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# SEX STEROID METABOLISM AND ACTION, INFLAMMATION AND PROSTATE CANCER: HYPOTHETICAL MECHANISMS AND NETWORKS


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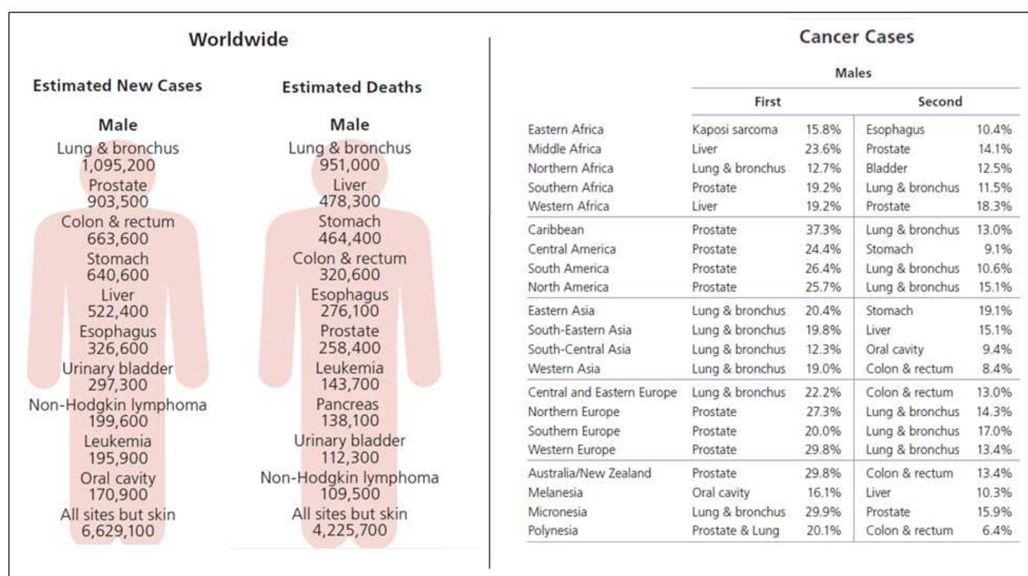
# *ABSTRACT*

In many developed countries, prostate cancer is the most common male tumor. Because of the high incidence and mortality rates, early diagnosis, along with prediction of clinical outcome and understanding of the pathogenesis with its typical metabolic aberrancies, is awaited with expectation. It is today widely accepted that inflammation has a role in many human cancers, including prostate cancer. Inflammation is thought to incite carcinogenesis by causing cell and genome damage, promoting cellular turnover and creating a tissue microenvironment that can foster cell replication, angiogenesis and tissue repair. Accordingly, several studies have suggested chronic inflammation of the prostate gland may be associated with an increased risk of developing prostate cancer. In this work I indicate that, in an inflammatory environment, the human prostate gland may synthesize high amounts of estrogens via the local over-expression of the aromatase enzyme, suggesting that these changes can be important in the emergence and/or the progression of prostate cancer. In this latter, the expression of key players, such as COX-2 and inflammatory cytokines, is significantly altered, favoring cell proliferation and minimizing programmed cell death. In addition, estrogens are able to induce amphiregulin expression that can, in turn, activate the EGFR signaling, becoming critical for the prostate cancer development. Taken together, these data strongly support a crucial role of estrogen in the malignant prostate. A better understanding of the mechanisms underpinning endocrine, paracrine and autocrine activity of sex steroids and their relationship with the inflammatory process in both normal and

diseased prostate gland is pivotal to develop new strategies for hormonal treatment of human prostatic carcinoma.

## 1.1 Epidemiology, risk factors and prevention

Prostate cancer represents the second most common cancer in men in the world and the sixth cause of cancer-related mortality, with 903,500 new cases and 258,400 deaths recorded worldwide in 2008 (Figure 1) [1]. The highest rates are observed primarily in Northern Europe, North America, and Oceania. In Europe, prostate cancer is the commonest type of cancer in men, although incidence varies considerably across Northern and Southern Europe, as shown in Figure 1[1]. Both genetic and environmental factors may contribute to explain this large geographic variation. Studies on populations migrating from countries with low incidence/mortality rates (e.g., China or Japan) to countries with higher rates of prostate cancer (United States), have revealed, within a generation, a significant increase in prostate cancer incidence/mortality as compared with their peers in the countries of origin[2-4].




| | Males | | | |
|---|---|---|---|---|
| | First | | Second | |
| Eastern Africa | Kaposi sarcoma | 15.8% | Esophagus | 10.4% |
| Middle Africa | Liver | 23.6% | Prostate | 14.1% |
| Northern Africa | Lung & bronchus | 12.7% | Bladder | 12.5% |
| Southern Africa | Prostate | 19.2% | Lung & bronchus | 11.5% |
| Western Africa | Liver | 19.2% | Prostate | 18.3% |
| Caribbean | Prostate | 37.3% | Lung & bronchus | 13.0% |
| Central America | Prostate | 24.4% | Stomach | 9.1% |
| South America | Prostate | 26.4% | Lung & bronchus | 10.6% |
| North America | Prostate | 25.7% | Lung & bronchus | 15.1% |
| Eastern Asia | Lung & bronchus | 20.4% | Stomach | 19.1% |
| South-Eastern Asia | Lung & bronchus | 19.8% | Liver | 15.1% |
| South-Central Asia | Lung & bronchus | 12.3% | Oral cavity | 9.4% |
| Western Asia | Lung & bronchus | 19.0% | Colon & rectum | 8.4% |
| Central and Eastern Europe | Lung & bronchus | 22.2% | Colon & rectum | 13.0% |
| Northern Europe | Prostate | 27.3% | Lung & bronchus | 14.3% |
| Southern Europe | Prostate | 20.0% | Lung & bronchus | 17.0% |
| Western Europe | Prostate | 29.8% | Lung & bronchus | 13.4% |
| Australia/New Zealand | Prostate | 29.8% | Colon & rectum | 13.4% |
| Melanesia | Oral cavity | 16.1% | Liver | 10.3% |
| Micronesia | Lung & bronchus | 29.9% | Prostate | 15.9% |
| Polynesia | Prostate & Lung | 20.1% | Colon & rectum | 6.4% |

**Figure 1.** Left: Estimated new cancer cases and deaths worldwide for leading cancer sites; Right: the two most common types of new cancer cases. Source: GLOBOCAN 2008.

This evidence suggests that lifestyle and dietary factors may play a central role in development and progression of human prostate cancer. Several studies have hypothesized that plant hormones contained in Asian diets, particularly the phytoestrogens present in soy products, might act as natural hormone antagonists and anticancer agents and that their intake could be associated with a decrease of prostate cancer risk. A recent review [5] of epidemiological studies on the association of soy and other nutrients containing phytoestrogens with the risk of developing prostate cancer has shown contradictory results, with only a few studies reporting a risk reduction associated with the intake of soy food, legumes, and isoflavones. In a meta-analysis of 8 epidemiological studies, Yan and Spitznagel indicated that the consumption of soy food is related to a nearly 30% reduction of prostate cancer risk, despite only 3 studies in the analysis showed statistically significant lower risk of prostate cancer [6]. Several studies in Asian men have also reported a trend toward a decreased prostate cancer risk with increased equol (a gut bacterial product of the isoflavonedaidzein) intake. In addition, both lower equol concentrations and a lower prevalence of equol-producers have been observed in Asian populations among men with prostate cancer as compared with controls, whereas studies in European populations have reported no association [7].

Several studies have supported the potential role of estrogen role in prostate cancer progression, with sex hormones behaving as intermediaries between exogenous factors and molecular targets (Figure 2). In this framework, endogenous sex steroids, along with genetic factors, environmental factors (including diet) host immune and inflammatory responses, are likely to concur in the pathogenesis of this disease. [8-11].

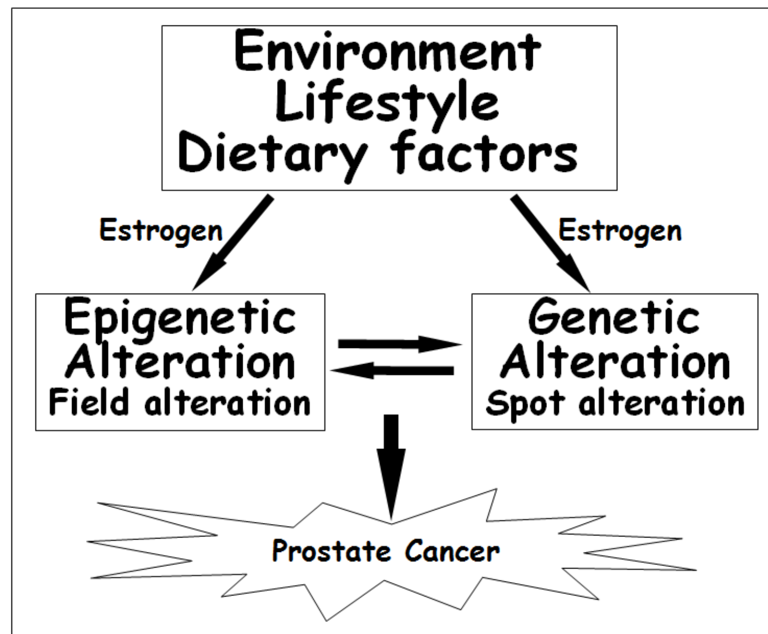



**Figure 2.** Main aspects affecting development and progression of prostate cancer.

Temporal trends in mortality rates of prostate cancer are easier to interpret than trends in incidence rates, as they are less or not affected by PSA testing. Incidence trends follow in fact a consistent pattern in countries with a widespread use of PSA, including Australia, Canada and United States, with a rapid rise in incidence in prostate cancer in the early 1990s, soon after the introduction of PSA testing, followed by a quick decline [12]. Death rates for prostate cancer have been decreasing in many developed countries, including Australia, Canada, Finland, France, Israel, Italy, Netherlands, Norway, Portugal, Sweden, United Kingdom and United States [12]. In contrast, mortality rates are rapidly rising in some Asian and Eastern European countries, such as Japan and Poland. While the decrease of prostate cancer death rates in Western European and North American countries has been ascribed mainly to earlier diagnosis and improved treatment, the increase in Asian and Eastern European countries has been thought to be a reflection of "westernization", including

increased consumption of animal fat, obesity and physical inactivity [13]. Some studies suggest that a diet high in processed meat may also be a risk factor [14,15] and that risk of dying from prostate cancer is increased in obese [16]. Nevertheless, the only well-established risk factors for prostate cancer are older age, race (black), and family history [17]. Recent genetic studies suggest that a strong familial predisposition may be an important factor to predict an increased risk of prostate cancer [18]. Although, modifiable risk factors for prostate cancer are not understood well enough to make definitive recommendations for preventive measures, factors that may reduce risk include maintaining a healthy body weight, getting regular physical activity and consuming a diet low in animal fat and high in fruits and vegetables. Evidence on the value of testing for early prostate cancer detection is insufficient to recommend for or against screening with PSA for men at average risk [19]. The American Cancer Society recommends that men who are at average risk of prostate cancer, do not have any major medical problems and have a life expectancy of at least 10 years, should be informed about the benefits and limitations of testing for early prostate cancer detection beginning at age 50 and have an opportunity to make an informed decision about testing [20].

Treatment options vary depending on age, stage, and grade of the tumor, as well as other medical conditions. Surgery, external beam radiation, or radioactive seed implants may be used to treat early stage disease. Hormonal therapy, chemotherapy and radiation, alone or in combinations, are used for metastatic disease and as a supplemental or additional therapy for early stage disease. Hormone treatment may control prostate cancer for long periods by shrinking the size or limiting the growth of the cancer, thus relieving pain and

other symptoms. Careful observation (*watchful waiting*) rather than immediate treatment may be appropriate for some men with less aggressive tumors, especially older men with limited life expectancy and/or other health considerations. Over the past 25 years, a dramatic improvement in survival has been observed, partly attributable to earlier diagnosis of asymptomatic cancers and improvements in treatment. The five-year relative survival rate for patients diagnosed with prostate cancer in the United States approaches 100% [21] and in Europe ranges from 48% (Denmark) to 87% (Austria) [22]. In sub-Saharan African and Southeast Asia the five-year survival rate is less than 40% in most countries [23].

## 1.2 Prostate cancer and sex hormones

### *1.2.1 Sex steroid metabolism*

Men and women synthesize both androgens and estrogens, but the relative ratio of the two hormones between the two sexes is markedly different. The importance of androgens to the male is unequivocal, whereas the roles of estrogens are less clear. Estrogen synthesis occurs via aromatization of androgens through the aromatase enzyme (cytochromeP450arom), with aromatase representing a critical regulator of the balance between androgens and estrogens and their circulating and tissue levels (Figure 3). In men, the balance between plasma levels of androgens and estrogens is significantly altered upon aging. Plasma androgen levels decline whereas estradiol levels remain relatively constant [24].In specific tissues of the body, the balance between androgens and estrogens differ significantly from that in the plasma

because it is dependent upon the expression and activity of steroid metabolizing enzymes, such as 5α-reductase and aromatase [11,25-27].

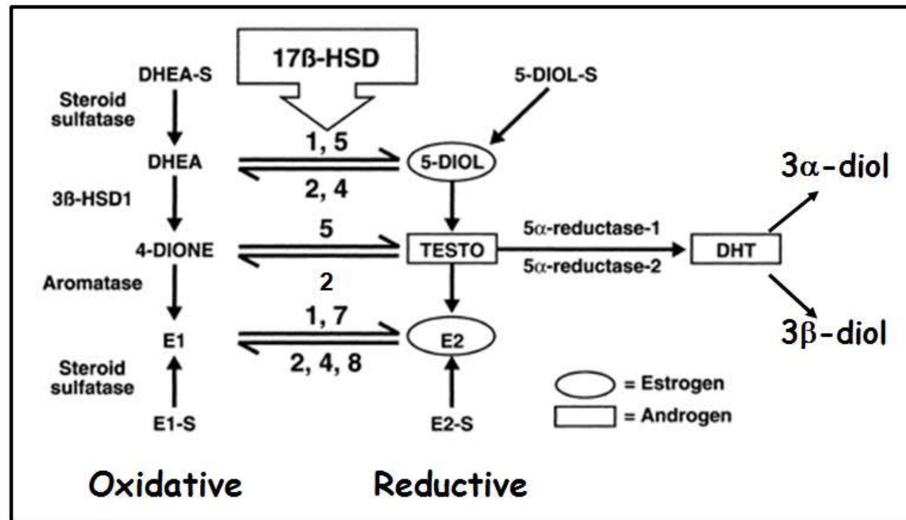



**Figure 3.** Pathways of sex steroid metabolism in peripheral target tissues. Abbreviations: TESTO, testosterone; 4-DIONE, androstenedione; DHT, dihydrotestosterone; 3α-diol, 3α-androstanediol;3β-diol, 3β-androstanediol; DHEA, dihydroepiandrosterone; 5α-DIOL, 5α-androstanediol; E2, estradiol; E1, estrone; E2-S, estradiol-sulfate; E1-S,estrone-sulfate.

The critical role of local synthesis of steroids has assumed increasing importance in some disease states, particularly in glandular tissue such as the breast, wherein abnormal levels of estradiol may promote either malignant transformation or cell proliferation in the early stages of tumor development [28,29]. Abnormal expression of aromatase is believed to contribute to the development and progression of human breast cancer [28,29]. As there is increasing evidence that the prostate is a direct target for estrogenic activity [30-32], it is important to determine whether or not aromatase is expressed locally and to identify any changes that may occur with prostate disease. The Rancho Bernardo study, conducted in California, revealed an association of elevated plasma estradiol and estrone with an increased risk of prostate cancer [33]. Two more recent nested case-control studies on serum levels of both androgens and

estrogens failed to show any association with prostate cancer risk [34,35]. Interestingly enough, one of the two studies has reported a positive association of plasma total testosterone with low-grade disease and an inverse association with high-grade disease [34].Recently, a limited but significant decrease of prostate cancer risk has been associated with increasing serum levels of total testosterone [36]. In a study on hypogonadal men, Morgentaler and colleagues [37] reported that subjects with PSA levels <4.0 ng/mL had a 15% overall rate of prostate biopsies positive for cancer. Interestingly, subjects with plasma levels of testosterone <250 ng/dL had a prostate cancer rate of 21%, as opposed to 12% for men with a testosterone level >250 ng/dL. Furthermore, the probability of cancer in men in the lowest tertile was over twice as much as that in men in the highest tertile of both total and free testosterone. Several studies have scrutinized the relationship between pretreatment serum levels of testosterone with clinical stage of prostate cancer and patient survival, suggesting that low serum testosterone could be used as a negative prognostic predictor for this neoplasia.

To date, investigators have raised the question why it has been so difficult to demonstrate that plasmatic androgens are associated to an increased risk of developing prostate cancer. The most obvious answer to this question is that circulating androgens are simply not associated with prostate cancer risk. It should be taken into consideration, however, that several issues related to measurement of plasma steroids, both androgens and estrogens, could be contemplated to explain this large inconsistency of data. They include the low statistical strength of most studies, the limited number of incident cases in prospective studies, the minor differences in sex steroid serum levels between

cases and controls, and the rather large intra- and inter-assay laboratory variations of serum hormone measurements [38]. On the other hand, several other variables, including obesity, physical activity, diabetes, metabolic syndrome and benign prostatic hyperplasia, that might have an impact on serum levels of hormones and/or have been related to prostate cancer, have not been adjusted for in previous nested case-control studies [39].

In any case, it is unlikely that a single assay of plasmatic androgens can be regarded as descriptive of average androgen levels over an etiologically relevant period of life. In this respect, since the length of prostate carcinogenesis and tumor progression can span 35-40 years or longer, the timing for the carcinogenetic activity of androgen and/or estrogen on human prostate should be counted 20-30 years (or even earlier) prior to the clinical manifestation of the disease, when serum androgens are higher and, hence, could be biologically relevant.

So far, aromatase expression in either *nontumoral* or malignant prostate is controversial, with various studies that have detected or failed to detect aromatase activity in prostatic tissues [40-43].

### *1.2.2 Estrogens in prostate tumor development and progression*

Cellular signaling of estrogens is mediated through two ERs, ERα and ERβ, both belonging to the nuclear receptor superfamily of transcription factors. Estrogen receptors contain specific and functionally distinct domains. The central and most conserved domain, the DNA-binding domain (DBD), is involved in DNA recognition and binding, whereas ligand binding occurs in the COOH-terminal multifunctional ligand-binding domain (LBD). Transcriptional

activation is facilitated by two distinct activation functions (AF), the constitutively active AF-1 located at the NH2 terminus of the receptor and the ligand-dependent AF-2, that resides in the COOH-terminal LBD. Both AF regions recruit a range of coregulatory proteins that are bound in complexes to the DNA-bound receptor. The two ERs share a high degree of sequence homology except in their NH2-terminal domains, and they have similar affinities for 17β-estradiol (E2) and bind the same DNA response elements (Figure 4).

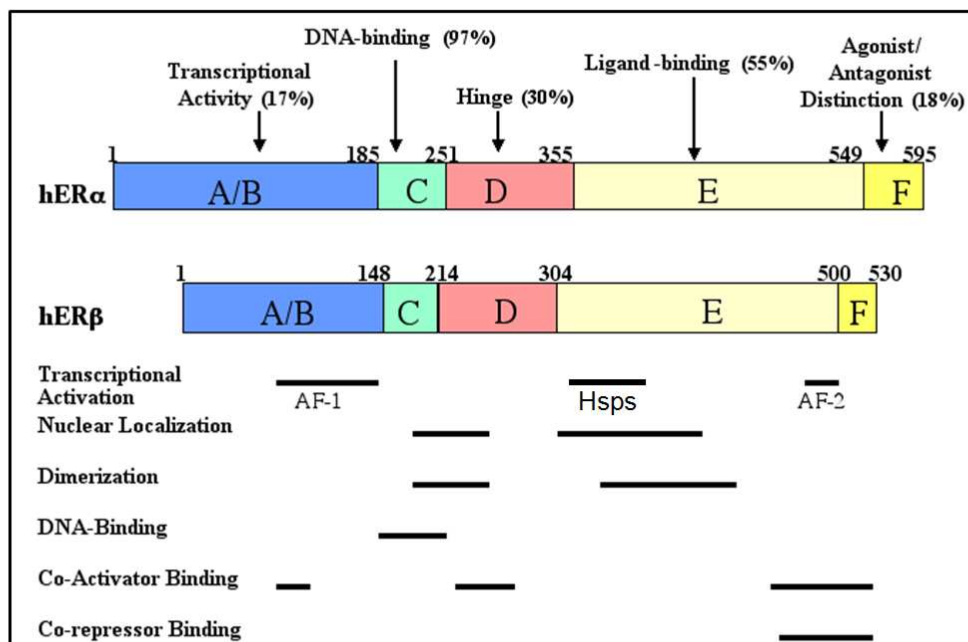



**Figure 4.** Functional domains of the two major estrogen receptor proteins.

Ligand-dependent estrogen signaling begins with the binding of estrogen to ERs. Afterward, the cell-specific transcriptional response to the estrogen depends on multiple factors, with the coregulatory complex being fundamental to activate estrogen-responsive genes. Since hormones are transcriptional modulators, the pattern of regulated genes also depends on other signaling pathways that are active in the cell at the time of hormone exposure [44-46].The more recent identification of a second ER, the ERβ, and the identification of

several receptor isoforms, has confirmed the complex nature of estrogen signaling and helped understanding estrogen activity in tissues that do not express ERα. Several splice variants have been described for both receptor subtypes (Figure 5), but it remains unclear whether these variants are expressed as functional proteins and endowed with biological functions.

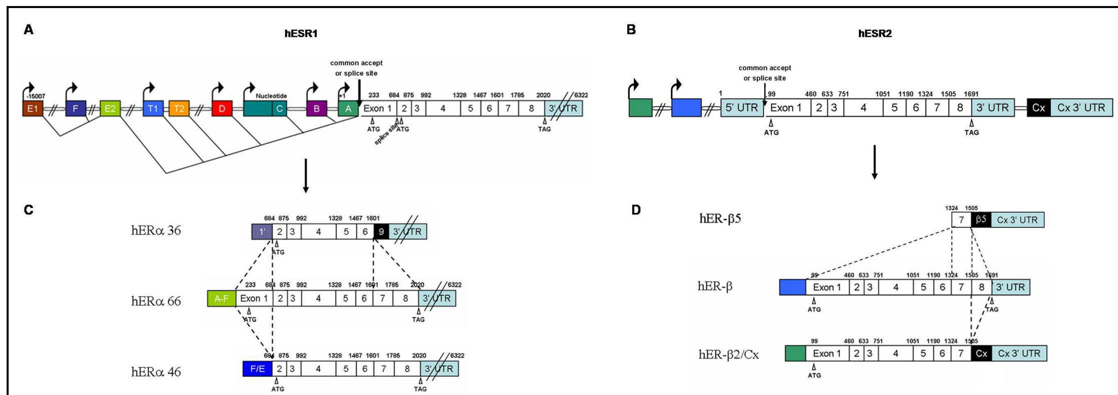


**Figure 5.** A) and B) Genomic organization of the human Esr1 and Esr2 gene respectively. The location of multiple promoters and corresponding upstream exons of the human ERα gene and human ERβ gene are shown left of +1. Upstream exons are represented by numbered boxes and their promoters shown as arrows. Downstream Exons are numbered in the corresponding blocked region with the nucleotide number above. The ATG start codon, splice sites and the TAG stop codon are also identified. C) and D) Some mRNAs splice variant of the human ERα and human ERβ from hEsr1 and hESR2 gene respectively.

Shorter hERα isoforms lacking exon 1 or exon 1 and 7-8, termed respectively hERα46 and hERα36, have been identified both in *vivo* and *vitro* [47-50], although their role in regulating estrogen effects remains to be determined. Functionally, hERα46 lacks the amino-terminal A/B domain present in the full-length hERα66 and is consequently devoid of the AF1 function. The hERα36, consists of a 36 kDa protein variant which lacks both ligand-dependent and-independent transactivation regions (AF-1 and AF-2), but retains the ligand- and DNA-binding domains [48]. Interestingly, both ERα variants have the ability to heterodimerize with the full-length hERα, thereby repressing AF-1-mediated

activity [47,49]. They may also localize to the plasma membrane, being implicated in mechanisms through which rapid, "*nongenomic*" estrogen signaling occurs [49,51]. In particular, ERα36 is expressed both on the plasma membrane and in the cytoplasm, where it mediates membrane-initiated effects of estrogen signaling, including activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), and stimulation of cell proliferation [49]. Today, there is convincing evidence that estrogens may exert an amazing array of biological activities through distinct, ER-related pathways (Figure 6) [52].

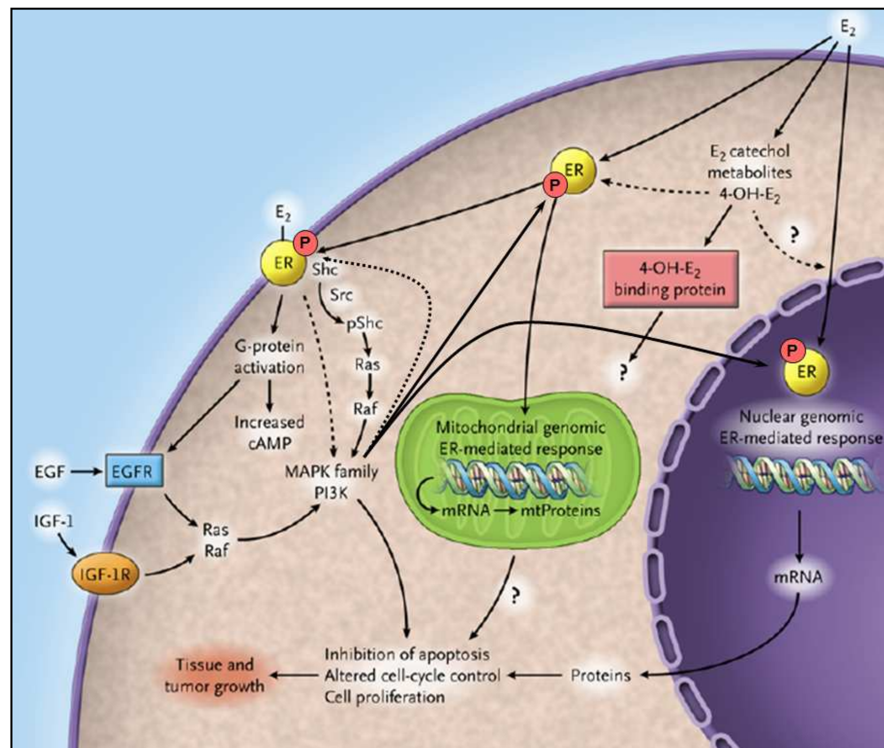



**Figure 6.** Major estrogen signaling mechanisms. Adapted from Yager & Davidson, 2006.

In fact, other than the classical transcriptional action of E2, rapid effects, that occur within seconds or minutes after addition of E2, have been described [53,54]. These rapid effects include activation of kinases and phosphatases and

the increase in ion fluxes across membranes. Also, growth factor signaling may eventually lead to activation of kinases that in turn phosphorylate and thereby activate ERs and/or recruit coregulators in the absence of ligand [55]. Although these rapid effects have been extensively studied, there is still no agreement as to whether or not the classical ERs are involved [56,57] or, rather, there is a distinct membrane-associated receptor [58].

The concept that human prostate cancer represents a paradigm of androgen-dependent tumor has endured for decades against a bulk of experimental evidence suggesting that estrogens and other growth factors may be at least equally important in prostate carcinogenesis and tumor progression [10]. The expression and functional status of ERs appear to play a significant role in the carcinogenesis of all hormone sensitive organs including prostate.

Recent experimental evidence suggests that prostate cancer originates from precancerous lesions, such as chronic proliferative inflammatory atrophy (PIA), as a consequence of prostate tissue injury [59]. Normally, in response to tissue injury, the prostate stem cell compartment, that represents a minority (1-3%) of basal epithelial cells and has been located at the basement membrane of the prostatic glandular epithelium, would give rise to a population of transit-amplifying/intermediate cells that would, in turn, terminally differentiate and generate luminal secretory and neuroendocrine epithelial cell types. It is speculated that tumor-initiating cells could arise during the prostate regeneration process within the pool of prostate stem cells, when their differentiation ability is somehow impaired by a mutation activating oncogenic and/or abrogating tumor suppressor signaling pathways [60]. The resulting progeny of cells would clonally expand and undergo the promotion and

progression phases of the multistep carcinogenetic process, eventually leading to create a population of cancer stem cells featured by unrestricted replicative potential and reduced apoptosis. In this context, estrogens have been reported to up-regulate both expression and activity of telomerase in human prostate epithelial cell lines, an event that is generally associated with unlimited cell proliferation [61].

Cavalieri and Rogan [62] have produced consistent experimental evidence in support of their hypothesis that selected tissue estrogen metabolites, notably the electrophilic catechol estrogen-3,4-quinones, may react with DNA and generate depurinating estrogen-DNA adducts. After adducts are released from DNA, error-prone base excision repair of the resulting apurinic sites may lead to mutations that can be critical to initiate breast, prostate, and several other human cancers.

Some studies have reported that long-term administration of testosterone to rats induces the development of prostate tumors, suggesting that testosterone acts as a complete carcinogen on the rat prostate, though in a limited proportion of cases and in some but not all rat strains [63-65]. However, when Noble rats were used as model system, the administration of testosterone and estradiol, in sequence or combined, resulted in the occurrence of both ductal and acinar epithelial dysplasia, followed within 1 year by the development of adenocarcinomas of the dorsolateral prostate in 90-100% of the animals [66]. If rats were treated with androgen alone, the incidence of prostate cancer dropped to 35-40% [67].

The mechanisms underpinning the hormonal carcinogenesis in the rat prostate remains largely undefined, but there is evidence to suggest that both receptor-

mediated and *nonreceptor* effects may be implicated. As far as estrogens are concerned, the development of dysplastic lesions in the dorsolateral prostate of rats exposed for 16 weeks to a combination of testosterone and estradiol was almost completely abrogated by the simultaneous administration of the pure antiestrogen ICI-182,780[68]. However, since ICI-182,780 also induces a block of the hyperprolactinemia produced in rats by estrogen treatment, it is difficult to establish whether the effects of this estrogen antagonist are a consequence of binding to estrogen receptor or not. Although most studies on hormonal carcinogenesis of the prostate have been conducted on rodents, it ought to be emphasized that the rat prostate, consisting of dorsal, lateral, ventral and anterior lobes, has embryology and anatomy distinct from human. Therefore, results of these studies should be interpreted with caution.

Both epidemiological and experimental evidence presented herein supports the view that prostate cancer arises in the aging male in an estrogenic environment. However, the ultimate biological impact of sex steroids, particularly estrogen, on prostate cancer cells is difficult to dissect as it is strictly dependent upon several variables, including the estrogen:androgen ratio in both plasma and prostate, the expression and activity of steroid enzymes, the binding to intracellular and/or membrane receptors, the exploitation of genomic and/or *nongenomic* mechanism(s) of action. Previous studies have assessed the proliferative effects of sex hormones in cultured prostate cancer cells. Although several reports have shown that androgens markedly stimulate prostate cancer cell growth [69,70], unequivocal evidence for a direct increase of DNA synthesis brought about by bioactive androgens in prostate tumor cell lines is surprisingly rare and often conflicting (if any). The inconsistency of the results obtained in

cell model systems does not allow to draw any truthful interpretation also because different variables, including culture and experimental conditions, age of cultured cells, and exposure to endogenous hormones and growth factors may considerably affect the results.

Various in vitro studies carried out on LNCaP cells have indicated that both androgen and antiandrogen stimulate growth of these cells [71]. It has been previously reported that the exposure to physiological estrogen concentrations may either stimulate or decrease growth of androgen-responsive LNCaP and -refractory PC3 prostate cells, respectively, and that these effects are predominantly receptor-mediated, being completely abrogated by the simultaneous addition of the pure estrogen antagonist ICI-182,780 [72,73]. This evidence implies that estrogen may affect proliferative activity of prostate cancer cells even if the cells have become androgen-resistant. This finding is also corroborated by the significant rates of clinical response to the systemic administration of estrogens observed in prostate cancer patients having a metastatic, androgen-refractory disease [74]. Other authors have revealed that tamoxifen (a *mixed antiestrogen*) and ICI-182,780 (a *pure antiestrogen*) inhibit growth of both DU145 and PC3 prostate cancer cell lines and have cytotoxic effect on DU145 cells. Based on the finding that the proliferative effects of estrogens on human prostate cancer cells in culture appear to be typically receptor-mediated, it would be important to assess the ER content and the balanced expression of different ER types and their variants *in vivo*.

## 1.3 The epidermal growth factor receptor axis in prostate cancer

In the human prostate, epithelium-lined ducts exist in intimate contact with smooth muscle cells and undifferentiated fibroblasts of the fibromuscular stroma. Moreover, homeostatic interactions between the epithelial compartment and the differentiated stromal compartment are believed to be important in the maintenance of prostate tissue function in the adult [75]. Stromal-epithelial interactions have also been proposed to determine the natural history of tumors arising from epithelial organs and descriptive observations of carcinomas support the relevance of a stromal-epithelial interaction in tumor progression [76-79]. Tumor stroma has been documented to “react” to the presence of associated carcinoma cells by altering(usually increasing) the expression levels of specific secreted proteins capable of paracrine signaling which have been identified as being up-regulated in the reactive stroma [80-82].

The epidermal growth factor receptor (EGFR) is a 170-kDatransmembrane glycoprotein that has been identified in normal, hyperplastic and malignant prostatic epithelium [83-85].Growth factors and their receptors have received much attention as potential targets for the treatment of prostate cancer [86,87]. Epidermal growth factor (EGF), the related transforming growth factor-α (TGFα) and amphiregulin (AREG), are the main autocrine/paracrine growth factors that have been identified in prostate cancer specimens and cell lines [88-90]. Autocrine activation of EGFR has been proposed as a mechanism to support growth and invasiveness of malignant prostate epithelial cells [91-93]. The epidermal growth factor receptor is the most extensively studied member ofthe ErbB family, which includes ErbB2 (HER-2/Neu) [94], ErbB3 (HER-3) [95], and ErbB4 (HER-4) [96]. Many cells co-express multiple ErbB receptors that

homodimerize and/or heterodimerize upon stimulation with respective ligands [97]. Activated ErbB receptors trigger a number of important intracellular signaling pathways, including the phosphoinositide3-kinase (PI3K) and the mitogen-activated protein kinase/extracellular-related kinase 1/2 (MAPK/ERK1/2) pathways. PI3K activity acts as a membrane-associated second messenger that binds and recruits a variety of cytosolic signaling enzymes to the cell membrane [98]. One of these, the serine/threonine kinase Akt, becomes phosphorylated at both Thr308 and Ser473 residues and promotes cell survival through multiple mechanisms [99]. The phosphorylation status of Akt (P-Akt), *in vitro* and*in vivo*, has been widely used to monitor aspects of malignant behavior such as proliferation, resistance to chemotherapy, irradiation, invasion, and metastasis [100,101].The MAPK cascade constitutes a signaling pathway that links surface receptor–mediated signals to nuclear events affecting cellular processes of growth, division, differentiation, and death [102]. Phosphorylation of the most downstream elements, p44 and p42 MAPKs (also called ERK1 andERK2), is a hallmark of MAPK activation and is increased in many human tumors, including prostate cancer [103,104].

Amphiregulin is an epidermal growth factor family member initially purified from conditioned media of MCF-7 cells treated with 12-0-tetradecanoylphorbol-13-acetate [105]. This peptide induces variable effects on growth in different cell types. It is an autocrine factor for normal human mammary epithelial cells [106] and promotes growth of normal fibroblasts and keratinocytes, as well as some ovarian and pituitary tumor cell lines. Amphiregulin has not been shown to interact directly with the gene products of other members of the c-erbB family of

receptors including c-erbB2, 3, or 4 [107], and thus, the EGFR (c-erb B1/HER1) is believed to be the sole cell surface receptor for AREG in epithelial cells [90]. Amphiregulin has been reported to be overexpressed in many types of cancers including prostate [108],where it is encoded by an estrogen-regulated gene [109,110]. It has been suggested that growth factor-based autocrine loops may contribute to hormone-refractory tumor growth and that these autocrine mechanisms may allow cells to survive after steroid deprivation in human prostate cancer cells [111].

### 1.4 Inflammation and prostate cancer

Both Benign Prostate Hyperplasia (BPH) and prostate cancer represent chronic diseases whose development and progression to become clinically manifest often requires lengthy time periods[112]. In both these diseases, changes in the prostate microenvironment, including growth factors, cytokines and steroid hormones, may result in an altered regulation of prostate cell growth consisting of elevated cell proliferation and reduced apoptosis [113,114].

Inflammation is thought to play a role in the causation of all human cancers. Virchow already hypothesized that the origin of cancer is at sites of chronic inflammation in 1863 [115]. Several sources of inflammation may influence the risk of prostate cancer, including dietary [116], genitourinary bacterial [117,118], viral infections[119], and intraprostatic urine reflux [120,121]. With regard to diet, a number of nutritional factors may reduce the risk and progression of prostate cancer through antioxidant and anti-inflammatory effects (figure 7) [116]. These include $\omega$–3 polyunsaturated fatty acids (PUFAs), fish, selenium, vitamins D and E, and lycopene [116].

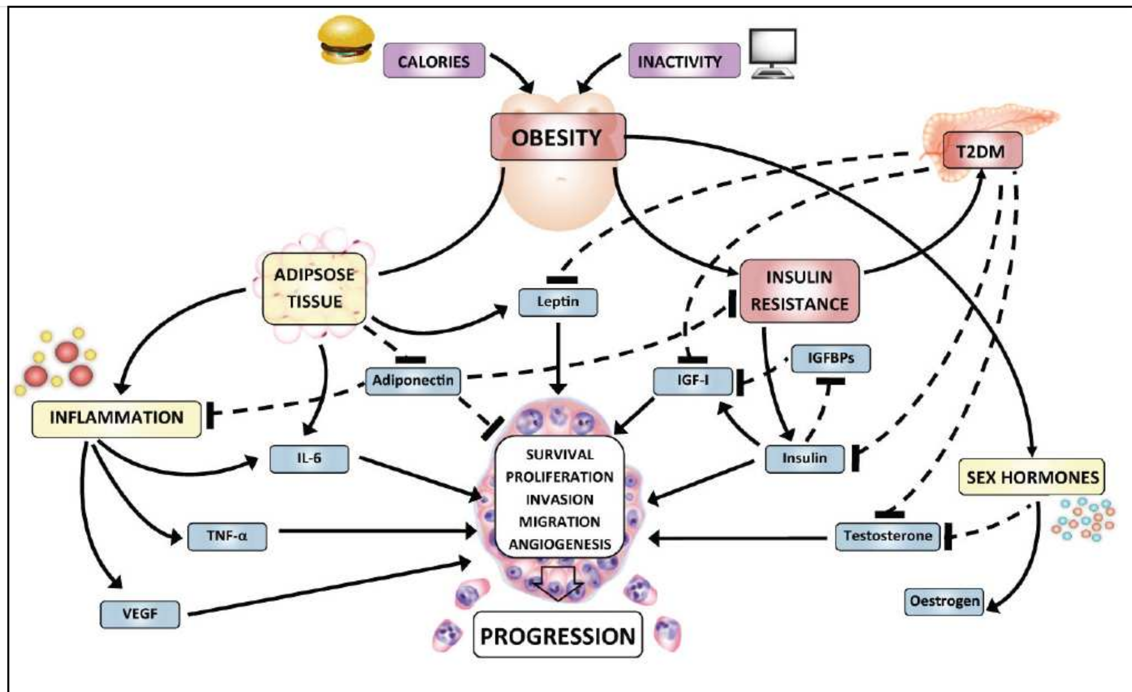



**Figure 7.** Overview of potential pathways linking metabolic disruption to prostate cancer progression. Arrows: stimulates/up regulates. Dashed lines: inhibits/down regulates. Abbreviations: T2DM, type 2 diabetes mellitus; IGF, insulin-like growth factor; IGFBP, IGF binding protein; IL-6, interleukin-6; TNF-α, tumour necrosis factor alpha; VEGF, vascular endothelial growth factor. From Anya J. Burton et al., 2010.

PUFAs are classified according to their molecular configuration: ω–3 or ω–6. The pro-inflammatory ω–6PUFAs, such as linoleic acid and arachidonic acid, are metabolized through the cyclooxygenase (COX) pathway into inflammatory eicosanoids, including prostaglandin E2, which has been linked to carcinogenesis in studies of prostate and other tumors [122,123]. In contrast, the anti-inflammatory ω–3 PUFAs, such as α -linolenic acid (ALA) 18:3, eicosapentaenoic acid (EPA) 20:5, docosahexaenoic acid (DHA) 22:6 and docosapentaenoic acid (DPA) 22:5,exhibit their anti-inflammatory properties by inhibiting competitively the arachidonic acid cascade, mainly at the COX pathway [124]. This inhibition reduces the production of pro-inflammatory prostaglandins derived from arachidonic acid, potentially preventing their carcinogenic effect. The long-chain ω–3 PUFAs, EPA, DPA and DHA, appear

to be the most potent inhibitors of the COX inflammatory pathway. Furthermore, PUFAs appear to be beneficial in the prevention and treatment of numerous disease states, including cardiovascular diseases, neurodegenerative disorders and cancer [125]. The role of inflammation in prostatic diseases is suggested by the presence of inflammatory cells within the prostate in BPH and prostate cancer patients and by the evidence that pro-inflammatory genotypes predispose to prostate cancer [126,127,128] (figure 8). Histopathological studies have confirmed that inflammation is much more common in the transition and peripheral zones of the prostate, where BPH and prostate cancer preferentially occur [129,130,131]. Inflammation is a complex phenomenon consisting of a humoral (cytokines) and cellular (leukocytes, monocytes and macrophages) components [123,132]. Inflammation is usually a self-limited event, with an initial release of pro-inflammatory cytokines/growth factors and angiogenesis, followed by an anti-inflammatory cytokine–mediated resolution [133].

In normal tissues, anti-inflammatory cytokines are synchronically upregulated after the pro-inflammatory cytokines are produced, leading to inflammation resolution, while chronic inflammation, mainly consisting of chronically activated T cells and mononuclear phagocytes, is based upon persistence of inflammatory stimuli or a failure in mechanisms required to resolve inflammation or both. This condition would result in a further release of inflammatory cytokines and various growth factors and attract additional immune cells to the inflammation site, thus amplifying the inflammatory response [133,134].

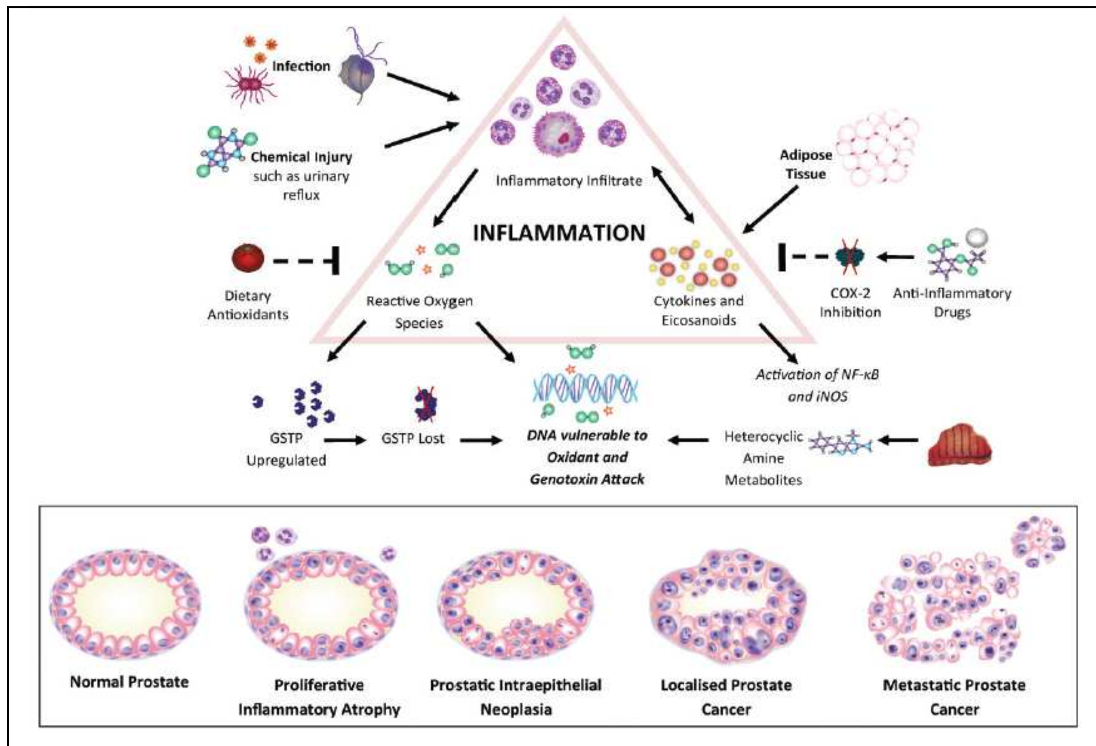



**Figure 8.** Inflammation in prostate carcinogenesis. Arrows: stimulates. Dashed lines: inhibits. Abbreviations: COX-2 cyclooxygenase-2, GSTP glutathione-S-transferase, NF-κB nuclear factor-κB, iNOS inducible nitric oxide synthase. From Anya J. Burton et al., 2010.

Stromal–epithelial interaction plays a pivotal regulatory role in the maintenance of homeostasis in healthy and diseased prostate [135,136]. In this context, Antigen Presenting Cell (APCs)and the expression of the Toll-like receptor (TLR) repertoire can produce pro-inflammatory cytokines and activate immune responses [130,137,138,139]. Most BPH tissues show a strong expression of TLR 4, 5, 7, and 9, whereas an increased expression of TLR 1, 2, and 3 is observed in prostate cancer [140]. T-cells, along with prostatic stromal and epithelial cells, secrete higher levels of pro-inflammatory cytokines, such as interleukins (IL-1, IL-1α, IL-2, IL-4, IL-6, IL-7, IL-8, and IL-17), the CXC-type chemokines and their receptors, in BPH and prostate cancer tissues when compared to normal prostate tissues [135,137,141]. These cytokines are thought to induce fibromuscular reaction and proliferation of prostatic stromal or

epithelial cell through autocrine or paracrine loops or via induction of COX-2 expression [130,139,141-143]. IL-1αproduced by epithelial cells induces in turn fibroblast growth factor-7 (FGF-7) in prostate stromal cells and this would result in benign growth of the prostate. IL-17 up-regulates the secretion of other proinflammatory cytokines, such as IL-8 and IL-6 as well as of TGF-β. IL-8 and IL-6 are recognized as two potent growth factors for prostatic epithelial and stromal cells, with IL-8 playing a major role in stromal proliferation by the induction of FGF-2 [126,144]. BPH and prostate cancer show a distinct expression of pro-inflammatory cytokines, with elevated IL-6 and IL-8 in cancer as compared to BPH tissues [137,140]. In addition, IL-6 regulates prostate tumor cell growth and activates androgen receptor-regulated genes in prostate cancer cells in the absence of androgen [137].

Chronic inflammation continuously induces COX-2 [113,115,145,146] and COX–2 in turn increases production of prostaglandin (PG) E2, concentrations of Bcl-2 protein (the product of a pro-apoptotic gene), and reduces the E-cadherin protein(with consequent loss of cell-to-cell adhesion). Overall, COX-2 favors the appearance of a malignant phenotype through oxidation of pro-carcinogens to carcinogens, increase of cell growth and decrease of apoptosis, reduction of immune response and over expression of matrix metalloproteinases with an associated increase of invasiveness [126,147-149]. COX-2 is up-regulated in a variety of malignancies including prostate cancer [145,150]. COX-2 over expression has been reported also in in Prostatic Intraepithelial Neoplasia (PIN)and poorly differentiated tumors [133,140,146,150].

Chronic inflammation also produces a free radical/oxidative stress, consisting of inducible nitricoxide (i-NOS)/reactive nitric species (RNS) and various reactive

oxygen species (ROS) [113,115,145,146,151]. This oxidative stress can induce vascular damage, protein and genomic alterations, and post-translational modifications, including those involved in DNA repair and apoptosis [113]. These may eventually lead to a variety of oxidative DNA damage, including point mutations, deletions, or chromosomal rearrangements, resulting in repetitive cycles of tissue damage and repair, release of cytokines and growth factors and an increase of epithelial or stromal cell proliferation [133,134,152,153]. Oxidative stress can also activate the transcription factor NF-kB (nuclear factor kappa-light chain-enhancer of activated B cells) through the TNFα/AP-1transduction pathway and NIK transduction pathway.NF-kB is known as a master inflammatory transcriptional regulator and is highly active in macrophages. Targets of NFkB include genes regulating immune response, inflammation, cell proliferation, cell migration, and apoptosis. The nuclear translocation of NF-kB can activate target genes involved in carcinogenesis [112,147]. Dysregulation of the transcription factor NF-kB has been proposed as one putative molecular mechanism leading to chronic inflammation and cancer.TheIL-1β-induced NF-kB pattern of intraprostatic chemoattractive signals might have the capability of maintaining the chronic inflammation and proliferative inflammatory atrophy (PIA) in the prostate, which are recognized as precursor lesions in the development of prostate cancer [154].

In the normal prostate, the transduction pathway from NIK/NF-kB seems to be inactive. In BPH, there is an increasingly high TNF-α/AP-1 transduction pathway, followed by a rise of apoptotic pathways to stop uncontrolled cell proliferation. Conversely, in prostate cancer the pro-apoptotic effect of TNF-α/AP-1 pathway decreases and Is accompanied by an increase of nuclear

translocation of NFkB, resulting in the stimulation of prostate tumor cell growth [112,134].

Another distinction between BPH and prostate cancer is gene polymorphism. There is consisting evidence that BPH has only rare genetic abnormalities [155]. Recently, multiple genes with regulatory roles in inflammatory pathways have been associated with prostate cancer risk, including Ribonuclease L(*RNASEL*), macrophage scavenger receptor 1 (*MSR1*),macrophage inhibitory cytokine-1 (*MIC-1*), interleukins (*IL-8, IL-10*), vascular endothelial growth factor(*VEGF*) and intercellular adhesion molecule (ICAM),ELAC2/HPC2, Machropaghe Scavenger Receptor(SR-A/MSR1), CHEK2, Breast Cancer Gene 2 (BRCA2), Paraoxonase (PON) 1, 8-oxoguanine glycosylase (OGG-1), TLRs and COX-2. Most of these genes are implicated in cellular responses against inflammation andoxidative stress, and defects in their function may be associated to an increased risk of developing prostate cancer [146,155,156,157-160].

An increased production of inflammatory mediators seen visceral fat of the obese reflects the ongoing chronic inflammation of the adipose tissue. Proinflammatory cytokines are produced by the adipose stroma, as well as by the adipocytes (Figures 6 and 7) [161]. Some studies have also found a positive association between body mass index and advanced, aggressive and/or fatal prostate cancer [162-164]. Adipose tissue produces the enzyme aromatase, that presides over conversion of androgens into estrogens. As a consequence, obesity is associated with lower total testosterone and higher estrogen levels [164-166], which is a challenging concept in the association of prostate cancer and obesity. It has been hypothesized that lower androgen concentrations may

provide a microenvironment that favors and selects more aggressive and/or androgen-independent tumor cells, indicating one potential mechanism by which obesity may influence disease progression [165,166,168].Alternatively, lower testosterone levels may represent a bystander effect of the metabolic imbalance, which is one basis to prostate carcinogenesis [165], or the association between obesity and prostate cancer could be masked by complex and inverse relationships with other metabolic hormones such as leptin, insulin and IGF-I [169]. However, the intraprostatic conversion of testosterone to the bioactive tissue androgen 5α-dihydrotestosterone may be more influential in prostate cancer development than androgens in the circulation [170].

## *2. PURPOSE OF THE STUDY*

Despite the recent advances in biotechnology have provided researchers with unprecedented potential to attain a deeper understanding of the molecular events leading to prostate cancer, this disease continues to be a significant healthcare problem world-wide. Prostate cancer remains a major health concern for the male population throughout the Western world. Several studies have suggested the association of chronic inflammation and prostate cancer, whereby prostatic inflammation may be responsible of and/or contribute to prostate carcinogenesis and/or tumor progression. Furthermore, high levels of endogenous sex steroids are considered as risk factors for prostate cancer. Interestingly, it is clear that elevated estrogen in the presence of testosterone results in a prostate-specific inflammatory response and that early inflammatory events, induced by sex hormones, may serve as a prerequisite for the onset of prostate cancer.

Human prostate cancer is generally considered a prototype of androgen-dependent tumor; however, estrogen role in both normal and malignant prostate appears to be equally important. The association between plasma androgens and prostate cancer remains contradictory and mostly not compatible with the androgen hypothesis. Apart from methodological problems, a major issue is to what extent circulating hormones can be considered representative of their intraprostatic levels.

This thesis is aimed to improve current knowledge on the mechanisms underpinning prostate cancer development and progression, with special emphasis on sex steroid (notably estrogen) mechanism(s) of action and

metabolism in relation to the expression and activity of inflammatory enzymes (COX). As the number of men afflicted by prostate cancer will continue to grow with the aging population, finding new preventive strategies and innovative therapeutic options for this disease is crucial.

# *3. MATERIALS AND METHODS*

## 3.1 Cell cultures treatments

The RWPE-1 (normal immortalized epithelial prostate cell line, expressing androgen receptor), LNCaP (prostate tumor metastasis derived from left supraclavicular lymph node, expressing androgen receptor) and PC-3 (prostate tumor metastasis of grade IV derived from bone, androgen receptor negative), were obtained from the American Type Culture Collection (ATCC, Virginia, USA). RWPE-1 cells, were routinely grown and maintained in the Keratinocyte Serum Free Medium (K-SFM) base medium added two additives required to grow of this cell line, bovine pituitary extract (0.05 mg/ml BPE) and human recombinant epidermal growth factor (5 ng/ml EGF), provided by Invitrogen (GIBCO, Grand Island, NY). LNCaP and PC-3 cells were routinely grown and maintained in RPMI medium containing 10% defined fetal bovine serum (Hyclone, Salt Lake City, UT). To all medium was added 1% antibiotic-antimycotic and 1% L-Glutamine (GIBCO, Grand Island, NY), and all cells were grown at temperature of 37℃ in an atmosphere of 5% CO2 and 95% air.

Subconfluent cell monolayers were rinsed twice in PBS-A (NaCl 170 mM, KC1 3.4 mM, and Na,PO, 2 mM, pH 7.2), harvested and seeded at a confluence of 40%. Cells were left undisturbed for 24 h and then growth for 24h in phenol red-free RPMI plus 10% charcoal treated-FCS (CT-FCS) and 1 nM 17β-estradiol (E2) or 10 μM prostaglandin E2 (PGE2) (Sigma-Aldrich, St. Louis, MO), or 1 nM E2 with or without 10 μM of SC-560 (Sigma-Aldrich, St. Louis, MO), a COX1 specific inhibitor, and/or 10 μM of NS-398 (Sigma-Aldrich, St. Louis, MO), a

COX2 specific inhibitor for RT-PCR assay. All cells were grown at temperature of 37℃ in an atmosphere of 5% CO2 and 95% air.

### 3.2 Prostate Tissues

Tissues from benign prostatic hyperplasia (BPH) or prostate cancer (PCa), were obtained from patients undergoing surgical resection and/or biopsy procedures. Written informed consent was obtained in all cases. Histologically BPH tissues were obtained from patients (n = 10; mean age 58 years; range 51-65 years) during biopsy procedures. Prostate cancer specimens were obtained from patients (n = 10; mean age 53 years; range 48-58 years) during prostate surgical resection. All tissue samples were collected immediately after surgery, snap frozen in liquid nitrogen and stored at 80℃ u ntil analysis.

### 3.3 RNA extraction, reverse transcription and Polymerase Chain Reaction

Total RNA were isolated from cells or tissue samples using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, California). The extracted amounts of RNA were determined by measuring the absorbance at 260 nm, and the RNA integrity was assessed by nondenaturating agarose gel electrophoresis. All the RNAs were treated with RNase-free DNase I to remove potential contamination of genomic DNA. The cDNAs were synthesized in the presence of random hexamer-primer, using SuperScript II reverse transcriptase according to the manufacturer's instructions (Invitrogen, Carlsbad, California).

The PCR was conducted with a GeneAmp® PCR System 9700 (Applied Biosystems). I used β-actin as internal standard for PCR analysis of estrogen receptor alpha 66 (ERα66), estrogen receptor alpha 46 (ERα46), estrogen receptor alpha 36 (ERα36), estrogen receptor beta 1 (ERβ1), estrogen receptor beta 2/Cx (ERβ2/Cx), estrogen receptor beta 5 (ERβ5), aromatase (Aro), amphiregulin (AREG), ADAM metallopeptidase domain 17, also called tumor necrosis factor-α-converting enzyme (TACE/ADAM17), cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2) and epidermal growth factor receptor (EGFR) genes. ERα and ERβ genes were amplified with specific primers for exclusive exons of each splice variant. Conventional amplification was performed using 35 cycles at 95℃ for 30s, specific temperature of annealing (51-62 ℃) for 45s, 72℃ for 45s, followed by 72℃ for 5 min respectively. The SM-PCR analysis was performed, as previously reported (171), a condition whereby PCR products accumulate exponentially and their quantity increases in an mRNA-dependent manner. All PCR products were analyzed by gel electrophoresis on 2% agarose gels with ethidium bromide staining, followed by fluorescence digitization using the software "ImageJ 1.38X" (National Institutes of Health, USA). Expression level of each transcript was quantified relative to β-actin expression level and expressed as arbitrary units. Discrete cDNA bands were semiquantitated by digitized evaluation of their optical density after subtraction of background. The results were expressed as ratios of the intensity of the band of the investigated transcripts to the intensity of the band used as internal standard. For semiquantitative analysis of the amplified products, a suitable number of PCR cycles for each gene and β-actin was determined so that it was within the exponential phase.

Exponential regression equations fitted to the curves were used to calculate the number of cycles necessary to reach a normalized intensity threshold value = 1 for each sample. The relative difference in abundance between two samples was taken as $2^n$ where n is the difference between the numbers of cycles required by the samples to reach the threshold. Three different RNA preparations from each experimental condition were pooled to make more significant the differences between the expression levels, if any. Oligonucleotide primer pairs were designed for human ERα66, ERα46, ERα36, ERβ1, ERβ2/Cx, ERβ5, Aro, AREG, TACE/ADAM17, COX-1, COX-2 and EGFR genes, using published literature or sequence information contained in the National Center for Biotechnology Information GenBank database (see Table 1). Oligonucleotide primers were tested using BLAST software to confirm gene specificity and to determine exon locations.

**Table 1.** *List of Primers for PCR*

| Gene | Sequence (5'-3') | Exon Localization (bp) | Accession no. |
|---|---|---|---|
| ERα66 | for-TAC TGC ATC AGA TCC AAG GG<br>rev-ATC AAT GGT GCA CTG GTT GG | 403-422<br>1052-1033 | NM000125 |
| ERα46 | for-CCA AAA CTG AAA ATG CAG GC<br>rev-CCT TGC AGC CCT CAC AGG AC | Promoter F<br>981-962 | NM000125 |
| ERα36 | for-CAA GGG AAG TAT GGC TAT GGA A<br>rev-CCA GAG GCT TTA GAC ACG AGG A | 224-245<br>1175-1154 | BX640939 |
| ERβ1 | for-CGA TGC TTT GGT TTG GGT GAT<br>rev-ACG TGG GCA TTC AGC ATC TCC | 1400-1420<br>1594-1574 | AB006590 |
| ERβ2/5 | for-CGA TGC TTT GGT TTG GGT GAT<br>rev-CTT TAG GCC ACC GAG TTG ATT | 1770-1790<br>1983-1963 | NM001040275 |
| AREG | for-AGA GTT GAA CAG GTA GTT AAG CCC C<br>rev-GTC GAA GTT TCT TTC GTT CCT CAG | 517-541<br>937-914 | NM001657 |
| TACE | for-CGC ATT CTC AAG TCT CCA CA<br>rev-TAT TTC CCT CCC TGG TCC TC | 924-943<br>1375-1356 | BC136783 |
| COX-1 | for-AGT ACA GCT ACG AGC AGT TCT TGT T<br>rev-GTC TCC ATA CAA TTC CTC CAA CTC T | 1204-1228<br>1493-1469 | M59979 |
| COX-2 | for-GAG AAA ACT GCT CAA CAC CG<br>rev-GCA TAC TCT GTT GTG TTC CC | 249-268<br>994-975 | M90100 |
| EGFR | for-TCC CCG TAA TTA TGT GGT GAC AGA TC<br>rev-ACC CCT AAA TGC CAC CGG C | 1131-1156<br>1380-1362 | NM201282 |
| Aro | for-CTG GAA GAA TGT ATG GAC TT<br>rev-GAT CAT TTC CAG CAT GTT TT | 562-581<br>1221-1202 | M18856 |
| β-Actin | for-CTG GCA CCA CAC CTT CTA C<br>rev-GGG CAC AGT GTG GGT GAC | 339-357<br>576-559 | NM001101 |

### 3.4 Immunocytochemistry

For immunocytochemistry staining, 1 x $10^5$ of both nontumoral and tumoral prostate cell lines were grown directly on slide cover glass. The cover glass was removed when the cells covered 80% of the slide. After, the cells were fixed in 4% formalin for 15 min at 4℃ and rinsed twice in 1 × phosphate-buffered saline (PBS, pH 7.4) for 5 min each. Endogenous peroxidase activity was removed by incubation in 3% hydrogen peroxide and non specific binding was blocked by incubation in "Novocastra™ Protein Block" (NovoLink™ Polymer Kit, Novocastra Laboratories, Newcastle, UK) for 5 minutes at room temperature. Primary antibodies used included: (a) a mouse anti-hERα (clone F-10) monoclonal antibody epitope mapping at the C-terminus of estrogen receptor alpha of human origin (dilution 1:50, Santa Cruz Biotechnology, San Diego, CA); (b) a goat anti-hERβ (clone L-20) polyclonal antibody directed against a peptide mapping near the C-terminus of estrogen receptor beta of human origin (dilution 1:50, Santa Cruz Biotechnology, San Diego, CA). The slides were incubated with diluted primary antibody for 16h at 4℃. Secondary HRP-coniugated antibody (NovoLink™ Polymer Kit) was added to slides for 30 min at room temperature and specific staining was identified following incubation with a solution of the chromogenic peroxidase substrate, diaminobenzidine (DAB)/hydrogen peroxide for 4 minutes. Slides were counterstained with 0.02% hematoxylin, followed by successive dehydration in ethanol and xylene before mounting of coverslips. Quantitative image analysis was performed using a Leica computerized image analysis system with a Qwin software (Leica Imaging System Ltd. Cambridge, England). Quantification of immunostaining was performed on digitized images representing at least 10 randomly selected fields

for each sample. The proportion (%) of positive stain was calculated as the ratio of the total area of positively stained cells over the total area of cell nuclei using a color discrimination software.

## 3.5 Androgen and estrogen incubation, steroid extraction and chromatographic analysis

Cell or tissue cultures were washed twice with phosphate-buffered saline (PBS)-A and incubated for 24 h in FCS-free phenol red-free RPMI medium containing 1 nM tritiated androgen ([1,2,6,7-3H(N)]-testosterone, S.A. 92.4 Ci/mmol, or [1,2,6,7-3H(N)]-androstenedione, S.A. 84.5 Ci/mmol; DuPont de Nemours Italiana SpA, Milan, Italy), or 2 μCi $ml^{-1}$ tritiated estrogen ([6,7-$^{3}$H(N)]-E2) as precursor. For cultured cells, following incubation medium was transferred to plastic tubes and stored at −80 ◦C until analysis. As far as tissue cultures are concerned, minced tissues were resuspended in the incubation medium, transferred to a plastic tube and centrifuged at 2000 rpm for 5 minutes. The resulting pellet was gently homogenized using a glass-glass Douncehomogenizer (Kontes Co., Vineland, NJ) in 3ml of PBS, while the supernatant (incubation medium) was transferred to a separate tube. Both the homogenate and the medium were stored at −80 ◦C until steroid extraction. Medium and cells were therefore processed as described below.

Steroid extraction was carried out on the incubation medium, since we have previously shown it contains proportionally greater amounts of radioactive steroids than those found in both cell and tissue homogenates. Extraction of steroids was performed with SPE method in Vac-Elut apparatus using C18

cartridges on 1ml aliquots of medium. Briefly, two fractions were collected: in the first, conjugate (sulfate and glucuronide) steroids were eluted using water–methanol solution (60:40, v/v); in the second, the free (unbound) steroids were eluted using water–methanol solution (15:85, v/v). The two fractions were dried in a SVC100H Speed Vac evaporator-concentrator (Savant Instruments Inc, Farmigdale, NY) and conjugate steroids were hydrolized at 37 ℃ for 18 h, in 1ml of a solution consisting of 970 µl of 0.2M acetate buffer (pH 5.0) and 30 µl of Glusulase enzyme mixture (duPont Co, Wilmingon, DE). The hydrolyzed steroids were extracted again with SPE method using ethylacetate and evaporated to dryness, as described above. Both free and conjugate steroids were analyzed in RP-HPLC using a Beckman 324 model HPLC system equipped with an UV detector set at 280 nm, and an on line Flo-One/beta (500TR Series) three-channelflow scintillation analyzer (Packard Instrument Co, Meriden, CT). Steroids were eluted under isocratic conditions using a Ultrasphere ODS column (250×4.6 I.D.mm) and an optimized mobile phase consisting of acetonitrile: tetrahydrofuran: 0.05 M citric acid (39: 6: 55, v/v/v) at a flow rate of 1ml/min. Radiometric detection was performed using a 1 ml flow cell and Ultima-Flo-M (Camberra-Packard) scintillation mixture at flow rate of 4ml/min. Routine data integration was achieved by the Flo-One radio-HPLC workstation software package (Packard) and computed in net cpm, after correction for both residence time and background subtraction.

### 3.6 Cell proliferation assay

The MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega Corporation, Madison, USA) was used to measure cell proliferation or viability in cultures. Cells were seeded at a confluence of 30% in 96-well microplates and then growth for 24h in phenol red-free RPMI plus 10% charcoal treated-FCS (CT-FCS) and 1 nM 17β-estradiol (E2) or 10 μM prostaglandin E2 (PGE2), or 1 nM E2 with or without 10 μg/ml of genistein or resveratrol (Sigma-Aldrich, St. Louis, MO), or 1 nM E2 with or without 2 μg/ml of amphiregulin antibody (R&D Systems, Minneapolis, USA), or 50 nM of amphiregulin protein (Sigma-Aldrich, St. Louis, MO). In brief, the MTS tetrazolium compound (Owen's reagent) is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. Assays are performed by adding a small amount of the CellTiter 96® AQueous One Solution Reagent directly to culture wells, incubating for 1–4 hours and then recording the absorbance at 490nm with a 96-well plate reader. The quantity of formazan product as measured by the absorbance at 490nm is directly proportional to the number of living cells in culture. All cells were grown at temperature of 37℃ in an atmosphere of 5% $CO_2$ and 95% air. Control cells received vehicle (0.1% ethanol) only and all experiments were performed in triplicates. Cell having a narrow range of passage number were used for all experiments.

### 3.7 Cell differentiation

Growth factor-reduced Matrigel (BD Biosciences, San Jose) was placed in the well of a pre-chilled 6-well cell culture plate and incubated at 37℃ for 1 hr to allow polymerization. RWPE-1, LNCaP and PC-3 cells, at concentrations of $4 \times 10^4$ per well, were plated into the growth factor-reduced Matrigel coated wells, and incubated at 37℃ in 5% $CO_2$ in the condi tioned media with or without 1 nM 17β-estradiol (E2). After 48-72 h incubation, the plates were photographed. Tube formation was quantified by counting the number of connected cells in five randomly selected fields at ×200 magnification. All experiments were performed in triplicates.

### 3.8 Statistics

The data were expressed as mean ± SD. Analyses were performed using computerized statistical software with the ANOVA test. When ANOVA revealed $P<0,05$ the data were further analyzed by Dunnet's t-tests. Differences were considered statistically significant at $P < 0,05$.

## 4.1 Expression of ERα and ERβ wild-type and splicing variants in *nontumoral* and malignant human prostate cell lines

The expression of ERα and ERβ mRNA, was investigated using an exon-specific RT-PCR analysis on a panel of *nontumoral* (RWPE-1) and malignant (LNCaP and PC-3) prostate epithelial cell lines.

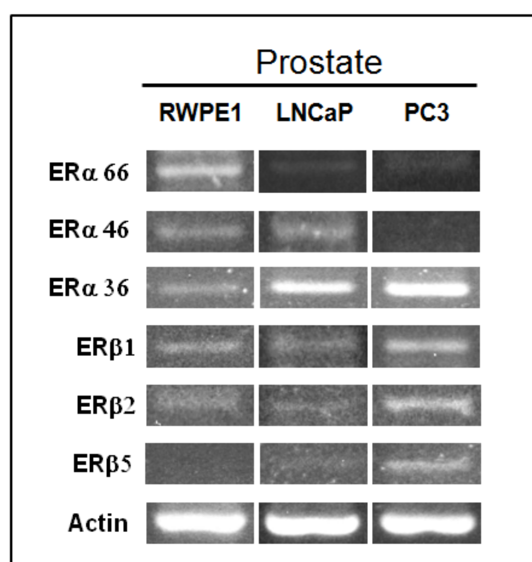



**Figure 9.** Expression of ERα and ERβ wild-type and splicing variants mRNA in *nontumoral* (RWPE-1) and malignant (LNCaP and PC-3) prostate epithelial cell lines.

In particular, ERα mRNA expression was inspected using RT-PCR with primer sequences located within regions A/B (exon 1) and D (exon 4) for ERα66 (wild-type mRNA), within promoter F and region C (exon 2) for ERα46mRNA and within region C (exon 2) and an additional region (exon 9) for ERα36 mRNA. Using ERα66-specific primers, the PCR product was revealed only in RWPE-1, while no ERα66 could be detected in LNCaP and PC-3 cell lines. ERα46 was

expressed both RWPE-1 and LNCaP, but not in PC3. Interestingly, ERα36 appeared to be inversely related to ERα66 expression, with very low levels in RWPE-1 cell line, intermediate levels in LNCaP cells, and high levels in PC-3 cells. ERβ mRNA expression was inspected using RT-PCR with primer sequences located within exon 7 and exon 8 for ERβ1 (wild-type mRNA), within exon 7 and an additional region exon Cx for ERβ2/Cx mRNA, within exon 7 and an additional region exon β5 for ERβ5 mRNA. The expression pattern of ERβ isoforms is rather variable, with ERβ5 being not expressed in RWPE-1 cells only, while PC-3 cells express high levels of all ERβ isoforms (Figure 9). The expression of ERα and ERβ isoforms was also determined at protein level using immunocytochemistry assay (ICA) on cells grown directly on glass slides. Overall, data of ICA were largely in accordance to what observed using RT-PCR analysis. In particular, using an antibody directed against a C-terminus epitope of ERα, shared by both ERα66 and ERα46 protein, but absent in ERα36 protein, ERα66 was detected in both RWPE-1 and LNCaP cells, while no ERα66 protein could be revealed in PC-3 cells. ERβ protein was expressed ubiquitously in all the cell lines tested (Figure 10).

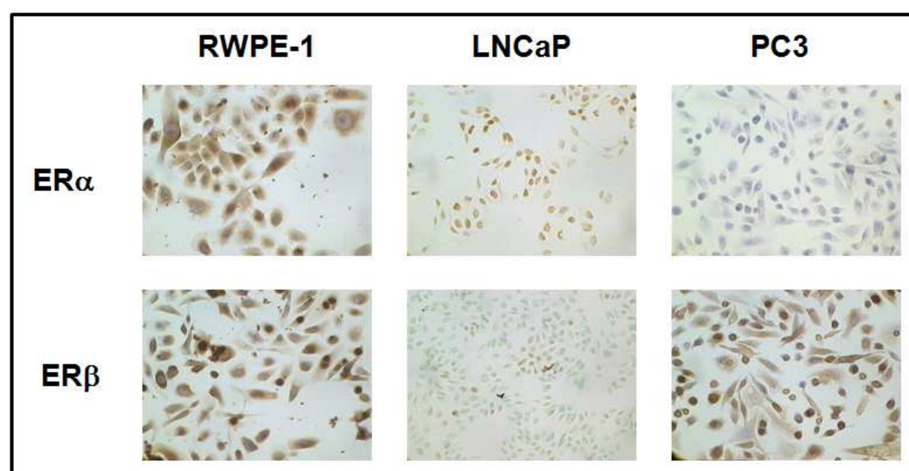



**Figure 10.** Expression of ERα and ERβ protein in *nontumoral* (RWPE-1) and malignant (LNCaP and PC-3) prostate epithelial cell lines.

## 4.2 Expression of aromatase, amphiregulin, TACE/ADAM17 and COX 1/2 in *nontumoral* and malignant human prostate cell lines

The expression of aromatase (Aro), amphiregulin (AREG), TACE/ADAM17, COX1 and COX2, was investigated using RT-PCR analysis in RWPE-1, LNCaP and PC-3 prostate epithelial cell lines. Aro expression levels corresponded to those of chromatographic analysis of aromatase activity, being very low or undetectable in all cell lines tested. AREG was detected only in PC-3 cells, while TACE/ADAM17 and COX1 were ubiquitously present in all cell lines. No COX 2 could be detected in LNCaP cells (figure 11).

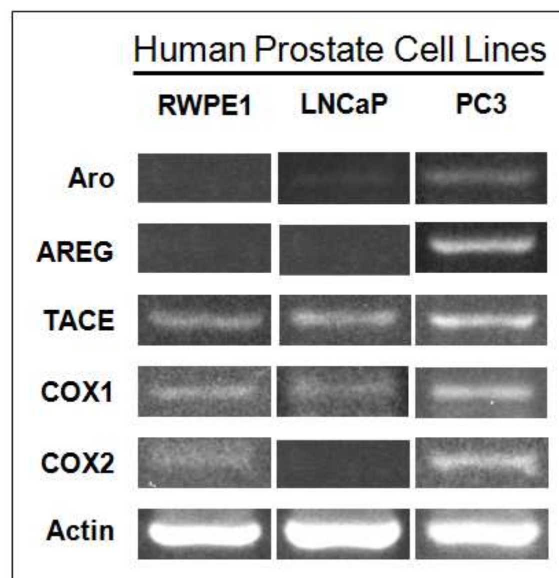



**Figure 11.** Expression of Aro, AREG, TACE, COX1 and COX2 mRNA in *nontumoral* (RWPE-1) and malignant (LNCaP and PC-3) prostate epithelial cell lines.

### 4.3 Effects of estradiol and prostaglandin E2 on the expression of Aro, AREG, TACE/ADAM17 and COX1/2 in *nontumoral* and malignant human prostate cell lines

The potential effect of both E2 and PGE2 on the expression of Aro, AREG, TACE/ADAM17, COX1 and COX2 genes was scrutinized in RWPE-1, LNCaP and PC-3 prostate epithelial cell lines. Treatment of cells with 1 nM E2 resulted in the up-regulation of all genes tested except COX-1. In particular, Aro and AREG mRNA were induced 3,2 fold and 5,1 fold respectively in PC-3cells. Interestingly, E2 induced aromatase expression also in Aro-negative LNCaP cells, suggesting that transcriptional activity of this gene remains potentially inducible. After E2 treatment, TACE/ADAM17 mRNA was 1,9 fold, 2,3 fold and 2,7 fold greater than in control in RWPE-1, LNCaP and PC-3 cells, respectively. COX-2 expression was up-regulated as well by E2, with expression levels 2,5 fold and 2,9 fold higher than in control in RWPE-1 andPC-3 cells, respectively (figure 12).

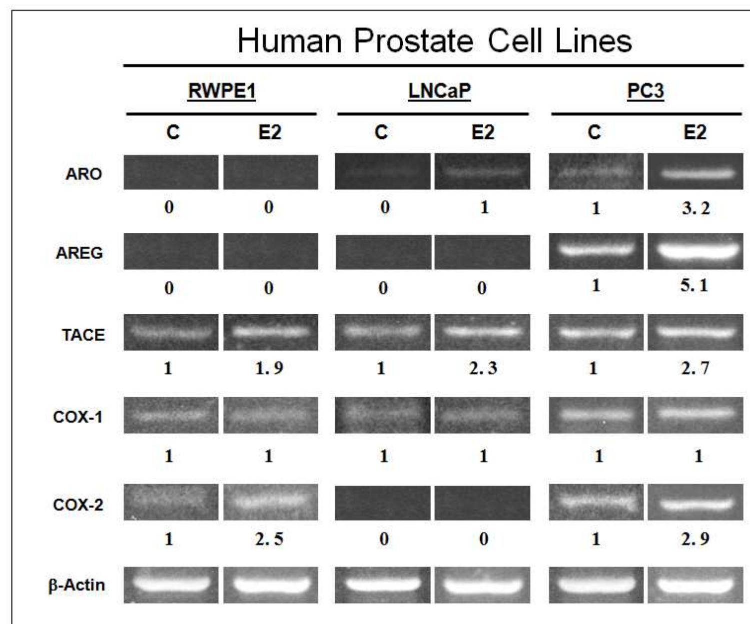



**Figure 12.** Expression of Aro, AREG, TACE, COX-1 and COX-2 mRNA after 1 nM estradiol (E2) treatment in *nontumoral* (RWPE-1) and malignant (LNCaP and PC-3) prostate epithelial cell lines.

Interestingly, also PGE2 was able to induce Aro expression in both PC-3 cells (3-fold) and Aro-negative LNCaP cells (Figure 13).

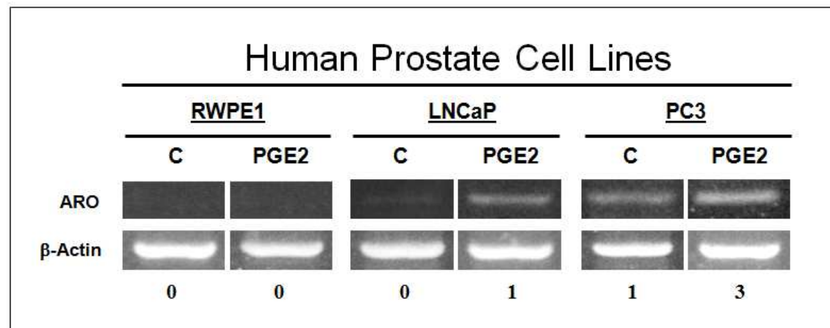



**Figure 13.** Expression of Aro after 10 μM prostaglandin E2 (PGE2) treatment in *nontumoral* (RWPE-1) and malignant (LNCaP and PC-3) prostate epithelial cell lines.

In addition, the use of specific COX-1/2 inhibitor (SC-560, a COX-1 inhibitor and NS-398, a COX-2 inhibitor),resulted in a decrease or in the abrogation of the E2-induced Aro expression in LNCaP and PC3 cells, suggesting that both COX1/2-dependent and -independent effects of SC-560 and NS-398 could be implicated in the regulation of Aro expression in human epithelial prostate cancer cells (Figure 14).

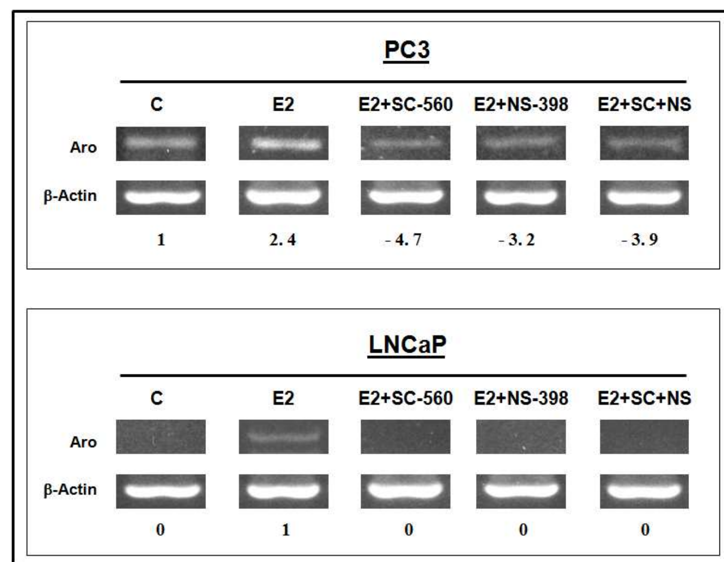



**Figure 14.** Expression of Aro after 1 nM estradiol (E2) treatment, with or without 10 μM SC-560 or NS-398, in LNCaP and PC-3 prostate cancer cell lines.

Lastly, aiming to evaluate the potential role of E2 on EGFR-axis in PC-3 cells, this cell line was treated with 1 nM E2 or 10 μM PGE2, and the potential effects on AREG, TACE/ADAM17 and EGFR expression were evaluated. PC-3 cells express significant amounts of epidermal growth factor receptor; E2 induced both AREG (5.1-fold) and TACE (2.7-fold), suggesting that estrogen is potentially able to trigger EGFR through the activation of expression/function of AREG (Figure 15).

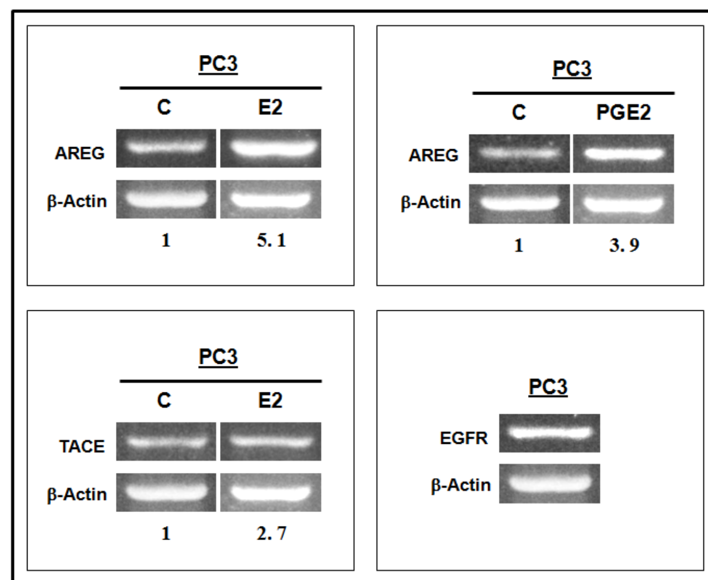



**Figure 15.** Expression of AREG, TACE and EGFR with or without 1 nM of estradiol (E2) treatment or 10 μM PGE2 treatment in PC-3 prostate cancer cell line.

## 4.4 Androgen and estrogen metabolism in *nontumoral* and malignant human prostate cell lines

Overall, data from *in vitro* studies reveal that activities of steroid enzymes, including aromatase, sulfotransferase, sulfatase, glucuronil-transferase, 17β-HSDand 5α-reductases, are markedly divergent in *nontumoral* (RWPE-1) and malignant (LNCaP and PC-3) human prostate epithelial cell lines. Results of 24h incubation of cells with either testosterone (T) or estradiol (E2) as androgen or estrogen precursor, are reported in Figure 16. In the first place, only PC-3 cell line shows a remarkable androgen metabolism, with on average only 1.9% testosterone remaining unconverted after incubation. By contrast, after 24h incubation with T in RWPE-1 and LNCaP cells, only a limited androgen metabolism was observed, with an average of 92% and 93% T remaining unconverted after incubation, respectively. This different metabolic aptitude is reflected in the prevalence of reductive metabolism in RWPE-1 and LNCaP androgen-responsive cells, while oxidative pathways are largely dominant in PC3 androgen-refractory cells. Also, only RWPE-1 produced quantifiable amounts (around 4,7%) of the biologically active androgen DHT and only PC-3 cell line showed a significant 5α-reductase activity, accounting on average for 28% of all androgen metabolites. Aromatase activity could not be detected in any of the cell lines tested. Interestingly, RWPE-1 cells, incubated for 24h with E2, gave rise to 75% estradiol-sulfate formation, while PC-3 cells produced 33,5% estrone (E1), 8% estradiol sulfate and 27% estrone sulfate. No E2 metabolism was observed in LNCaP cells showing instead a massive (97%) activity of the glucuronil-transferase enzyme when incubated with T (Figure 16).

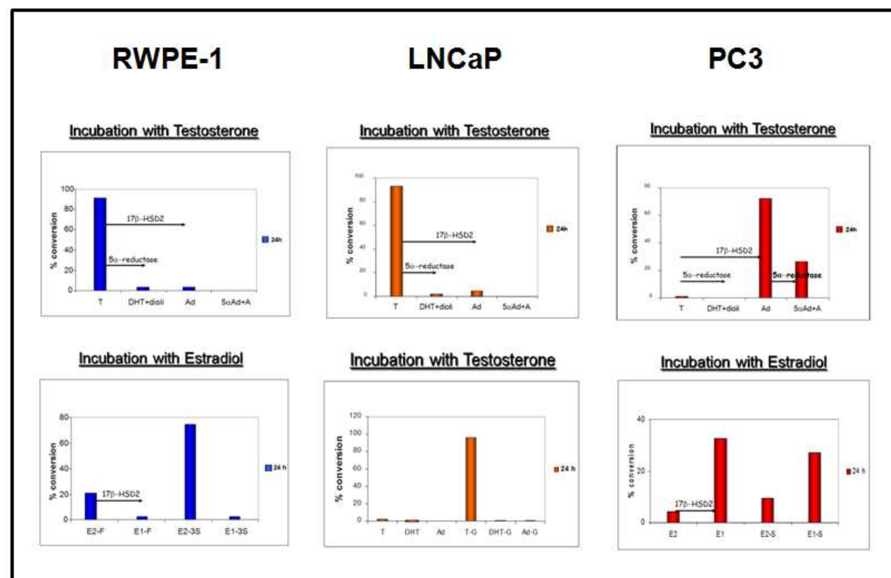



**Figure 16.** Androgen an Estrogen metabolism in *nontumoral* (RWPE-1) and malignant (LNCaP and PC-3) prostate epithelial cell lines after 24 h incubation with 1 nM tritiated testosterone (T) and 1 nM tritiated estradiol (E2). Data represent mean ±SD values of conversion rates (% conversion) from triplicate experiments.

### 4.5 Androgen and estrogen metabolism in benign prostatic hyperplasia and prostate cancer tissues

Patterns of androgen metabolism were also investigated in minced prostate tissue samples, under exactly the same experimental conditions used for *in vitro* studies. These studies were conducted on a series of hyperplasic (BPH) and malignant human prostate tissues. As reported in Figure 17, both BPH and cancer tissues exhibited a significant proportion of T conversion into dihydrotestosterone (DHT) and 3α/3β-diols (biologically active androgen metabolites), with an average of 69% and 89% respectively. Overall, the formation of 5α-androstanedione (5αAd) and androsterone (A) was limited to 20% and 5% in BPH and cancer, respectively (Figure 17). Both BPH and prostate cancer specimens did not exhibit detectable amounts of estrogen and/or intermediate products of the aromatase enzyme (data not shown).

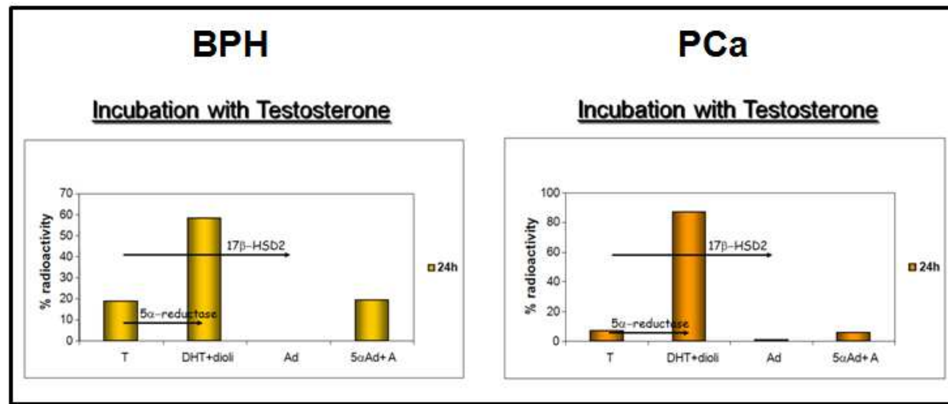



**Figure 17.** Androgen metabolism in hyperplasic (BPH) and malignant (PCa) human prostate tissues after 24h incubation with 1 nM tritiated testosterone (T).Data represent mean ±SD values of conversion rates (% conversion) from triplicate experiments.

Interestingly, when androgen metabolism was investigated in separate epithelial and stromal cell of BPH, aromatase activity was present in stromal cells only, with a 3.6% E1 production (data not shown). In addition, results obtained in from primary cultures of stromal and epithelial cells from BPH tissues revealed a prevalent oxidative androgen metabolism, leading to the formation of 17keto androgen metabolites in either cell type (Figure 18).

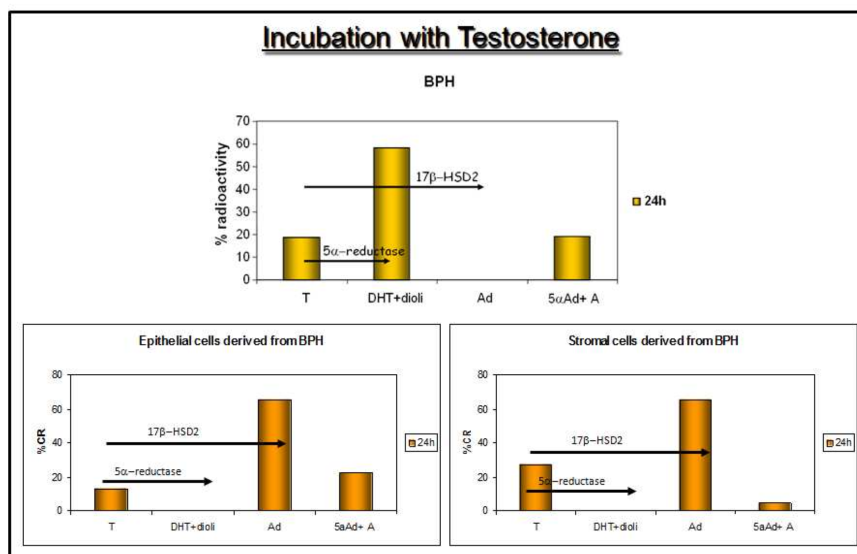



**Figure 18.** Androgen metabolism in BPH tissue vs primary cultures of stromal and ephitelial cells derived from BPH. BPH tissues and their epithelial and stromal cells were incubated for 24h with 1 nM tritiated testosterone (T). Data represent mean ±SD values of conversion rates (% conversion) from triplicate experiments.

## 4.6 Growth effects of estradiol, Genistein and Resveratrol on *nontumoral* and malignant human prostate cell lines

To establish the effect of estradiol, genistein and resveratrol on cell proliferation of epithelial prostate cells with different ER status, a series of experiments were conducted on RWPE-1, LNCaP and PC3 prostate epithelial cell lines. RWPE-1 cells, which express all the ERα and ERβ splice variants except ERβ5, displayed a significant induction of growth with E2 (78%), with E2 plus genistein (25%) and E2 plus resveratrol (51%). Resveratrol alone induced a nearly 20% growth increase in RWPE-1 cells, while genistein and genistein plus resveratrol inhibited growth of RWPE-1 cells respectively by 18% and 17%. A corresponding figure was observed in LNCaP cells, that express all ERα and ERβ splice variants except ERα66 (E2=+37%; E2 and genistein=+3%; E2 and resveratrol=+21%; resveratrol=+18%; genistein and resveratrol=-49%), while all treatment inhibit cell proliferation in PC-3 cells, expressing only ERα36 mRNA and all ERβ splice variant mRNA tested (E2=-15%; genistein=-42%; resveratrol=-52%; E2 and genistein=-23%; E2 and resveratrol=-4%; genistein and resveratrol=-58%). These effects were observed after 24h exposure to concentrations of 1nM estradiol, 10 µg/mlgenistein and 10 µg/ml resveratrol (Figure 19).

## 4.7 Growth effects of estradiol, AREG and PGE2 on *nontumoral* and malignant human prostate cell lines

Measurement of cell proliferation showed that growth of RWPE-1 cells was stimulated by 1 nM E2 (65%), while non significant proliferative effects were

observed after exposure to AREG (50 nM) and PGE2 (10 μM) slightly induced cell proliferation (17%).

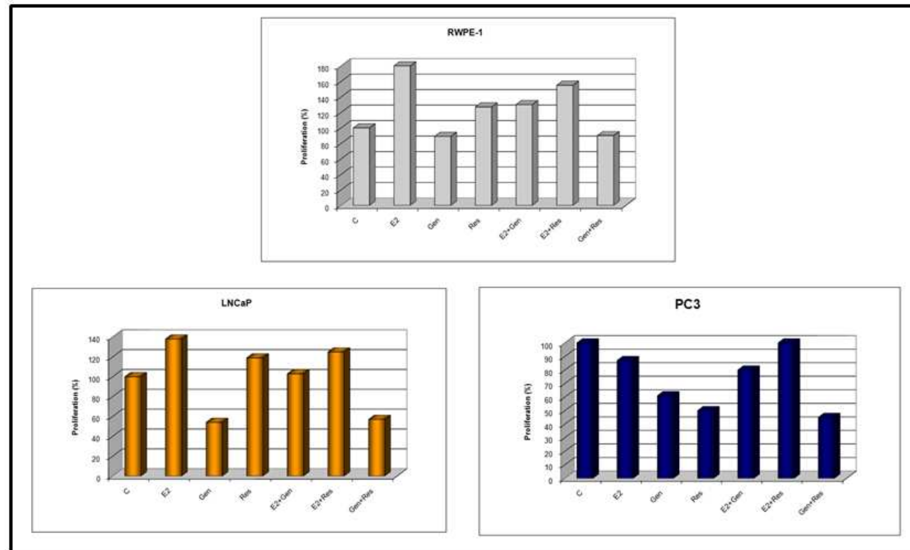



**Figure 19.** Effect of estradiol (1 nM), genistein (10 μg/ml) and resveratrol (10 μg/ml) on cell proliferation of *nontumoral* (RWPE-1) and tumoral (LNCaP and PC3) epithelial prostate cell lines. All experiments were conducted in triplicate.

E2 increased cell growth also in LNCaP cells and this effect could be abrogated by the addition of a neutralizing antibody (2 μg/ml) directed against AREG. A comparable effect was observed when LNCaP cells were treated with AREG along with an anti-AREG antibody, while AREG alone induced a 30% increase of LNCaP cell growth. On the contrary, PGE2 induced LNCaP cell proliferation only limitedly (8%). Lastly, PC-3 cells, that express high levels of EGFR, showed an increase (63%) of their proliferative activity in response to AREG. Once again, this effect could be abolished after addition of neutralizing antibody anti-AREG. In this cell line, PGE2 induced a significant increase of cell proliferation (43%) (Figure 20).

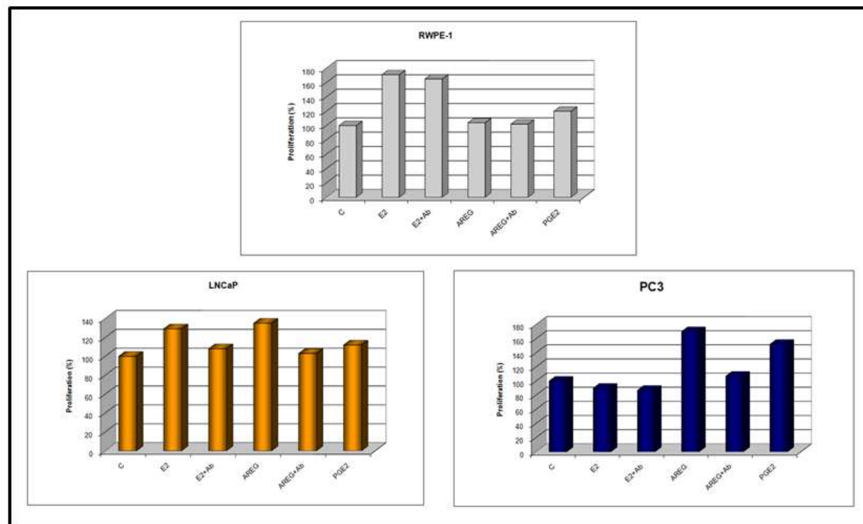



**Figure 20.** Effect of estradiol (1 nM), amphiregulin protein (50 nM) prostaglandin E2 (10 μM) genistein (10 μg/ml) and amphiregulin antibody (2 μg/ml) on cell proliferation of *nontumoral* (RWPE-1) and malignant (LNCaP and PC3) epithelial prostate cell lines. All experiments were conducted in triplicate.

## 4.8 Effects of estradiol on differentiation of *nontumoral* and malignant human prostate cell lines

The potential of E2 to sustain glandular (acini) formation of *nontumoral* (RWPE-1) and malignant (LNCaP and PC-3) prostate epithelial cell lines was assessed in a differentiating environment (Matrigel). This condition, normally, induce cell lines to self-organize in athree-dimensional tubular network wherein cells are arranged in aduct-like pattern around a central space (differentiation process). Significant differences were observed in the patterns of duct-like structures of the three cell lines after 2-3 days in culture. Interestingly enough, only RWPE-1 after E2 showed the formation of “branching end buds” or “ductular-alveolar outgrowths”, while neither control (untreated) RWPE-1 cells, nor the other two cells lines (LNCaP, PC-3 cells), under any condition, produced this kind of three-dimensional process. Treatment with 1 nM E2 increased growth in LNCaP and PC-3 cells, but failed to induce any differentiation, independent of the

presence or absence of E2 (Figure 21). To better dissect the E2 role in the regulation of ductal-alveolar morphogenesis, no sera and only phenol red-free media were used.

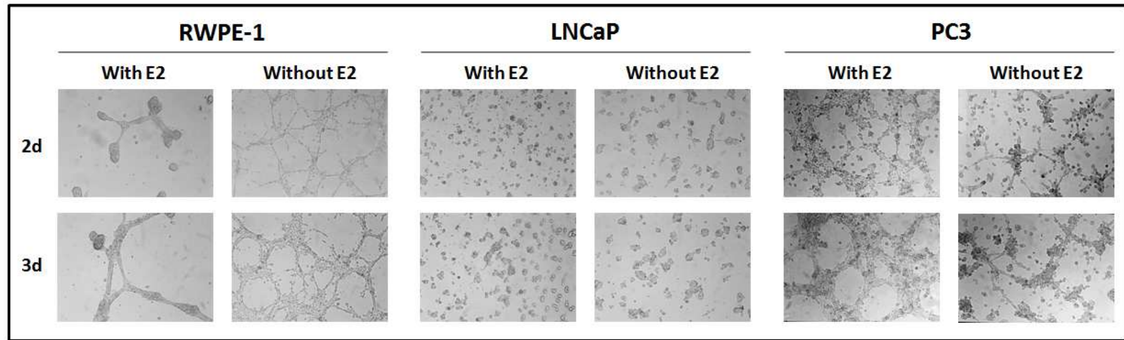



**Figure 21.** Effect of 1 nM estradiol (E2) on differentiation of *nontumoral* (RWPE-1) and malignant (LNCaP and PC3) epithelial prostate cell lines.

While androgens have been historically considered male hormones and the sole responsible for both normal and malignant prostate growth, sex steroids exert similar, yet different, effects in the prostate, and it is becoming clear that a finely tuned balance between estrogen effects mediated by ERα and ERβ, is required for the maintenance of prostate health. The action of estrogens is complex, having both adverse and beneficial roles via ERα and ERβ respectively. The present study indicates that estradiol is an important factor to induce differentiation only in *nontumoral* RWPE-1 prostate cells, while estradiol regulates cell proliferation in LNCaP and PC3 prostate cancer cells. The adverse effects, namely malignant transformation, aberrant cell proliferation, and chronic inflammation, all seemingly require the presence and activity of ERα, providing a rationale for the use of ERα-specific antagonists in the chemoprevention of human prostate cancer. Conversely, ERβ appears to promote beneficial effects by antagonizing the carcinogenetic process and preventing the development of hyperplastic lesions and inflammatory response, thus providing the rationale for the use of ERβ agonists as a therapeutic option for both BPH and prostate cancer [172]. Although the beneficial effects of modulating estrogen receptor activity as a target for treatment of prostate diseases have been demonstrated by several studies in vitro and in animal model systems, the translation of this information into potential therapeutic applications, particularly for prostate cancer, is likely to be highly challenging. This complication lies in a number of factors. In the first place, ERβ expression is variable and appears to be down-regulated or even silenced during prostate

cancer progression. In addition, little is known about the expression and/or function of the ER splice variants, gene mutations, ligand-dependent and independent receptor activation and the role of genomic versus non-genomic signaling in various target tissues and cells [49,56,173,174]. Despite this complexity, there is significant potential for the use of targeted ER therapies in prostate diseases and this clearly warrants further investigation.

Overall, the potential role played by estrogens in the neoplastic transformation of prostate epithelial cells, as well as in prostate cancer progression remains controversial. Exposure of humans or rodents to estrogens generally induces a proliferative lesion, called squamous metaplasia, in their prostates [175-177], whereas prolonged treatment of rats with androgen plus estrogen causes a high incidence of prostate cancer lesions in the dorsolateral prostates of the treated animals [178-180]. Paradoxically, diethylstilbestrol, tamoxifen, and other estrogenic drugs have been used as treatment for advanced, metastatic prostate cancer [181-186]. Other than acting as chemical castration agents, both estrogens and antiestrogens are believed to exert direct growth-inhibitory effects on prostatic cancer cells via the induction of apoptosis or cell cycle arrest [187-190]. Precisely how estrogens/antiestrogens elicit these actions remains uncertain. Traditionally, the activities of estrogens/antiestrogens are thought to be mediated via the classical pathways, namely by binding to ER localized in the stromal compartment and basal epithelial cells of human and rodent prostates [191-194]. Because ERα is not expressed in the normal glandular epithelium of rat or human prostate [188,192-194], it is generally believed that the effects of estrogen/antiestrogen on normal prostate epithelial cells are indirect, presumably mediated via estrogen-induced stromal factors.

Results of the present study reveal that amphiregulin (AREG), a EGF-like growth factor, is expressed and induced by both E2 and PGE2 in PC3 cell line. In addition, E2 is also able to induce TACE/ADAM17 (a transmembrane proteolytic enzyme that produces the bioactive forms of AREG) in RWPE-1, LNCaP and PC3 cells. In this respect, AREG may be considered an estrogen-regulated factor that could mediate estrogen stimulation of cell proliferation via the activation of EGFR signaling.

After the discovery of ERβ [195] and its localization to the epithelial compartment of rodent prostates [196], a distinct possibility has been raised that estrogen/antiestrogen could affect prostate epithelial cells function via an ERβ signaling pathway. Although some author [192] has evaluated the expression of ERβ transcripts and proteins in human prostate and found undetectable levels in both normal and diseased tissues, this study unequivocally demonstrates the expression of wild-type ERβ and its splicing variants ERβ2/Cx and ERβ5 in *nontumoral* RWPE-1 and malignant LNCaP and PC3 epithelial prostate cells. Several investigators have detected ERα expression in human prostate cancer cell lines, including LNCaP, PC-3, and DU-145 cells [72,73], whereas others did not [197]. Reports on ERα expression in prostate cancer specimens were equally controversial. Bonkhoff *et al.* [192] reported that ERα expression was infrequent in low-to-moderate grade prostatic adenocarcinomas, but common in high-grade and metastatic cancers. Conversely, Konishi *et al.* [198] revealed ERα immunopositivity in well-differentiated adenocarcinomas, but not in poorly differentiated prostate tumors. This issue becomes even more intricate when the expression pattern of ERβ is taken into consideration. Additionally, several variants of ERα are often found to coexist with the wild-type transcript in normal

and malignant prostate tissues [50]. These variants, produced by alternative splicing, are whole exon variants that may have “outlaw functions”. In the current study, wild-type ERα66 was expressed only in RWPE-1, while no ERα66 could be detected in LNCaP and PC-3 cell lines. ERα46 was expressed both RWPE-1 and LNCaP, while no expression was observed in PC3 cells. Intriguingly, ERα36 appeared to be inversely related to ERα66 expression, with very low levels in *nontumoral* RWPE-1 cells, intermediate levels in hormone-responsive LNCaP cells and high levels in androgen-refractory PC-3 cancer cells. The expression pattern of ERβ isoforms was rather variable with RWPE-1 cells only expressing no ERβ5 and PC-3 expressing high levels of all ERβ isoforms studied. This complex framework implies that, to understand the role played by ER-mediated signaling in prostate carcinogenesis, an accurate assessment of both wild-type and variants ERs should be conducted comparing normal and cancerous prostate gland. As a matter of fact, this study clearly indicates that diverse ER ligands can have distinct effects on the proliferative activity of prostate epithelial cells. Estradiol alone and estradiol plus genistein or estradiol plus resveratrol all induced a significant increase of cell proliferation in RWPE-1 cells, that express all ERα and ERβ isoforms, except ERβ5 and in LNCaP cells that express all ERα and ERβ isoforms, except ERα66. In these two cell lines, resveratrol alone stimulated, while genistein and genistein plus resveratrol reduced prostate cell growth. Conversely, all treatment inhibited cell proliferation in androgen-resistant PC-3 cells that express only ERα36 and all ERβ splice variants.

Steroid hormones play a significant role in growth and function of the normal prostate as well as in the development of BPH. Although serum estrogen levels

are low in healthy men [200], intraprostatic E2 levels increase in men with age and this increase is accompanied by the rise in the prostate volume [201,202]. Estrogens are synthesized from androgens through the aromatase enzyme and, therefore, an increased expression/activity of aromatase disrupts the balance of estrogen/androgen in the prostate. Many studies have detected aromatase expression and function in human prostate respectively using RT-PCR and biochemical assay of potential enzyme activity [26,43,47]. Estrogen levels in the stromal compartment of BPH increases with age [24] and this increase has been associated with an elevated expression of aromatase in prostatic stromal cells, especially around hyperplastic glands of BPH patients [199]. Recent studies have reported that PGE2 up-regulates aromatase expression in breast cancer [203], endometriosis and uterine leiomyomata [204]. COX-2 is a key enzyme in PGE2 biosyntheses. In this work, using RT-PCR, an E2-inducible COX-2 expression was observed in RWPE-1 and PC3 cells, while no COX-2 could be detected in LNCaP cells. Aromatase expression was very low or undetectable in all cell lines tested (RWPE-1, LNCaP and PC-3 cell lines) and this evidence was confirmed by chromatographic analysis of Aro activity. Treatments with 1 nM E2 resulted in the up-regulation of Aro expression in Aro-negative LNCaP and PC3 cells. Interestingly, PGE2 was also able to induce Aro expression in the two cell lines. In addition, the use of specific COX 1/2 inhibitor, SC-560 or NS-398, resulted in a decrease or in the abrogation of the E2-induced Aro expression, suggesting that both COX1/2-dependent and independent effects of SC-560 and NS-398 could be implicated in the regulation of Aro expression in human prostate cancer cells. Furthermore, when androgen metabolism was investigated in separate epithelial and stromal cells from BPH,

aromatase activity was present only in stromal cells. It is conceivable that in the prostate of elderly men, characterized by an inflammatory environment, increased aromatase expression would eventually led to locally elevated estrogen that could in turn stimulate proliferative activity of prostate epithelial cells in a paracrine fashion.

The assessment of androgen and estrogen metabolism revealed that distinct metabolic patterns of both estrogen and androgen are encountered in cultured *nontumoral* and malignant human prostate epithelial cells and that this different metabolic aptitude is reflected in the prevalence of reductive metabolism in *nontumoral* (RWPE-1) and cancer (LNCaP) androgen-responsive cells, while oxidative pathways are largely prevalent in neoplastic, androgen-refractory cells (PC3). Furthermore, different enzyme activities, including 17β-hydroxysteroid dehydrogenases (HSDs), sulfotransferase/sulfatase and glucuronil-transferase, appear to be significantly associated with steroid receptor status. In vivo studies conducted using exactly the same experimental conditions have revealed that both BPH and prostate cancer tissues exhibit a predominantly reductive androgen metabolism, eventually leading to the formation of large amounts of bioactive androgen derivatives (DHT, diols), though reductive metabolism was remarkably higher in cancer than in BPH tissues. However, results from primary cultures of stromal and epithelial cells separated from BPH tissues showed a dominant oxidative androgen metabolism, leading to the formation of 17keto androgen metabolites in both cell compartments. This combined evidence suggests that bioactive androgens are being actively produced in both BPH and prostate cancer tissues and that stromal-epithelial interaction appears to be crucial in determining the ultimate androgen metabolic pathways.

In summary, this study indicates that human *nonmalignant* prostate epithelial cells express exclusively wild-type ERα66 and that, hence, estrogen are likely to regulate growth and function of these target cells via a receptor-mediated signaling. On the contrary, prostate cancer cells exhibit a variable pattern of ER expression and, therefore, their response to estrogen or antiestrogen depends upon the differential expression of individual ER subtype(s). Overall, data from this study support to the conception that ER splice variant may play a central role in estrogen/antiestrogen signaling in *normal* and malignant human prostate. In addition, the evidence presented herein suggests that, in an inflammatory environment, prostate epithelial cells may potentially induce stromal aromatase through the secretion of PGE2 and aromatase overexpression could in turn lead to locally elevated E2. This latter, on one hand, induces directly COX-2, which produces high concentrations of PGE2, generating a vicious-circle. On the other hand E2 increases AREG synthesis/shedding, creating a rapid, *nongenomic* mechanism that ultimately leads to stimulate cell proliferation through activation of EGFR signaling. This mechanism could be triggered by either ligand-dependent or –independent stimuli (figure 22).

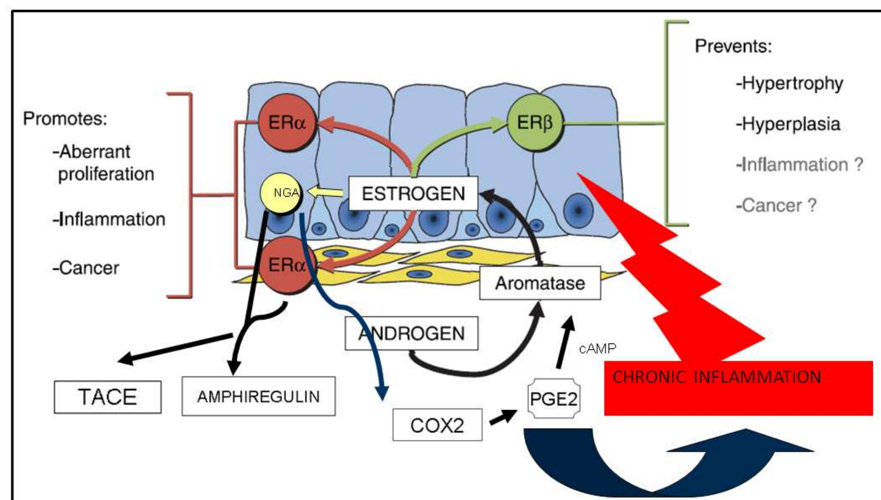



**Figure 22.** Estrogen signaling mechanisms in human prostate. Modified from Gail P Risbridger et al 2007.

# 6. CONCLUSIONS

In spite of the recent, significant advances in the research on prostate cancer, mechanisms underpinning development and progression of the malignant prostate remain undefined. Several networked factors, including the balance of estrogen and androgen, changes and polymorphisms in the enzymes responsible for the biosynthesis and transformation of intraprostatic hormones, alteration of hormone signaling or local balance between estrogen receptor types and variants, may all be markedly affected by lifestyle factors (notably diet), genetic determinant and exposure to environmental chemicals. Presently, the lasting conception that androgens are the key determinants in prostate carcinogenesis and tumor progression appears to be a never-ending persuasion that has, faultily led to neglect different areas of research with promising perspectives for both treatment and prevention of this disease. In particular, steroid enzyme inhibitors [205], as well as ER subtype selective agonists/antagonists or SERMs [206,207], have been in turn proposed as potential agents for both chemoprevention and treatment of prostate cancer. In a review, Williams [208] proposes that several distinct factors may significantly affect hormone balance in the organism, including up-regulation of the P450 aromatase enzyme and the resulting unopposed excess of endogenous estrogen, alteration of insulin receptor machinery and leptins, exposure to elevated environmental xenoestrogens. This unbalanced hormonal milieu may represent a common condition for development of life-threatening diseases, including, diabetes, obesity, Alzheimer’s disease and many type of cancer including prostate.

The evidence presented in this work strongly supports the concept that, likewise for breast cancer, changes in aromatase expression and/or activity can be important for development and/or progression of human prostatic carcinoma and that locally elevated estrogen may have a significant impact on the proliferative and functional regulation of prostate cells also through rapid (*nongenomic*) signaling mechanisms. In this respect, a better knowledge and understanding of estrogen-driven mechanisms in different processes related to human health and disease would be of primary importance to design and exploit original preventive and therapeutic strategies also in prostatic carcinoma.

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