested in G0/G1 phase of the cell cycle after 24hours incubation with androgen. It was postulated

that this affect was due to androgens inducing the synthesis of a gene product, termed androcyclone-II, which prevented entry of target cells into the cell cycle (Szelei *et al.* 1997).

In the present study, despite 5α-dihydrotestosterone inhibiting the proliferation of MCF7 and T47D breast cancer cell lines over longterm incubations no affect on progression of cells through the cell cycle was observed over 48 hours. These findings are consistent with those of Poulin *et al* and Birrell *et al* who observed that the inhibitory actions of 5α-dihydrotestosterone on breast cancer cell lines were not observed until 6 days incubation with androgen (Birrell 1995) (Poulin 1988). Further studies are necessary to determine how androgens affect the growth of breast cancer via the androgen receptor, and in particular whether they affect the expression of cyclin-dependent kinases and cyclin-dependent kinase inhibitors in breast cancer cell lines.

The present study demonstrates that pharmacological levels of 5α-dihydrotestosterone stimulate MCF7 and T47D cells to enter the S phase and enhance proliferation. In MCF7 cells this action is blocked by co-incubation with faslodex implying an oestrogen receptor mediated mechanism, consistent with previous observations (Zava 1978) (Lippman 1976). Faslodex does not block this action in T47D cells, suggesting an alternative pathway is involved in mediating the proliferation of T47D cells by pharmacological levels of 5α-dihydrotestosterone.

7.7 The actions of 5-androstene-3β,17β-diol on the proliferation of breast cancer cell lines

The oestrogenic actions of androgens are established and are the subject of a review by Rochefort and Garcia (Rochefort *et al.* 1984). Much attention has centred on 5-androstene-3β,17β-diol, an androgen termed "hermaphrodiol" due to its relatively high affinity for the oestrogen and androgen receptor (Poortman *et al.* 1975) (Adams 1998). 5-Androstene-3β,17β-diol induces the secretion of a 46K oestrogen-dependent protein and stimulates the proliferation of MCF7 (Adams *et al.* 1981) (Hackenberg 1993) (Boccuzzi *et al.* 1992) and ZR751 (Poulin *et al.* 1986) breast cancer cell lines at physiological concentrations. This action has subsequently been demonstrated to occur via a direct interaction with the oestrogen receptor (Maggiolini *et al.* 1999).

In addition to its oestrogenic actions, at physiological levels 5-androstene-3β,17β-diol antagonises the oestrogen-induced proliferation of MCF7 cells by an androgen receptor mediated mechanism (Boccuzzi *et al.* 1992) (Hackenberg 1993).

5-Androstene-3β,17β-diol is formed by peripheral conversion of DHEA, which is in equilibrium with DHEAS thus providing a buffer to maintain levels of its metabolites (Longcope 1986). In contrast to 5-androstene-3β,17β-diol, DHEA has a very low and DHEAS a negligible affinity for the oestrogen receptor (Rochefort *et al.* 1984). However, DHEAS and DHEA have both been shown to stimulate the proliferation of hormone-dependent breast cancer cell lines, the former at physiological levels (Adams *et al.* 1981)

(LeBail *et al.* 1998) and to be elevated in the serum of postmenopausal women who develop breast cancer (Cauley 1999) (Dorgan 1997) (Gordan 1990).

The mechanism of action of DHEAS was unknown. However recently, it has been shown that sulfatase inhibitors block the DHEAS-stimulated growth of MCF7 cells (Billich *et al.* 2000). This suggests that the mitogenic affect of DHEAS on breast cancer cell lines is mediated via its metabolism to 5-androstene-3β,17β-diol, rather than aromatisation of its metabolites androstenedione and testosterone to oestrogens. This pathway is of clinical significance as it enables the ongoing stimulation of hormone-dependent breast cancers by adrenal androgens in patients on aromatase inhibitors.

The interim findings of the Arimidex, Tamoxifen, alone or in Combination (ATAC) trial (Baum 2001) demonstrate that aromatase inhibitors are superior to tamoxifen as the adjuvant therapy for postmenopausal women with breast cancer. In light of these findings the evidence-base for the routine use of aromatase inhibitors in clinical practice is currently being established (Winer *et al.* 2002). The properties of 5-androstene-3β,17β-diol are therefore of particular significance and it is timely therefore to examine the effects 5-androstene-3β,17β-diol on breast cancer growth.

Several previous studies have reported that 5-androstene-3β,17β-diol stimulates the proliferation of hormone-responsive breast cancer cell lines via interaction with the oestrogen receptor at physiological levels (Adams *et al.* 1981) (Poulin *et al.* 1986) (LeBail *et al.* 1998) (Boccuzzi *et al.* 1992) (Hackenberg 1993) (Maggiolini *et al.* 1999).

The present study demonstrates that within 24 hours 5-androstene-3β,17β-diol stimulates MCF7 cells to enter S phase of the cell cycle thereby enhancing cell proliferation. Furthermore, this affect was blocked by co-incubation with faslodex, confirming that this action is mediated via the oestrogen receptor.

5-Androstene-3β,17β-diol is a steroid, which has affinity for both the oestrogen and androgen receptor and has therefore been termed "hermaphradiol" (Poortman *et al.* 1975) (Adams 1998). Consistent with its affinity for the androgen receptor, the present study confirms previous findings demonstrating that 5-androstene-3β,17β-diol like 5α-dihydrotestosterone inhibits the oestrogen-induced proliferation of hormone-dependent breast cancer cell lines (Boccuzzi *et al.* 1992) (Boccuzzi *et al.* 1994) (Hackenberg 1993).

7.8 Implications for the role of androgens in breast cancer

Androgens are metabolised to substrates, which inhibit (5α-dihydrotestosterone) or stimulate (5-androstene-3β,17β-diol, oestrogens) the proliferation of hormone-dependent breast cancer cells at physiological levels. The net affect of androgens *in vivo* seems likely to be a balance between the stimulatory and inhibitory affects of their metabolites. The findings by several groups that serum androgens are elevated in postmenopausal women who develop breast cancer suggest that the net activity *in vivo* is stimulatory. In premenopausal women, the hormonal milieu is rich in ovarian oestrogens. In the present study both 5α- dihydrotestosterone and 5-androstene-3β,17β-diol antagonise the

oestrogen-induced proliferation of breast cancer cells. Elevated levels of androgens in premenopausal women by antagonising the oestrogen-induced proliferation of hormone-dependent breast cancer may therefore protect against the development of breast cancer. This mechanism may underlie the observations of Bulbrook *et al* and others that premenopausal women who develop breast cancer have subnormal androgen levels (Bulbrook 1986).

The observation that 5-androstene-3β,17β-diol stimulates the proliferation of hormone-dependent breast cancer cell lines at physiological levels has implications for patients on aromatase inhibitors, as this pathway provides a mechanism for the ongoing stimulation of hormone-dependent breast cancer. Evidence that this pathway may have importance *in vivo* is suggested by a recent study. This found that elevated serum dehydroepiandrosterone sulphate levels predict breast cancer progression in postmenopausal women on aromatase inhibitors (Morris *et al.* 2001).

Results and Discussion

A lectin ELISA for the cerbB-2 oncoprotein

Results

Following the development of a lectin ELISA for the cerbB2 oncoprotein, which showed encouraging results in a pilot study as an assay for the diagnosis of breast cancer (Cook *et al.* 1999), serum was collected from women with breast cancer and controls, and steps were taken to re-establish the assay *in vitro*. It was the intention to apply the assay to test serum taken from women with primary operable breast cancer and controls, expanding the results of the pilot study.

In order to re-establish the assay *in vitro* it was necessary to demonstrate that each step of the assay was functioning satisfactorily *i.e.* cerbB2 antigen was captured by antibody (OM-11-954) and lectin (wheat germ agglutinin) bound carbohydrate. Having established that these steps were working a lysate was prepared from a breast cancer cell line SKBR3 known to express high levels of the cerbB2 protein (Leitzel *et al.* 1992). The cerbB2 lectin ELISA was performed on this lysate. Modifications were then carried out including the addition of human serum, to investigate different aspects of the assay.

8.1 Biotinylation of a synthetic p185 peptide (OP-11-3549)

A synthetic peptide OP-11-3549 (Cambridge Research Biochemicals Ltd, Cheshire, UK) consisting of an eighteen peptide sequence, which forms part of the extra-cellular domain of cerbB2 which was used as the antigen for producing the monoclonal antibody OM-11-954 (Genosys Biotechnolgies, Cambridge, UK) was biotinylated as outlined in materials and methods.

The following graph illustrates the optical density of 1ml fractions collected after passing a mixture of synthetic p185 peptide OP-11-3549 and EZ-link™ NHS-LC biotin through sephadex beads. Optical density was determined using a spectrophotometer and reflects the protein content of the fraction.

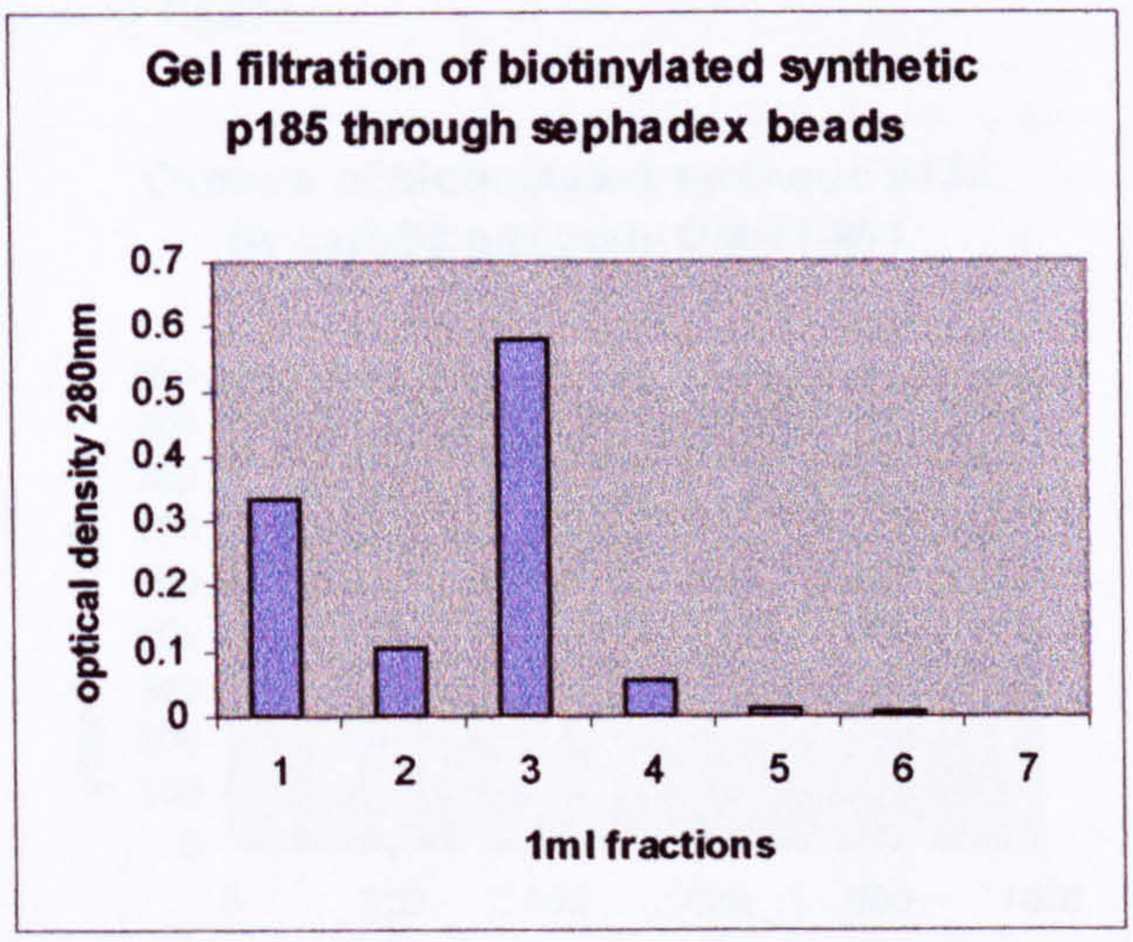


Figure 8.1: Gel filtration of synthetic p185 peptide (OP-11-3549) and NHS-LC biotin through sephadex beads

It was likely that the biotin-p185 was contained in the fractions with the highest optical density *i.e.* the first and third fractions. In the following experiment both fractions were therefore tested to investigate which contained the biotin-p185.

8.2 Capture of biotinylated synthetic p185 peptide OP-11-3549 by monoclonal antibody to cerbB2 (OM-11-954)

This experiment was performed to demonstrate that the monoclonal antibody used in the cerbB2 lectin ELISA, OM-11-954 bound cerbB2 protein and would therefore capture this antigen when applied to the serum samples. Following the steps outlined in the materials and methods section the first and third fractions obtained above by gel filtration were investigated. No response was obtained with the first fraction, but the third fraction showed an increase in absorbance with higher concentrations of biotin p185. This is illustrated in the following figure.

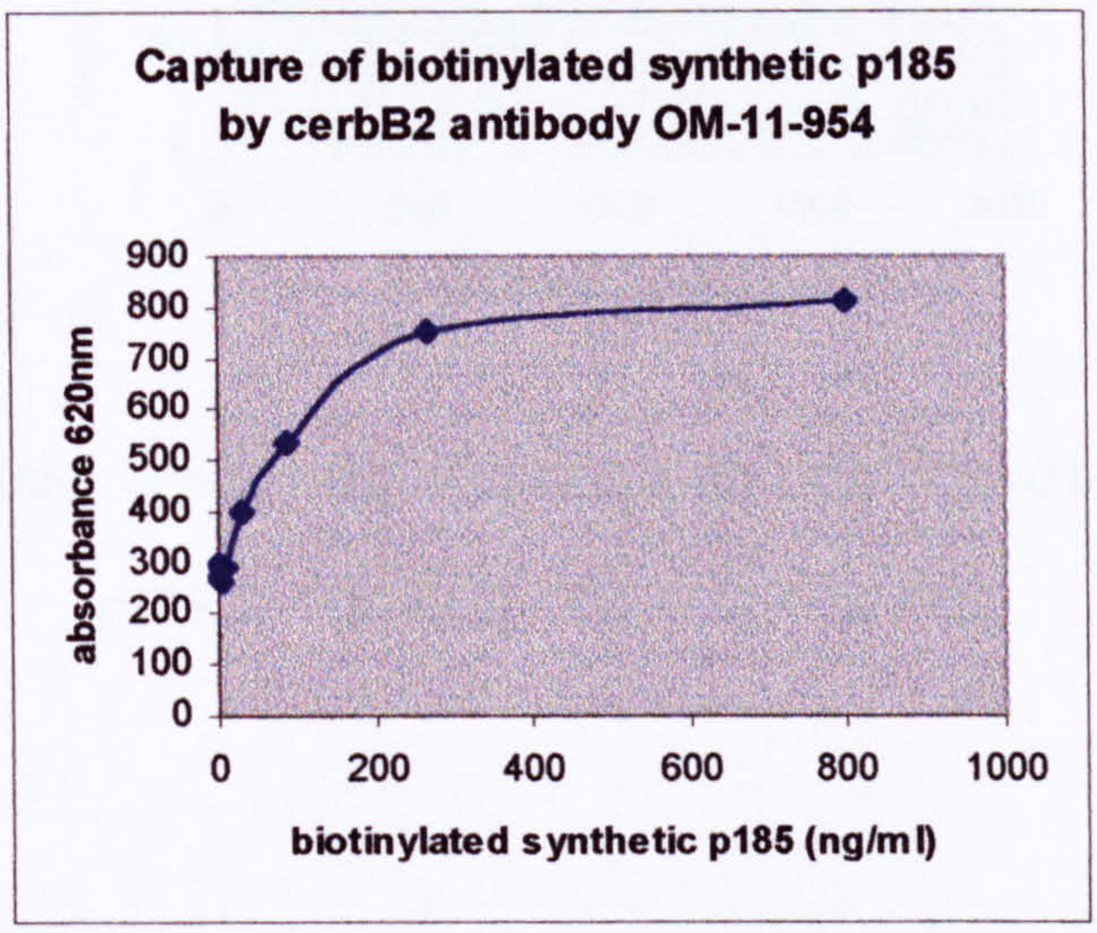


Figure 8.2: Capture of biotin synthetic p185 by cerbB2 monoclonal antibody OM-11-954

It was assumed that all the synthetic biotin p185 was contained in the third fraction, giving a final concentration of about 75µg/ml of synthetic p185.

Furthermore, a competition experiment was performed to confirm that unlabelled synthetic p185 competes with biotinylated synthetic p185 for antibody binding sites. Following the method outlined in the materials and methods section, the following results were obtained. These demonstrate that competition between unconjugated synthetic p185 and biotinylated synthetic p185 does occur.

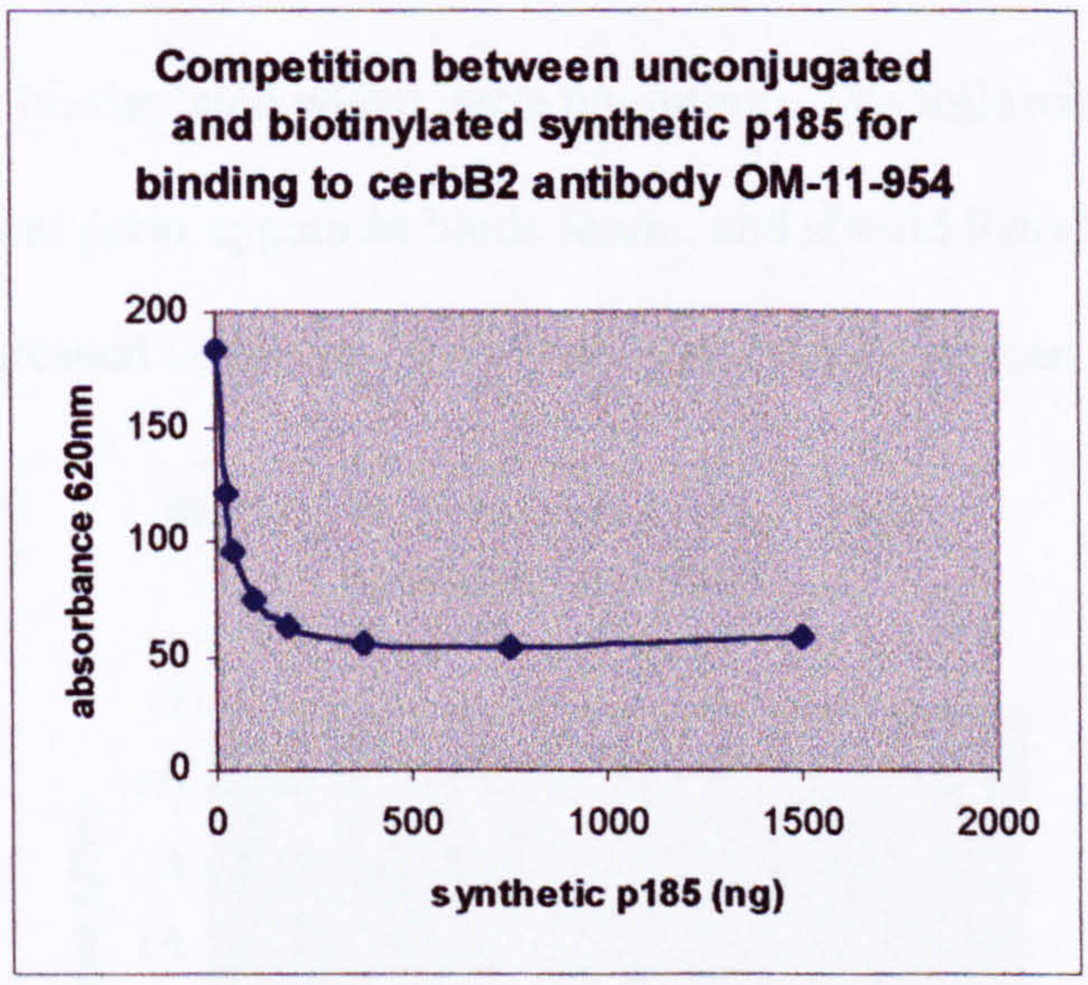


Figure 8.3: Competition between unconjugated synthetic p185 and biotinylated synthetic p185 for binding to OM-11-954 antibody

8.3 Binding of lectin (wheat germ agglutinin) to glycoprotein

In the first step of the lectin ELISA antigen is captured by the monoclonal antibody OM-11-954. In the second step lectin binds to carbohydrate expressed on the surface of the cerbB2 protein. In order to test whether the lectin used in this assay, wheat germ agglutinin, binds carbohydrate, fetuin a heavily glycosylated glycoprotein was used. Following the steps outlined in materials and methods increasing concentrations of fetuin were incubated with biotinylated wheat germ agglutinin. The following figure illustrates that biotinylated wheat germ aggutinin binds fetuin, and should therefore bind to glycoconjugates expressed on the surface of captured cerbB2 antigen.

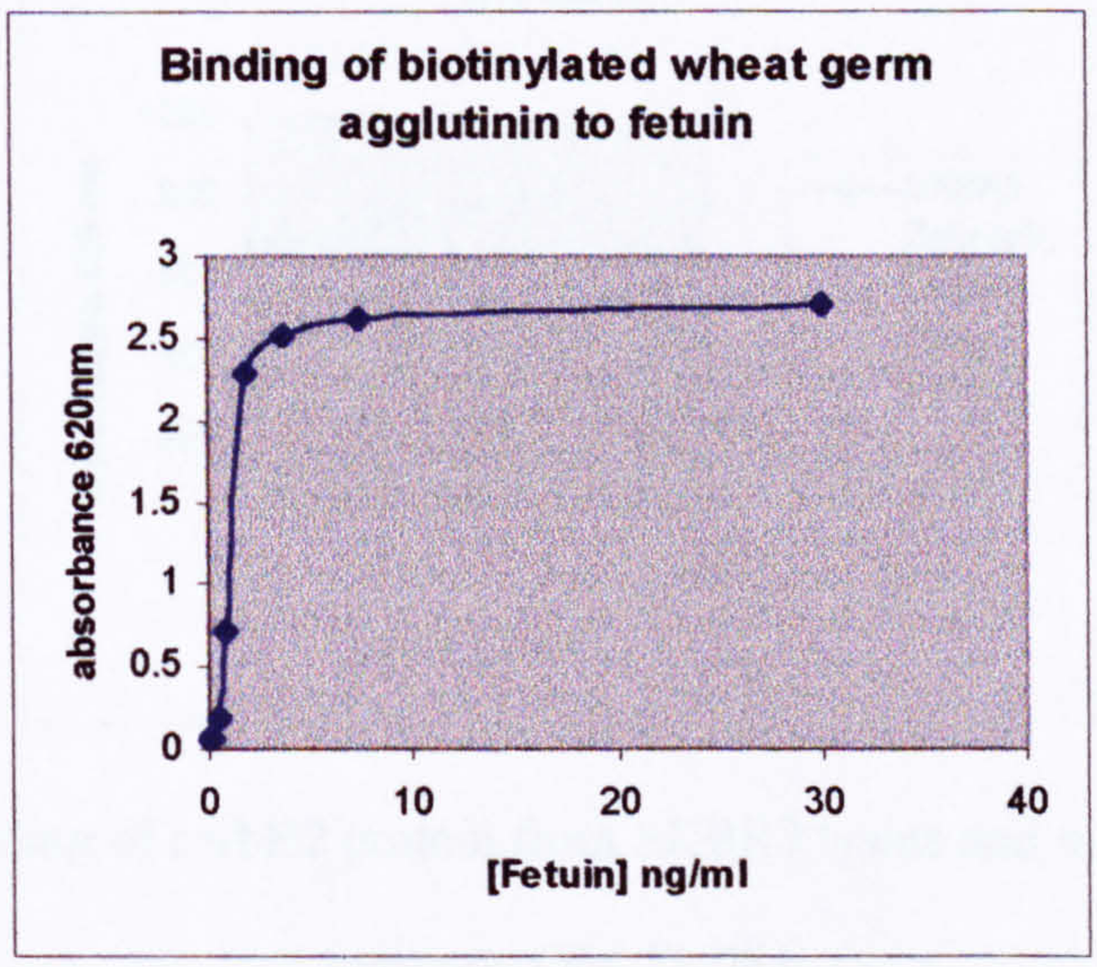


Figure 8.4: Binding of biotinylated wheat germ agglutinin to fetuin

8.4 Binding of monoclonal antibody OM-11-954 to extra-cellular domain of cerbB2 in a lysate of SKBR3 cells

A lysate to be used as a standard against which serum samples could be compared was prepared by a method outlined in materials and methods from a breast cancer cell line, SKBR3, known to express high levels of cerbB2 (Leitzel *et al.* 1992). In order to test that the antibody OM-11-954 bound to cerbB2 in SKBR3 lysate, an experiment was performed in which antibody was incubated with dilutions of SKBR3 lysate immobilised in the solid phase, see section 4.5 in materials and methods. The following figure illustrates that a substantial quantity of receptor was present in the lysate.

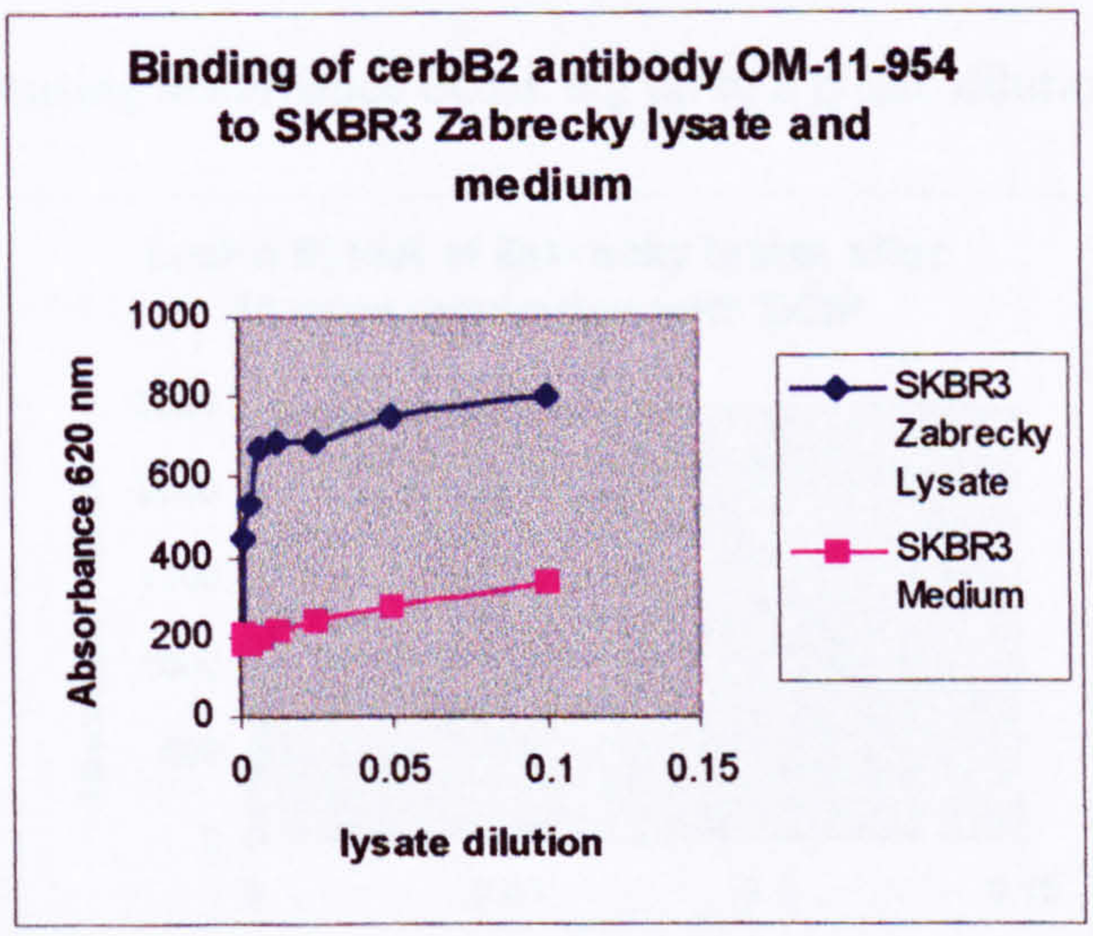


Figure 8.5: Binding of cerbB2 protein from SKBR3 lysate and medium by cerbB2 antibody OM-11-954

The response levels off at a SKBR3 lysate dilution of 1/50. Failure of the signal to increase at higher concentrations of lysate may be due to steric hindrance or saturation of

the plastic. It can also be seen that the medium in which the SKBR3 cells were cultured does not contain significant quantities of the cerbB2 antigen.

8.5 A lectin ELISA for cerbB2

Having demonstrated that the monoclonal antibody OM-11-954 binds to a synthetic cerbB2 protein and to a protein from a lysate of SKBR3 cells, and that biotinylated wheat germ agglutinin binds to carbohydrate groups on the heavily glycosylated glycoprotein fetuin. The different components of the lectin ELISA were put together as outlined in materials and methods. The following figure illustrates that a good response was achievable with increasing absorbance occurring up to a lysate dilution of 1/20.

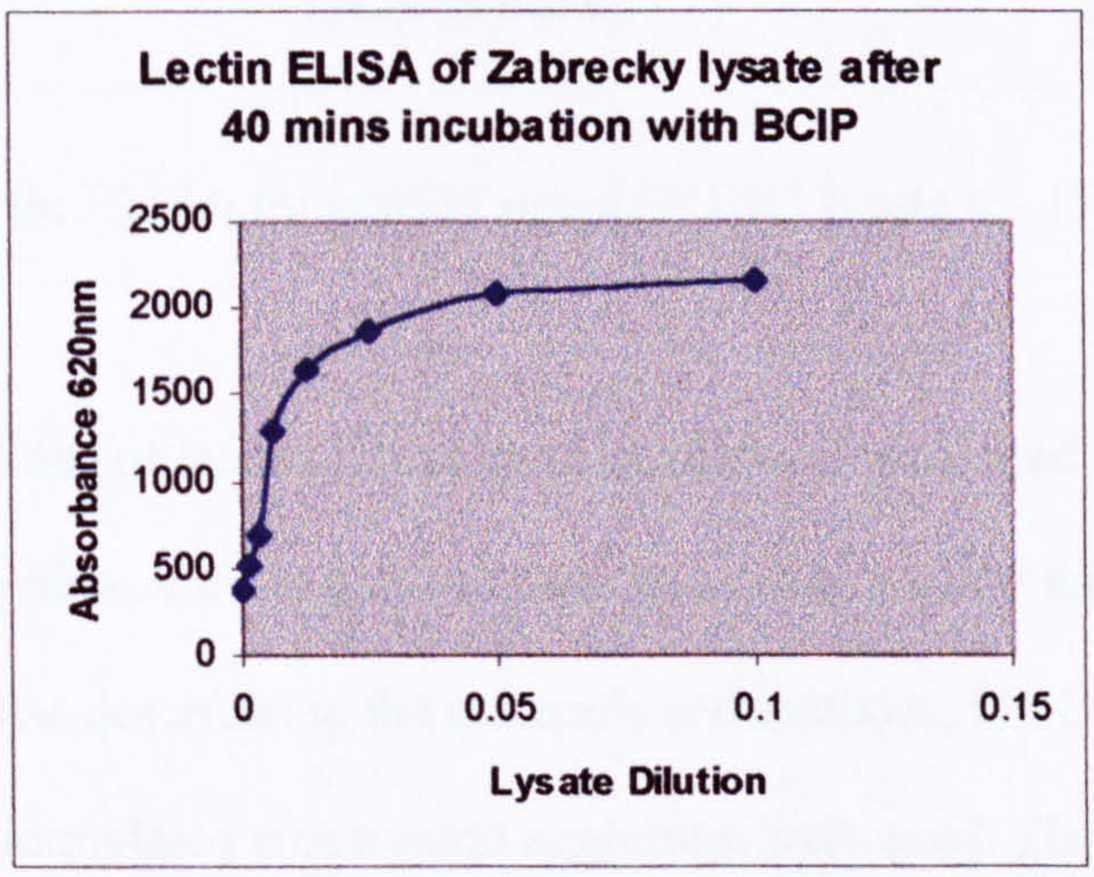


Figure 8.6: Lectin ELISA for cerbB2 using SKBR3 lysate

Various aspects of the assay were then determined. The effect to the response of the assay of adding human serum was investigated. This was done to simulate the serum samples,

in which non-specific proteins in the serum by competing for binding of cerbB2 antigen to antibody and lectin may interfere with steps in the assay. The lectin ELISA was performed as outlined above, but the lysate contained 15% human serum taken from one of the investigators. The following figure illustrates that serum does suppress the response of the assay.

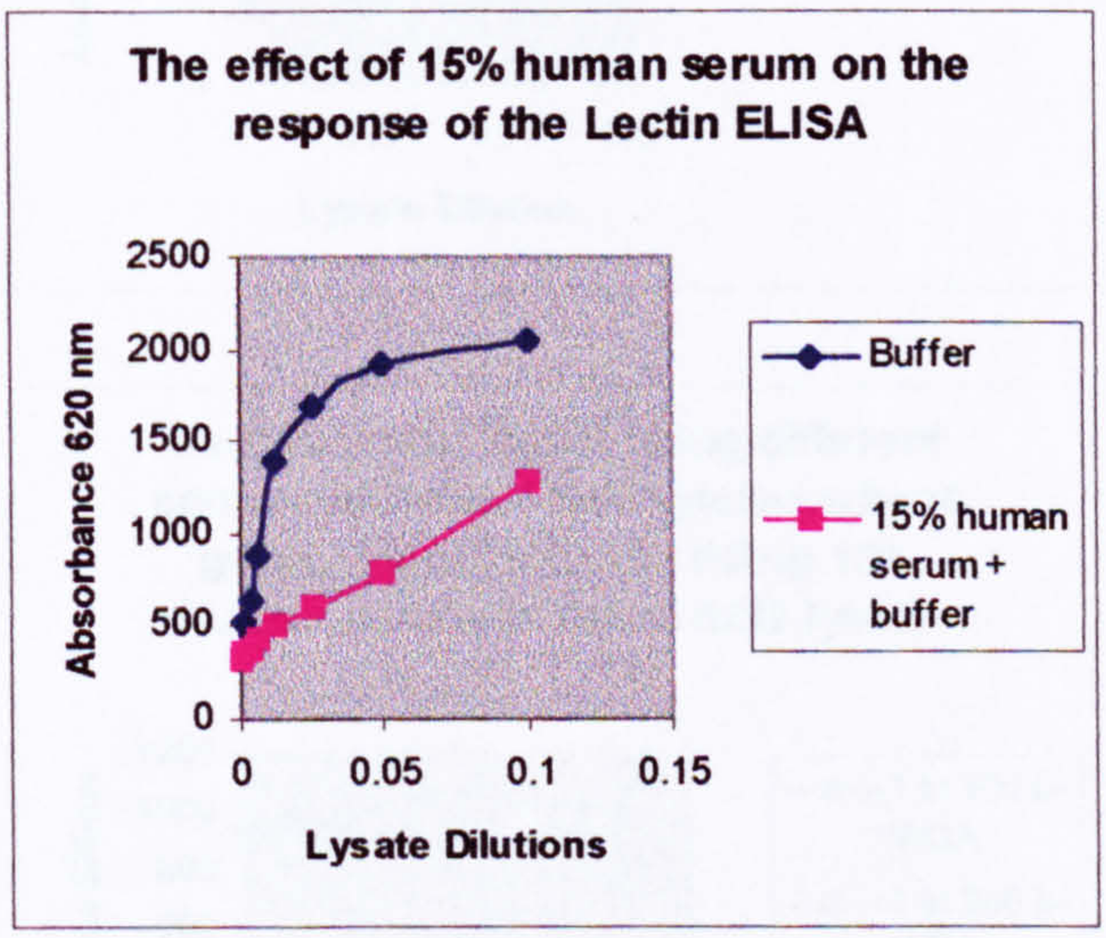
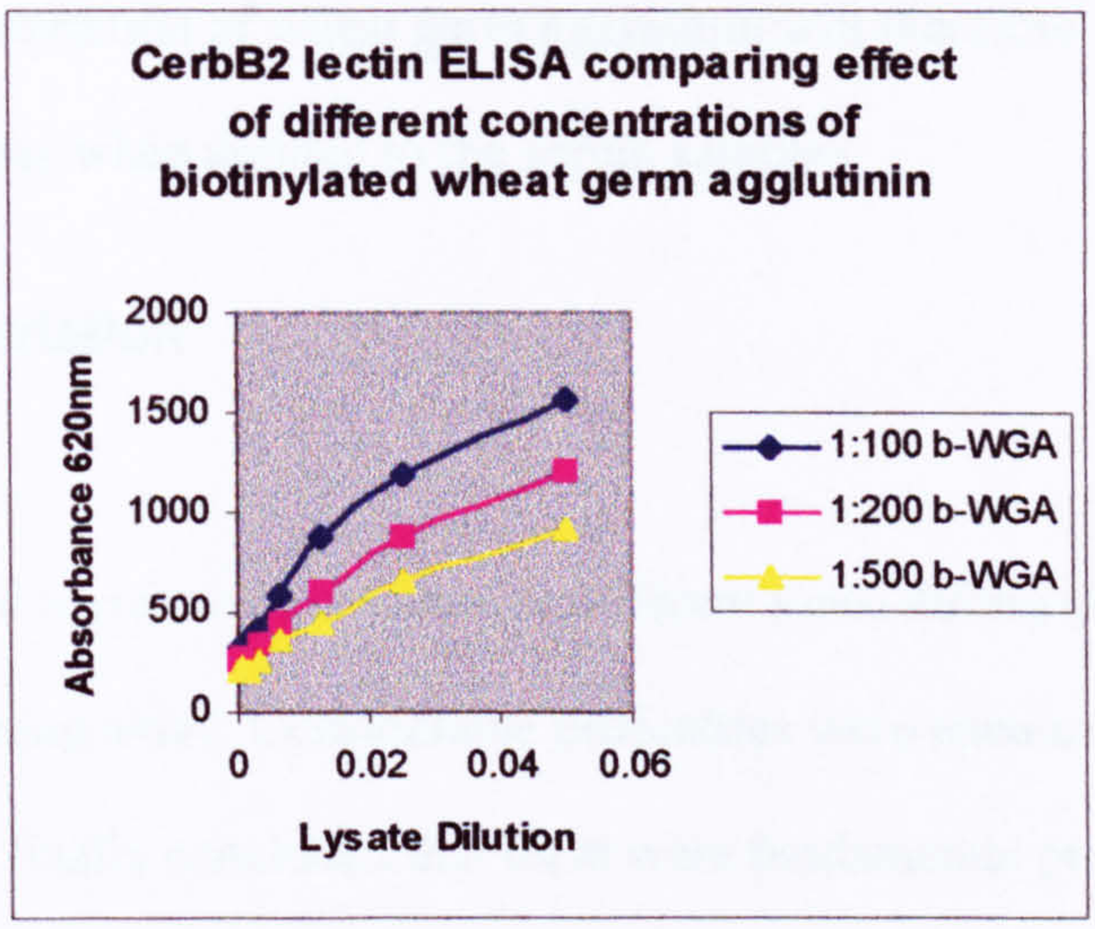


Figure 8.7: Lectin ELISA for cerbB2 using SKBR3 lysate +/- 15% human serum

Different concentrations of lectin (wheat germ agglutinin) were used to investigate whether this would enhance the response of the assay. The cerbB2 lectin ELISA was therefore performed as described in the materials and methods, but 1/100, 1/200 and 1/500 dilutions of biotinylated wheat germ agglutinin were used. The following figures show that higher concentrations of wheat germ agglutinin will enhance the response of the assay, however on addition of 15% human serum to the lysate a flat response curve was obtained.



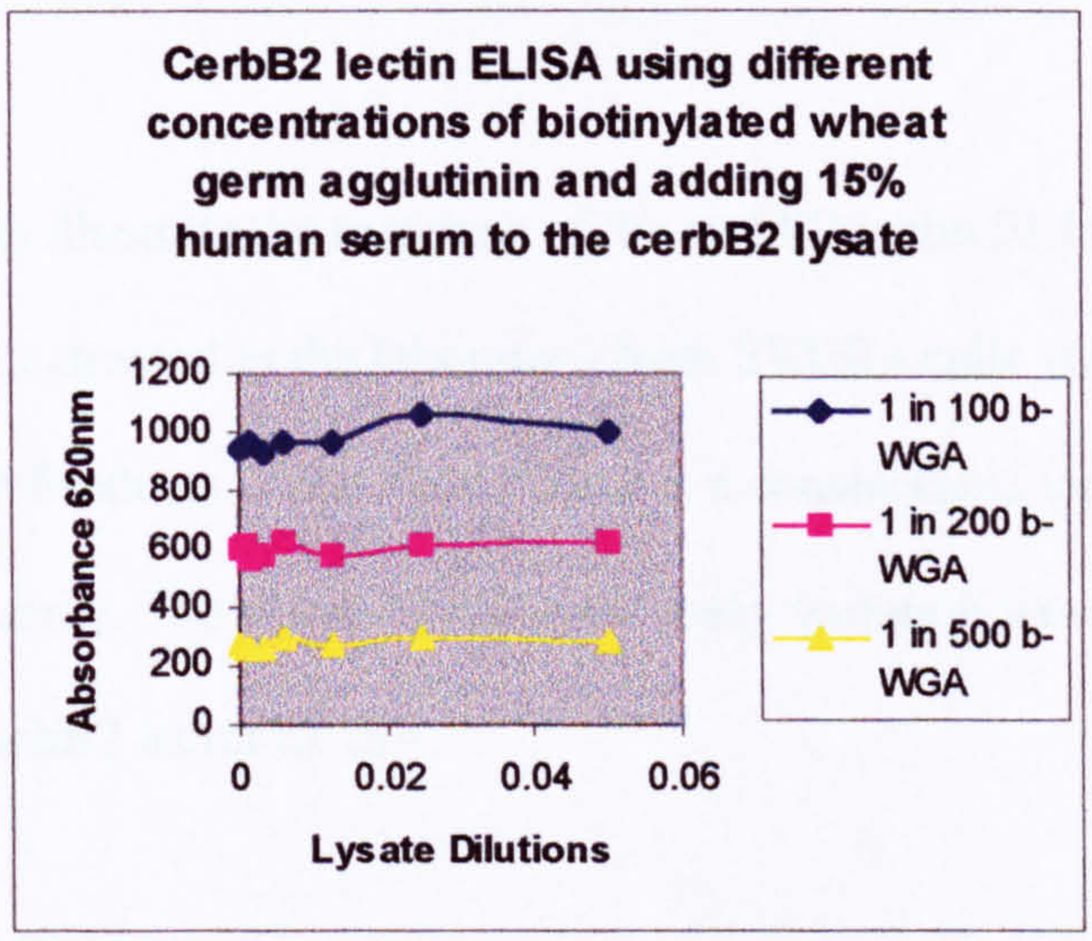


Figure 8.8: Lectin ELISA for cerbB2 using different concentrations of wheat germ agglutinin +/- 15% human serum

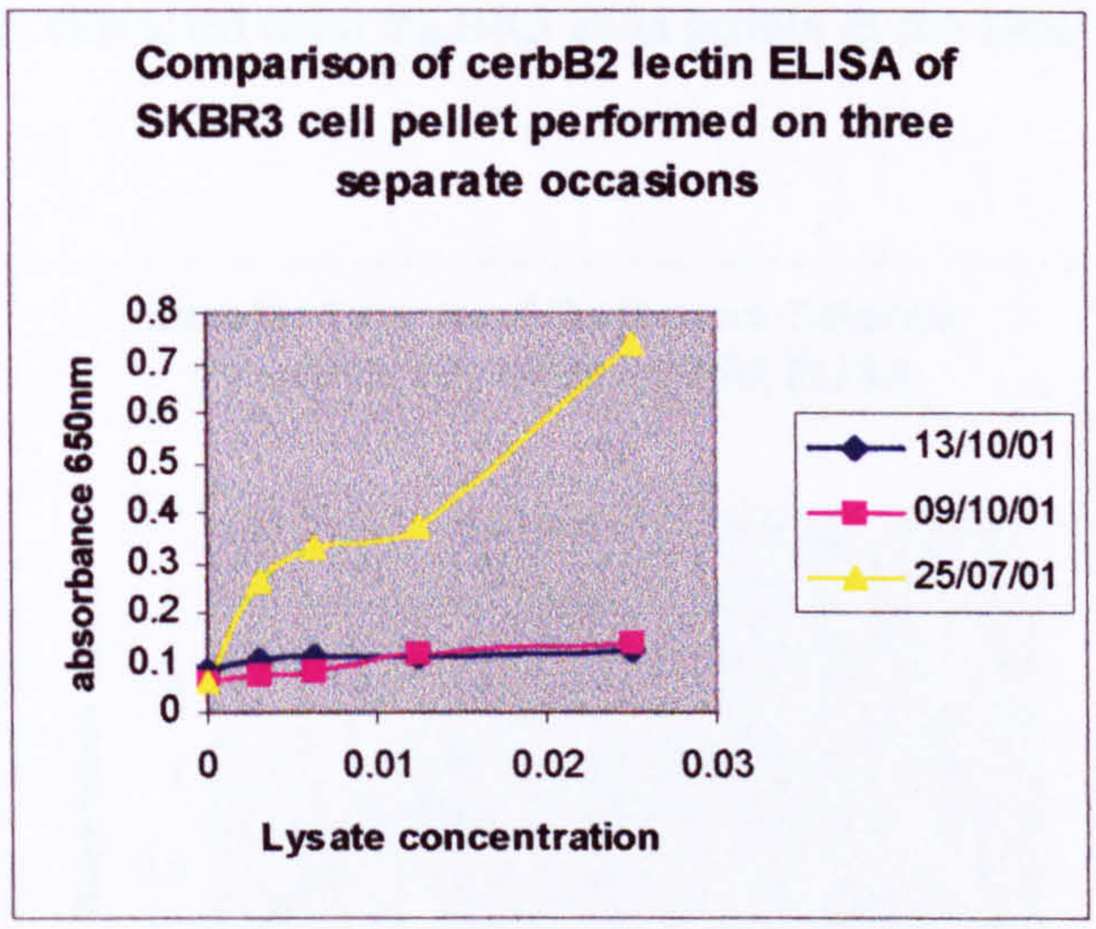
Lectins, though they have specificity for particular sugars, will bind to glycoconjugates expressed by non-specific proteins present in the serum. It is probable that the high response observed on increasing the concentration of lectin is due to wheat germ agglutinin binding to non-specific serum proteins, absorbed to the well, or antibody.

Using a higher concentration of wheat germ agglutinin will therefore decrease the specificity of the assay when applied to the serum samples.

8.6 Inter-assay variation

The results presented above were obtained at different times during the research project and imply a functioning assay. Considerable difficulties were encountered in reproducing these results. It was finally concluded that there were fundamental problems with the assay. The experiments upon which this conclusion was drawn are outlined below.

The following graphs illustrate the response of the cerbB2 lectin ELISA on three separate occasions on lysates extracted in the laboratory from SKBR3 cells and a SKBR3 pellet (Oncogene Research Products). This shows there is a considerable variation in the performance of the assay. The extent of this inter assay variation would preclude the clinical use of the cerbB2 lectin ELISA.



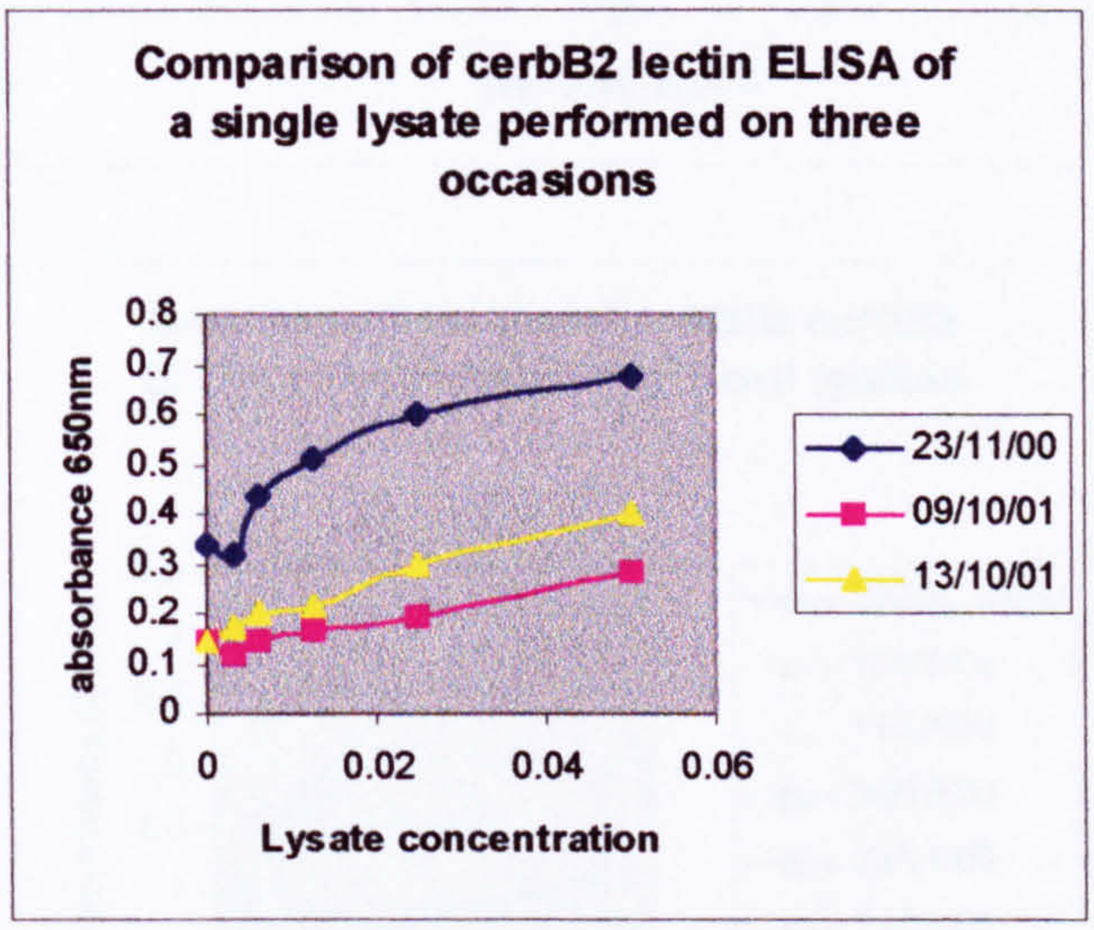


Figure 8.9: Comparison of cerbB2 lectin ELISA performed on three separate occasions on SKBR3 lysates

It was then decided to determine how much cerbB2 protein was present in the collected lysates that had been extracted from SKBR3 cells. For this purpose an Oncogene Science Research Products cerbB2 ELISA was used. This shows that the lysate extracted from the SKBR3 pellet contained a considerable quantity of cerbB2 antigen, however with one

exception, the lysates extracted from SKBR3 cells grown in the laboratory contained little cerbB2 protein.

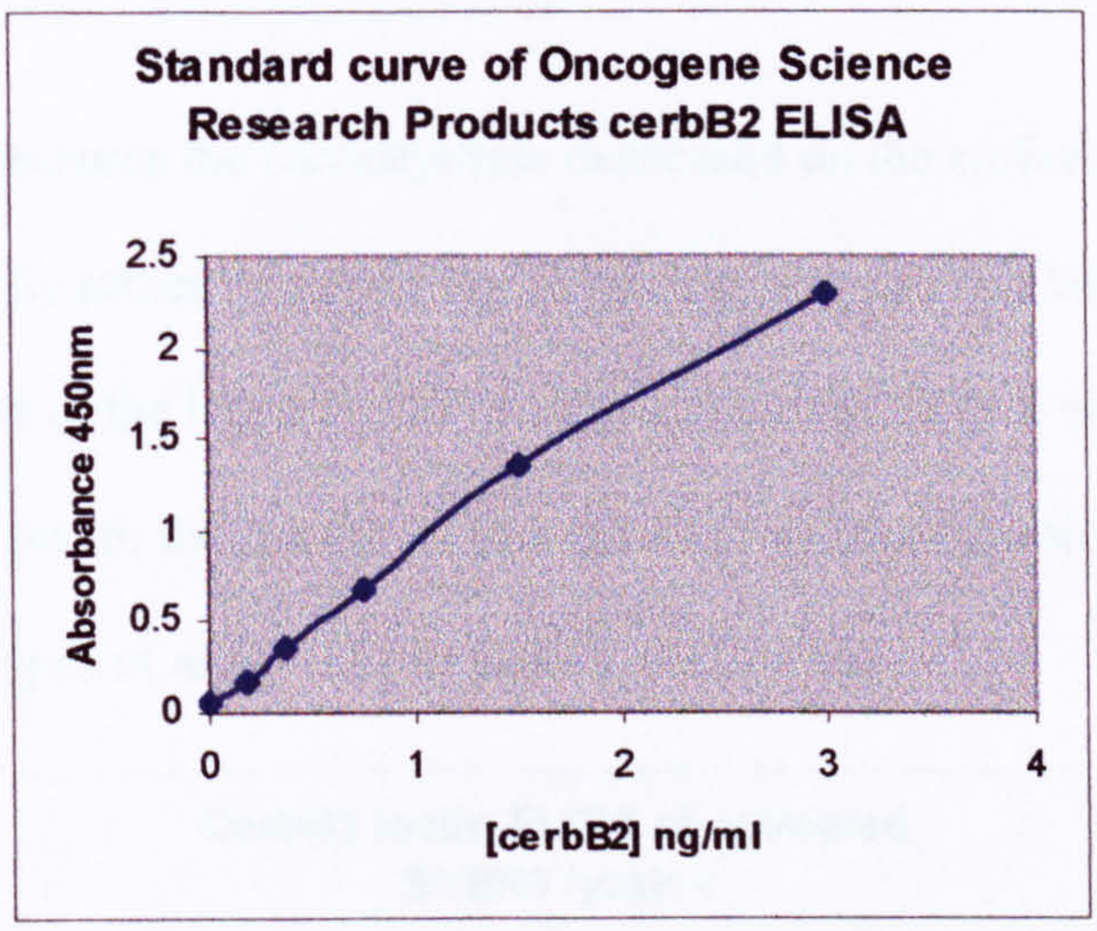


Figure 8.10: Oncogene Science Research Products cerbB2 ELISA of collected lysates

In view of these results a comparison of the cerbB2 lectin ELISA of the collected lysates was performed. This illustrates that there was no correlation between the response of the cerbB2 lectin ELISA and the quantity of cerbB2 protein present in the lysate. The lysate

prepared from a SKBR3 cell pellet contains significantly more cerbB2 protein than the other lysates yet doesn't give a higher response in the cerbB2 lectin ELISA.

The lectin ELISA measures the carbohydrate expressed on the surface of proteins bound to the capture antibody rather than quantity of protein present. The lack of correlation between the response of the lectin ELISA and conventional ELISA was therefore not an entirely unexpected result, though other studies have reported significant correlations between these two types of assay (Madiyalakan *et al.* 1996).

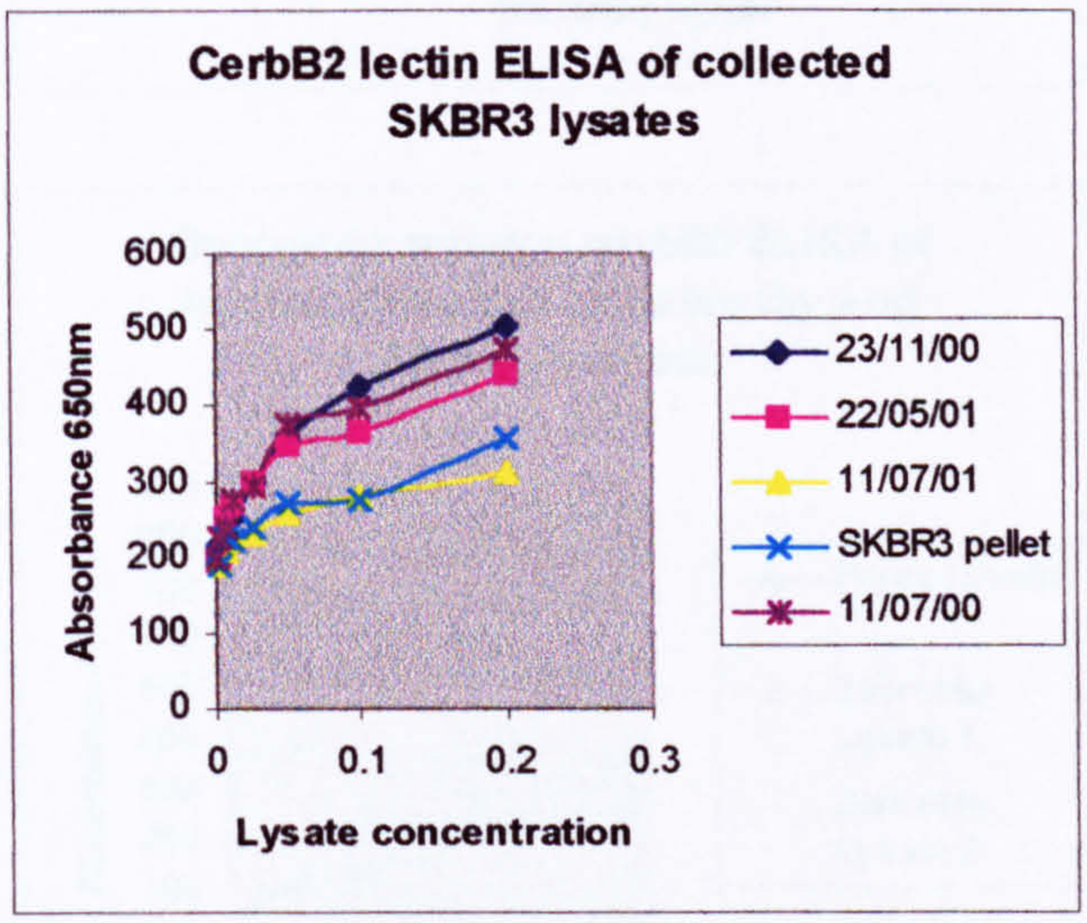
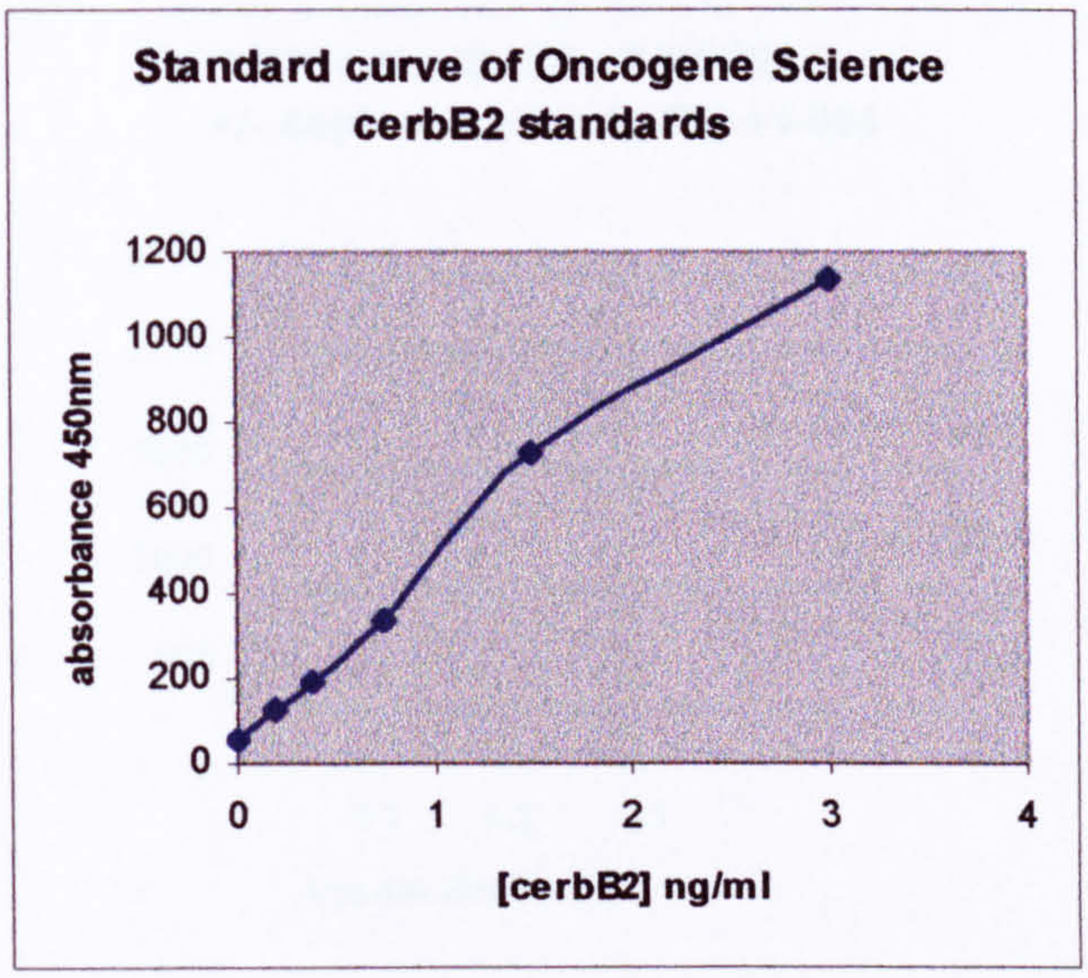


Figure 8.11: CerbB2 lectin ELISA of collected lysates

Lysates were then prepared from using 4-5 × number of cerbB2 cells in the hope that they contain more cerbB2 protein. Two different extraction methods were compared. These were the Zabrecky method described above and a commercial protein extraction kit (Pierce, USA). The following figures demonstrate that these lysates contained significant amounts of cerbB2 protein.



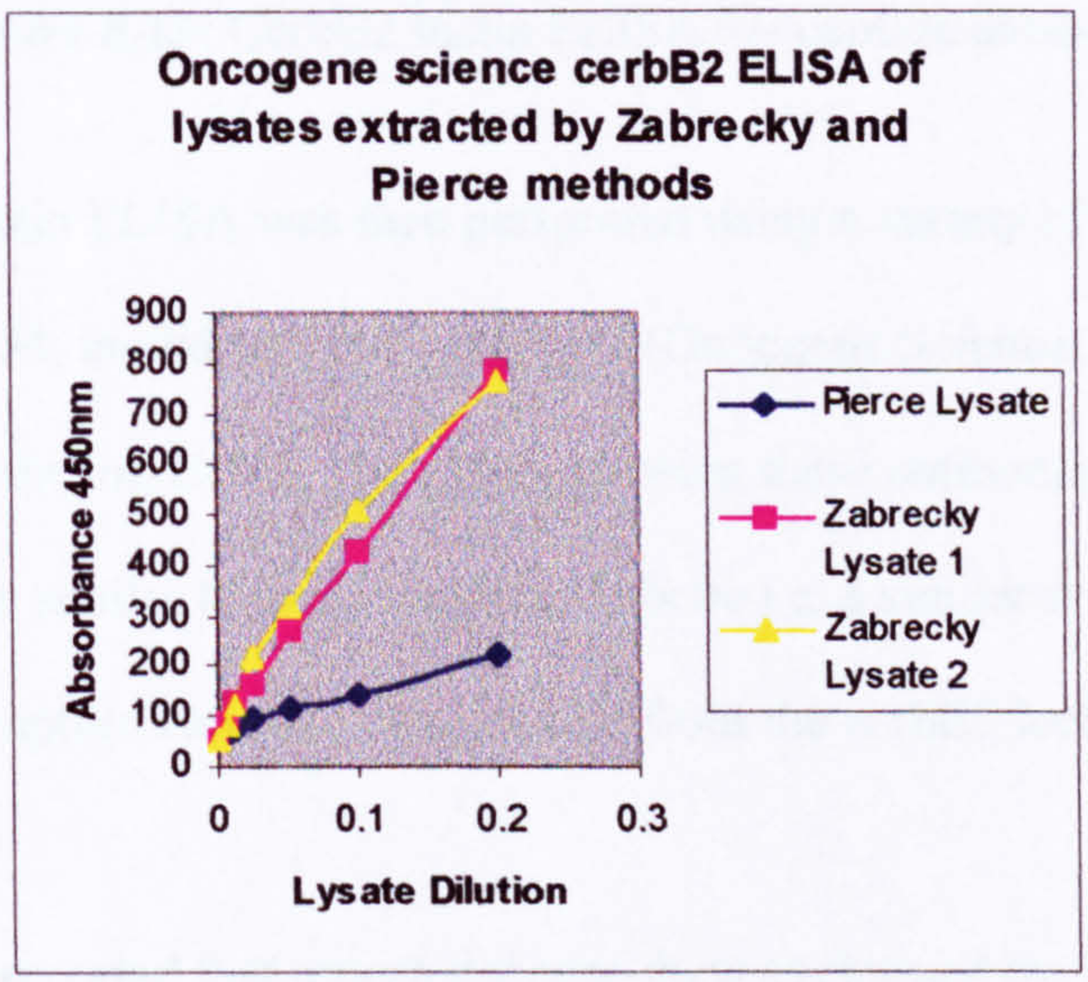


Figure 8.12: Oncogene Science cerbB2 ELISA of lysates extracted by Zabrecky and Pierce methods

The cerbB2 lectin ELISA was performed on these lysates as described in materials and methods, though for comparison capture antibody was omitted from one strip of wells. The following graph illustrates that this did not change the response observed.

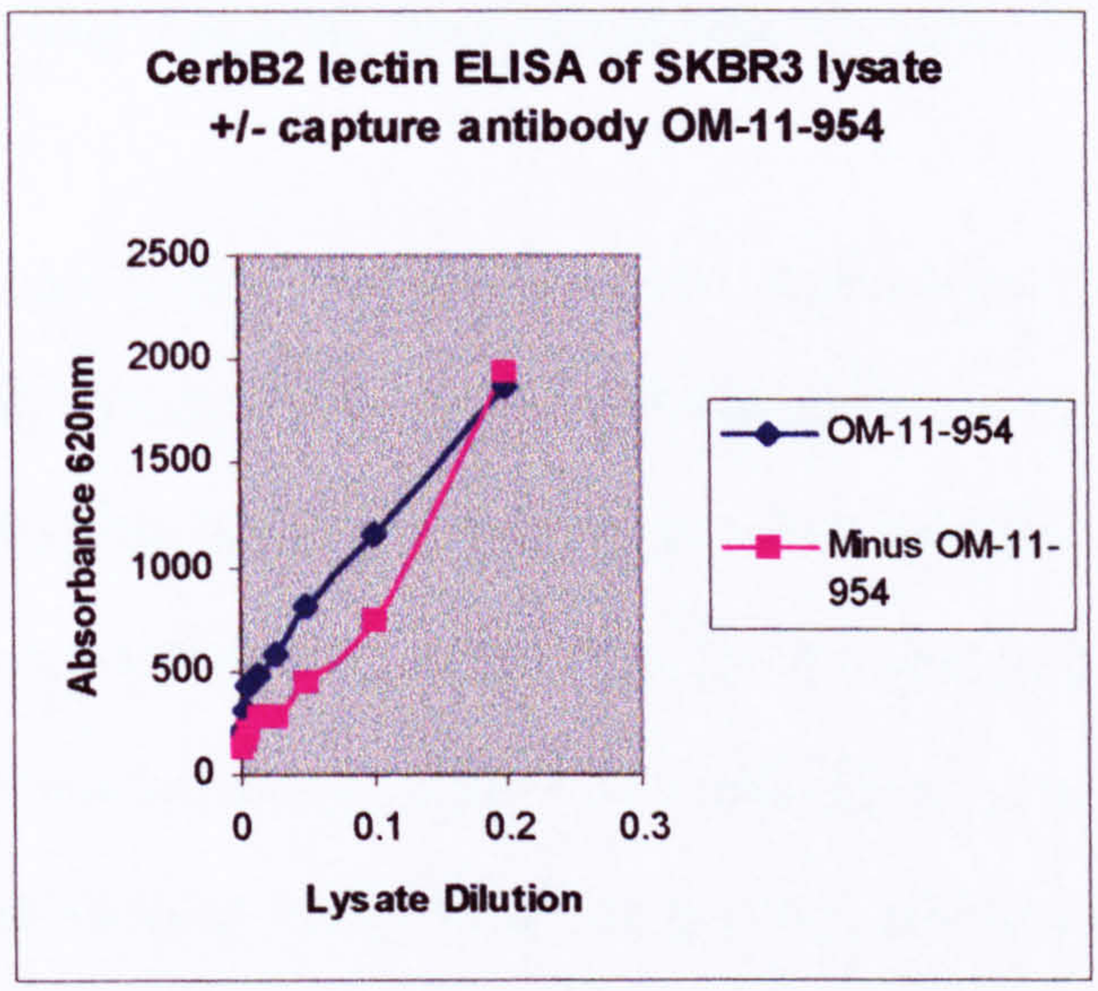


Figure 8.13: CerbB2 lectin ELISA +/- capture antibody

A similar cerbB2 lectin ELISA was then performed using a variety of cerbB2 antibodies instead of OM-11-954, including Ab-5 and Ab-7 (Oncogene Science, USA) and Ab-3, Ab-19 and Ab-21 (Abcam, USA). The response using these antibodies to capture the cerbB2 antigen were similar to those illustrated above i.e. a similar response curve was obtained when the capture antibody was omitted from the cerbB2 lectin ELISA.

Finally, it has been reported that repeated freeze-thaw cycles and the length of time specimens are stored may affect the conformation of glycoproteins. Thompson *et al* in a study of sera from ovarian cancer patients reported that abnormal forms of alpha-1-proteinase inhibitor could not be extracted from fresh sera by the lectin *lotus tetrogonolobus* unless specimens had been subjected to repeated cycles of freezing and thawing. Thompson *et al* also attributed discrepancies between the measurement of

abnormal forms of alpha-1-proteinase inhibitor between this and previous studies to the length of time specimens were stored prior to analyses (Thompson *et al.* 1990).

In the current study, the protein concentration in the lysates required to achieve a given response in the cerbB-2 lectin ELISA was much higher than that employed by Bustamam *et al* (Bustamam 1998). The affect of subjecting the serum sample to repeated freeze thaw cycles was therefore examined. The cerbB-2 lectin ELISA was performed on a fresh serum sample and a sample from the same patient subjected to 10 freeze thaw cycles. The steps outlined in section 4.7 in the materials and methods section were followed, however wells were incubated overnight at 4°C with 100µl of a 1 in 10 dilution of serum in 1% BSA PBS-Tween instead of SKBR3 Zabrecky lysate. The following figure demonstrates that repeated freeze thaw cycles did not alter the response from the cerbB-2 lectin ELISA.

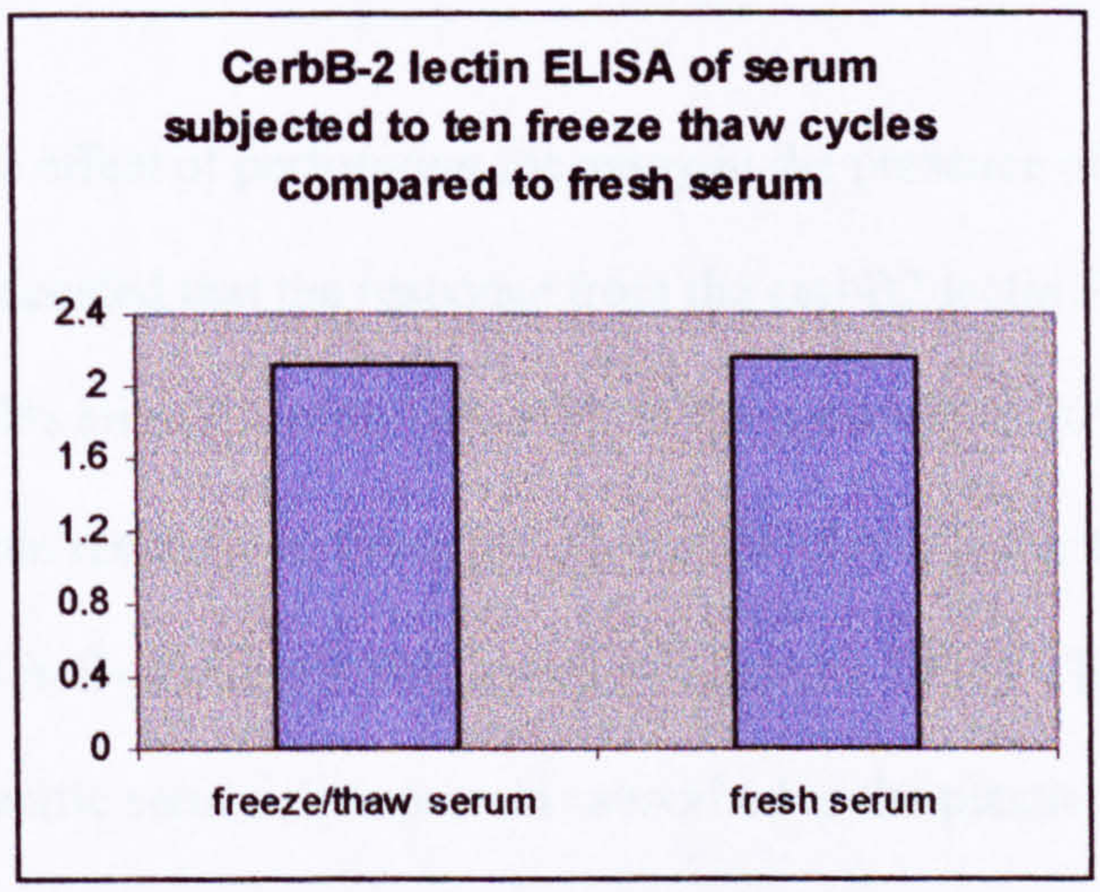


Figure 8.14: CerbB-2 lectin ELISA on a serum sample to investigate the affect of repeated freeze-thaw cycles

8.7 Discussion: A lectin ELISA for cerbB2

The cerbB2 lectin ELISA was established by BustamamAA *et al* as a means of detecting the extracellular domain of cerbB2 in the serum, at a time when only one antibody to the extracellular domain was commercially available (Bustamam 1998). This necessitated that a lectin was used as a probe in the second part of the antibody-lectin sandwich to detect the cerbB2 extracellular domain (p185) bound to antibody.

The assay was investigated quite extensively including competition experiments to confirm the specificity of the assay, see figure 82 in Bustamam's PhD thesis (Bustamam 1998). These experiments demonstrated that a synthetic p185 protein competed with lysate protein for antibody binding sites. These experiments were repeated in the present study and imply that the antibody was successfully capturing cerbB2 protein.

In the pilot study, the effect of performing the assay in the presence of human serum was investigated. It was decided that the response from the cerbB2 lectin ELISA was optimum in about 20% human serum. However, in the presence of 20% human serum approximately half the response of the cerbB2 lectin ELISA was due to the addition of serum, see figure 98 in Bustamam's PhD thesis (Bustamam 1998). This affect was attributed to non-specific serum glycoproteins absorbed to the plastic. These results should have alerted the investigators to question the suitability of the assay for use on clinical samples.

Bustamam *et al* did not perform any experiments to assess the reproducibility of the assay. The present study has demonstrated a wide variation in the response from the assay performed on different occasions on the same SKBR3 lysate. The results presented above demonstrate that each step of the assay functions satisfactorily and that a lysate has been successfully extracted that contains sufficient quantities of cerbB2 protein. The final experiment demonstrates the response from the cerbB2 lectin ELISA does not reflect glycosylation of cerbB2, but glycosylation of non-specific proteins present in the lysate absorbed to the sides of the wells, albeit the protein concentration in these lysates was much higher than that employed by Bustamam *et al* (Bustamam 1998). Rebeski *et al* have reported on unacceptable background caused by non-specific protein adsorption to immunoassay plates using ELISAs concluding that this could be a factor in the development and application of ELISAs (Rebeski *et al.* 1999).

The difficulties that have been encountered in this study may be attributed to the extent and variation of glycosylation of cerbB2 and the nature of lectins. Several lectin ELISAs have been published which have reported low coefficients of inter-assay variation 11.2% (Parker *et al.* 1992), <5% (Madiyalakan *et al.* 1996) and 16.4% (Nagata *et al.* 1991). Madiyalakan *et al* reported a correlation between the lectin ELISA and conventional two-site antibody sandwich for the determination of CA-125 in the serum of patients with ovarian cancer (Madiyalakan *et al.* 1996). These studies have investigated proteins, which are heavily glycosylated. Parker *et al* measured a mucin, a group of molecules, which are heavily glycosylated (Kim *et al.* 1996) and Nagata *et al* measured carcinoembryonic antigen (CEA), which is known to comprise of 50% carbohydrate

(Nagata *et al.* 1991). In contrast little is known about the extent and nature of glycosylation of cerbB2.

Thompson *et al* have reported that abnormal forms of alpha-1-proteinase inhibitor present in the sera of women with ovarian cancer could not be extracted by the lectin *lotus tetrogonolobus* unless samples had beeen stored for 6-9 years or artificially aged by being subject to repeated freeze-thaw cycles (Thompson *et al.* 1990). In the present study repeated freeze-thaw cycles did not affect the response of the cerbB-2 lectin ELISA (see figure 8.14), however the affect of the length of time for which samples were stored on the response of the lectin ELISA could not be excluded.

SKBR3 cells were cultured in the same laboratory using similar experimental techniques in the present study and the pilot study. However, there was a time lapse of 3-4 years between studies. The nature and extent of glycosylation of glycoproteins may vary with time and repeated passage of cell lines. However, differences in the glycosylation of glycoproteins are difficult to quantify without lengthy and time-consuming techniques, which were out of the scope of this project. These factors may have contributed to the differences between performance of this assay in the present and pilot study. In addition, these factors presented difficulty in producing a reliable standard with known cerbB2 protein and constant glycosylation against which the samples could be compared.

Lectins have specificity for sugar groups present on glycoproteins (Walker 1989). However, when used in lectin ELISAs, they may bind to non-specific glycoproteins

adherent to the side of the wells. A conventional two-site double-antibody ELISA has capture and detection antibodies with specificity for the protein under investigation. This confers on them a high degree of reproducibility. In contrast the response from a lectin ELISA may vary to a greater extent on the presence of other non-specific proteins present in the sample to which the lectin binds. This may be overcome to an extent when the protein under investigation is heavily glycosylated, such as a mucin (Kim *et al.* 1996).

The results from the present study do not support the use of lectin ELISAs for the measurement of cerbB2 in the serum of women with breast cancer. Results from the pilot study revealed shortcomings in the assay, which may have become apparent if the reproducibility of the assay had been investigated at this stage. Further investigation of this area should commence with an investigation of the nature and extent of glycosylation of cerbB2 using techniques such as mass spectroscopy or nuclear magnetic resonance analysis.

The Role of Androgens in Breast Cancer

9.1 Central Hypothesis

In this study, serum and tumour samples were collected from women with primary operable breast cancer and controls. Serum samples were used to measure androgen levels (dehydroepiandrostene sulphate, androstenedione and testosterone levels) using conventional ELISA techniques. As "crosstalk" occurs between steroid hormone and growth factor pathways in breast (and prostate) cancer, it was proposed to measure cerbB-2 levels using a lectin ELISA format. This technique was chosen as a lectin ELISA for cerbB-2 had previously been developed in our laboratory, which showed encouraging results as a diagnostic test for breast cancer.

Elevated levels of serum androgens in postmenopausal women and reduced levels of serum androgens in premenopausal women with breast cancer have been reported by several recent studies though there is disagreement on the significance of levels of different androgens. On the basis of these observations and the results of cell line studies it has been proposed that androgens may have a role in the aetiology of breast cancer by stimulating growth of hormone-dependent breast cancer in postmenopausal women and inhibiting the growth of hormone-dependent breast cancer in premenopausal women (Adams 1998).

In order to test this hypothesis, in the present study, the levels of three androgens dehyroepiandrosterone sulphate, androstenedione and testosterone were measured in a

sample of women with primary operable breast cancer and controls. Two further relationships, between serum androgen levels, body mass index and age have been investigated.

Cell line studies have been performed to examine the effect of two androgens, 5α-dihydrotestosterone and 5-androstene-3β,17β-diol, on the proliferation of hormone-dependent breast cancer cell lines MCF7 and T47D and the hormone-independent cell breast cancer cell line MDAMB231. 5α-Dihydrotestosterone and 5-androstene-3β,17β-diol have been chosen as they are true androgen metabolites, which act at the tissue level *in vivo* (Rochefort *et al.* 1984).

A flow cytometric technique has been used to investigate the effect of these androgens on DNA cell cycle analysis in 48-hour incubations and cell numbers in 9-day incubations. Cell line models have been set up to mimic the hormonal environment in pre and postmenopausal women in order to investigate mechanisms by which androgens may stimulate the proliferation of hormone-dependent breast cancer in postmenopausal and inhibit the proliferation of hormone-dependent breast cancer in premenopausal women.

A positive correlation between breast cancer incidence throughout the world and nutritional status has been demonstrated (DeWaard 1969). Nutritional status has been reported to affect androgen production and metabolism and therefore it has been proposed that androgens are implicated in the development of "Western-environmental" type breast cancer (Adams 1977). In order to investigate this hypothesis a correlation

between serum androgen levels and body mass index has been sought. Secondly, a correlation between serum androgen levels and age has been investigated, as it has been proposed that although serum androgen levels fall with age in healthy women they are independent of age in women with breast cancer (Adams 1998).

Flow cytometry is an established method for the determination of cell surface (Loken *et al.* 2000), cytoplasmic and nuclear antigens (Larson 2000). Multiparameter flow cytometry enables the simultaneous determination of expression of several antigens on tumour specimens. Flow cytometric methods for the determination of oestrogen receptor alpha (Brotherick *et al.* 1995), oestrogen receptor beta (Girdler *et al.* 2001) and epidermal growth factor receptor expression (Brotherick *et al.* 1994) have been established in our laboratory. In the present study a flow cytometric method for the determination of androgen receptor expression in breast cancer has been described.

A correlation between androgen receptor expression, determined by flow cytometry and a more established technique, immunohistochemistry, has been investigated. In order to investigate the clinical significance of androgen receptor expression in breast cancer, androgen receptor expression has been correlated with existing prognostic markers Bloom Richardson grade (Bloom *et al.* 1957) and lymph node status (Fisher *et al.* 1978).

Oestrogen receptor expression in breast cancer has been found to increase with age (Elwood *et al.* 1980). Expression of steroid hormone receptors is regulated by their ligands, by a mechanism known as "negative autoregulation". In order to determine

whether negative autoregulation determines androgen receptor expression in breast cancer *in vivo* a correlation between androgen receptor expression and serum dehydroepiandrosterone, androstenedione and testosterone levels have been investigated. In addition, an association between androgen receptor expression and age has been investigated.

In the present study, in addition to androgen receptor expression, oestrogen receptor alpha and epidermal growth factor receptor expression has been determined. The relationship between androgen receptor, oestrogen receptor and epidermal growth factor receptor expression has been examined.

The rational for investigating an association between androgen receptor and epidermal growth factor expression was that crosstalk occurs between steroid receptor and growth factor receptor pathways in breast (and prostate) cancer (Nicholson *et al.* 1999). An inverse correlation has been reported between oestrogen receptor expression and epidermal growth factor expression in breast cancer (Walker *et al.* 1999), though a relationship between androgen receptor and epidermal growth factor expression has not hitherto been investigated.

9.1 The role of androgens in breast cancer

The investigation of the role of androgens in breast cancer has been divided into three sections. Firstly the serum levels of androgens in women with breast cancer and controls

have been measured. A correlation of serum androgen levels in women with breast cancer and controls with age and body mass index has been sought. The results presented above demonstrate that serum testosterone, androstenedione and DHEAS levels fall with increasing age, in both women with breast cancer and controls. These findings conflict with previous published data on the association between serum androgen levels and age, which suggested that serum androgen levels were independent of age in women with breast cancer (Zumoff 1981).

No association was demonstrated between serum androgen levels and body mass index in women with breast cancer or controls. These findings may have importance in the epidemiology of breast cancer. Obesity is implicated in the aetiology of breast cancer. The results of the present study demonstrate that serum androgen levels are independent of body mass index. Therefore the higher incidence of breast cancer in obese postmenopausal women is more likely to be due to higher serum oestrogen levels (Cauley *et al.* 1989) due to greater aromatisation of androgens in peripheral adipose tissue rather than a direct affect of body mass index on serum androgen levels.

In this study, serum DHEAS levels were observed to be higher in postmenopausal women with breast cancer. This suggests a role for adrenal androgens in the aetiology of breast cancer in postmenopausal women. These results are supported by the cell culture experiments which demonstrated that 5-androstene-3β,17β-diol a metabolite of DHEAS stimulates the proliferation of hormone receptor positive breast cancer cell lines at physiological levels via an interaction with the oestrogen receptor. On the other hand, 5α-

dihydrotestosterone a metabolite of DHEAS with high affinity for the androgen receptor was found to inhibit the basal proliferation of hormone receptor positive breast cancer cell lines. Thus it is apparent that androgens are metabolized to steroids which may stimulate (5-androstene-3β,17β-diol, 17β-oestradiol) or inhibit (5α-dihydrotestosterone) the growth of hormone dependent breast cancer.

In longterm tissue culture both androstene-3β,17β-diol and 5α-dihydrotestosterone inhibited the oestrogen-induced proliferation of hormone receptor positive breast cancer cell lines. This suggests androgens may protect against breast cancer in premenopausal women. They are supported by the results of Bulbrook *et al* who measured urinary androgen metabolites in a sample of women on the island of Guernsey who subsequently developed breast cancer (Bulbrook 1986). In the present study, serum androgen levels did not differ between premenopausal women with breast cancer and controls. The results of this study do not entirely support a protective role against breast cancer for androgens in premenopausal women. However, fewer numbers in the premenopausal group and failure to account for the variables outlined above may have accounted for the failure to observe a difference between serum androgen levels in premenopausal women with breast cancer and controls.

By using a flow cytometric technique, tissue culture experiments in this study have demonstrated for the first time the effect of androstene-3β,17β-diol and 5α-dihydrotestosterone on DNA cell cycle in hormone receptor positive and negative breast cancer cell lines. Szelei *et al* demonstrated that androgens inhibit the proliferation of a

MCF7 breast cancer cell line transfected with an androgen receptor (Szelei *et al.* 1997). There was no effect on DNA cell cycle during forty-eight hour incubation of hormone receptor positive breast cancer cells with physiological levels of 5α-dihydrotestosterone in this study. However, longterm incubations demonstrate that 5α-dihydrotestosterone inhibits basal proliferation of hormone receptor positive cells after 6 days incubation.

A flow cytometric method for the determination of androgen receptor expression in breast cancer has been established in this study. The potential for flow cytometry to simultaneously measure several antigens on tumour samples suggests a clinical utility for flow cytometry in the management of breast cancer. The level of androgen receptor expression and the co-expression of androgen receptors with oestrogen receptors in breast cancer demonstrated in this study are in agreement with previous immunohistochemical studies (Kuenen-Boumeester *et al.* 1992) (Isola 1993). Androgen receptor expression was shown to increase with age. Serum androgen levels declined with increasing age in women with breast cancer. Negative autoregulation of steroid receptor levels by their ligands is thought to be the phenomenon common regulating steroid receptor levels in tissues. The negative correlation between serum DHEAS, androstenedione and tumour androgen receptor expression demonstrated in this study supports this concept.

Crosstalk between steroid receptor and growth factor pathways has been demonstrated to occur in both breast and prostate cancer. A correlation between androgen receptor and epidermal growth factor receptor expression was investigated in the present study as indirect evidence of a functionally important interaction between these receptors in breast

cancer. Expression of androgen and epidermal growth factor receptors was positively correlated in breast cancer specimens. This supports the concept of a functionally important interaction between these receptors in breast cancer. However, further work in this area, to establish the role of the androgen receptor in breast cancer is now indicated.

9.2 A lectin ELISA for cerbB-2 / HER-2

Bustamam *et al* have established a lectin ELISA for the determination of cerbB-2/ HER-2 in our laboratory (Bustamam 1998). Only one cerbB-2 antibody was available at this time, therefore a lectin was used to detect captured cerbB-2 antigen in the assay. Results from applying this assay to the serum of women with breast cancer and controls, suggested the assay had value as a diagnostic test for breast cancer. Despite cerbB-2/ HER-2 being over-expressed/ amplified in only 25-30% of breast cancers (Slamon *et al.* 1987) it was hypothesized that as the assay did not measure cerbB-2 protein levels, but the glycosylation of cerbB-2/HER-2, it would be worthwhile investigating a larger sample of patients.

Preliminary experiments to establish the assay *in vitro* uncovered fundamental problems with the assay that precluded its use on serum samples collected from patients with breast cancer. As discussed in chapter 8 these problems may relate to lectins and the glycosylation of cerbB-2/HER-2. Lectins have specificity for sugar groups expressed by complex carbohydrates. They are therefore able to bind to glycoconjugates expressed by

non-specific proteins present in the serum or lysate, which inevitably bind to the sides of the well during incubation stages of the assay. In addition lectins may bind directly to the sides of the wells. This phenomenon has been reported by Rebeski *et al*, who concludes that non-specific binding of lectins to immunoassay plates could be a serious impediment to development of lectin ELISAs as diagnostic assays (Rebeski *et al.* 1999).

On review of experiments performed by Bustamam *et al* it is apparent that non-specific binding of lectins occurred during establishment of the cerbB-2/HER-2 lectin ELISA.This phenomenon certainly occurred in the course of the present study. This is demonstrated by the final experiment in chapter eight, which demonstrated that omission of the capture antibody from the lectin ELISA did not affect the response.

Secondly, problems were encountered with the production of a standard with constant glycosylation. It is unknown how storage or tissue culture conditions affect the composition and structure of complex carbohydrates expressed on the surface of antigens such as cerbB-2/HER-2. As lectin-ELISAs measure the glycosylation of proteins, rather than the quantity of protein present, this presents considerable difficulties for the production of a reproducible standard against which the serum samples can be compared.

Thompson *et al* reported that artificially ageing samples by repeated freeze-thaw cycles and the length of time samples were stored affected the analysis of glycoproteins by lectins (Thompson *et al.* 1990). Although in the present study repeated freeze-thaw cycles were not shown to significantly affect the response of the cerbB-2 lectin ELISA,

differences in response of the cerbB-2 lectin ELISA between this study the pilot study may be attributed to the length of time the samples were stored.

Finally, the extent and nature of the glycosylation of cerbB-2/HER-2 is not known. Further work in this field should be directed at establishing the glycosylation of cerbB-2 by techniques discussed in the introduction to this thesis.

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