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In Touch with your Feminine Side: How Oestrogen Metabolism Impacts Prostate Cancer

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Abstract

Prostate cancer is the primary male cancer with increasing global incidence rates making this malignancy a significant healthcare burden. Androgens promote normal prostate maturity but also influence the development and progression of prostate cancer. Intriguingly, evidence now suggests endogenous and exogenous oestrogens, in the form of phytoestrogens, may be equally as relevant as androgens in prostate cancer growth. The prostate gland has the molecular mechanisms, catalysed by steroid sulphatase (STS), to unconjugate and utilise circulating oestrogens. Furthermore, prostate tissue also expresses enzymes essential for local oestrogen metabolism, including aromatase (CYP19A1) and 3β- and 17β-hydroxysteroid dehydrogenases. Increased expression of these enzymes in malignant prostate tissue compared to normal prostate indicates oestrogen synthesis is favoured in malignancy and thus may influence tumour progression. In contrast to previous reviews, here we comprehensively explore the epidemiological and scientific evidence on how oestrogens impact prostate cancer, particularly focusing on pre-receptor oestrogen metabolism and subsequent molecular action. We analyse how molecular mechanisms and metabolic pathways involved in androgen and oestrogen synthesis intertwine to alter prostate tissue. Furthermore, we speculate on whether oestrogen receptor status in the prostate affects progression of this malignancy.

Introduction

In the UK prostate cancer is the number one male malignancy accounting for 25% of all new cancer diagnoses in men (Siegel, et al. 2012). In 2011, there were almost 42,000 new cases with an age-standardised incidence rate of 104.7 per 100,000. Prostate cancer is the second leading cancer killer in UK men and 4th most common cause of cancer death in the general population. Similarly, in Europe prostate cancer is the most common cancer in males and third most common cancer overall (Jacob and Henrik 2006) . It is the third most common cause of cancer deaths in men and sixth overall. Currently, prostate cancer is the second most common cancer in males worldwide after lung cancer. However, it is predicted that prostate cancer will become the most common cancer in men globally (Parkin, et al. 2001).

Survival statistics from prostate cancer have improved dramatically over the last four decades which may be attributed to earlier detection and treatment granted by prostate specific antigen (PSA) testing and transurethral resection of the prostate (TURP). The UK 10-year survival has improved from 25% when diagnosed in 1970 to 84% in 2010 (Quaresma, et al. 2015). Prostate cancer primarily affects the elderly with 99.9% of patients diagnosed over the age of 50 and the mean age at diagnosis being 73 (Parkin, et al. 1997). Furthermore, from autopsy studies of non-cancer-related deaths, there is histological evidence of prostate neoplasms in more than 50% of men in their 50s (Sakr, et al. 1993). As average male life expectancy gradually increases, it is foreseeable that men will live longer with the disease and may experience a poorer quality of life.

There are significant geographical variations between prostate cancer incidences around the world with up to a 24-fold difference between the regions with the highest rates (in Australia, North America and Western Europe) and the lowest rates (in India, Japan and China) (Center, et al. 2012). While some of the discrepancies might be explained by disparities in healthcare

access, diagnostic methods, screening programmes and reporting systems; environment and lifestyle remain considerable factors. Studies comparing the incidence of prostate cancer in first and second generation Asian immigrants to USA with age-matched controls in their native countries have found that migrants travelling from low risk countries to high risk countries adopt the higher risk (Cook, et al. 1999). This advocates that environmental risk factors may have a higher precedence than genetic associations in determining risk of prostate cancer. Furthermore, environmental and lifestyle factors, diet in particular, fundamentally alter endogenous hormones including sex steroids (Barazani, et al. 2014). Indeed, factors such as smoking, increased physical exercise and a vegetarian diet increased serum androgen concentrations in British men while obesity, high fat diet and sedentary occupation reduced serum androgen concentrations (Allen et al. 2002). Such hormonal changes have the propensity to subsequently affect tumour initiation and progression (Kolonel, et al. 2004).

Sex Steroids and Prostate Cancer

Both males and females produce sex steroid hormones; the predominant androgens are testosterone and the more biologically active dihydrotestosterone (DHT) and the predominant oestrogens are oestrone (E_1) and the more biologically active oestradiol (E_2). However, the ratio of the two hormones differs between the sexes significantly. In the prostate, androgens are required for normal development and function. However, the role of oestrogens in normal prostate development is ill defined as biochemical mechanisms are still under investigation; the current dogma being that oestrogens are involved in the differentiation of epithelial tissue (Chen, et al. 2012; Francis, et al. 2013) and regulation of prostatic angiogenesis (Montico, et al. 2013).

Androgens have been implicated in prostate carcinogenesis since 1941 when Huggins published his Nobel winning study showing testosterone injections exacerbate prostate cancer in patients with late-stage disease and androgen deprivation alleviated the disease (Huggins and Hodges 1941), this suggested prostate cancer as an androgen-dependent malignancy. The primary source of androgens in males is testosterone secreted by the testicles, however, the adrenal glands secrete 100-500 times greater amounts of dehydroepiandrostrone sulphate (DHEAS), a testosterone precursor which can be converted peripherally in the prostate into testosterone and DHT (Labrie, et al. 2005). Androgen ablation therapy is initially successful in the vast majority of prostate cancers but relapse is common as tumours become castration resistant; they still however continue to express androgen receptors which respond to very low concentrations (as low as 10 pM) of peripherally synthesised testosterone and DHT (Chen, et al. 2004; Mohler, et al. 2004). Using microarray experiments on LNCaP and LAPC4 cell lines, Chen et al. (2004) showed an increase in androgen receptor mRNA and protein expression *in vitro* and *in vivo* in castrated xenograft murine models which correlated with tumour growth. Increased expression of androgen receptors amplified signals from low levels of androgen ligands to confer castration resistance. Mohler et al (2004) demonstrated using immunostaining and radioimmunoassays that activation of androgen receptors occur even in human prostate cancer samples retrieved from chemically castrated patients. This explains why surgical or medical castration is not 100% effective.

Previously, second-line hormonal therapy has proven to improve survival in patients with castration-resistant disease, both before and after docetaxel chemotherapy. Both inhibition of steroidogenic enzyme CYP17A1 using abiraterone and androgen receptor antagonism by enzalutamide have successfully ablated continued androgen receptor activation and prostate cancer growth (Beer , et al. 2014; de Bono , et al. 2011; Ryan , et al. 2013; Scher , et al.

2012). However, as with other androgen ablation therapy, resistance to abiraterone and enzalutamide inevitably develops.

Even though molecular mechanisms were not elucidated, oestrogens were traditionally considered to protect against prostate cancer. Therapeutic use of oestrogens was based on their anti-androgenic effects. Huggins reported exogenous oestrogens had protective properties mediated by a negative feedback effect on the hypothalamic-pituitary-gonadal (HPG) axis which reduced stimulation for androgen secretion from the testes (Huggins and Hodges 1941). Diethylstilbestrol (DES), a synthetic non-metabolised oestrogen is still used in certain clinics as a non-first line therapy to chemically castrate patients with metastatic prostate cancer (Bosset, et al. 2012; Clemons, et al. 2013). DES negatively feedbacks on the pituitary gland to reduce secretion of luteinizing hormone which reduces the stimulus for the testes to synthesise sex hormones In addition to the effects oestrogens have on the HPG axis, demonstrated by quantitative PCR,DES inhibits androgen-stimulated telomerase activity and gene expression and induces apoptosis in LNCaP and PC3 prostate cancer cell lines in both the presence and absence of androgens (Geier, et al. 2010). On the contrary, while DES is still licensed in the UK for treatment of prostate cancer it is infrequently used as secondary treatment due to the accompanied high rates of cardiovascular toxicity (Malkowicz 2001).

Importantly, the interactions of oestrogens on androgen receptors should be considered. For example, E_2 can activate both wildtype and, with greater efficacy, mutated (T877A) androgen receptors in LNCaP cells (Susa et al J Cell Physiolog 2015; Yeh et al. 1998; Veldscholte et al J Steroid Biochem Mol Biol. 1992). Mutations of the androgen receptor are uncommon in the early stages of prostate cancer but are much more frequent in late-stage disease. In one study, out of 99 patients diagnosed with early stage prostate cancer none were found to have

mutations in the androgen receptor. On the contrary, eight tumours out of 38 patients with advanced prostate cancer were found to harbour androgen receptor mutations (Marcelli, et al. 2000; Brooke and Bevan 2009). There is, however, mounting evidence that oestrogens may be involved in the initiation and progression of prostate cancer, although compelling evidence confirming oestrogen binding affinity to AR is lacking.

Impact of Endogenous Oestrogens in Prostate Cancer

Males are exposed to a high oestrogen/androgen (E/T) ratio twice in their lifetime. The first is as a foetus, during the third trimester when the maternal E_2 levels increase and foetal androgen levels decrease. Raised E_2 levels stimulate the developing epithelial cells of the prostate to proliferate but also cause morphological changes. For example, the prostate glands of neonatal rats and mice show abnormal proliferation and cell structure when the pregnant mother is injected with E_2. (Wernert, et al. 1990). This early exposure may imprint intracellular changes by modulating expression pathways of steroid enzymes and receptors as shown in rat models where the response to endogenous androgens and oestrogens becomes abnormal, thus predisposing the animal to prostate cancer after sexual maturation (Rajfer and Coffey 1978). Moreover, studies in mice show that when exposed to high levels of oestrogens in utero, foetal prostate tissue develops abnormalities including intraepithelial neoplasia and predisposition to carcinogenesis in adult life (Prins, et al. 2006). This hypothesis is supported by epidemiological evidence obtained from African-American men having twice as high a risk of developing prostate cancer than comparable Caucasian men which correlates with African-American women having a higher serum oestrogen level during pregnancy compared to Caucasian women (Henderson, et al. 1988).

The second time men are exposed to a high E/T ratio is during old age when serum testosterone decreases, partly due to a dampened HPG axis and partly due to reduced Leydig cell function in the testes. In addition to this, sex hormone-binding globulin (SHBG), which has a higher affinity to testosterone than E_2 (Knochenhauer, et al. 1998), also increases with age which further decreases free serum testosterone relative to free serum E_2 (Samaras, et al. 2012). Furthermore, there is evidence that E_1 and E_2 not only remain at the same level, but in fact increase with age even when accounted for BMI and other metabolic diseases (Jasuja, et al. 2013). While the evidence for an association between serum oestrogen concentration and risk of prostate cancer is unclear and inconsistent, increased serum oestrogen concentrations may stimulate the prostate stroma and epithelia to proliferate and subsequently become neoplastic. Indeed a higher oestrogen:androgen ratio stimulates proliferation of normal prostate stromal (PrSC) and normal epithelial (PrEC) cell lines in vitro (King, et al. 2006).

Another interesting population which is exposed to a high E/T ratio are transsexual male to female individuals. Often in this group of former males, individuals are orchiectomised and then supplemented with anti-androgens to relinquish masculine secondary sex characteristics. They are also supplemented with oestrogens to acquire and enhance feminine characteristics. Their prostates, however, remain unadulterated. A study observing such a cohort of transsexual persons for over 30 years has not identified any increase in risk for prostate cancer (Gooren and Morgentaler 2014). However the study has suggested that when presenting these patients are more likely to be diagnosed with a later stage disease. One limitation admitted by the authors is that the majority of the cohort has not reached the mean age at which prostate cancer is typically diagnosed (Gooren and Morgentaler 2014). Observations made to this cohort over the next two or three decades will be most

enlightening in ascertaining whether oestrogens have any significant effects in the development of prostate cancer.

Oestrogen Metabolism in Adipose and Prostate Cancer

While in pre-menopausal females the primary source of oestrogens are the ovaries, in males there is no central organ which produces substantial quantities of E_2. Instead, peripheral conversion of oestrogen precursors is the main source of oestrogen in men. Local synthesis of E_1 and E_2 is regulated by a plethora of enzymes. DHEA secreted from the zona reticularis of the adrenal glands, and stored in the blood as a reservoir as DHEAS, is the ultimate precursor. Adipose tissue is another notable source of oestrogen synthesis (Cui, et al. 2013). White adipose tissues (the predominant type in obesity) express significant quantities of cytochrome P450 aromatase enzyme (*CYP19A1*) in the abdominal adipose fat of male human samples, which is the final catalyst in the conversion of androgens to oestrogens (Polari, et al. 2015; Wang, et al. 2013). There is also a positive correlation between the amount of visceral adipose tissue and serum E_2 levels as shown in a study of 229 man with a mean age of 53.6 years where visceral fat was measured using magnetic resonance imaging (Gautier, et al. 2013).

There have been conflicting reports as to whether obesity is a risk factor for prostate cancer as some suggest it decreases risk while others have found the opposite. Allott *et al.* have summarised the findings published between 1991 to 2012 in their review and conclude obesity is associated with aggressive prostate cancer (Allott, et al. 2013). There is further

221 robust evidence that obese patients are more likely to present with aggressive high-grade
222 prostate cancer (De Nunzio, et al. 2013; Vidal, et al. 2014). It is possible that the risk
223 associated with obesity may in fact be due to elevated circulating oestrogen levels secondary
224 to increased adipose deposition. If this is the case, it would parallel the effects of oestrogen
225 that have been observed in colorectal cancer where oestrogen exposure in the form of
226 hormone replacement therapy or oral contraceptives are initially protective against colorectal
227 cancer but when patients present, they present with a later stage disease (Foster 2013). The
228 intra- and extracellular handling and metabolism of oestrogens within the prostate gland may
229 clarify what effects oestrogens have on tumours. However, studies are lacking regarding the
230 exact intra-tumoural metabolism of oestrogens in prostate cancer cells and human prostate
231 cancer tissue.

232

233 **Impact of Exogenous Oestrogen on Prostate Cancer**

234 Exogenous oestrogen intake and subsequent availability to the prostate should be considered
235 when determining whether oestrogens affect the development and progression of prostate
236 cancer. A Western diet comprising of high meat, saturated fat, and dairy products has been
237 associated with increased risk of prostate cancer as highlighted by numerous epidemiological
238 studies (Grönberg 2003; Howell 1974; Whittemore, et al. 1995). Additionally, it has been
239 observed that such a Western diet is more likely to cause men diagnosed with prostate cancer
240 to die from the disease when compared to a diet rich in fruits, vegetables, and whole grain
241 cereals (Yang, et al. 2014). Supporting this, it has been widely speculated that dietary
242 oestrogenic compounds from plant sources, termed phytoestrogens, are protective against
243 prostate cancer and are the reason behind lower incidence rates in East Asia where per capita
244 consumption of phytoestrogen-rich foods, such as soya beans, are considerably higher than

the Western world (Adlercreutz, et al. 2000; Goetzl, et al. 2007; Strom, et al. 1999). It is possible that phytoestrogens reduce the risk of prostate cancer through multiple mechanisms. In rodent models phytoestrogens can upregulate SHBG synthesis in the liver leading to a higher circulating concentration (Pilšáková, et al. 2010). Increased SHBG is anti-androgenic as it binds to free testosterone with a higher affinity than oestrogens (Knochenhauer et al. 1998) implementing a net reduction of testosterone relative to E_2 (Ronde, et al. 2005). This reduction in androgen is thought to be important in the reduction of risk. In addition to chelation of free testosterone via SHBG, phytoestrogens have a negative feedback effect on the HPG axis directly leading to reduced secretion of luteinising hormone and consequently reduced stimulation of androgen and oestrogen synthesis (Goetzl et al. 2007).

Phytoestrogen compounds are similar enough to endogenous oestrogens to be able to bind to oestrogen receptors (ER) and evoke ligand-specific intracellular responses (Usui 2006). Preference for different types of nuclear ER varies between phytoestrogens (see section on oestrogen receptors). Isoflavones and coumestans are two main categories of phytoestrogens and are structurally similar to E_2 (Figure 1). The prostate cancer cell lines LNCaP and DU145 are more sensitive to apoptotic factors when treated with isoflavones *in vitro*. A dose-response relationship between concentration of biochanin A and apoptosis was observed using cytotoxicity and lactate dehydrogenase release assays, flow cytometry and fluorescence microscopy (Szliszka, et al. 2013). Coumestans are able to induce caspase-dependent apoptosis in LNCaP, DU145 and PC3 cells. When treated with wedelolactone, a plant derived coumestan, there was dose-dependent apoptosis in androgen-sensitive cell lines (LNCaP) and androgen-independent cell lines (DU145 and PC3). However, normal non-cancerous PrEC prostate epithelial cells were not affected as harshly showing 90% cell viability compared to circa 20% in cancerous cell lines at concentrations of 30μM. (Sarveswaran, et al. 2012).

While in vitro evidence argues that phytoestrogens are protective against prostate cancer, clinical trials looking at the relationship between consumption of dietary phytoestrogens and progression of prostate cancer have been inconclusive (Goetzl et al. 2007). One double blind randomised control trial in which 81 healthy men were either given a soy protein drink with high isoflavone concentration (83mg/day) or a drink with low isoflavone concentration (3mg/day) showed no significant difference in PSA over 12 months (Adams, et al. 2004). Another trial offering men with confirmed prostate cancer who had either failed medical/surgical therapy or had chosen active surveillance a high dose (450mg/day) oral isoflavone supplement for 6 months showed only a clinically insignificant improvement in PSA in the active surveillance group with no difference in the failed therapy group (deVere White, et al. 2004). Furthermore, a study following up 3628 men with diagnosed prostate cancer for a median duration of 11.5 years showed an increased risk of advanced prostate cancer (HR: 1.62) but a reduced risk of non-advanced prostate cancer (HR: 0.88) in the higher dietary intake of isoflavones group. Dietary intake of phytoestrogens was measured using a validated food frequency questionnaire and so exact doses of phytoestrogens are subject to variation (Reger et al. 2015). This preliminary evidence could infer that dietary phytoestrogens might protect against initiation of prostate cancer, however may promote the progression of advanced prostate cancer.

Steroid metabolism in the prostate

Androgens

The metabolism of oestrogens and oestrogen precursors is important for availability of biologically active E_2 to prostate cancer cells. Oestrogens are synthesised from androgens which themselves are synthesised from progestogens (Khurana 2008). In addition to circulating androgens secreted from the testes, normal prostate tissues have the potential to produce androgens from circulating C19 steroids DHEA and androstenedione (Figure 2). There have been conflicting reports on the possibility of prostate cancer to synthesize androgens *de novo* through the conversion of progestogens via cytochrome P450 17A1 (17-hydroxylase and 17, 20 lyase enzyme [*CYP17A1*]). In prostate cancer, the expression of cytochrome P450 17A1 was reportedly increased in LNCaP and LuCaP cells and human prostate tissue samples ascertained by PCR and immunoblotting (Locke, et al. 2008; Montgomery, et al. 2008); however not all studies support this (Ellem and Risbridger 2009; Hofland, et al. 2010). Although DHT formation from cholesterol was detected using mass spectrometry in castration-resistant prostate cancer (CRPC) models in one study (Locke et al. 2008) these steroid fluxes have not been confirmed quantitatively to date in either *in vitro* or *in vivo* models.

Another key enzyme in the synthesis of biologically active androgens and oestrogens is 3-betahydroxysteroid dehydrogenase (3β-HSD) which converts dehydroepiandrosterone and androstenediol to androstenedione and testosterone, respectively (White, et al. 2013). 3β-HSD is expressed in the normal human prostate, with immunoblotting revealing that the highest concentrations are found in basal epithelial cells (Luu-The, et al. 2008). Certainly, in mouse xenograft studies using the CRPC LAPC4 cell line, expression of 3β-HSD is increased within the tumour in addition to AKR1C3 and 17β-HSD3 (Chang, et al. 2011), although its mRNA expression almost completely mutually excludes that of CYP17A1 (Hofland et al. 2010).

Inhibitors of 3β-HSD have been explored as an androgen deprivation technique as they are effective in decreasing proliferation in androgen sensitive LNCaP or CRPC cell lines 22Rv1, VCaP and PC346C *in vitro* (Evaul, et al. 2010; Kumagai, et al. 2013). Furthermore, abiraterone was found to inhibit 3β-HSD activity in addition to CYP17A1 in prostate cancer cell lines and isolated yeast microsomes (Li, et al. 2012). This mechanism might rely on abiraterone being converted to the more active Δ(4)-abiraterone (D4A) within the prostate gland by 3β-HSD itself (Li, et al. 2015b). Further research into 3β-HSD inhibition are currently being pursued, however alternative pathways which bypass androstenedione synthesis exist and so 3β-HSD function is not strictly necessary.

An alternative pathway has been demonstrated by which synthesis of DHT within the prostate may bypass testosterone and instead be synthesised by reduction of androstenedione by 5α-reductase SRD5A1 to 5α-androstanedione which is converted to DHT by 17β-HSD5. Mass spectrometry has shown that even in patients on anti-androgen therapy with very low serum testosterone levels, intratumoral DHT concentrations remain at the pre-treatment level (Chang et al. 2011; Sharifi and Auchus 2012). 17β-HSD-5, also known as AKR1C3, appears to be the key enzyme responsible for intratumoural androgen production in CRPC. Its expression in LNCaP, DU145 and PC3 cellsare potently stimulated by androgen deprivation *in vitro* and in humans *in vivo* (Ellem and Risbridger 2009; Ellem, et al. 2004) and this secures continued production of testosterone and DHT from circulating adrenal androgens. Local growth factor activin A was shown to be a key intermediate in the castration-induced rise of *AKR1C3* expression levels and intratumoural testosterone production as observed in

LNCaP, VCaP and PC3 cells. The concentration of activin A and testosterone were also shown to be increased in the cultured supernatants, as measured by ELISA and mass spectrometry (Hofland, et al. 2011). 17β-HSD-5 has also been implicated in ezalutamide resistance to anti-androgen therapy. Knockdown of 17β-HSD-5 using shRNA or inhibition with indomethacin has shown to resensitise enzalutamide-resistant cells *in vitro* and *in vivo* (Liu, et al. 2015).

Peripheral Oestrogen Metabolism in Prostate Cancer

As mentioned previously, aromatase is a key enzyme required for oestrogen synthesis from androgen precursors. Aromatase converts androstenedione and testosterone to E_1 and E_2, respectively (White et al. 2013). The local synthesis of E_2 within the prostate has previously been debated as not all experiments have identified aromatase expression in normal prostate tissue (Ellem et al. 2004). However, it has been demonstrated in human samples by substrate conversion assays and mass spectrometry that E_2 synthesis does occur in prostate cancer cells (and benign prostatic hyperplasia) via aromatisation (Ellem and Risbridger 2009; Härkönen and Mäkelä 2004). In normal prostate, aromatase is expressed by the stromal tissue but not the epithelial cells, however once malignant, epithelial cells also express aromatase (Ellem and Risbridger 2007). Aberrant expression and activity of aromatase is crucial in the pathophysiology of endometrial and breast cancers where an imbalance of oestrogen is a key factor in tumour growth (Chen 1998; Cunha 1994). As with the developmental similarities between breast and prostate tissues (Ellem and Risbridger 2010), abnormal aromatase activity also plays a major role in breast and prostate tumourigenesis (Ellem and Risbridger 2010). Tumourigenic growth factors including epidermal growth factor and transforming growth factor-1 can modulate aromatase activity in androgen-sensitive LNCaP cells lines leading to decreased oestrogen synthesis (Block, et al. 1996). Furthermore, the expression of aromatase is up to 30-fold greater in metastatic prostate cancer compared to primary tumours

(Miftakhova, et al. 2016). In addition, overexpression of aromatase increased the progression of bony metastasis in xenograft experiments where nude mice were injected with PC3 cell lines transfected to overexpress aromatase (Miftakhova, et al. 2016). Consequently, the use of aromatase inhibitors for the treatment of prostate cancer has been investigated many times in patient cohorts. The first generation aromatase inhibitor aminoglutethimide is non-selective and showed poor objective responses including serum PSA levels and disease stability in some studies while showing a significant increase in survival in others (Santen, et al. 1997). One study treated 58 castrated men with advanced prostate cancer resistant to conventional therapy with 500-750mg daily aminoglutethimide; 11 men showed an objective response with a mean remission of 10 months and a further two showed disease stabilisation for a mean seven months (Murray and Pitt 1985). The second generation aromatase inhibitor, 4-hydroxyandrostenedione showed good subjective responses in 18 out of 25 patients with advanced CRPC, particularly alleviation of bone pain in prostate metastases. However the objective responses were still poor with a reduction in tumour volume seen in only three patients and all patients progressed to have skeletal metastasis. (Davies, et al. 1992). A Phase II clinical study looking at the effects of oral letrozole, a third-generation aromatase inhibitor more commonly used in the treatment of hormone-dependent breast cancer, in 43 men with CRPC showed no significant disease regression with serum PSA decreasing by more than 50% in only one patient and decreasing by less than 50% in one further patient (Smith, et al. 2002). A very similar conclusion was drawn from clinical studies looking at anastrazole, another third generation aromatase inhibitor, where out of 14 patients with CRPC none showed a decrease in serum PSA and mild bone pain relief was reported by only two patients (Santen, et al. 2001). While aromatase is of utmost importance in local oestrogen synthesis, it appears as though therapeutic approaches targeting aromatase may be futile in treating prostate cancer. An alternative possibility is that E_2 is not synthesised from androgens within

the prostate but instead is converted from systemic sulphated E_1 within the prostate via steroid sulphatase (STS).

STS is widely expressed in almost all peripheral tissues and is responsible for hydrolysing sulphate moieties off of circulating sulphate-conjugated steroids in order to make them biologically active (Mueller, et al. 2015). Oestrone sulphate (E_1S) is the most abundant circulating oestrogen in adult humans (Muir, et al. 2004) with plasma levels between 2-4nmol/L in men (Mueller et al. 2015) and while oestradiol sulphate also exists, plasma levels are very low. Furthermore, serum E_1S levels have been correlated with increased risk of prostate cancer. In a cohort study of 5995 men aged over 65 where the mean serum E_1S levels in the 275 patients who developed prostate cancer was significantly higher than those who did not develop prostate cancer (Daniels, et al. 2010).

Before sulphated oestrogens can be unconjugated by intracellular STS, transport of sulphated oestrogens into cells requires the expression of organic anion transporter peptides (OATP) (Raftogianis, et al. 2000) and indeed several different OATPs involved in the transport of oestrone sulphate are expressed in prostate cancers (Buxhofer-Ausch, et al. 2013; Giton, et al. 2015; Wright, et al. 2011). STS has been shown to be expressed in normal human prostate tissue (Reed, et al. 2005), prostate cancer cell lines LNCaP, DU-145 and PC3 (Nakamura, et al. 2006) and in primary prostate homogenates (Klein, et al. 1989). Furthermore, one study found that STS is expressed in the majority of localised prostate cancers showing higher expression in malignant tissues compared to benign (Nakamura, et al. 2006). The activity of STS has been proven within the human prostate for the desulphation of dehydroepiandrosterone sulphate (DHEAS) into DHEA, an androgen precursor (Farnsworth

1973). Moreover, E_1 synthesis from desulphation of E_1S within the prostate is putatively 10-fold greater than synthesis via aromatase (Nakamura et al. 2006). The relevance of STS in cancer has been more extensively studied in breast cancer where there is significantly higher expression of STS than in normal breast (Utsumi, et al. 2000). Consequently, several STS inhibitors have been developed for the treatment of breast cancer, some of which have shown early promise (Stanway, et al. 2006). Moreover, first and second generation STS inhibitors have been effective pre-clinically against breast cancer (Foster et al. 2006; Foster et al. 2008; Purohit and Foster 2012). Meanwhile, investigations into the efficacy of STS inhibitors in prostate cancer have been undertaken. It has been observed that middle-aged rats treated with oral STS inhibitor, STX64 decreased conversion of E_1S to E_1 (Giton et al. 2015; Roy, et al. 2013). Neither study presented evidence of STS inhibition affecting any proliferative markers of proliferation, however the latter study did demonstrate that STS inhibition in middle-aged rats prevented increase of prostate mass when treated with E_1S + STX64 *vs* E_1S alone where prostate mass increased (Giton et al. 2015). An alternative conjugate of circulating oestrogens is glucuronide (Raftogianis et al. 2000), however, research into oestrogen glucuronide transport into prostate cells and evidence of glucuronidase enzymes within the prostate is lacking.

Conversion of E_1 to E_2 (and androstenedione to testosterone) requires 17-betahydroxysteroid dehydrogenase (17β-HSD) enzymes (White et al. 2013). 17β-HSDs enzymes are alcohol oxidoreductases which catalyse reduction (E_1 to E_2) and oxidation (E_2 to E_1) at carbon atom 17. There are over 14 different isozymes of 17β-HSDs (17β-HSD *1-14*) and certain 17β-HSDs have a higher propensity to catalyse the reaction in a certain direction, for example 17β-HSD-1 favours reduction whereas 17β-HSD-2 favours oxidation (Lukacik, et al. 2006; Oduwole, et al. 2003). 17β-HSDs play an important role in hormone sensitive cancers.

Increased expression of 17β-HSD-1 in breast cancers of post-menopausal women helps maintain high intratumoural E_2 levels (Lukacik et al. 2006). Moreover, expression of 17β-HSD-2 and 17β-HSD-3 mRNA is significantly higher in malignant prostatic tissues compared to normal prostate tissues (Day, et al. 2013) with one study reporting prostate cancer biopsies showing 30-fold higher mRNA expression than normal. In addition to converting androstenedione to testosterone, 17β-HSD 5 can convert E_1 to E_2. Inhibitors of 17β-HSD 5 have been explored in castration-resistant prostate cancer and breast cancer, in the latter where androgens are not considered to play an important role (Adeniji, et al. 2013). The study found no appreciable decrease in E_2 synthesis in breast cancer cell lines when treated with a 17β-HSD 5 inhibitor and only a moderate decrease in E_2 synthesis in some subpopulations of prostate cancer cell lines. Interestingly, inflammation associated with tumours modulates the expression of 17β-HSD-2 and 17β-HSD-5 (and also 3β-HSD). Treatment of prostate cancer stromal cell lines PrSC with TGFβ1 showed a marked down-regulation in mRNA expression of 17β-HSD-2 and 17β-HSD-5 in a dose-dependent manner (Piao, et al. 2013). The counterintuitive action of TGFβ1 again demonstrates how little is understood about oestrogenic pathways in prostate cancer. Regardless of the mechanisms by which oestrogens become available within the prostate gland, tumour-promoting or tumour-suppressing effects must be mediated by activation of oestrogen receptors (ER).

Oestrogen receptors (ER) in the prostate

The effects of oestrogens on tissues are mediated via activation of oestrogen receptors (ER). There are two well studied ERs; ER alpha (ERα) and ER beta (ERβ) encoded by two separate genes *ESR1* and *ESR2*, respectively. ERα and ERβ are members of the nuclear receptor superfamily (Robinson-Rechavi, et al. 2003). When bound and activated, ERs interact

directly with the genome acting as transcription factors (or activating transcription factors) which act directly on oestrogen response elements (Deblois and Giguere 2013). As well as E_2, ERs can be stimulated by phytoestrogens, and different classes of phytoestrogens have selected preferences for each type of ER. In general, phytoestrogens show agonistic activity towards ERβ at lower concentrations than towards ERα using hamster uterine cells (Takeuchi, et al. 2009). When human cells are examined, the relative binding affinity (RBA) of genistein to ERβ is approximately 20-30 times greater than for ERα as shown in MCF-7 breast cancer cell lines (Pilšáková et al. 2010). The affinity of phytoestrogens for ER widely varies with most molecules having an RBA to ERβ 1000-fold lower than E_2. However, molecules such as genistein and coumesterol have an RBA 100-fold lower than E_2. Genistein and coumesterol are able to activate transcriptional activities of ERα and ERβ at concentrations of 1-10nM compared to physiological E_2 concentrations of 20-40pM in males (Kuiper, et al. 1998; Mueller et al. 2015). Of course, the ability of phytoestrogens to bind to ER also depends on the existing levels of E_1 and E_2 as these molecules are direct competitors with phytoestrogens.

ERs have been studied more extensively in the context of breast cancers, a neoplasm that has been likened as the sister disease to prostate cancer, especially in regards to their hormonal responses and sensitivities (Risbridger, et al. 2010). In breast cancer, activation of ERα promotes tumour growth as it initiates anti-apoptotic (Chaudhri, et al. 2014; Razandi, et al. 2000) and mitogenic effects (Bhatt, et al. 2012; Yamnik and Holz 2010). This anti-apoptotic effect of ERα makes ERα positive breast cancers more likely to metastasise (Ross-Innes, et al. 2012). In fact, a review of ERs in breast and ovarian cancers has found ERα expression correlates with worse prognosis whereas ERβ expression correlates with better clinical

489 outcomes (Burns and Korach 2012). Generally, ERα activation promotes proliferative
490 pathways whereas ERβ activation leads to apoptotic pathways (Acconcia, et al. 2005).

491

492 Expression of ERα and ERβ in the normal prostate has been determined as the role of
493 oestrogens in prostatic development was identified (Ho 2004). Recently it has been reported
494 that prostate progenitor stem cells, while lacking expression of androgen receptor, express ER
495 abundantly. Indeed, the expression of ERβ is putatively 6-fold greater and ERα 125-fold
496 greater in progenitor cells compared to LNCaP mature cells (Di Zazzo, et al. 2016). Although
497 this supports the importance of oestrogens in embryonic and neonatal development of
498 prostate gland, it has been hypothesised that lack of androgen receptor expression could be an
499 imprint which later predisposes to CRPC in the elderly. In non-cancerous prostate ERα is
500 predominantly expressed in the stromal compartment and ERβ is predominantly expressed in
501 basal-epithelial cells. However in prostate cancer, ERα expression is down-regulated in
502 stromal cells and upregulated in the cancerous epithelial cells. ERβ expression is down-
503 regulated in epithelial cells as seen by immunostaining in human prostate tissue (Yeh, et al.
504 2014). Indeed there is evidence that down-regulation of ERβ promotes activation of NF-κB
505 mediated by hypoxia-inducible factor 1 (HIF-1). In immortalised normal prostate epithelial
506 cell line PNT1a, loss of ERβ using shRNA showed an increase in NF-κB mRNA expression
507 and activity. This mirrors what is seen in high grade, late stage prostate cancer (Mak, et al.
508 2015). Consequently, it appears that an increase in ERα expression and decrease in ERβ
509 expression is what shifts the balance between protective effects of oestrogens and
510 proliferative effects of oestrogens as has been suggested in other cancers (Barzi, et al. 2013;
511 Burns and Korach 2012). Figure 3 summarises the difference in ERα and ERβ expression
512 between non-cancerous and cancerous prostate tissue. Single nucleotide polymorphisms
513 (SNP) in the ER genes have been investigated and associations have been made between

514 certain polymorphisms and the risk of prostate cancer (Holt, et al. 2013; Jurečeková, et al.
515 2015). In both studies, the genomes from histologically confirmed human prostate cancer
516 samples were analysed using polymerase chain reaction restriction fragment length
517 polymorphism (PCR-RFLP) based analysis and compared to age-matched healthy control
518 subjects. A meta-analysis exploring the results of 24 published studies that include
519 Caucasian, Asian and African participants concluded that *ESR1* rs9340799 polymorphism is
520 allied to increased risk in the general population of Caucasians and Africans whereas ESR2
521 rs1256049 polymorphisms has been linked to increased risk only in Caucasians (Fu, et al.
522 2014).

523

524 Research into ERβ has been more extensive than in ERα. McPherson et al. (2007) highlighted
525 the potential significance of ERβ manipulation when they treated prostate hyperplasia in
526 oestrogen depleted mice with a selective ERβ agonist and found it to induce apoptosis and
527 shrink the size of the prostate. Hussain et al. (2012) carried forward this research and initial
528 studies have found ERβ agonist treatment with 8β-VE_2 can induce apoptosis in primary
529 human and murine prostatic basal cells, a lineage considered to be the cells of origin for
530 prostate cancers (Taylor, et al. 2012). The mechanism behind how ERβ activation induces
531 apoptosis in prostate cancer cells lines may be via up-regulation of p53-upregulated
532 modulator of apoptosis (PUMA) and consequent intrinsic caspase-9 mechanisms. Dey, et al.
533 overexpressed ERβ in LNCaP, PC3 and 22Rv1 prostate cancer cell lines *in vitro*, the latter
534 which does not express ERβ, and treated with E_2 and agonist 3β-adiol. Immunofluorescence
535 revealed that cells which expressed ERβ were more likely to undergo apoptosis following
536 expression of PUMA independent of p53 (Dey, et al. 2014). (Dey, et al. 2014). It has even
537 been reported that ERβ activation impedes on the epithelial-mesenchymal transition process
538 thereby reducing the risk of invasion and metastasis. In human tissue samples and LNCaP

and PC3 cell lines, treatment with E_2 and high concentration of ERβ1 agonist 3β-adiol resulted in inhibition of VEGF and destabilisation of HIF-1 in vitro thus suppressing the factors that drive epithelial-mesenchymal transition necessary for metastasis. Furthermore, loss of ERβ1 expression by means of shRNA transfection resulted in significant increase in migration and invasion (Mak, et al. 2010). Mounting evidence also suggests that pharmaceutical targeting of ERβ pathways may be effective in treating prostate cancer. However, recently a 'switching roles' theory has been proposed suggesting the effects of ERβ activation switches from protective to proliferative as cancer progresses (Savoy and Ghosh 2013). The theory is based on the observation that castration-resistant prostate cancers have higher expression of ERβ compared to hormone-naïve prostate cancers. It is possible that decreased levels of circulating androgens and up-regulation of androgen receptors may be important in this switch however the actual mechanisms and processes are yet unknown.

Splice variants of ERβ are also important as it has been shown that at least 5 different isoforms exist, many of which are expressed in the prostate (Leung, et al. 2006). Activation of different isoforms may have opposing effects; for example $ERβ_1$ is tumour-suppressing whereas $ERβ_2$ is tumour-promoting in LNCaP cells (Chen, et al. 2009). In a study of primary prostate cancer samples from 144 patients who underwent radical prostatectomy, two particular isoforms $ERβ_2$ and $ERβ_5$ have been identified to promote invasion and metastasis of prostate cancer and thus correlate with worse outcomes while others continue to be studied (Leung, et al. 2010; Nelson, et al. 2014). Certain ERβ isoforms, such as $ERβ_2$ and $ERβ_3$, when activated interact with transcription factors which enable and promote the epithelial mesenchyme transition and hence might be why advanced prostate cancers have higher expression of ERβ (Leung et al. 2010). More research needs to be carried out to understand the mechanisms of the complex downstream pathways of ERβ activation in prostate cancer.

The tumour promoting effects of ERα within the prostate are not as well defined. ERα is expressed in significant quantities in the stromal tissue of prostate cancer where they have been associated with cancer-associated fibroblasts (CAF) (Slavin, et al. 2015). Da, et al. isolated CAF from adenocarcinoma of mouse prostate lentivirally transduced ERα. Conditioned media from ERα+ CAF promoted proliferation of LNCaP, PC3, C4-2 and 22Rv1 cells. Furthermore, in xenograft experiments mice co-implanted with ERα+ CAF showed a higher growth rate of tumour mass compared to injection of prostate cancer cell lines alone (Da, et al. 2015). Activation of ERα on CAFs stimulates the release of tumour-promoting factors which act on prostate epithelia in a paracrine manner. Slug (SNAI2), a transcription factor with anti-apoptotic pathways can repress ERα expression by binding to gene promotor regions and consequently promote epithelial-mesenchymal transition in prostate cancer cells and human breast cancer samples (Li, et al. 2015a). In contrast, downstream pathways of ERα activation can inhibit metastasis by down-regulating expression of matrix metalloproteinase 3 and upregulating expression of thrombospondin 2 as seen in a range of breast cancer cell lines and LNCaP cell line, however this is not evidence in primary human prostate tissue (Li et al. 2015a). This may be an effect of ERα activation which diverts cell resources towards growth of prostate cancer rather than spread and invasion (Hanahan and Weinberg 2011). A study investigating the role of ERα in prostate cancers of PTEN-deficient mice has shown expression of ERα correlates strongly with the expression of Ki67,- a proliferative marker. In addition, inhibition and knockdown of ERα decreases proliferation but has no effect on cell viability thus the tumour mass remained static. This further demonstrates that ERα regulates cell proliferation through PI3K and MAPK signalling (Takizawa, et al. 2015).

589 Human trials in 1590 men with high grade intraepithelial neoplasia of the prostate has shown
590 no significant decrease in risk of prostate cancer when treated with daily toremifene, a
591 selective oestrogen receptor modulator (SERM) used for the treatment of metastatic breast
592 cancer, compared with placebo. Of the 1467 men who underwent a biopsy during the three-
593 year study, cancer was detected in 34.7% in the placebo group compared to 32.3% in the
594 treatment group (p= 0.39) (Taneja, et al. 2013). Conversely, experimental use of toremifene,
595 in cell lines and nude mice models have suggested that ERα antagonists can repress the
596 tumorigenicity of prostate cancer (Hariri, et al. 2015). Intriguingly, there is recent evidence
597 that abiraterone, used frequently in advanced prostate cancer is able to activate ER. Capper,
598 et al. demonstrated an increase in proliferation of MCF-7 and T47D breast cancer cell lines
599 when treated with abiraterone. The proliferative effects were diminished when the cells were
600 treated with ER antagonist ICI 182,78 (Capper, et al. 2016). ER-mediated progression of
601 prostate cancer might thus constitute a novel mechanism of resistance to abiraterone that
602 warrants further investigation. The signalling mechanisms of ERα and ERβ are summarised
603 in Figure 4.

604

605 In addition to the two nuclear ERs, ERα and ERβ, another relatively recently discovered ER
606 exists. G-protein coupled oestrogen receptor (GPER), alternatively known as GPR30, is a
607 membrane-bound receptor discovered in 1998 (O'Dowd, et al. 1998). GPER is found in 50%
608 of breast cancers and is believed to be critically involved in how Tamoxifen (a SERM)
609 resistance is developed (Mo, et al. 2013). Tamoxifen can bind and stimulate GPER in breast
610 cancer (Prossnitz, et al. 2008a) activating downstream cancer promoting pathways. GPER has
611 also been shown to be expressed in various hormone-sensitive tissues in the body including
612 the prostate (Prins and Hu 2013; Prossnitz, et al. 2007) and has very similar affinity for E_2 as
613 ERα and ERβ with almost no interaction with androgens or glucocorticoids (Prossnitz, et al.

2008b). In addition to being activated by endogenous E_2, GPER can also be activated by phytoestrogens with similar RBA as phytoestrogens have to ERβ and elicit an oestrogenic signalling pathways (Thomas and Dong 2006).

Evidence of changes in GPER expression within prostate cancer is scarce, though it has been established with immunofluorescence and immunoblotting that GPER is expressed LNCaP, DU145 and PC3 cellswhich have varying degrees of invasiveness (Maier, et al. 2006). In addition, expression of GPER has been identified by immunohistochemistry and immunoblotting in prostate adenocarcinomas and in pre-neoplastic lesions in 50 patients with confirmed prostate cancer of varying grades of aggressiveness and in 5 patients with benign prostatic disease (Rago, et al. 2016). Naturally, more research has been conducted in aggressive cell lines and primary tissues. In contrast to the effects of GPER activation in breast and ovarian cancers where it promotes growth, it has been identified that treatment of castration-resistant prostate cancer with a specific GPER agonist, G1, actually inhibits the growth of prostate cancer in PC-3, DU145 and LNCaP cell lines *in vitro* and *in vivo* PC3 xenografts (Chan, et al. 2010; Lam, et al. 2014). While most studies only reported tumour inhibition in castration-resistant cell lines, Lam et al. found that G1 treatment has no effect on androgen-sensitive LNCaP cells *in vitro* and *in vivo* xenograft mouse models whereas it had a significant effect on castration-resistant tumours without apparent toxicity to the host (Lam et al. 2014). Furthermore, GPER expression is significantly increased in androgen-deprived environments compared to androgen-replete milieus (Prins and Hu 2013)with increased GPER expression also evident in cells isolated from distant metastases in patients with CRPC CRPC compared to tissue from primary prostate cancers (Lam et al. 2014). Androgen receptor activation downregulates GPER expression thus explaining why expression of GPER is greater in androgen deprived environments (Lam et al. 2014). The mechanisms by

639 which the GPER agonist G1 has anti-tumour effects has been explored in PC3 cell line *in*
640 *vitro* and *in vivo* xenograft castrated mice models and is reported to be via up-regulation of
641 p21 and consequent cell cycle arrest at G2 phase (Chan et al. 2010). Although GPER
642 activation inhibits growth of prostate cancer, it increases proliferation of other tissues
643 including testicular germ cells and urothelial cells of the bladder and urinary tract (Chevalier,
644 et al. 2011; Huang, et al. 2015). The fact that GPER activation can have opposing effects in
645 different tissues through the same pathway illustrates the complexity of intracellular
646 oestrogen signalling. Figure 4 grossly summarises GPER signalling pathways that have thus
647 far been identified in prostate cancer.

648

649 **Conclusion**

650 This review has presented evidence that suggests an imbalance of circulating oestrogens and
651 androgens may be responsible for changes to the development and progression of prostate
652 cancer. In addition to endogenous oestrogen availability, exposure to exogenous oestrogens
653 in the form of phytoestrogens may also have a profound effect. However, there is substantial
654 evidence that intratumoural synthesis of oestrogens, and indeed androgens, plays a significant
655 role as the prostate is endowed with the ability to express key enzymes required for oestrogen
656 synthesis. There is a relationship between stage of disease and level of expression of these
657 enzymes, as is evident from the emergence of resistance to anti-androgen therapy further
658 supports this hypothesis.

659

660 Changes in the expression pattern of ERα and ERβ greatly affect whether oestrogens are
661 tumour promoting or tumour suppressing. In normal prostate and during early stages of

prostate cancer where ERβ is the prominent ER, oestrogens may be beneficial as ERβ activation initiates apoptotic pathways. Perhaps this is why a lifetime of increased phytoestrogen consumption can reduce the risk of prostate cancer development. In late stage prostate cancer where ERα is the dominating ER within the prostate, oestrogens are deleterious as ERα activation regulates cell proliferation through PI3K and MAPK signalling. Activation of GPER inhibits growth of prostate cancer however, GPER is not uniformly expressed in all prostate cancer and thus any GPER targeted therapy will be of benefit to a limited number of patients. Figure 5 summarises how the expression of ERs change during the progression of prostate cancer.

Before any definitive conclusions can be drawn over whether oestrogens are good or bad for prostate cancer, further research has to be conducted exploring the signalling pathways of ER within prostate tissue. In addition an understanding of the mechanisms behind abiraterone (Romanel, et al. 2015) and enzalutamide resistance (Claessens, et al. 2014), and whether this is linked to altered androgen and oestrogen metabolism, will be required before the next big step is taken towards development of endocrine therapy for prostate cancer.

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Figure Legends

Figure 1: Molecular similarities between phytoestrogens and E_2. E_2 contains the cyclopenta[α]phenanthrene ring structure common to all steroid molecules. Isoflavonesand coumestans are two common categories of phytoestrogens and have a molecular structure similar to E_2. As a result phytoestrogens can also bind and activate the oestrogen receptors.

Figure 2: Oestrogen and Androgen synthesis pathways.

Intratumoural E_2 can be formed from desulfation and reduction of circulating oestrone-sulphate (E_1S) by steroid sulphatase (STS) and 17β-hydroxysteroid dehydrogenase (HSD). Alternatively, oestrogens can be produced from androstenedione or testosterone by aromatase. Aromatase competes with 5α-reductase (SRD5A1), responsible for potentiating androgens, for these substrates. DHEA, the precursor for androstenedione, is most likely derived from the large pool of circulating DHEAS by STS, as intratumoural synthesis from progestogens remains disputable.

Figure 3: The expression of ERα and ERβ changes during prostate cancer progression.

During development of prostate cancer the ERβ isoform is downregulated in epithelial cells. On the other hand, ERα is upregulated in tumour cells as well as the surrounding environment. The remainder of the 'normal' prostate retains its existing expression of ERα and ERβ

Figure 4: Signalling pathways in prostate cancer through ERα, ERβ and GPER. ERα and ERβ bind to the oestrogen response elements (ERE) of DNA and regulate transcription. Activation of ERα induces mitogenic pathways via PI3K which in turn promotes HIF-1α which activates anti-apoptotic pathways; whereas activation of ERβ induces apoptosis, cell cycle arrest and inhibits dedifferentiation pathways. GPER activation in prostate cancer is anti-tumourigenic as it upregulates p21 and induces cell cycle arrest.

Figure 5: The altered expression of ERs during prostate cancer development. Changes in ERα and ERβ have been studied throughout the evolution of prostate cancer; however, expression of GPER in normal prostate and early stages of prostate cancer is currently unknown.

Fig. 1

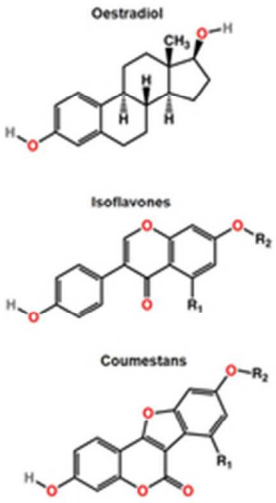

Figure 1
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Fig. 2

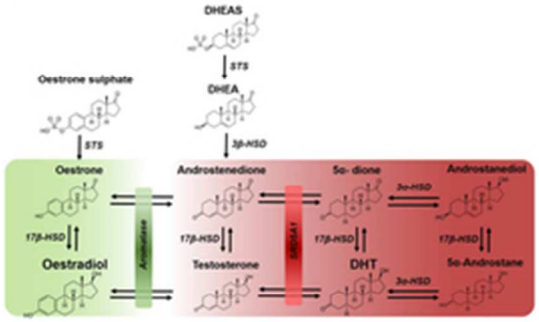

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Fig. 3

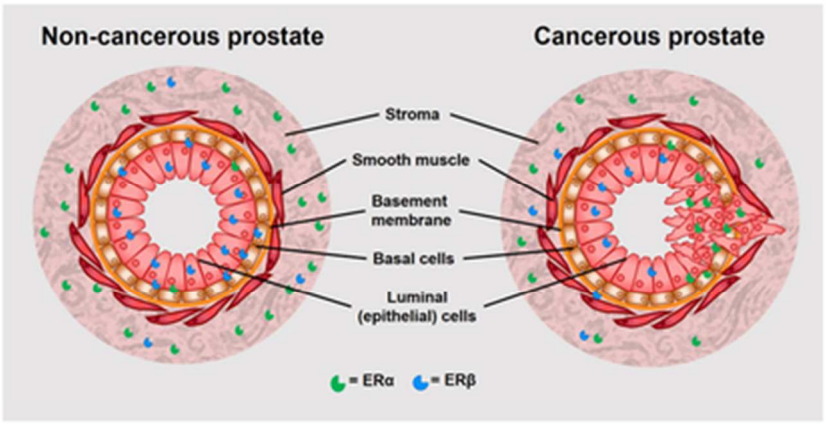

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Fig. 4

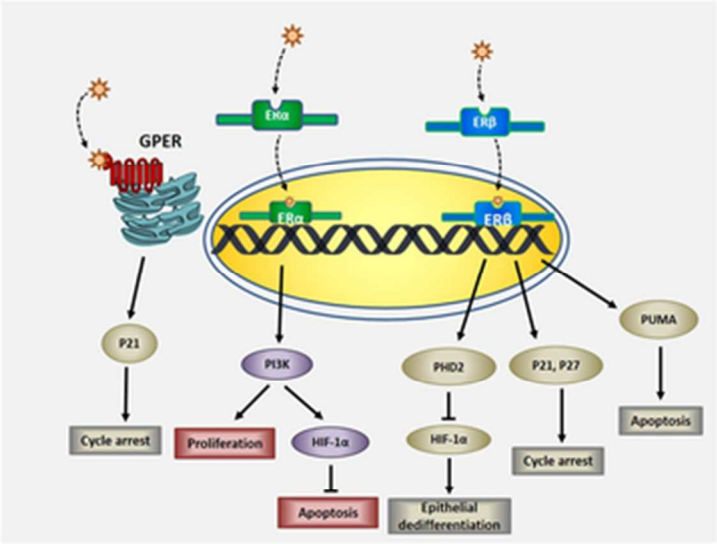

Figure 4
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Fig. 5

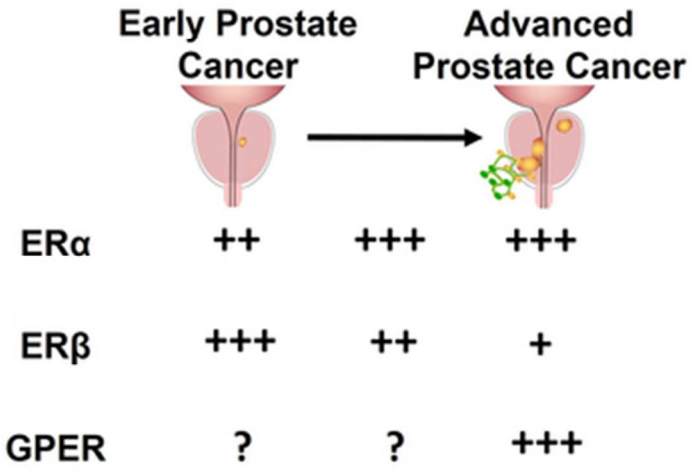

Figure 5
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