Inflammation and Prostatic Carcinogenesis

- a morphological study of the human prostate

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天行健，君子以自强不息

-《易经》

As heaven maintains vigor through movements,
a gentle man should constantly strive for self-perfection.

-《*I Ching: Book of Changes*》

To my family

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The Prostate 61:60 - 72 (2004).

II. Wanzhong Wang, Anders Bergh, and Jan-Erik Damber
Cyclooxygenase-2 expression correlates with local chronic inflammation and tumor neovascularization in human prostate cancer.
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INFLAMMATION AND PROSTATIC CARCINOGENESIS – A MORPHOLOGICAL STUDY OF THE HUMAN PROSTATE

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ABSTRACT: Chronic inflammation has been suggested to be linked to cancers. Inflammatory infiltrates are often found in and around foci of prostatic atrophy. These foci, called proliferative inflammatory atrophy (PIA), are proposed as precursors of prostate cancer (PCa) or prostatic intraepithelial neoplasia (PIN). Up-regulated cyclooxygenase-2 (COX-2) may play a role in influencing cell proliferation, differentiation, apoptosis, and angiogenesis. In the present studies, we found that COX-2 was overexpressed in the PIA lesions. Epithelium in these PIA lesions had high proliferation index and increased level of anti-apoptosis protein Bcl-2. The association between COX-2 and the focal chronic inflammation, dominant T-lymphocytes and macrophages infiltration, was clearly shown. This study suggests that chronic inflammation and the related oxidative stress might play crucial roles in inducing COX-2 overexpression, which could be involved in the pathogenesis of prostate disorders.

Transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) plays a major role during the initial stage of COX-2 transcription. In the present study we report a novel finding that C/EBPβ was overexpressed in PIA lesions and in relation to COX-2. The data also demonstrates that chronic inflammation appeared to play a role in inducing C/EBPβ expression in atrophic prostate epithelial cells.

Using a similar technique, we investigated COX-2 expression in human PCa tissue and found that COX-2 expression correlated with Gleason score. The focal chronic inflammation in the cancer areas seems to induce COX-2 expression, since the COX-2 expression was significantly related to inflammation density. This study provides the first evidence of a direct link between COX-2 and angiogenesis in PCa tissues.

Morphological transition from PIA to HGPIN and PCa was found in radical prostatectomy specimens, although it was not very common. Atrophic epithelial cells are easy to recognize and clearly delineated by CK5 and GSTP1 immunostaining. One striking finding of this study is that clusters of cells that show nuclear atypia were found in some PIA lesions. Such focal atypical epithelial cells fulfil the criteria for HGPIN and expressed both CK5 and GSTP1. This study suggests that PIA lesions may develop into HGPIN and prostate cancer directly or via some intermediate process.

Key words: prostate, carcinogenesis, chronic inflammation, atrophy, morphology, immunohistochemistry

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ABBREVIATIONS

AAH	atypical adenomatous hyperplasia
AMACR	alpha-methylacyl coenzyme A racemase
AR	androgen receptor
BPH	benign prostatic hyperplasia
C/EBPβ	CCAAT/enhancer binding protein beta
CK	cytokeratin
c-MET	mesenchymal epithelial transition factor
COX	cylooxygenase
COXIBs	COX inhibitors
DHT	dihydrotestosterone
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	estrogen receptor
FGFR	fibroblast growth factor receptor
GM-CSF	granulocyte-macrophage colony stimulating factor
GSTP1	glutathione-*S*-transferase pi
HGPIN	high grade prostatic intraepithelial neoplasia
IGF	insulin like growth factor
IHC	immunohistochemistry
IL	interleukin
iNOS	inducible nitric oxide
LGPIN	low grade prostatic intraepithelial neoplasia
LPS	lipopolysaccharide
NO	nitric oxide
NSAIDs	nonsteroidal anti-inflammatory drugs
PA	proliferative atrophy
PAH	post-atrophic hyperplasia
PCa	prostate cancer
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PG	prostaglandin
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
PIA	proliferative inflammatory atrophy
PIN	prostatic intraepithelial neoplasia
PSA	prostate specific antigen
PSCA	prostate stem cell antigen
PZ	peripheral zone
ROS	reactive oxygen species
SA	simple atrophy
STD	sexually transmitted disease
TNFα	tumour necrosis factor alpha
VEGF	vascular endothelial growth factor

BACKGROUND

Prostate Gland

Anatomy

The prostate is an accessory genital gland with the size and shape of a chestnut. The gland is located in the pelvis, inferior to the bladder, where it surrounds the prostatic part of the urethra. It consists of 30 to 50 tubuloalveolar glands arranged in three concentric layers: an inner mucosal layer, an intermediate submucosal layer, and a peripheral layer containing the main prostatic glands [1-3]. The glands of the mucosal layer secrete directly into the urethra; the other two layers have ducts that open into the prostatic sinuses, located on either side of the urethral crest on the posterior wall of the urethra [3].

The adult prostate gland can be divided into four anatomical zones [4].

- **The peripheral zone (PZ):** The PZ is located predominantly in the posterior and lateral aspects of the gland, extends to the apex variably and anteriorly, and surrounds the central zone towards the base. Its ducts open into the distal prostatic urethra. The PZ contains the majority (70%) of the glandular tissue in the normal prostate, and represents the most frequent site of prostate cancer (PCa) origin. It is also the predominant site for the occurrence of the PCa precursor lesion, prostatic intraepithelial neoplasia (PIN), including high grade PIN (HGPIN). This zone is the one most susceptible to inflammation [5] **.**
- **The central zone (CZ)** contains about 25% of the glandular tissue and is resistant to both carcinoma and inflammation. In comparison with the other zones, cells in the central zone have distinctive morphologic features: more prominent and slightly basophilic cytoplasm and larger nuclei displaced at different levels in adjacent cells. Recent findings suggest that this one originates embryologically from the inclusion of mesonephric duct cells into the developing prostate.
- **The transitional zone (TZ)** contains the mucosal glands. It is composed of lobules of glands with shorter ducts compared to those reaching out to the PZ and is often separated from the PZ by an indistinct band of collagenous tissue, which becomes more pronounced as the TZ is expanded by benign prostatic hyperplasia (BPH). In the young, post-pubertal adult, architectural and histologic differences in the glands of the TZ and the PZ are not well defined. The TZ is the exclusively site of BPH in the human prostate. In older individuals, the parenchymal cells of this zone frequently undergo extensive division and form nodular masses of epithelial cells. Since this zone is in close proximity to the proximity to the prostatic urethra, causing difficulty in urination. This condition is known as BPH.
- **The periurethral zone** contains mucosal and submucosal glands. In later stages of BPH this zone may undergo pathological growth, mainly from the stromal components. Together with the

glandular nodules of the transitional zone, this growth causes increased urethral compression and further retention of urine in the bladder.

Histology

Approximately 70% of the prostate consists of glandular elements, while the remaining 30% is a fibromuscular stroma. Normal prostatic gland is composed of three distinct cell types: secretory (luminal) cells, basal cells, and neuroendocrine cells. The secretory cells line the glandular ducts and have a polarized intracellular organization, which is lost during carcinogenesis. The predominant secretory cells are characterized by expression of androgen receptor (AR), cytokeratins 8 and 18, CD57, and prostate-specific antigen (PSA). The basal cells adjacent to the basal membrane, contain a small number of cells considered the stem cells for the prostate gland. They express cytokeratins 5 and 14, CD44, and low levels of AR [6-8]. Neuroendocrine (NE) cells of the prostate are spread among the epithelial cells and are of neurogeic origin. NE cells are androgen-independent and express chromogranin A and a variety of peptide hormones. NE cells have a role in both prostatic growth and differentiation as well as in the exocrine secretory process and pathogenesis of both prostatic cancer and hyperplasia [9, 10]. The stromal cells in the prostate gland are composed of both smooth muscle cells and fibroblasts.

The entire duct-acinar system, with the exception of the main lateral ejaculatory ducts, is lined by columnar secretory cells, separated from the prostatic stroma by a layer of basal cells belonging to the basement membrane [1-3].

Secretions

The ejaculate from the human prostate is a slightly acid (pH 6.5) serous fluid in which several major secretory products can be identified, notably acid phosphatase, citrate, zinc, soluble fraction proteins, carbohydrates, electrolytes, polyamines, hormones, lipids and growth factors [11, 12].

Up to 57 major protein groups, of which 27 non-serum proteins (i.e. presumably excluded by the epithelial cells) have been identified. Major prostatic-specific proteins are prostatic acid phosphatase (PAP), PSA, and prostate binding protein (PBP), which are expressed at pubertal and adult ages. Proteolysis is the major function of prostate secretion, being rich in exopeptidase and endopeptidase. The most extensively studied protease is PSA, also known as seminin, seminal protease or chymotrypsin-like protease [3, 13].

PSA

This enzyme is secreted into the alveoli and is ultimately incorporated into seminal fluid. The alveoli secretion is pumped into the prostatic urethra during ejaculation by contraction of the fibromuscular tissue of the prostate. The fibrinolysin in the secretion serves to liquefy the semen. Normal individuals have a low serum concentration of PSA. Circulating PSA is produced by the liver, not by the prostate gland which, in normal individuals, releases PSA only into prostatic secretion. However, in prostate cancer, serum concentration of PSA increases. In this case, the additional PSA is produced and released into the circulation by the prostate gland. Therefore, the elevated levels of PSA are directly related to increased activity of the prostate cancer cells. Increased blood levels of PSA, as well as PAP, are used as markers of the presence and progression of the disease.

Prostate Disorders

There are three pathological processes affecting the prostate gland with sufficient frequency to merit discussion: inflammation, benign nodular enlargement, and tumours.

Prostatitis

The term prostatitis refers in its strictest sense to histological inflammation of the tissue of the prostate gland, although historically the term has been used loosely to describe a set of widely differing conditions.

Incidence and prevalence: Prostatitis can affect men of any age, and it is estimated that 50% of men experience the disorder during their lifetime. Prostatitis is the most common urological disorder in men over the age of 50 and the third most common disorder in men younger than 50. According to the National Institutes of Health (NIH), prostatitis accounts for 25% of all office visits involving the genitourinary system by young and middle-aged men [14]. Nonbacterial prostatitis and prostatodynia are the most common diagnoses. Bacterial prostatitis (acute and chronic) accounts for less than 5-10% of cases. Acute bacterial prostatitis occurs most often in men under age 35, while chronic bacterial prostatitis primarily affects men between the ages of 40 and 70.

Risk factors and causes: Risk factors include bladder outlet obstruction, (e.g., stone, tumour, BPH), diabetes mellitus, a suppressed immune system, and urethral catheterization. Some sexually transmitted diseases (STDs) increase the risk of developing bacterial prostatitis.

Bacterial prostatitis is caused by the growth of bacteria normally found in prostatic fluid, such as *Escherichia coli* and *Klebsiella*. Urine reflux entering the prostate can also cause the

condition. There is no known cause of nonbacterial prostatitis or prostatodynia, but atypical organisms (e.g., viruses, chlamydial organisms) have been suggested.

Classification: Prostatitis may be divided into several categories: acute and chronic bacterial prostatitis and chronic abacterial prostatitis and granulomatous prostatitis. According to the 1999 NIH Classification [15], there are four categories of prostatitis: I: Acute prostatitis (bacterial); II: Chronic bacterial prostatitis; III: Chronic prostatitis/chronic pelvic pain syndrome: Subdivisions of IIIa (inflammatory) and IIIb (non-inflammatory) exist based on levels of pus cells in expressed prostatic secretions, but these subcategories are of limited use clinically; IV: Asymptomatic inflammatory prostatitis.

Benign prostatic hyperplasia (BPH)

BPH refers to the increase in size of the prostate in middle-aged and elderly men. It occurs almost exclusively in the transitional and periurethral zones. It is characterized by hyperplasia of prostatic stromal and epithelial cells, resulting in the formation of large, fairly discrete nodules in the periurethral region of the prostate. When sufficiently large, the nodules compress the urethral canal to cause partial, or sometimes virtually complete, obstruction of the urethra, which interferes with the urine flow. This leads to symptoms of urinary hesitancy, frequent urination, increased risk of urinary tract infections and urinary retention.

Epidemiology. BPH can be seen in the vast majority of men around the world as they age, particularly in men over the age of 70 years. The prevalence of histologically diagnosed prostatic hyperplasia increases from 8 percent in men ages 31 to 40, to 40 to 50 percent in men ages 51 to 60, to over 80 percent in men older than 80 [16].

Etiology. Androgens (testosterone and related hormones) are considered to play a permissive role in BPH [17]. Dihydrotestosterone (DHT), a metabolite of testosterone, is a critical mediator of prostatic growth and also of BPH. Therapy with 5α-reductase inhibitor markedly reduces the DHT content of the prostate and in turn reduces prostate volume. There is growing evidence that both estrogens and inflammation play roles in the etiology of BPH [18, 19].

Histopathology. The hallmark of BPH is nodularity attributable to glandular proliferation or dilation and to fibrous or muscular perforation of the stroma. The proportion of these elements varies from nodule to nodule, ranging from purely stromal fibromuscular nodules to fibroepithelial nodules with glandular predominance. Glandular proliferation takes the form of aggregations of small to large to cystically dilated glands, lined with two layers, an inner columnar and an outer cuboidal or flattened epithelium, based on an intact basement membrane. The diagnosis of BPH cannot usually be made on needle biopsy, as the histology

of glandular or mixed glandular-stromal nodules of BPH cannot be appreciated on this limited sampling. Two other histological changes are associated with BPH, namely foci of squamous metaplasia and small areas of infarction [20, 21].

Prostate Cancer

Epidemiology

PCa remains one of the most common cancers afflicting men today. It is the third most common cancer in the world and the most common non-cutaneous malignant neoplasm, as well as the second cause of cancer death in men in Western countries, with a world age-standardised rate of 104 cases per 100, 000 men [22]. PCa is responsible for the death of approximately 30,000 men per year in the United States [23]. In Sweden, approximately 10, 000 new cases were detected in 2004 and 2,549 men died of PCa (Swedish National Board of Health and Welfare). The incidence has increased over the last two decades, with the PCa mortality rate remaining unchanged at a level of 59.03 cases per 100,000 men in 2003 in Sweden.

Compared with the high rates in Western countries, PCa has low rates in Asian countries. However, with aging populations and increasing use of PSA screening, the incidence of prostate cancer in the high-risk countries has risen sharply in the past decade [24]. It is revealing a more rapid increasing incidence of PCa in Asia than high-risk countries in recent data, such as in Japan, Singapore, and even in some Chinese cities, such as Beijing, Shanghai, and Hong Kong [24]. The age-adjusted incidence rate has risen from 6.6 to 14.4 per 100,000 person-years from 1978 to 1997 in Singapore Chinese [24].

Pathogenesis - the Possible Causes of Prostate Cancer

Less is known about the cause of PCa than about any other common cancer in the human body. A number of risk factors for PCa have been proposed, although the findings are often weak and controversial. The well-established risk factors include: increasing age, race, and family history of PCa. Risk factors that have no been well elucidated include: dietary, obesity, physical inactivity, hormonal, occupational, social, smoking, infection and/or inflammation, and sexual factors.

Old age: It is uncertain why PCa is an almost inevitable consequence of old age. Perhaps there is breakdown of the immune system. It is also possible that the immune system is overwhelmed by an increased number of genetically altered cells in the body which occurs naturally with age. The number of altered or damaged cells may increase because of progressive failure of the enzymes acting on a cell-by-cell basis to deal with environmental

damage such as carcinogens, a high fat diet, and other factors. The cumulative exposure to mutagens and carcinogens could influence all of these factors, resulting in cancer.

Family history and genetics: Heredity appears to be one of the most consistent and strongest risk factors for the development of PCa [25]. Genetic studies suggest that a strong familial predisposition may be responsible for as many as 5-10% of PCa cases [26]. A man's risk is two-fold higher if one first degree relative such as a father or brother has PCa, and the risk is 5-11 folds higher if 2 or 3 first degree relatives have PCa.

Diet: An increased intake of animal fat and possibly red meat has been associated with an increased risk of PCa. In contrast, vegetables may protect against PCa. High intake of cruciferous vegetables containing the chemoprotective isothiocyanate sulforaphane was correlated with a diminished risk of PCa [27]. But the relationship is complex and ill-defined, perhaps due in part to the influence of diet on the production of sex hormones, including increased androgen levels with high fat diets as well as the weak estrogen activity of soy compounds in Asian diets [28]. Obesity and alcohol abuse may also be risk factors [29].

Smoking: PCa is one of the few cancers in the human body which has not been strongly linked to smoking.

Male hormones (testosterone): The prostate requires hormones for growth and development, but hormones are also essential for cancer maintenance and growth. However, their role in the initiation of prostate cancer is unknown [30]. Men whose testicles are never developed or are removed, never develop PCa [31].

Sexual Activity: Investigators have suggested that sexual abstinence may contribute to PCa risk, but there is no consistent evidence to support this contention despite multiple studies [32]. Recent studies suggest that prior sexual practices, exposure to sexually transmitted microbial agents and a history of prostatic infection may contribute to the risk of PCa [33].

BPH: BPH is frequently seen in association with PCa, and there are a number of compelling similarities, including increasing incidence and prevalence with age, concordant natural history, and hormonal requirements for growth and development. However, no causal relationship has been established.

Infections and inflammations (see below in detail): The cause of inflammation may be microbial infection or a noninfective physical, or chemical irritant, or a hormonally induced inflammation.

Molecular pathology

PCa, like other types of cancer, is a result of the accumulation of both genetic and epigenetic alterations that transform normal glandular epithelium to preneoplastic lesions and then to invasive carcinoma. Recent advances in molecular research enable us to understand the functions of many of the protein products of oncogenes and tumour suppressor genes in great detail.

Currently, it is difficult to determine whether PCa is caused by the inheritance of one or more specific genetic traits. Less than 10% of PCa are inherited; most appear to be sporadic [26]. It is therefore suggested that the multiple clinical subsets presented may be the effects of different genetic mechanisms, and that multiple genetically controlled factors are involved in the evolution of sporadic tumours [34].

Loss of heterozygosity (LOH) and tumour suppressor genes: Cytogenetic studies reveal consistent chromosomal abnormalities in particular stages of PCa. Most of these abnormal chromosomal regions harbor several important genes involved in tumour development and progression. In PCa, chromosomal losses are more common than gains: losses of chromosomes 6q, 7q, 8p, 10q, 13q, 16q, 17, and 18q are particularly common events [35-37] (Table 1).

Table 1. Abnormal chromosomal regions and the candidate genes involved in prostate cancer development and progression:

Abnormal chromosomal regions	**candidate genes**
Chromosome 6q	*Insulin-like growth factor II receptor (IGF2R)*
	Cyclin C (CCNC)
Chromosome 8, 8p23 and 8p12–22	*NKX3.1.*
Chromosome 10q24	*PTEN*
Chromosome 10q24–25	*Negative regulator of c-myc proto-oncogene, MXI1*
Chromosome 11p11.2	*Metastasis suppressor gene KAI1*
Chromosome 13q	*Retinoblastoma (RB) gene*
	$p15^{INK4B}$, p16/CDKN2, $p21^{WAF1}$ and $p27^{KIP1}$
Chromosome 16q22	*E-cadherin*
Chromosome 17p13	*p53*
Chromosome 17q21.3–22	*Metastasis-associated genes, nm-23 H1 and H2*

Data from Lijovic M [35], Karan D [36], De Marzo AM [37], De Marzo AM [38]

Oncogenes: Information on the participation of oncogenes in PCa is limited, and the role of the expression or amplification of oncogenes remains unclear. *c-myc*, *ras*, *c-erbB2* (Her-2 neu), and Bcl-2 have received attention for their properties.

- ***c-myc:*** Several studies have demonstrated increased *c-myc* expression in PCa and a significant correlation to Gleason grade [39].

- ***Ras***: The frequency of *ras* mutations reported in PCa is variable. Mutations in K-, H-, and N-*ras* were found in less than 5% of tumours in Western populations but were detected in 26% of prostate cancers in Japanese men [40, 41].
- ***c-erbB2 (HER-2/neu)***. There is some controversy over the role of *c-erbB2* in PCa. Some studies reported *c-erbB2* over-expression in PCa and suggested that expression increases as PCa progresses to androgen independence [42]**.** Other studies have failed to identify HER-2/neu amplification or overexpression in PCa [43]**.**
- **Bcl-2**, which encodes an anti-apoptotic protein, is overexpressed in approximately half of prostate cancers, particularly in androgen-independent tumours [44]**.**

DNA methylation: Aberrant epigenetic events such as DNA hypermethylation, DNA hypomethylation and histone acethylation have been observed in PCa. Apart from specific genetic mutations, recent studies have demonstrated silencing of tumour suppressor genes by promoter hypermethylation to be the most common feature in human tumours. Hypermethylation in the promoter regions of $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$, glutathione-*S*-transferase *(GST)*, *E-cadherin* and *VHL* genes has been described in PCa [45]. The most common epigenetic change in PCa is hypermethylation of glutathione-*S*-transferase π (GSTP1), one of the major enzymes protecting against reactive oxygen species damage.

Diagnosis

Screening by digital rectal examination (DRE), biopsies and imaging: Patients with PCa show few early symptoms. Severe symptoms such as hematuria, urinary obstructive and bone pain are only present in advanced disease or cancer with metastases. Blood level of PSA, prostate palpation, and ultrasound-directed biopsy are the most common combinations of diagnostic tools [46]. Digital rectal examination and imaging by ultrasound give information about the size and shape of the prostate. The most widely accepted indication for prostate biopsy is a PSA value in serum greater than 4.0 ng/ml [46]. 10-12 cores of tissue from the gland under ultrasound guidance can be obtained from a single transrectal prostate biopsy. The biopsies are examined by a pathologist and routinely graded to the Gleason system.

Gleason score

A widely acknowledged method of grading the aggressiveness of PCa was developed by DF Gleason between 1969 and 1974. The Gleason system was based on histopathological data from over 4000 PCa biopsies and resections between 1960 and 1975 [47-49]. Architectural patterns seen at low magnification were recorded without preconception and independently

correlated with mortality data. Patterns that occurred frequently together and were associated with the same outcomes were grouped together, resulting in five grades (Fig 1):

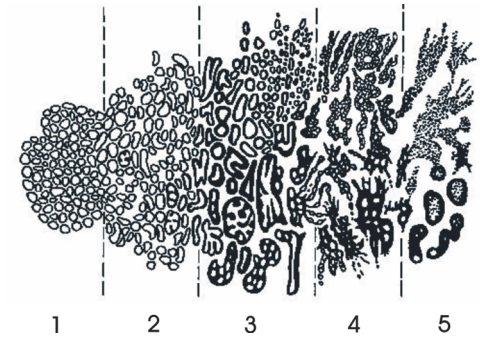

Fig 1. Gleason grade of prostate cancer

- **Grade 1** (well differentiated), circumscribed mass of evenly spaced, closely packed, uniform shaped glands, with no evidence of infiltration of the stroma.
- **Grade 2** (well differentiated), some infiltration into the surrounding stroma and more variation in gland size and spacing, although this was limited.
- **Grade 3** (moderately differentiated), most common grade with more variation in size, shape, and separation of the glands, less defined boundaries, and less intervening stroma.
- **Grade 4** (poorly differentiated), fusion of the glands forming a solid anastamosing network with a ragged invasive edge.
- **Grade 5** (undifferentiated), characterised by a complete absence of gland formation with sheets or clusters of cells.

Because of the histological variation within each tumour, two grades, the predominant, or primary, grade and the less extensive, or secondary grade, were recorded as the Gleason sum score in each case. For consistency, if only one grade was present, this was doubled. The primary and secondary grades showed similar correlations with mortality but the sum of these two grades showed the strongest correlation with cancer-specific mortality, hence, a low Gleason score (≤ 6) is indicative of a more indolent malignancy with a good prognosis whereas a high Gleason score (> 8) is associated with aggressive biological behaviour and an increased risk of occult systemic disease.

Over many years the Gleason system has been shown to be a powerful predictor of prostate cancer behaviour and outcome after either prostatectomy [50, 51], radiotherapy, or in patients managed with surveillance [52].

Biomarkers

There are several groups of established biomarkers, but many of them are still under investigation, and a number of new promising markers are being identified by gene expression analysis, e.g. hepsin, AMACR, EZH2, cell cycle proteins, autoantibody signature [53, 54]. Among them, PSA, AMACR, and established tissue markers HMW (34βE12) and p63, which are both basal cell markers, are routinely used in clinical laboratories. A triple antibody

cocktail staining, AMACR in combination with p63 and 34βE12, is currently widely used in diagnosis of PCa.

HMW 34βE12: With the advent of immunohistochemistry, 34βE12 has become an often useful adjunct in the diagnosis of prostatic adenocarcinoma [55]. Specifically, 34βE12 has been shown to highlight the sometimes inconspicuous basal cell layer in benign glands and has conventionally been considered negative in carcinomatous glands. 34βE12 can occasionally be essential in differentiating carcinoma from its many benign mimickers, including adenosis, basal cell hyperplasia, and atrophy [56].

Alpha-methylacyl-CoA-racemase (AMACR): AMACR is a recently discovered tumour marker whose expression is significantly upregulated in PCa. It has been shown to be a highly sensitive marker for the diagnosis of PCa. The majority of prostatic ductal and cribriform acinar carcinomas strongly expressed AMACR [57]. Two antibodies, P504S and AMACR-p, were usually used for immunohistochemistry [57].

P63: Basal cell marker. P63 is used as a tool to determine the state of the basal cell layer and to distinguish the regions of PIA from those of PIN and PCa [58].

Alternative Treatments

Despite recent advances in early diagnosis and treatment, PCa remains the second most lethal cancer type for men in the Western World [59, 60]. Initially, the majority of prostate cancers are responsive to androgen ablation therapy, but most of the tumours eventually progress to the androgen-refractory state and then do not respond to hormonal treatment and often develop metastatic phenotypes [61].

Prostatectomy is a surgical approach the treatment of PCa, used to remove all or part of the prostate. Typically, men with early-stage disease or cancer that is confined to the prostate will undergo radical prostatectomy, or surgical removal of the entire prostate gland plus some surrounding tissue [62].

Radiation Therapy involves the killing of cancer cells and surrounding tissues with directed radioactive exposure, and is an initial treatment for PCa. Some forms of radiation therapy can also be used in men with advanced or recurrent PCa [63].

Hormone Therapy is designed to stop testosterone from being released or to prevent the hormone from acting on the prostate cells. Hormone therapy plays an important role in men with advancing PCa, and it is increasingly being used before, during, or after local treatment as well. The majority of cells in PCa respond to the removal of testosterone. But some cells grow independently of testosterone, and therefore remain unaffected by hormone therapy. As

these hormone-independent cells continue to grow unchecked, over time, hormone therapies have less effect on the growth of the tumour. Hormone therapy is therefore not a perfect strategy, but remains an important step in the process of managing advancing disease, and is likely to be a part of therapeutic regimen at some point in recurrent or advanced PCa [62, 64].

New Therapies

Chemotherapy: Systemic chemotherapy may be of value for men with advanced high stage PCa who have failed to respond to hormone therapy. However, all chemotherapeutics, either as single agents or in combination, are associated with toxicity [65]. Recently, doxetaxel was shown to improve survival in hormone refractory PCa [66]**.**

Thermal therapy: Direct application of high temperatures will destroy cancer cells, and this method has been exploited with laser therapy, microwave hyperthermia, and electrocautery and electrovaparization. These techniques are of proven value for treatment of BPH, while none has been shown to be of value in PCa [67].

Gene therapy: Intensive research about the molecular changes in androgen-independent PCa led to the identification of several interesting genes, which may be useful as targets for gene therapy. In fact, there is a broad range of different gene therapy approaches in the field of PCa, some of which have already progressed to clinical evaluation in patients (Phase I/II clinical). These agents have aimed to be immunomodulatory (i.e., to stimulate tumour recognition by the immune system), to target an oncogene, replace a defective tumour-suppressor gene, or to directly lyse tumour cells. The Granulocyte-macrophage colony-stimulating factor (GM-CSF) gene-engineered prostate cancer vaccine (Prostate Cancer GVAX® Vaccine) has been used in a phase II study of hormone refractory prostate cancer and suggested that the Vaccine delays progression and prolongs survival in patients with bone metastatic prostate cancer [68].

Anti-angiogenesis therapy: Several anti-angiogenic agents are currently under investigation in clinical trials, and the results are promising [69, 70]. One advantage of anti-angiogenic therapy over conventional therapy is the low frequency of drug resistant mutations in endothelial cells. Current anti-angiogenic strategies target endothelial cells either directly, by inhibiting their proliferation and migration or inducing apoptosis, or indirectly, by inhibiting the production of angiogenic factors by tumour cells. Therapeutic strategies include the delivery of endogenous inhibitors of angiogenesis, agents that prevent the degradation of the basement or extracellular membrane, agents that interfere with or block the action of pro-angiogenic factors, and small molecule inhibitors of angiogenic factor receptors found in prostate cancer [71].

Tyrosine Kinases inhibition: Tyrosine kinases have been implicated in prostate epithelial cell transformation and tumour progression. Implicated tyrosine kinases include fibroblast growth factor receptor (FGFR), epidermal growth factor receptor (EGFR), and platelet-derived growth factor receptor (PDGFR). It has been reported that tyrosine kinase inhibitors (TKIs) assert their anti-tumour effects against PCa by a hormone-independent mechanism [72]. The combination of TKIs therapy and anti-hormone therapy can be synergistic [73].

Androgen receptor (AR)

Androgens are indispensable for the development and regulation of the male reproductive system and are also involved in the development of PCa [74]. The effects of androgens are mediated through the AR.

AR, a member of the subfamily of steroid receptors, has a pivotal role in the regulation of prostate growth and secretory responses. After binding of its ligand, the AR undergoes a conformational change and acquires an active form which regulates transcription of androgen-responsive genes. Thereby its functional activity is modulated by interactions with receptor-associated tissue-specific co-regulatory proteins, called co-activators and co-repressors [75, 76].

It is well known that PCa is driven by androgen. Indeed, neither cancer nor hypertrophy will occur in the absence of androgen. Men castrated before puberty or those with 5α-reductase deficiency do not develop PCa [77]. Interestingly, high levels of AR are associated with aggressive clinicopathologic features and with decreased biochemical recurrence-free survival in PCa patients treated with radical prostatectomy [78]. These observations are consistent with reports demonstrating that increases in AR mRNA and protein levels are both necessary and sufficient to convert PCa growth from the hormone-sensitive to hormone-refractory stages, and is dependent on a functional AR ligand-binding domain [79]. It may be that activity of AR and levels of AR, rather than testosterone, are the driving forces for PCa. It was shown that low levels of testosterone was associated with higher androgen receptor density in PCa, as well as higher Gleason score [78].

AR in androgen-independent PCa [80]: Analysis has shown that AR continues to be expressed in androgen-independent PCa and that AR signalling remains intact, as demonstrated by the expression of the AR regulated gene, *PSA*. Androgen-independent PCa has demonstrated a variety of AR alterations, including AR amplification, AR point mutation, and changes in expression of AR co-regulatory proteins [81]. These AR changes result in a "super AR" that can respond to lower concentrations of androgens or to a wider variety of agonistic ligands. There is also evidence that AR can be activated in a ligand independent

fashion by compounds such as growth factors or cytokines working independently or in combination, such as insulin-like growth factor-1(IGF-1), keratinocyte growth factor (KGF), and epidermal growth factor (EGF) [80].

AR down-regulation: Studies have shown the AR expression decreased after castration in the CWR22 human prostate cancer xenograft model [82]. AR expression decreased from 100% before androgen deprivation therapy to 88% after ADT, and the loss of AR was associated with a tendency to high Ki-67 index (from 17.4% to 26.3%). Reduced expression of AR appeared in 20–30% of androgen-independent PCa [83]. Methylation of the AR gene promoter has been reported in advanced hormone-independent PCa tissue and may be one of the causes of AR down-regulation in PCa [84]. AR down-regulation is more common in PCa cells. In androgen-independent PCa cell lines, such as DU145 and PC3, the loss of AR expression often occurs during the clinical evolution of PCa [83, 85]. *In vivo* studies have shown that AR mRNA and protein levels in the LNCaP cells could be down-regulated by EGF [86], basic fibroblast growth factor (bFGF) [87], and tumour necrosis factor α (TNFα) [88]. These results suggested that inflammatory cytokines may play roles in the initiation of an androgen-independent state in PCa through their ability to inhibit AR sensitivity in PCa. Moreover, IL-1β is also responsible for down-regulation of AR protein expression [89].

Chronic Inflammation and the Pathogenesis of Neoplasia

Epidemiology and Clinical Studies

In 1863, Virchow noted leucocytes in neoplasitc tissues and hypothesized that malignant neoplasms occurred at sites of chronic inflammation. Virchow reasoned that various "irritants" caused tissue injury, inflammation, and increased cell proliferation [90, 91].

Recently, the association between inflammation and cancer was illustrated in epidemiological and clinical studies [90-92]. There is increasing evidence supporting the association between chronic inflammation and cancer development. For instance, the risk of colorectal cancer was 10-fold greater when associated with inflammatory bowel disease, such as ulcerative colitis and Crohn's disease [90, 93]. Moreover, the control of colitis by certain anti-inflammatory agents reduced colon cancer incidence[93]. Persistent gastric infection with *Helicobacter pylori* and stomach cancer [94], human papillomavirus (HPV) infection and cervical cancer [90,95] are also examples of such process.

The proportion of cancer deaths attributable to infectious agents has been estimated to range from 20% to 25% in developing countries and 7% to 10% in more industrialized countries,

that meaning about 1.8 million cases of cancer were attributable to infectious agents in the year 2000 [90-92] (Table 2)

Table 2. Chronic Infection and/or Inflammation and Human Tumours

Causal Mechanisms	Types of Tumour
Helicobacter pylori and chronic gastritis	Adenocarcinoma of stomach
Epstein-Barr virus	B-cell lymphoma Non-Hodgkin lymphoma
	Hodgkin lymphoma
	Nasopharyngeal carcinoma
Human papillomavirus	Anogenital carcinoma
	Oropharyngeal carcinoma
Hepatitis B or C virus	Hepatocellular carcinoma
HIV/AIDS	Non-Hodgkin lymphoma
	Kaposi sarcoma
Liver flukes (eg, Clonorchis sinensis)	Cholangiocarcinoma
Schistosoma haematobium	Squamous carcinoma of urinary bladder
Gastroesophageal reflux	Adenocarcinoma of the distal esophagus and gastric cardia
Ulcerative colitis	Adenocarcinoma of the large intestine
Crohn granulomatous colitis	Adenocarcinoma of the large intestine
Chronic obstructive lung disease	Carcinoma of the lung
Chronic lung infections	Carcinoma of the lung
Chronic diffuse infiltrative lung diseases (eg, asbestosis, silicosis)	Carcinoma of the lung
Chronic cholecystitis	Gallbladder carcinoma
Inflammatory atrophy of prostate	Prostate carcinoma

Data from Balkwill F [90], Coussens LM [91], Schottenfeld D [92]

Causes of Inflammation

The cause of inflammation may be microbial infection, such as *Helicobacter pylori* in gastric cancer, hepatitis B virus (HBV) in hepatocellular carcinoma, or a non-infective physical and/or chemical irritant, such as gallstones in gallbladder cancer. In addition, autoimmune and inflammatory reactions of uncertain etiology were also be involved (Table 2).

Epidemiologic Support for a Relation between Inflammation and Prostate Cancer

There is evidence indicating that prostatic inflammation may contribute to prostate growth either in terms of BPH or PCa changes and, therefore, several clinical prevention trials for neoplasms have focused on antioxidants or anti-inflammatory agents. Several clinical trials have demonstrated an association between chronic inflammation, including prostatitis and sexually transmitted infections/diseases (STDs), and PCa.

Prostatitis: Various epidemiological studies have shown a significant association between prostatitis and prostate carcinoma [96-98]. A cross-sectional analysis from a prospective cohort study of 5821 men aged ≥65 years found positive associations for a self-reported history of prostatitis with a history of PCa [96]. Another case-control study investigated 409 cases at diagnosis of PCa and 803 control subjects from 1980 to 1996 [97]. The odds ratio of PCa in men with any type of prostatitis was 1.7, with acute bacterial prostatitis was 2.5, with chronic bacterial prostatitis 1.6, and with chronic pelvic pain syndrome 0.9. Therefore, infectious prostatitis might be associated with PCa. A meta-analysis of 11 case-control studies revealed a statistically significant summary odds ratio of prostate cancer of for ever having had prostatitis [98]. They found an increased risk of PCa among men with a history of prostatitis (OR = 1.6), particularly in population-based case-control studies (OR = 1.8). More recently, MacLennan et al.[99] analyzed 177 prostate needle biopsies from patients with clinical parameters suspicious for malignancy, including 144 patients with and 33 without chronic inflammation in the initial biopsies. In repeated biopsies within 5 years, in patients with chronic inflammations, 29 new PCa were diagnosed, representing a new cancer incidence of 20%. In contrast, in 33 patients initially showing no inflammation, PCa was subsequently found in 2 (6%).

However, some studies show no compelling evidence to support the hypothesis that prostatitis is a risk factor for PCa[97]. Karakiewicz et al. [100] examined the association between inflammation and coexistent PCa, as well as HGPIN, in 4,526 patients assessed with systematic prostate biopsies. Results showed that men with chronic inflammation exhibited HGPIN (2.7% vs. 20.3%, $p < 0.01$) and PCa (13.6% vs. 43.5%, $p < 0.01$) less frequently than their counterparts without chronic inflammation. In the study, the OR of 0.20 indicated that inflammatory aspects on needle biopsy are 80% less likely to appear with coexistent PCa than men without chronic inflammation. Similar results were found between inflammation and HGPIN (OR = 0.11) [100].

Goldstraw et al. analyzed the possible confounding factors leading to these different results in epidemiologic studies [101]: (1), the true incidence of prostatitis or prostatic inflammation is uncertain. Men who have prostatitis may have the condition without clinical symptoms; (2), symptomatic men with prostatitis are more likely to be screened for PCa or to undergo a needle biopsy. Therefore, PCa may be over-diagnosed in this population.

Sexually transmitted infections/diseases (STDs): STDs have been inconsistently associated with PCa, with positive associations being reported with syphilis, gonorrhoea and HPV infections in various studies [102]. Dennis and Dawson [103] found a greater relative risk of PCa

among men with a history of STDs, using both random and fixed-effects models (OR = 1.4; 17 studies), especially for syphilis (OR = 2.3; six studies). These results indicate an association between PCa and STDs, suggesting that infections might represent one mechanism through which PCa develops. A similar meta-analysis of 29 case-control studies showed a significantly high odds ratio for PCa for any STD (OR = 1.48), gonorrhoea (OR = 1.35) and HPV (OR = 1.39) [104]. Another study demonstrated that the OR between sexually transmitted (most evidence involves syphilis and gonorrhea) prostatic infections and PCa was significant (OR = 1.4; $p = 0.003$) in a meta-analysis [103].

The causes of prostatic inflammation

Although various epidemiological studies have shown a significant association between prostatitis and prostate carcinoma, the causality in these epidemiological studies is unclear, because of potential confounders. There are various potential sources for the initial event, including direct infection, urine reflux inducing chemical and physical trauma, dietary factors, estrogens, and combinations of two or more of these factors. Furthermore, any of these could lead to a disruption of immune tolerance and the development of an autoimmune reaction to the prostate.

Infectious agents: Many different pathogenic organisms have been observed to infect and induce an inflammatory response in the prostate [38]. These include sexually transmitted organisms [102], such as Neisseria gonorrhoeae, Chlamydia trachomatis [105], and non-sexually transmitted bacteria such as Propionibacterium acnes and those known to cause acute and chronic bacterial prostatitis, primarily Gram-negative organisms such as *Escherichia coli*. Although each of these pathogens has been identified in the prostate, the extent to which they typically infect this organ varies [38].

Bacterial DNA sequences were detected in 19.6% of patients with PCa [106]. In another analysis with prostate samples from organ donors, PCa or BPH, there was a strong association between inflammation and positive bacterial 16S rRNA gene PCR findings [107]. Concordance between inflammation and positive PCR results suggests that bacteria might often have a role in histologically inflammatory prostatitis caused by bacteria detectable through 16S rRNA [107]. Recently, a study of mouse model of chronic bacterial prostatitis induced by *E. coli* suggested that chronic bacterial prostatic infection and inflammation can lead to neoplastic tissue alterations in the prostate: from varying degrees of atypical hyperplasia to severe dysplasia in the prostate. In particular chronic inflammation could produce histologic changes similar to a PIN lesion [108].

Viruses can also infect the prostate. Human papillomavirus (HPV), human herpes simplex virus type 2 (HSV2), cytomegalovirus (CMV) and human herpes virus type 8 (HHV8) have been detected in the prostate [38, 109]. How frequently these agents infect the prostate, and whether they elicit an inflammatory response, is largely unknown.

Estrogens: Estrogenic exposures may be another cause of prostate inflammation and PCa. Increased levels of estrogens have long been linked to the development of PCa [110]. Estrogens affect the growth and development of the prostate, by direct effects mediated by estrogen receptor (ER)-α in the stroma, and ER-β in the epithelium or through indirect routes [110, 111]. Brief exposure of rats to high-dose estrogen during the neonatal period result in an "imprinted state" or "developmental estrogenization" in which there are developmental defects, including a reduction in prostatic growth. This treatment also results in the development of lobe-specific inflammation, hyperplasia and dysplasia or PIN [111]. Therefore, it is quite plausible that chronic inflammation in the adult human prostate might reflect an autoimmune reaction caused, at least in part, by estrogens. In adult animals, exposure to estrogens with or without DHT also results in prostate inflammation [112, 113].

Dietary factors: Epidemiological studies have revealed a link between PCa incidence and mortality and the consumption of red meat and animal fats [114, 115]. One mechanism could be related to the formation of heterocyclic amines (HCAs). Exposure of rats to dietary 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) results in carcinomas of the intestine in both sexes, in the mammary gland in females and in the prostate in males [116]. More interestingly, a processes from atrophy in prostate to HGPIN was observed directly in the atrophic epithelium [116].

Urine reflux, chemical, physical trauma and others: Chemical irritation from urine reflux has been proposed as an etiological agent for chronic inflammation in the prostate [117]. A study suggested that in mice the bacterial colonization of the prostate, possibly through the reflux of urine into the prostatic ducts of the peripheral zone, could play a role in the genesis of chronic inflammation and in prostatic tumorigenesis [38]. Urine reflux of injurious chemicals can function in conjunction with infectious agents to increase prostate inflammation [118]. Another manner by which prostate inflammation might occur is the development of corpora amylacea [119]. Corpora amylacea have been proposed to contribute to prostate inflammation, persistent infection and prostate carcinogenesis, since they are frequently observed adjacent to damaged epithelium and focal inflammatory infiltrates. Intraprostatic spermatozoa is another cause of prostate inflammation and is related to prostate atrophy [38,120].

Inflammatory Microenvironment Associated with the Subsequent Development of Cancer

Chronic inflammation is characterized by sustained tissue damage, damage-induced cellular proliferation, and tissue repair. Histopathological features of chronic inflammation include the predominance of macrophages and lymphocytes, proliferation of nurturing structurally heterogeneous and hyperpermeable small blood vessels, fibrosis, and necrosis [121]. The tumour inflammatory microenvironment is characterised by the presence of host leucocytes, high levels of reactive oxygen and nitrogen species both in the supporting stroma and in tumour areas, and inflammatory cytokine network [90, 91].

Host leucocytes. Macrophages, mast cells, neutrophils, eosinophils, lymphocytes, and dendritic cells have been found to be key components in the epithelial-originated tumours [122].

- **Tumour-associated macrophages** (TAM) contribute to tumour development through several mechanisms. TAMs release interleukin (IL)-10 and prostaglandin E_2 (PGE2), which suppress antitumour response [123]. TAMs also facilitate tumour growth by releasing angiogenic factors, such as VEGF, endothelin-2, and urokinase-type plasminogen activator [121, 124]. TAMs produce IL-1, which up-regulates VEGF transcription [125]. TAMs also facilitate tumour cell invasion and metastasis by releasing matrix metalloproteinases (MMP-2 and MMP-9) [126]. In addition, TAMs induce TNF-α and iNOS, which damage DNA and inhibit DNA repair. Moreover, TAMs release epidermal growth factor and other epidermal growth factor receptor family ligands to promote tumour cell proliferation and migration [123].
- **Activated mast cells** generate angiogenic growth factors, such as VEGF/vascular permeability factor and bFGF, specific angiogenic regulators histamine and heparin, MMP-9, and mast cell–specific proteases MCP-4 and MCP-6 [127, 128]. Therefore, activated mast cells are suggested to be involved in tumour angiogenesis, invasion, and metastasis. Inflammatory mast cells also enhance tumour progression by releasing cytokines and chemokines.
- **Tumour-associated neutrophils** enhance tumour angiogenesis, invasion, and metastasis in a similar manner to TAMs and mast cells [128]. Neutrophils may also play a role in genetic instability of tumours [128].
- **T-lymphocytes** are recruited to tumours by a series of chemokines. At the premalignant lesion stage in a skin cancer model, the knockout of T cells resulted in decreased leukocyte infiltration and reduced levels of MMP-9 [129]. The increase of $CD4^+$ T cells was consistently positively correlated with poor prognosis in both renal cell cancer and colorectal cancer [130].
- **Tumour-associated dendritic cells (TADC)** have an immature phenotype with defective ability to stimulate T cells [90].

Reactive oxygen and nitrogen species: Chronic inflammation and the metabolic products of phagocytosis are often accompanied by the excessive formation of reactive oxygen and

nitrogen species that are potentially damaging to DNA, lipoproteins, and cell membranes. Inflammatory cells also release metabolites of arachidonic acid, or eicosanoids, including prostanoids or prostaglandins and leukotrienes. Cyclooxygenase (COXs) are key enzymes that control rate-limiting steps in prostaglandin synthesis.

Table 3. Key Molecular Players Linking Cancer to Inflammation

Potential linkers	Functions in linking inflammation to cancer
Cytokines	
IL-6	Promote tumour growth
TNF-α	Induce DNA damage and inhibit DNA repair
	Promote tumour growth
	Induce angiogenic factors
Chemokines	Promote tumour cell growth
	Facilitate invasion and metastasis by directing tumour cell migration and promoting basement membrane degradation
NF-κB	Mediate inflammation progress, promoting chronic inflammation
	Promote the production of mutagenic reactive oxygen species
	Protect transformed cells from apoptosis
	Promote tumour invasion and metastasis
	Feedback loop between proinflammatory cytokines
iNOS	Downstream of NF-B and proinflammatory cytokines
	Induce DNA damage and disrupt DNA damage response
	Regulate angiogenesis and metastasis
COX-2	Produce inflammation mediator prostaglandins
	Promote cell proliferation, antiapoptotic activity, angiogenesis, and metastasis
HIF-1α	Promote chronic inflammation
	Induced by proinflammatory cytokines through NF-κB
	Enhance the glycolytic activity of cancer cells
	Contribute to angiogenesis, tumour invasion, and metastasis by transactivating VEGF
STAT3	Activated by proinflammatory cytokines
	Promote proliferation, apoptosis resistance, and immune tolerance
Nrf2	Anti-inflammatory activity
	Protect against DNA damage
NFAT	Regulate proinflammatory cytokine expression
	Required in cell transformation

Date from Lu H [121]. HIF-1α, hypoxia-inducible factor-1alpha; STAT3, Signal Transducer and Activator of Transcription 3; Nrf2, NF-E2-related factor-2; NFAT, nuclear factor of activated T-cells.

Inflammatory cytokine network: The cytokine network is rich in inflammatory cytokines (lymphokines, ILs, interferons), growth factors, stress proteins, and chemokines, which might be produced by the tumour cells and/or tumour-associated leucocytes and platelets, and which may directly or indirectly contribute to malignant progression. This cytokine network may influence survival, growth, mutation, proliferation, differentiation, and movement of both

tumour and stromal cells. Moreover, these cytokines can regulate communication between tumour and stromal cells, and tumour interactions with the extracellular matrix [90, 91] (Table3).

Mechanism of Inflammation and/or Infection and Carcinogenesis of Prostate

Genetic aspects. Nelson et al. analyzed the molecular basis of the association between inflammation and prostate proliferative diseases from a genetic view point [131]. PCa has the greatest inherited contribution of any common cancer. PCa genes appear to confer increased susceptibility to PCa in certain families. The possibility that viral or bacterial infections or inflammation might lead to PCa has been linked with the identification of two candidate susceptibility genes familial PCa genes [131]. ***RNASEL,*** which encodes an enzyme that degrades viral RNA on viral infection, is linked to PCa in specific families [132] and is associated with an increased PCa risk [133]. ***MSR1*** encodes subunits of a macrophage-scavenger receptor capable of binding bacterial lipopolysaccharides [131]. *MIC1* gene is a member of the TGFβ superfamily and is recognized to have an important role in inflammation by regulating macrophage activity [131]. In a study a series of *MSR1* mutations appeared to be linked to PCa susceptibility in some families at high risk for PCa [134].

Oxidative stress: Chronic inflammation can induce proliferative events and posttranslational DNA modifications in prostate tissue through oxidative stress. In fact, repeated tissue damage and oxidative stress related to this event may provoke a compensatory cellular proliferation with the risk of hyperplastic growth or also of neoplastic modifications [135, 136]. It is well accepted that regions of prostatic inflammation can generate free radicals, such as nitric oxide (NO) and various species of oxygen. In particular, macrophages and neutrophil infiltrations provide a source of free radicals that can induce hyperplastic or precancerous transformations through the oxidative stress to the tissue and DNA [135]. The inducible NO synthase (iNOS) is the principal factor activating reactive nitrogens that can damage cells [137]. Gradini et al. [138] characterized NOS expression in human prostate tissue and, particularly for iNOS, they found an increased immunostaining in the epithelial cells of cases with BPH and more with HGPIN and PCa, when compared to normal tissue. NO also enhances COX activity [135].

Glutathione-*S*-transferase activity: Normally, prostate tissue is protected by oxidative stress reactions, free radicals, and highly reactive oxygen species by the superoxide-dismutase and the GST enzymes systems. GST gene methylation produces the loss of this protective enzyme system and it could be implicated in the transition from inflammation to preneoplastic lesions and therefore to PCa [135]. Lee, et al [139] identified GST methylation in nearly 70% of HGPIN lesions and in >90% of PCa.

COX-2 and Cancer

Cyclooxygenase

Cyclooxygenase (COX), also referred to as prostaglandin endoperoxide synthase, is a key enzyme in the conversion of arachidonic acid to prostaglandins (PGs) and other eicosanoids. It exists as three isoforms, COX-1, COX-2, and COX-3. COX-1 and COX-2 are encoded by separate genes located on separate chromosomes [140]**.** They are highly related at the DNA, RNA, and protein level. COX-1 and COX-2 consist of 576 and 587 amino acids, respectively, and they share approximately 60% primary sequence homology. COX-1 and COX-2 exist as integral membrane glycoprotein homodimers and are found on the luminal surfaces of the endoplasmic reticulum and nuclear envelope [141, 142]. Traditional NSAIDs, such as aspirin and ibuprofen, act by inhibiting COX activity.

Although COX-1 and COX-2 genes are similar, they are under profoundly different mechanisms of control and fulfil different physiological functions. COX-1 is essentially a "housekeeping gene", expressed constitutively in most normal tissues and is required for their normal physiological function. It is important for mediating various normal physiologocial processes, including the preservation of renal blood flow and function, platelet aggregation and hemostasis, and cytoprotection of the gastrointestinal mucosa. COX-2, on the other hand, is an "immediate to early gene" [143, 144]. It is frequently undetectable at baseline in normal tissues, but can be induced in response to mitogenic agents such as growth factors, hormones, bacterial endotoxin, tumour promoters, cytokines, hypoxia, and its role has been connected to inflammation and carcinogenesis [145]. The third form of COX, termed COX-3, has been identified. It encodes a protein with a completely different amino acid sequence than COX-1 or COX-2, and its exact function is still to be determined [146].

COX-2 expression in human tumours

Expression of the COX-2 is elevated in a variety of human malignancies and premalignant lesions, including colon, head and neck, lung, bladder, stomach, breast, and prostate cancer. In colon cancer**,** for example, several studies reported an inverse correlation between colon cancer incidence and regular use of NSAIDs and NSAIDs are known to function by inhibiting COX enzyme activity [147, 148]. COX-1 levels were not increased in colorectal tumours. In contrast, striking COX-2 upregulation was observed in colon cancer and in some adenomas [149, 150]. Increased COX-2 staining correlated with larger polyp size and progression to invasive carcinomas as well [149, 150]. Treatment with the NSAIDs causes a decrease in the size and number of polyps in familial adenomatous polyposis patients [151]. Thus, overexpression of

COX-2 appears to contribute to colorectal cancer and COX-2 inhibitors are likely to be useful chemopreventive agents.

COX-2 overexpression in gastric cancer is another example. Normal gastric mucosa scarcely expresses COX-2. It has been shown that *H pylori* infection induces COX-2 mRNA/protein levels with the production of PGE_2 in gastric premalignant and malignant lesions[152-154]. The COX-2 expression also correlates with to poor prognostic parameters, tumour size, depth of invasion, lymph node metastasis, lymphatic invasion, and angiogenesis [155, 156]. COX-2 expression also correlated with p53 accumulation, indicating that COX-2 is induced by p53-mediated activation[157].

COX-2 and inflammogenesis of cancer

There are increasing evidences from molecular, animal, and human investigations support the hypothesis that aberrant induction of COX-2 and up-regulation of the prostaglandin cascade play a significant role in carcinogenesis, and reciprocally, blockade of the process has strong potential for cancer prevention and therapy [158]. Supporting evidence includes:

1) Expression of constitutive COX-2-catalyzed prostaglandin biosynthesis is induced by most cancer-causing agents, including mitogens, growth factors, proinflammatory cytokines, microbial agents, tumour promoters, essential polyunsaturated fatty acids, and other epigenetic factors [158, 159].
2) COX-2 expression is a characteristic feature of both neoplasms and premalignant neoplasms, including in colon cancer and some adenomas [149, 150]**;** in pancreatic carcinoma and its pre-malignant lesion, intraductal papillary mucinous adenomas [160]; in esophageal adenocarcinoma and Barrett's esophagus, as well as dysplasia [161]**;** in gastric cancer and its premalignant lesions[153]; and in prostate cancer and HGPIN [162-171]**.**
3) Most of the essential features of carcinogenesis (mutagenesis, mitogenesis, angiogenesis, reduced apoptosis, metastasis, and immunosuppression) are linked to COX-2-driven PGE-2 biosynthesis [172-175]**.**
4) Animal studies show that COX-2 up-regulation is sufficient to stimulate the transformation of normal cells to invasive cancer and metastatic disease [176, 177].
5) Non-selective COX-2 inhibitors, such as aspirin and ibuprofen, and selective COX-2 inhibitors, such as celecoxib, reduce the risk of human cancer and precancerous lesions [178, 179].

Proposed mechanism for the role of COX-2 in carcinogenesis

Multiple lines of compelling evidence support that COX-2 plays a crucial role in carcinogenesis. Since COX-2 is a PG synthase, the most obvious consequence of COX-2 overexpression is increased PG production, and indeed high PG levels have been detected in many cancers. PGs are believed to be important in the pathogenesis of cancer because of their effects on cell proliferation, angiogenesis, immune surveillance, and apoptosis [171, 180] (Fig 2).

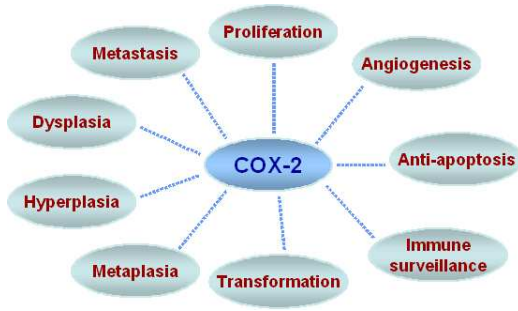

Fig 2. Schematic representation of roles of COX-2 in carcinogenesis. Adjusted from **C**hun[181]**,** Pruthi[171], and Zha[180]

PGs stimulate proliferation. Inappropriate stimulation of cellular proliferation by PGs may contribute to tumorigenesis. Enhanced PG synthesis may contribute to carcinogenesis in several ways, including direct stimulation of cell growth [172]. PGE can stimulate mitogenesis in Balb/c 3T3 fibroblasts in synergy with epithelial growth factor (EGF) [172]. Both PGE1 and PGE2 stimulate proliferation of mammary epithelial cells in the presence of EGF [182, 183].

Effects on angiogenesis. Tumour angiogenesis includes destabilization of pre-existent blood vessels, proliferation of vascular endothelial cells, invasion by endothelial cells into the extracellular matrix (ECM) and finally the migration and positioning of endothelial cells. COX-2 is known to play an important role in tumour-induced angiogenesis through the synthesis of angiogenic PGs such as PGE2, which induces matrix metalloproteinase (MMP) and VEGF [184-186]. Subsequently, numerous studies showed co-localization of angiogenesis factors, such as VEGF, PDGF, bFGF and TGF-β with COX-2 by immunohistochemical staining in different cancer types [187]. In breast cancer, the density of microvessels was higher in patients with COX-2 expression than in those without COX-2 expression [188]. Studies of colon cancer cell lines co-cultured with vascular endothelial cells demonstrated that COX-2 supported angiogenesis at multiple steps both directly and indirectly [189]. First, COX-2 up-regulation leads to PGs production, and PGs have distance roles for angiogenesis. Second, overexpression of COX-2 in tumour cells directly stimulates the production of angiogenic

factors, including VEGF, PDGF, bFGF and TGF-β, from these cells [189, 190]. Through these angiogenesis mediators and their receptors on the endothelial cells, COX-2 increased vascular permeability and induced endothelial cell proliferation and migration. In breast cancer, the density of microvessels was higher in patients with COX-2 expression than in those without COX-2 expression. COX-2 expression was also associated with the LN metastasis [173].

COX-2 mediated resistance to apoptosis. The suppression of apoptosis associated with COX-2 overexpression could be an important factor in tumorigenesis, although the precise mechanistic basis remains uncertain. The first hint came from the observation that NSAIDs could induce apoptosis in cultured cell [191]. Since then numerous studies using cultured cells and animal models have supported a role for COX-2 in promoting cell survival under unfavourable growth condition [192]. Several hypotheses have been advanced to account for suppression of apoptosis in response to COX-2 overexpression. The ability of PGE2 to inhibit apoptosis caused by a selective COX-2 inhibitor, and concomitantly to induced Bcl-2, suggests that PG-mediated upregulation of Bcl-2 may suppress apoptosis [192]. Multiple NSAIDs, including selective COX-2 inhibitors, induce apoptosis in a variety of cells [193]. The further studies have shown that NSAIDs stimulate apoptosis may via both COX-dependent and -independent mechanisms [194].

Immune suppression. PGs do not act as mitogens for all cell types, and in fact depress proliferation of some cells, particularly those immune cells [174, 195], and thereby contribute to the immune suppression. PGE2 inhibits T and B cell proliferation and cytokine synthesis, and diminishes the cytotoxic activity of natural killer cells. PGE2 also inhibits the production of TNFα while inducing IL-10 production, which itself has immunosuppressive effects [175], and inhibit antigen processing by dendritic cell [174]. Thus, PG-mediated immune suppression may contribute to tumorigenesis, since this may allow tumours to avoid immune surveillance.

Effects on cell motility and invasiveness. PGs may have effects on cell motility and adhesion. COX-2 expression leads to increased tumour invasiveness and adhesiveness to extracellular proteins, perhaps by the alteration of cellular dynamics, including increased MMP-2 expression and decreased e-cadherin expression [196]. Rat intestinal epithelial cells with stable overexpression of COX-2 show several altered characteristics including increased adhesion to extracellular matrix [197]. COX-2 inhibitors have been shown to decrease cell migration, cell adhesion, and tumour invasiveness *in vitro* and *in vivo* studies [192].

Transcriptional regulation of COX-2 expression

Overexpression of COX-2 appears to be a consequence of both increased transcription and enhanced mRNA stability [198]. Lipopolysaccharide (LPS) was the first inducer of COX-2 expression to be identified [199]. It is now known that most pro-inflammatory mediators stimulate COX-2 transcription via different signaling [198], including: growth factors, such as IGF, TGFα and EGF [181]; nitric oxide (NO) and reactive oxygen species (ROS) [200]; and several pro-inflammatory cytokines such as IL-1 or IFN-γ [201]. COX-2 protein levels are also regulated at a prost-transcriptional level via modulation of the stability of its mRNA. Signals from cytokines such as IL-1β and TNF-α affect COX-2 mRNA stability. Hypoxia represents another regulator of COX-2 mRNA. It increases its stability most likely via induction of TNF-α [202] (Table 4).

Table 4: Regulators of COX-2 expression

Increased Expression (up-regulators)	**Decreased Expression (down-regulators)**
LPS	p53
Interleukin-1α	Fish oil
Interleukin-6	Estrogens
Epidermal growth factor	Glycogen synthase kinase 3
Transforming growth factor-β	Glucocorticoids
Tumor necrosis factor-α	
Ultraviolet B	
Benzopyrene	
Androgens	
Inducible NO synthase	
reactive oxygen species (ROs)	
Wnt	
ras	
src	
Nuclear factor-κB	
cAMP-ERP	

Data from E. Fosslien [187], Turini [203] and Pruthi [171]

Glycogen synthase kinase 3 (GSK3) and glucocorticoids represent the most important negative regulator of COX-2 transcription. They achieve this effect via inhibition of NF-κB [204]. Wild-type p53 markedly suppressed transcription of COX-2 [205].

NF-κB is a crucial positive regulator of COX-2. NF-κB is activated in response to a wide variety of stimuli, such as LPS. A growing body of evidence indicates that NF-κB plays a central role in general inflammatory as well as immune responses. The promoter region of COX-2 contains two putative NF-κB binding sites. NF-κB has been shown to be a positive regulator of COX-2 expression in murine macrophages and human colon adenocarcinoma cell lines exposed to LPS [206,207] (Table 4).

CCAAT/enhancer Binding Protein Transcription Factors and COX-2 Regulation

The CCAAT/enhancer binding proteins (C/EBPs) are members of the basic region – leucine zipper (bZIP) class of transcription factors and play important roles in fundamental cellular processes including proliferation, growth arrest, and differentiation in a cell-type specific manner. There is a C/EBP binding site on the human COX-2 promoter region and C/EBPs are involved in regulating the activity of the COX-2 promoter [208].

The functions of C/EBPs in the regulation of COX-2 transcription are cell type- specific and differentiation stage-specific. Even the same C/EBP isoform displays opposite effects depending on the cell type [208-210]. C/EBPα is known to be involved in the regulation of cell proliferation and differentiation. C/EBPα can induce growth arrest in various cell types, for example, inhibits hepatocyte proliferation and also suppresses colony growth in mouse fibroblasts [211]. Unlike C/EBPα, C/EBPβ transcription factor has been shown to play a pivotal role in regulating key biological processes, such as cell proliferation, differentiation, and apoptosis, which are thought to be crucial in tumourigenesis [208]. In mammary epithelial cells and hepatocytes primed by the agents causing acute phase response, C/EBPβ was predominantly expressed, whereas the level of C/EBPα was quite low [212]. A few experimental data have shown that C/EBPβ and NF-*κ*B are the most dynamical transactivators for COX-2 promoter activation by diverse pro-inflammatory mediators [213]. The transfection with C/EBPβ led to increased COX-2 expression in gastric cancer cell lines [214,215]. In human tumour, for instance, in gastric carcinoma, a positive relationship between the expression of C/EBPβ and COX-2 has also been reported [216].

COX-2 Expression in Prostate Cancer

Epidemiology

The potential role of COX-2 in PCa has received considerable attention in the last several years. As in colon cancer, initial suspicion of the potential role of COX-2 in PCa came in the form of epidemiological evidence. Roberts et al. [217] observed in their cohort of 1,362 patients that the daily use of nonsteroidal anti-inflammatory drugs (NSAIDs), including aspirin, resulted in lower relative odds of PCa [217]. Nelson and Harris noted a decreased relative risk in patients on over-the-counter and prescription NSAIDs [218]. Interestingly of patients diagnosed with PCa those on NSAIDs were noted to be at somewhat lower risk for advanced cancer than age matched controls [219]. In a recent meta-analysis of 12 published series evaluating NSAID use and PCa risk, Mahmud *et al.* [220] demonstrated that NSAID users have a lower relative risk

of PCa development than do nonusers. Patients with a diagnosis of PCa, those who took NSAIDs were noted to have a lower risk (risk ratio 0.73) of developing "advanced" PCa compared with age-matched controls [219]. These findings were recently corroborated by a large cohort questionnaire study that examined the association between NSAID use and PCa incidence among 70,144 men [221]. The results demonstrated that regular NSAID users had an 18% lower overall risk of PCa and a 33% reduced risk of advanced disease [221]. However, Langman et al. were unable to identify such a correlation in their British cohort [222].

COX-2 mRNA and protein expression in prostate tissues

In addition to these epidemiological observations, molecular and immunohistochemical studies have demonstrated that COX-2 overexpressed in human PCa.

O'Neill and Ford-Hutchinson [223] firstly analyzed COX-1 and COX-2 mRNA expression in various human issues and found the highest levels were detected in the prostate where COX-1 and COX-2 transcripts were present in approximately equal levels [223]. Kirschenbaum et al.[168] investigated the COX-1 and COX-2 in the human fetal and adult male reproductive tracts. COX-2 was strongly expressed in epithelial cells of both fetal and adult seminal vesicles and ejaculatory ducts. No COX-2 was found in the fetal prostate. In BPH samples, COX-2 was strongly expressed in smooth muscle cells.

Evidence that increased COX-2 levels may be important in PCa development comes from preliminary results in human and canine prostates [163, 167, 224]. Gupta et al. [163] reported that COX-2 mRNA level were 3.4-fold higher in PCa tissue compared with the paired benign tissue [163]. Madaan [167] reported that COX-2 expression in the PCa was significantly different from those in BPH and were increased with increasing tumour grade. In contrast, only weak to moderate COX-1 expression found in tumour cells and its expression did not correlate with tumour grade. Several studies have similarly noted that human PCa overexpresses COX-2 mRNA and protein compared with benign epithelium, as measured by immunohistochemistry, quantitative RT-PCR, and Western blot analysis [163-171]. A few reports have shown that COX-2 expression correlates with PCa stage, grade and progression [163, 164, 166-169]. COX-2 expression is also increased in PCa lymph node metastasis [163, 164, 166-168], suggesting that in the prostate COX-2 may act in tumor promotion and progression [165, 171]. Cohen et al. [225] reported that COX-2 expression is an independent predictor of prostate cancer progression following radical prostatectomy. Hormonal therapy may induce the COX-2 expression in PCa as well, since one study found that neoadjuvant hormonal therapy induced the expression of COX-2

protein in PCa cells [226]. COX-2 expression in the epithelial cells of HGPIN to a variable degree was also reported [165, 166, 168, 169].

However, although most such studies have demonstrated increased COX-2 expression in the prostatic epithelial cells, some investigators have attributed the increased COX-2 expression to the accompanying inflammatory cells found in the neoplastic tissue and not from the cancer cells themselves [227]. They found that the expression of COX-2 was not elevated in HGPN or in established PCa. In limited cases, when staining for COX-2 was observed in PCa, the extent of positive staining did not correlate with established clinical and/or pathological risk factors-Gleason score or pathological stage. By contrast to the neoplastic tissue, the consistent expression of COX-2 protein in PIA lesions was present [227].

COX-2 expression in prostate cell lines

In addition to immunohistochemistry and mRNA studies correlating COX-2 expression with PCa presence and behaviour, an increasing number of *in vitro* pharmacological studies have likewise implicated COX-2 expression in PCa progression. In PCa cell lines, COX-2 is expressed in androgen responsive (LNCaP) and androgen resistant (PC-3) cell lines [228-230]. Furthermore, exposure of these cell lines to COX-2 inhibitors results in apoptosis induction in LNCaP and PC-3 cells in a time and dose dependent manner [184, 228-231]. While COX-2 inhibitors could suppress the proliferation of LNCaP and PC-3 cells, PrSC cells (stromal cells) were not affected by COX-2 inhibition [231]. TNFα is a strong inducer of COX-2 expression. It induced the COX-2 expression both in prostate epithelial cells and stromal cells [232].

Tjandrawinata et al. provided evidence that increased PG synthesis has both growth-promoting and positive feedback effects in PC-3 cells. The study suggested that the increased expression of COX-2/PGE2 contributes to prostate cancer development and this positive feedback activity of PGE2 may be responsible for the existence of COX-2 in these cells as compared to the short-life of the inducible COX-2 in other cells [233].

In COX-2 cDNA transfected LNCaP cells (LNCaP-COX-2), the level of COX-2 mRNA and protein and the COX activity was significantly increased. Both cell proliferation *in vitro* and tumour growth rate *in vivo* were increased. LNCaP-COX-2 cells also had increased secretion of VEGF protein, suggesting that the contribution of COX-2 on PCa progression was partly through increased VEGF [184]. Another study showed that PC-3 high invasive cells produce higher PGE2 than PC-3 low invasive cells and PGE2 may enhance the cell invasion of the invasive cells[234]. Indomethacin, a non-specific COX inhibitor, and NS-398, a specific COX-2 inhibitor, both inihibited the prostaglandin synthesis and cell invasion [234].

Treatment strategies based upon novel molecular targets in inflammatory pathways

New treatment strategies based upon novel molecular targets in inflammatory pathways have been suggested and are currently being evaluated in experimental and clinical trials. Inflammatory components targeted by these therapies include agents that inhibit or block cytokines, NF-κB activity, oxidative damage, or COX-2 (Fig 3) [90, 235].

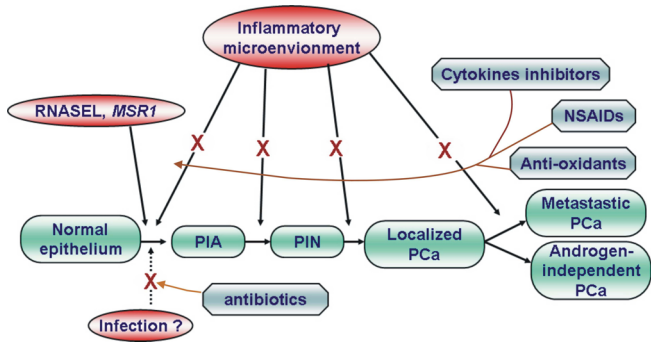

Fig 3. Possibilities for prevention of prostate cancer. Data from Yegnasubramanian [236], Pruthi[237], and Lu [121].

TNF blockade

Two TNF antagonists (etanercept, and infliximab) have been licensed for clinical trial in the treatment of rheumatoid arthritis and Crohn's disease [238]. There is clinical evidence for these actions of the anti-TNF antibody in rheumatoid arthritis joint tissue: inhibition of cytokine/chemokine production, reduced angiogenesis, prevention of leukocyte infiltration, inhibition of matrix metalloproteases, and improvement of bone-marrow function. All these actions would be useful in a biological therapy for cancer.

IL-6 antagonism

Monoclonal antibody to IL-6 was given to patients with myeloma. There was evidence of biological effect: deceased C-reactive protein, lower IL-6 production, and resolution of low-grade fever [239].

Anti-cytokines

A new generation of vaccines directed against cytokine activity could be beneficial in the treatment of cancer [240]. These new vaccines could be targeted to agents that directly stimulate tumour cell growth, such as TGF-β or IL-10, or could remove inappropriate suppression of immunefunction by aberrantly expressed cytokines. Clinical trials using vaccines directed

against EGF and VEGF in cancer patients have shown some potentially encouraging results. Further studies directed at cytokines and inflammatory pathways may further improve the potential benefit of these vaccines.

NF-κB inhibition

Therapeutic options directed at inhibiting NF-κB function could possible reduce inflammation and subsequent repair processes. Salicylates and corticosteroids are known to inhibit NF-κB function [241], but their effects are non-specific. A number of different agents have been developed that directly inhibit NF-κB activation by blocking proteasomes, inhibiting normal NF-κB activation pathways, or blocking NF-κB transcription. Anti-oxidants have also been used as NF-κB inhibitor, since ROS are known to activate NF-κB.

Anti-oxidants

The potential role for anti-oxidants is very appealing. Intake of fresh fruit and vegetables appears to be inversely correlated with cancer of the esophagus, stomach, and pancreas [242], although the specific factors involved remain undefined. A potential protective effect of specific vegetable components, suggested by a reduction of urinary ROS excretion, might be due to phytochemicals that induce enzymes scavenging electrophiles.

- **Selenium:** Epidemiology studies have correlated low selenium with an increased risk of PCa [243-245]**.** In addition, a clinical trial of selenium supplementation for the prevention of recurrent nonmelanoma skin cancer revealed a decrease in incident PCa, especially in men with low selenium at trial entry [246].
- **Vitamin E**: Inverse correlations between vitamin E (α-tocopherol and γ–tocopherol) and PCa risk have also been reported [247]**.** A randomized clinical trial of α-tocopherol and β carotene supplementation for the prevention of lung cancer in male smokers showed a 32% decrease in PCa incidence and a 41% decrease in PCa mortality in men who received α-tocopherol [248].
- **Lycopene**: Lycopene is a carotenoid that is found in a variety of foods, including watermelon and tomato, and it has been found in epidemiological studies to be associated with a lower risk of PCa [29, 249]**.** Consumption of vegetables containing the carotenoid lycopene and high lycopene blood levels have been associated with low PCa risk [250]**.** A clinical trial in which men were fed tomato based pasta showed a decrease in oxidative genome damage in the prostate [251]**.**
- **Sulforaphane**: Consumption of cruciferous vegetables containing isothiocyanates, such as the chemoprotective compound sulforaphane, has been reported to reduce PCa risk [27]. Preclinical data suggest that this micronutrient may decrease the risk of PCa [252]. Sulforaphane can act as an antioxidant by inducing a plethora of carcinogen detoxification enzymes via a mechanism involving the cysteine rich protein Keap1 and the transcription factor Nrf2 [253]**.**

NSAIDs and COX-2 inhibitors in prostate cancer

NSAIDs have been available during the past century for their analgesic and antiinflammatory properties. However, it was not until 1971, that Vane JR demonstrated that the anti-inflammatory effects of NSAIDs occurred through inhibition of the COX enzyme [254].

Typical NSAIDs (eg, aspirin, ibuprofen) function to nonselectively inhibit both isoforms of COX. It is through COX-2-specific inhibition that the antiinflammatory and analgesic properties of such medications occur. However, it is the nonselective inhibition of COX-1 that produces the well-known side effects of NSAIDs, including gastrointestinal irritation, impairment in renal blood flow, and reduced hemostasis [255].

The discovery of the two different COX isoforms and their unique physiologic properties led to the intensive search for drugs to selectively inhibit COX-2 alone - a drug that can achieve the antiinflammatory effects of COX-2 inhibition without the toxicities that can result from COX-1 inhibition. Such selective COX inhibitors (COXIBs) have since entered mainstream medical practice, with approval by the Food and Drug Administration (FDA) of celecoxib in 1998 and rofecoxib in 1999.

Celecoxib appeared to be the most effective NSAID at clinically relevant concentrations against human prostate carcinoma cells *in vitro* [256]. *In vivo* studies demonstrated a dramatic antitumour effect of selective COX-2 inhibition in nude mice models which injected with PC-3 cells [228]. This effect occurred via a combination of tumour cell apoptosis induction and down-regulation of tumour VEGF. In another animal study, celecoxib suppressed progression in the transgenic adenocarcinoma of the mouse prostate model [178].

Potential Clinical Uses of COX-2 Inhibitors: Celecoxib has been evaluated in patients with biochemical progression after definitive therapy with beneficial effects [257]. This study demonstrated a consistent, inhibitory effect on PSA progression in an androgen-independent fashion [257]. The results suggest that COXIBs may help delay or prevent disease progression after biochemical failure and, perhaps, prolong the time until institution of androgen-deprivation therapy.

Perhaps the most important is the potential role of COXIBs in the treatment of hormone-refractory disease. Preclinical studies have suggested an androgen-independent mechanism for the antitumour action of COXIBs in PCa [228]. Investigators have shown that inhibition of COX-2 may work synergistically with certain chemotherapeutic drugs (eg, docetaxel) to inhibit growth in hormone-refractory xenografts [258].

COXIBs may also play a role as a radiosensitizer in PCa. Wen *et al.* [259] demonstrated that the COX-2 inhibitors enhanced the antitumour effect of radiation *in vitro* and *in vivo* in PCa.

Another potential application of COXIBs is for bone metastases. A recent investigation has demonstrated that COX-2 is overexpressed in a mouse model of metastatic breast cancer and that COX-2-derived PGs may contribute to bone osteolysis [260]. Correspondingly, administration of NSAIDs inhibited osteolysis due to bone metastases. In a sarcoma-based model of metastatic disease, selective inhibition of COX-2 decreased cancer pain, bone destruction, and even tumour growth [260]. These provocative results warrant further study in bone metastases from PCa.

COX-2 inhibitors in PIN: COX-2 inhibitors were also used for reducing the PIN number in transgenic mouse prostate model (TRAMP), which exhibits many similarities to human PCa. The results showed that the effectiveness of celecoxib and exisulind in reducing the PIN lesions by modulating a cascade of molecular targets involved in COX-2-dependent and -independent mechanisms [177].

COX-2 inhibitors have cardiovascular side effects: Although COXIBs offer efficacy while minimizing the unwanted side effects that are attributable to COX-1 inhibition, e.g. gastric ulceration, COXIBs may invariably cause an imbalance between PGI_2 and thromboxane (TX), thus indicating that multiple and opposing cardiovascular influences may be operative during COX-2 inhibition. McGettigan and Henry performed a meta-analysis of controlled observational studies, which including 23 case- or cohort-controlled population studies, to compare the cardiovascular risks associated with COXIBs and other NSAIDs. The results showed that COXIBs (celecoxib and rofecoxib) have a lower safety profile than NSAIDs (naproxen and ibuprofen) [261].

Precursor Lesions of Prostate Cancer

Definition of prostate cancer precursor lesions

The development of a solid tumour is generally thought to be a multistep process, whereby successive genetic events occur in a normal cell to render it increasingly malignant. The prototype for this model of carcinogenesis in solid tumours is colon cancer, but similar studies have confirmed this model in other diseases including breast and cervical cancers. In PCa, the genetic and epigenetic phenomena that are occurring in cancer development are not well understood, but there is evidence that premalignant lesions in the prostate may precede the development of cancer by many years.

There are several criteria that should be met in order to consider a prostatic lesion as premalignant[262]: an epidemiological relationship must be revealed, the precursor lesion should be present at an earlier age than the cancer, and clear morphological (for example, cellular,

histological, architectural) similarities should be present. Also, premalignant lesions should be close to their presumed malignant equivalents.

HGPIN

In 1965, McNeal described lesions with possible premalignant features in prostatic epithelium [263]. In 1986, McNeal and Bostwick described the first criteria for the diagnosis of "intraductal neoplasia" [264]. In 1987, Bostwick and Brawer introduced the term PIN - prostatic intra-epithelial neoplasia [9]. At the beginning, PIN was categorized into three grades with regard to architectural and cytological characteristics, taking into account that the alterations cover a continuum. In 1989, the classification was altered to low-grade PIN (LGPIN) and high-grade PIN (HGPIN), respectively [265].

Morphologically, there are four basic patterns of HGPIN: flat, tufting, micropapillary, and cribriform [266]. These patterns often merge with each other. Other than diagnostic utility, these architectural patterns have no known clinical significance.

Several studies indicate that HGPIN is the most likely precursor lesion of PCa [267-270], because of the similarities between them:

1) Age. The frequency and extent of HGPIN lesions increase with age, and this increase is similar to the increase in diagnosis of PCa with age [271].
2) HGPIN is found significantly more frequently in prostates with cancer [264].
3) Coexistence. HGPIN often coexist with PCa in the same samples [267].
4) HGPIN is predominantly located in the peripheral zone, the zone in which most clinically important prostate tumours are found [271].
5) Morphological similarities. HGPIN is characterized by cellular crowding and stratification. There is inequality in cell and nuclear size. Hyperchromatism is frequently seen with an enlarged nucleus, often containing prominent nucleoli lines. These changes are also seen in Gleason grade 1-4 PCa [272].
6) Histologically, the atypia observed in HGPIN is virtually indistinguishable from that of PCa except that in HGPIN the basal membrane is still intact [273]. As HGPIN progresses, the likelihood of basal cell layer disruption increases. In HGPIN, the basal cell layer is disrupted or fragmented as demonstrated by 34βE12. In PCa, there is complete loss of the basal cell layer.
7) Both in HGPIN and PCa, collagenase type IV expression is increased as compared with normal prostate epithelium. This enzyme is responsible for basal membrane degradation and thus facilitates invasion [10, 274].
8) HGPIN and PCa share several nuclear properties, such as amount of DNA, chromatin texture, chromatin distribution, nuclear perimeter, diameter, and nuclear abnormalities [275].

9) Molecular and genetic similarities. Molecular abnormalities in HGPIN are mostly intermediate between benign gland and cancer, reflecting an impairment of cell-differentiation and regulatory control [276]. Several genetic changes encountered in PCa cells can be found in HGPIN [275]. Allelic loss is common in HGPIN and PCa [273]. The frequent 8p12-21 allelic loss commonly found in PCa is also found in microdissected HGPIN. Other examples of genetic changes found in PCa already existing in PIN include loss of heterozygosity at 8p22, 12pter-p12, and 10q11.2, and gain of chromosomes 7, 8, 10, and 12. Alterations in oncogene Bcl-2 expression and replication error (RER^+) phenotype are similar for HGPIN and PCa [277].
10) PCa and HGPIN have similar proliferative and apoptotic indices [278]. Mitotic figures and apoptotic bodies increase progressively from nodular hyperplasia to HGPIN [278]. Greater cytoplasmic expression of Bcl-2 is observed in HGPIN and PCa than in benign and hyperplastic epithelium [273].
11) Neovascularization is greater in HGPIN and PCa than in normal prostate. The available data indicate that angiogenesis has an important role in the progression of prostate neoplasia [279].

Atypical adenomatous hyperplasia (AAH)

Apart from HGPIN, several morphological lesions or conditions have been proposed that may act as potential precursor lesions of PCa. These are the morphologically distinct entities of atypical adenomatous hyperplasia (AAH), low grade PIN (LGPIN), and focal atrophy [269].

AAH is a lesion characterized by a proliferation of small acinar structures that mimics adenocarcinoma because of histological similarities[280]. AAH appeared from 0.8% in needle biopsy specimens to 7.3% in TURP specimens without cancer [281]. AAH is most often located in the transition zone of the prostate in intimate association with BPH [282]. It can also be found near the apex and in the periurethral area [10, 274]. In AAH, the basal cell layer is discontinuous and fragmented on 34βE12 cytokeratin immunostaining[283]. AAH is often associated with low-grade adenocarcinoma arising in the transition zone [284], since there are morphological similarities. Cytogenetic analyses have detected abnormalities of chromosome 8 in very small proportions (4 - 7%) of AAH cases [282, 283].

LGPIN

LGPIN is quite difficult to recognize, as it has common features with normal and hyperplastic epithelium. It has the similar morphology as HGPIN, but most of the cells lacked prominent nucleoli. The nuclei are enlarged, vary in size, have normal or slightly increased chromatin content, and possess small or inconspicuous nucleoli. More prominent nucleoli, when observable, comprise less than 10% of dysplastic cells [267, 285]. The basal cell layer normally surrounding secretory cells of ducts and acini remains intact [269, 270]. The distinction between HGPIN and LGPIN is based primarily on the extent of cytological abnormalities (that is,

prominence of the nucleoli) and secondarily on the degree of architectural complexity [286, 287]. Immunostaining studies of microvessel density may help to differentiate HGPIN from LGPIN [279].

Prostatic Atrophy

Atrophy in prostate related to aging and presumably attributed to androgen withdrawal is a consistent finding only in patients over the age of 70. There is no explanation for the great variation in the rate of involution seen among individuals below 70, but severe debilitating disease may produce advanced atrophy even in young men [2, 288].

Classification of Prostatic Atrophy

Atrophy of the prostate is identified as a reduction in the volume of preexisting glands and stroma and can be divided into two major patterns: diffuse and focal.

Atrophy due to aging is characteristically diffuse**.** In its advanced stage, reduced secretory cell volume is usually accompanied by markedly reduced or absent staining for PSA and PAP. Diffuse atrophy can also result from a decrease in circulating androgens, most commonly produced by ‘‘total androgen blockade,’’ which consists of orchiectomy combined with antiandrogens, or luteinizing hormone-releasing hormone agonists combined with antiandrogens. This hormonal type of atrophy involves the prostate in a relatively uniform manner and shows unique histological features [289, 290].

Focal atrophy lesions, by contrast, occur as heterogeneous patches, and contain a nonprominent basal cell layer, often attenuated. Although the term “focal” is used to indicate that these atrophic areas are limited to specific patches or ‘‘foci,’’ at times these patterns of atrophy may encompass very large areas of the prostate, especially in the peripheral zone.

The biological significance of prostatic atrophy has been an area of interest since at least the 1930s. Franks [291] indicated that focal atrophy occurs chiefly in the ‘‘outer’’ portion of the prostate, now referred to as the ‘‘peripheral zone’’, and that it increases with advancing age.

There are several morphological variants of focal prostate atrophy. These were first classified in detail by Franks who described four main patterns [291]: 1, simple atrophy (SA) with/without cyst formation; 2, sclerosing atrophy; 3, post-atrophic hyperplasia (PAH): 3.a, lobular hyperplasia; 3.b, sclerosing atrophy with hyperplasia (post-sclerotic hyperplasia); and 4, secondary hyperplasia.

McNeal et al. [2] found that almost all the focal atrophy were consequences of previous inflammation. They classified the focal atrophy into two groups: 1), postinflammatroy atrophy, 2), cystic atrophy.

In more contemporary literature, several descriptions of various morphological patterns of focal prostate atrophy have been presented. To simplify the classification of these lesions, Ruska et al. [292] referred to most of them as SA or PAH. They defined the PAH lesions as the crowded focus of small atrophic acini. PAH contained more frequent prominent nucleoli and had the highest MIB-1 label index, higher than that in simple SA and benign nonatrophic glands.

Proliferative inflammatory atrophy (PIA)

De Marzo [293](1999) proposed the term PIA to designate discrete foci of proliferative glandular epithelium with the morphological appearance of SA and/or PAH, occurring in association with inflammation. The key features of PIA are: the presence of two distinct cell layers; mononuclear and/or polymorphonuclear inflammatory cells in both the epithelial and stromal compartments; and stromal atrophy with variable amounts of fibrosis.

In 2006, a new classification was proposed [294]: most focal atrophy lesions could be sub-classified into the following four distinct subtypes: (i) SA, (ii) SA with cyst formation, (iii) PAH, and (iv) partial Atrophy. Apart from the definition of PIA, lesions in which the same prostate atrophy patterns do not contain increased inflammation were referred to as "proliferative atrophy" (PA). A more recent study showed that PIA is a more frequent finding in prostate with carcinoma and the simple atrophy was the most common type of PIA [295].

PIA as a Precursor to HGPIN and Prostate Cancer

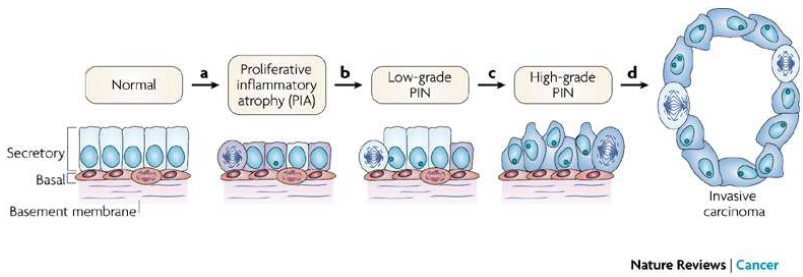

Fig 4. Data from De Marzo et al [38]

In 2003, Nelson et al. [131] proposed a model of the transition between PIA to HGPIN and prostate cancer. In 2007, De Marzo et al. [38] fulfiled the hypothesis of “injury and regeneration”. The morphological process was assumed to start from the initiated stage of the “stem cell”, then to intermediate cell differentiation, and at last, to the development of HGPIN or prostate cancer (Fig 4) [38]. This hypothesis is mainly based on the evidences from morphological, cellular, and molecular studies.

Proliferation index in PIA

Cell proliferation is a fundamental aspect in a number of prostatic diseases ranging from hyperplasia to neoplasia and can be studied using antibodies directed against nuclear antigens expressed in certain phases of the proliferation cycle, such as Ki-67 and PCNA.

Feneley et al. [296] found the proliferation index of benign acini is consistently lower (0.19-4.0%) than that of malignant acini (1.6-16%). They found that atrophic lobules showed a high proliferation index. Ruska et al. [292] studied the cellular kinetics in prostate needle biopsy specimens. Both SA and PAH had higher proliferation ratios and lower apoptosis than the nonatrophic glands. PAH lesions have the highest proliferation ratio and lowest apoptosis [292]. De Marzo [293] noted that in PIA lesions, both basal and secretory-type cells showed an elevated Ki-67 staining index. Shah et al. [297] concluded that there was a significant increase in nuclear proliferation from normal prostate along a continuum: benign < SA < PAH < HGPIN < PCa ($P < 0.001$).

Morphological transition between PIA, PIN, and PCa

Franks considered a specific subtype of PAH (sclerotic atrophy with hyperplasia) as a putative neoplastic precursor given its close association with PCa [291]. Liavag [298] demonstrated a similar topographical association between PAH and PCa. Several recent studies have re-examined the role of PAH as a potential neoplastic precursor. Shah et al.[297] noted that SA and PAH were both frequently found and topographically located near or adjacent to PCa in the peripheral zone of the prostate gland. Two other studies examining the topographic location of PAH with respect to PCa concluded that PAH is extremely common in the periphery of the prostate gland. Putzi and De Marzo [299] identified the two-dimensional topographical relation between PIA and PCa. It was described as merging when the neoplastic-appearing epithelium of the HGPIN or carcinoma merged directly with PIA within a given acinus or duct. In a study with radical prostatectomies, morphological transitions between HGPIN and PIA frequently occur: 42.5% of the HGPIN lesions were merged with PIA. Carcinoma did not merge with PIA. Another more recent study [300] reported that PIA lesions appeared to be directly merging with small foci of adenocarcinoma. Twenty five percent of the PIA lesions were found to merge directly with HGPIN and 31.3% of the PIA lesions contained at least some cells with prominent nucleoli (low-grade PIN). This finding supports a model in which the proliferative epithelium in PIA may progress to HGPIN.

However, it should be noted that not all the pathologists have noted morphologic transition of PIA, HGPIN, and invasive adenocarcinoma [301-305] and that not all HGPIN or small carcinoma

lesions are associated with atrophy. It is not clear how these parameters are interrelated and what intermediates are involved. At the WHO Consensus Conference in Stockholm, it was emphasized that no relationship between atrophy and prostate carcinoma or HGPIN has been proven [267].

Phenotype of PIA

Cell populations in PIA: Based on the expression of keratins, four cell populations can be discriminated in the human prostate epithelium. Basal cells express high levels of CK5, CK14, p63, and low levels of AR, PSA, CK8, and CK18. Luminal secretory cells lack p63, CK5, and CK14, but express high levels of CK8, CK18, AR, and PSA. Additionally, cells have been identified with a keratin phenotype intermediate between basal and luminal cells, which co-express high levels of CK5 and CK18 (CK5/18) as well as hepatocyte growth factor receptor c-MET [306, 307].

Many of the atrophic epithelial luminal cells in PIA are candidates for intermediate cells. van Leenders et al. [306] reported that all the atrophic luminal cells are strongly positive for CK8/18. About 40% of atrophic luminal cells in PIA lesions expressed CK5. c-MET positive staining was present in 44% luminal cell in PIA. The double-staining immunofluorescence also showed that luminal cells in PIA often co-expressed CK5 and Ki-67. The results suggested that cells phenotypically intermediate between basal and secretory are enriched in PIA lesions.

GSTP1: GSTs are an important class of enzymes that protect cells against genome damage mediated by oxidants and electrophiles from inflammation or dietary exposures. There are five major families of cytosolic GST isoenzymes. The most extensively studied GST in the human prostate is the pi-form of GST (GSTP1). In human prostate, GSTP1 is expressed in most basal cells in normal prostate [293], and was lost in HGPIN and PCa, which is associated with hypermethylation of the CpG island encompassing the *GSTP1* promoter[139, 308]. Methylation changes at this site have been detected in up to 100% of PCa DNA specimens [139, 308]. This suggests that GSTP1 may serve as a "caretaker" gene [309], the decreased expression of which might render prostate cells vulnerable to malignant progression.

In comparison with the increased expression in basal cells of normal prostate, GSTP1 is not usually expressed in normal secretory luminal cells. In contrast, many of the luminal epithelial cells in PIA lesions express increased level of GSTP1, although some do not [293]. Nakayama et al. [300] hypothesize that some PIA cells may acquire GSTP1 CpG island hypermethylation leaving these cells vulnerable to progress to HGPIN and/or PCa. In a study of microdissected tissues, GSTP1 CpG island hypermethylation was not detected in normal epithelium or in

BPH, but was found in 6.3% of PIA lesions, in 68.8% of HGPIN lesions and in 90.9% of adenocarcinoma lesions. It is possible that increased expression of GSTP1 in PIA may result from the presence of an ongoing oxidative insult to this tissue, with silencing of GSTP1 function related to the development of cancer [293].

Chromosome 8p22 loss and 8 centromere (8c) gain: PIA and HGPIN also share some chromosomal changes. Both lesions had significantly higher percentages of chromosome 8p22 loss than did normal prostate epithelia. Gain of centromere 8 (8c) was also detected in a few atrophic lesions [297, 310, 311].

Bcl-2: In the benign prostate, Bcl-2 immunohistochemical staining was consistently observed in the basal layer in the ducts and acini, but was absent in luminal cells [44]. In PCa, increased Bcl-2 protein levels are associated with high-grade tumours, advanced stage, metastasis, and androgen-independent [312, 313]. Baltaci et al. [277] reported detectable Bcl-2 in both LGPIN and HGPIN and its absence in normal and BPH prostate tissues. However, in the BPH glands within an area of prostatitis, both the basal and luminal cells expressed Bcl-2 indicating that inflammation was associated with Bcl-2 expression [314]. An inverse relation between Bcl-2 and AR was found in many PIA glands [293]. There was also a significant relation between Bcl-2 expression and the type of lesion: PAH lesions show higher overall levels of Bcl-2 than lesions not containing PAH [293].

P27^{kip1} belongs to the Cip/Kip family, and functions as an important cell cycle gatekeeper. Down-regulation of p27^{Kip1} occurs in the vast majority of PCa and reduced levels correlate positively with Gleason grade [315, 316]. Down-regulation of p27^{Kip1} was detected in HGPIN [317]. The absence of p27^{Kip1} was also reported in PIA lesions [293, 306].

COX-2: Zha et al.[227] reported COX-2 overexpression in PIA lesions. In their study, COX-2 was not elevated in HGPN or in established PCa. By contrast, there was consistent expression of COX-2 protein in PIA lesions.

P53: Tumour suppressor gene p53 is the most commonly mutated gene in human cancers. In common with many other human cancers, p53 mutations are also seen in PCa with a wide range of 5-65% [318]. [319]. In PIN cases, p53 mutation was reported from 5% to 70% [320-324]**.** The frequency of p53 mutations in microdissected PAH lesions was 5.3%, which was similar to that in HGPIN (4.2%) in the same report [325].

c-MET: c-MET has been implicated in embryogenesis, tissue reorganisation, and tumour progression. c-MET is overexpressed in several human malignancies including prostate carcinoma [326-328]. The c-MET protein was detected in a substantial number of PCa and more

often in metastatic growths of prostate carcinoma and in androgen insensitive PCa cell lines [329, 330].

Interestingly, levels of c-MET are also raised in some inflammatory lesions, such as ulcerative colitis and obstructive cholangiopathy [331]. In these inflammatory lesions c-MET might be involved in cell proliferation, migration, and differentiation as part of a regenerative process. In agreement with the stem cell model of Isaacs and Coffey [332], increased expression of c-MET together with their high proliferative activity supports the concept of intermediate cells as putative progenitor cells for prostate carcinogenesis. In a study by van Leenders GJ et al. [306], overexpression of c-MET was documented in PIA lesions and it was suggested that PIA is enriched with intermediate cells.

P63, a basal cell marker, is used as a tool to determine the state of the basal cell layer and to distinguish the regions of HGPIN from PCa [58]. In PIA lesions, however, P63 negative and P63 positive staining were detected in both the luminal and basal cell layers [333].

PSCA: Prostate stem cell antigen (PSCA) is a cell surface antigen expressed in normal prostate and associated with human and murine PCa. In normal prostate epithelial cells (PrEC), PSCA-positive cells were characterized by a more differentiated morphology and a slower proliferative rate than PSCA-negative cells, and expressed CD44, PSA, and AR, but lost expression of p63. PSCA may be a unique marker of an intermediate cells [334].

P16/CDKN2, a cyclin-dependent kinase inhibitor, is frequently altered in prostate cancer [335]. Increased expression of p16 was also reported in PIA lesions [336].

AR down-regulation: AR was found to be down-regulated in PIA [293]. Although the mechanism(s) for the loss of AR expression are not completely understood, it is possibly that the local inflammation and its inflammatory microenvironment, including the local high level of inflammatory cytokines, initiate a serious of molecular alterations in prostate atrophic epithelial cells, including AR down-regulation [88]. Very few articles analyzed the down-regulation of AR in non-malignant prostate epithelial cells. Bonkhoff noticed that AR status and Bcl-2 showed inverse correlation [278]. *In vivo* studies suggested that inflammatory cytokines may play roles in down-regulate AR expression. AR mRNA and protein levels in the LNCaP cells could be down-regulated by EGF [86], basic fibroblast growth factor (bFGF) [87], and TNFα [88]. Moreover, IL-1β is also responsible for down-regulation of AR protein expression [89].

AIM OF THE STUDY

The main aim of the studies in this thesis was to investigate the association between chronic inflammation and the carcinogenesis of prostate cancer, with a specific focus on the role of the molecular variation of PIA lesions and the morphological transition between PIA lesions and the malignancies in human prostate samples.

Specific aims of studies

- To examine the expression of COX-2 in atrophic lesions and to investigate the correlation between COX-2 expression, the anti-apoptosis protein Bcl-2, and the proliferation state of prostatic epithelial cells,
- To assess focal chronic inflammation densities and its association with COX-2 expression in prostatic epithelial cells in PIA lesions,
- To detect COX-2 expression in human prostate cancer tissues and to assess the microvessel density in prostate cancer tissues and the relation to COX-2 expression in tumor cells,
- To elucidate the possible role of focal chronic inflammation in human prostate cancer tissues in inducing COX-2 overexpression,
- To detect the expression of the transcription factor CCAAT/enhancer binding protein β (C/EBPβ) in PIA lesions and to investigate the association with COX-2 expression, focal chronic inflammation, and the epithelial cell proliferation state,
- To elucidate the morphological transition among PIA, HGPIN, and prostate cancer in radical prostatectomy specimens and try to find useful markers to help to detect atrophic epithelial cell in malignancy areas.

MATERIALS AND METHODS

Human Materials

Benign Prostatic Hyperplasia (BPH) specimens: Sixty-eight BPH specimens were obtained from the Department of Urology, Shandong Provincial Hospital, China. The patients' ages varied between 51 and 82 years (median age 71). All of the tissues were fixed in formalin, embedded in paraffin. Serial sections were cut at 4-μm thickness.

Radical prostatectomy specimens: The material consisted of randomly chosen tissue samples obtained from radical prostatectomy specimens from 93 patients with prostate adenocarcinoma, including 78 cases from the Department of Urology, Sahlgrenska University Hospital, Göteborg, and 15 cases from Department of Pathology, Shandong Provincial Hospital, China. All the preoperative stages were T1c (n = 78) and T2a (n = 15). None of the patients had received prior hormone therapy, chemotherapy, or radiation therapy. The age range of the patients was between 52 and 78 years (median age, 65). Specimens were fixed in 10% buffered formalin, embedded in paraffin.

Histological Identification

PIA: PIA lesions were classified into the following types: simple atrophy (SA), postatrophic hyperplasia (PAH), or mixed simple atrophy/postatrophic hyperplasia [291-293]. The morphologic definitions of SA, PAH, and mixed SA/PAH were as below:

> **SA**: consists of atrophic cells lining acini of relatively normal calibre, having no papillary fronds, and where the number of glands per unit area does not appear to be increased relative to normal tissue. SA usually involves an entire lobule, although isolated acini may be affected. The acini are small and show a lower than normal height of the epithelial cells, and the surrounding stroma may or may not show fibrosis.
>
> **PAH**: consists of acini that are small and round and appear in a lobular distribution, often surrounding a somewhat dilated duct with an apparent increase in the number of small glands as compared with normal tissue. Fibrosis may or may not be present in the stroma. When present, the proliferation is irregular and can result in distortion of the acinar lumen.
>
> **Mixed lesions**: the above patterns are found in the same region, appearing to merge.

PIN: The identification and classification of HGPIN are based mainly on the cytological characteristics of the cells [269, 274]. HGPIN is composed of cells with histologic features of prostate adenocarcinoma occurring in pre-existing prostatic acini/ducts. Cells are characterised with large nuclei of relatively uniform size, increased chromatin content, which

might be irregularly distributed, and prominent nucleoli that are similar to those of carcinoma cells. The basal cell layer may have frequent disruptions. Four basic patterns of HGPIN, flat, tufting, micropapillary, and cribriform, were identified as described by Bostwick et al. [266].

Lesions with similar morphology, but in which most of the cells lacked prominent nucleoli, were classified as LGPIN. The nuclei of cells composing LGPIN are enlarged, vary in size, have normal or slightly increased chromatin content, and possess small or inconspicuous nucleoli.

PIA-merging HGPIN: The histological definition of PIA-merging HGPIN was any malignant cell with fraction within a single PIA lesion. The periglandular stroma in these lesions often showed mononuclear inflammatory infiltrates mainly composed of lymphocytes, macrophages, and occasionally plasma cells. At times, mononuclear cells were prominent both intraluminally and in the epithelium as well.

PIA-merging adenocarcinoma (PIA-merging PCa): Any PIA lesion with a fraction of carcinoma was considered as PIA-merging PCa. Presence of adenocarcinoma was diagnosed according to the criteria of Mostofi and Price [337].

Histopathological classification for chronic prostatic inflammation: The histopathological classification system for chronic prostatic inflammation (Nickel et al.) [338] was used to classify prostatic inflammation into three grades of severity: mild, moderate, and severe, and three inflammation patterns: glandular, peri-glandular, and both peri-glandular and glandular (Table 5)

Table 5. The classification of prostatic inflammatory infiltrates (Nickel et al.).

Feature	Details
Anatomical location	Histological pattern
glandular	Inflammatory infiltrates lie within duct/gland epithelium and/or lumens.
periglandular	Inflammatory cells lie within prostatic stroma but not centred on prostatic glands/ducts and lie ≥ 50 μm from them.
Extent	Tissue area involved in inflammatory cell infiltrates
focal	< 10%
multifocal	10–50%
diffuse	> 50%
Grade	Morphological description (typical inflammatory cell density, cells/mm2)
1/mild	Individual inflammatory cells, most of which are separated by distinct intervening spaces (< 100).
2/moderate	Confluent sheets of inflammatory cells with no tissue destruction or lymphoid nodule/follicle formation (100–500).
3/severe	Confluent sheets of inflammatory cells with tissue destruction or nodule/follicle formation (> 500).

Antibodies

The antibodies and conditions used in this study are indicated in Table 6.

Table 6. Antibodies and conditions.

Antibody	Company	Clone, code	Dilution
COX-2	Cayman Chemical Co.	PAb, 160126	1:100
COX-2*	NeoMarkers Co.	SP21	1:200
COX-2 blocking peptide	Cayman Chemical Co.	360106	10μg/ml
COX-1	Cayman Chemical Co.	PAb, 160110	1:50
PCNA	NeoMarkers Co.	PC10	1:200
AR	NeoMarkers Co.	Ab-1	1:50 – 1:100
Ki-67	NeoMarkers Co.	MB67	1:100
Ki-67*	NeoMarkers Co.	SP6	1:100
HMW keratin	DAKO Co.	34βE12	1:200
CK5	Abcam Ltd.	ab24647	1:200
LMW keratin, CK8	NeoMarkers Co.	35 H11	1:200
Bcl-2	NeoMarkers Co.	Ab-1	1:100
CD3	NeoMarkers Co.	PS1	Ready to use
CD3	Abcam Ltd.	PAb	1:25
CD20	NeoMarkers Co.	L26	Ready to use
CD31	NeoMarkers Co.	JC/70A	1:25
CD68	NeoMarkers Co.	KP1	Ready to use
C/EBPβ	Sant Cruz Bio.	H-7	1:200
c-MET	Santa Cruz Bio.	C-28	1.500
GSTP1	MBL	PAb	1:4000
p27	NeoMarkers Co.	DCS-72.F6	1:200
P53	Santa Cruz Bio.	DO-1	1:100
P53*	NeoMarkers Co.	SP5	1:100
p63	NeoMarkers Co.	4A4	1:100
PSCA	NeoMarkers Co.	PAb	1:200
Chromogranin A	NeoMarkers Co.	LK2H10	1:500

*: Rabbit monoclonal antibody. PAb, polyclonal antibody.

Immunohistochemistry (IHC)

IHC staining: Two immunohistochemistry systems were used in this study: ABC kit (Vector) and EnVision™ system (DAKO Co.).

COX-2 IHC staining was performed using the ABC kit (Vector). Briefly, sections were rinsed with methyl alcohol-hydrogen peroxide and then microwaved in citrate buffer (pH 6.0) to induce epitope retrieval. Diluted COX-2 primary antibody (1:100) was incubated on slides at +4^{o}C overnight and then incubated with biotinylated secondary antibody at room

temperature. For localization, avidin-biotin complex was applied at room temperature for 30 minutes followed by 3,3'-diaminobenzidine tetrahydrochloride (DAB) as the chromagen. Slides were counterstained with Mayer hematoxylin.

As a negative control for nonspecific staining, COX-2 blocking peptide was added to the diluted COX-2 antibody at a final concentration of 10 µg/ml to the antibody mixture and incubated for 1 hour at room temperature before the application of the COX-2 antibody to the slides. Then manual IHC staining was performed as described above.

Ki-67 IHC staining was performed with the EnVision™ system. Briefly, after microwaving in citrate buffer, the section was incubated with primary antibody at room temperature for 30 min and then incubated with horseradish peroxidase (HRP) labelled polymer for 30 minutes at room temperature. After washing with PBS buffered, DAB was used as the chromagen under the control by microscopy. Slides were then counterstained with Mayer hematoxylin.

Double IHC staining: In order to accurately evaluate the expression of two reagents *in situ*, double IHC staining was performed with various combinations of antibodies.

- To detect the COX-2 expression and its association with focal inflammation: COX-2/CD3, COX-2/CD20, and COX-2/CD68.
- To detect the COX-2 expression on different epithelial cell layers: COX-2/34βE12 and COX-2/CK8.
- To evaluate the proliferation state: COX-2/Ki67 and COX-2/PCNA.
- To assess the microvessel density and the local COX-2 expression: COX-2/CD31.

DAKO EnVision Doublestain System (DAKO Co.) was used in this procedure. Briefly, serially consecutive sections at a thickness of 4 µm were treated for epitope retrieval as described above. After being incubated with peroxidase block for 5 minutes at room temperature, the slides were exposed to the first primary antibody (1:100) and incubated at +4^{o}C overnight or 30 minutes at room temperature. The slides were then incubated with horseradish peroxidase (HRP) labeled polymer for 30 minutes at room temperature. The first antibodies immunostaining was labeled by applying DAB for 1-5 minutes. Next, Doublestain Block was added to the slides, 'and the slides were incubated for 3 minutes. Then the slides were incubated with the second primary antibody for 30 minutes at room temperature followed by application of alkaline phosphatase (AP) labeled polymer for another 30 minutes. The second substrate-chromagen solution, Fast Red, was incubated on the slides for 1-5 minutes. Slides were counterstained with Mayer hematoxylin and cover slipped with DAKO Glycergel.

Evaluation of IHC staining:

- **COX-2 IHC staining**: COX-2 positive staining was identified by the presence of marked diffuse brown (DAB) cytoplasm or perinuclear staining in prostate epithelial cells. If any portion of epithelia in a prostate gland showed immunoreactivity, then the gland was considered as COX-2 positive stained.
- **HMW keratin 34βE12** and **CK8** both label the cytoplasm of epithelium. In normal prostatic acini and ducts, 34βE12 only appears in the basal cell layer and CK8 in luminal cells. Similar to 34βE12, P63 also only appears in the basal cell layer, but it shows nuclear immunostain instead of cytoplasm.
- **CD3, CD20,** and **CD68** are all expressed in the cytoplasm staining.
- **Ki-67** and **PCNA** are both the proliferation markers, and both show nuclear immunostaining.
- **C/EBPβ**: C/EBPβ is only expressed in the nucleus.
- **AR** and **p27** are mainly expressed as in nuclear staining and occasionally in cytoplasm. Only nuclear staining was evaluated as positive.
- **Bcl-2, GSTP1, PSCA**, and **c-MET**: cytoplasm IHC staining.

Evaluation of double IHC staining: The two antigens staining in double IHC staining slides were identified by color: the first antigen was stained brown (DAB) and the second red (Fast Red). For example, in COX-2/CD3 double IHC staining slides, apart from the brown immunostaining of COX-2 on prostate epithelium, the T-lymphocytes were labeled with cytoplasm red staining. PCNA and Ki-67 immunostaining were identified by the nuclear red immunostaining. When only one cell coexisted both the cytoplasmic brown staining for COX-2 and nuclear red staining for PCNA or Ki-67 were recorded as the PCNA or Ki-67 labelled COX-2 positive cell.

Quantitation of IHC Staining

- **COX-2 IHC staining**: Quantitation of COX-2 immunostaining was performed on 5 to 10 ocular measuring fields per slide chosen randomly, under a microscope at a power of x200.
- **Proliferation index**: PCNA and Ki-67 label counting were performed by counting 10 to 20 randomly selected microscopic fields at X400 magnification of each slide on COX-2/PCNA and COX-2/Ki-67 double IHC staining sections. The labeling index is the percentage of labeled cell nuclei over the total number of counted epithelial cell nuclei.
- **Bcl-2**: In COX-2/Bcl-2 double IHC staining slides, Bcl-2 labeling was classified into two score groups based on the location of the Bcl-2 immunostaining in prostate epithelia: Bcl-2 score I:

only the basal cell layer of prostate epithelia stained with Bcl-2; Bcl-2 score II: not only the basal cell but also the luminal cell layer were detected with Bcl-2 immunostaining. Similar evaluation was also used for GSTP1 and c-MET immunostaining.

- **CD3, CD20, and CD68 for chronic inflammation** (paper I, II, III, and IV): In COX-2/CD3, /CD20, and /CD68 double staining slides, severity of inflammation grades was evaluated by counting inflammatory cells in the area around each gland within 50μm [338] .
- **C/EBPβ**: In each case, 5 to 30 normal-appearing glands and 1-6 PIA lesions were selected at random to assess the IHC staining. If any portion of epithelia in a prostate gland showed C/EBPβ nuclear staining, then the gland was considered positive.
- **AR down-regulation**: AR down-regulation was defined as when ≧ 1/3 luminal epithelial cells lost the AR nuclear staining or/and ≧ 2/3 luminal epithelial cells had weak AR nuclear staining in a single gland compared with the adjacent normal-appearing glands.
- **Microvessel density assessment**: Any single endothelial cell or cluster of endothelial cells labeled with CD31 was regarded as a single microvessel. The quantification of microvessels was performed by counting 20 to 40 randomly selected microscopic fields of prostate cancer at 400 x magnification (high power fields, HPF) for each slide. The values of the second antigen staining were regarded as inflammatory cell density or MVD (/ HPF, 400 x).

Immunofluorescence

To determine whether the CK5 positive epithelial cells showed increased proliferative activity and decreased $p27^{kip1}$ expression, double-staining immunofluorescence of Ki-67 and CK5, as well as $p27^{Kip1}$ and K5 was performed.

Statistics

Statistical analysis was carried using SPSS for Windows software. The Pearson Chi-Square test was used to analyze the significance of COX-2 expression among various kinds, grades, and patterns of inflammation, and Bcl-2 expression (paper I). Chi-square test and/or Fisher exact test was also used to analyze the differences in C/EBPβ expression in relation to inflammation grades, COX-2 expression, and AR down-regulation (paper III) and in relation to the differences between proportions in paper IV. The Mann-Whitney U test was used for comparison of different groups, such as the significance of COX-2 expression, PCNA indices, and Ki-67 indices among different groups in atrophic lesions (paper I); COX-2 expression IHC score and the relationship with Gleason score, the inflammatory cell density, and MVD

(paper II), and the C/EBPβ expression ratio and the relationship with inflammatory cell density (paper III), and the comparison of HGPIN and PIA-merging HGPIN among groups (paper IV). Spearman rank correlation coefficient test was used to analyze the strength of association between the COX-2 IHC score and inflammatory cell density or MVD in paper II.

RESULTS AND DISCUSSION

Over-expression of COX-2 in Prostate Atrophic Lesions of BPH Samples and the Relation to Proliferation Index and Association to Apoptosis (paper I)

COX-2 over-expression and co-expression with CK8 in PIA

The expression and function of COX-2 in prostate has been the subject of recent multiple reports. In general, COX-2 expression in normal prostate tissue is either weak or nonexistent, and prostate cancer tissue shows a marked overexpression of COX-2. The results in paper I show that COX-2 was overexpressed in the atrophic lesions, both in SA and PAH, in comparison with the low COX-2 level in normal prostatic acini/ducts. The double IHC staining of COX-2/34βE12 and COX-2/CK8 clearly showed that COX-2 was co-expressed with CK8, suggesting the luminal phenotype.

These results were basically in line with other studies that COX-2 was seldom expressed in normal luminal epithelium. Only a few articles studying COX-2 expression in BPH were published and the results were contradictory. Furthermore, no consensus has been reached concerning which cell types express COX-2 in the prostate and at which stage of the disease this expression becomes elevated. Kirschenbaum et al.[168] have made a systematic investigation of COX-2 expression in both fetal and adult male reproductive tracts, including normal prostate and BPH. In prostate, COX-2 was negative in fetal prostate, was strongly expressed in the smooth muscle cells and had no expression in the luminal epithelial cells [168]. However, Madaan reported that COX-2 was constitutively expressed in the luminal cells of the BPH samples [167]. In the present study, we found that COX-2 immunostaining mainly appeared in the atrophy glands (PIA lesions), suggesting that in comparison with the normal acini and ducts, the PIA lesions showed a different phenotype. This support the hypothesis that such atrophic cells are intermediate cells that have been identified between basal and luminal cells and that co-express both CK5 and CK8 as well as hepatocyte growth factor receptor c-MET, and lack p63 [306]. According to the stem cell hypothesis [339], these cells may develop into PIN or PCa.

COX-2 expression and correlation to inflammation in BPH

Another interesting point made in this study is that COX-2 expression was related to the focal chronic inflammation. COX-2 immunostaining was found closely adjacent to the focal chronic inflammation, both inside and surrounding the prostate foci. The predominant

inflammatory cells are T-lymphocytes and macrophages. These data indicate that focal chronic inflammation might play an important role in inducing COX-2 expression in BPH.

BPH is one of the most common diseases in aging men. Nonetheless, the etiology of BPH is far from being completely understood, despite the major efforts made by molecular and clinical researchers. Most of the initial research studies focused on hormonal factors, considering that advanced age and the presence of functioning testes to be the two best-established conditions for the development of BPH. There is emerging evidence that prostatic inflammation may contribute to prostate growth either in terms of BPH or neoplastic changes. The Medical Therapy of Prostatic Symptoms (MTOPS) study showed that men with inflammation had a significantly higher risk of BPH progression and acute urinary retention [340]. The use of COX-2 inhibitor in combination with a 5-alpha-reductase inhibitor could increase the apoptotic index in BPH tissue [340]. Studies have also addressed the correlations between inflammation, apoptosis, and the development of prostate cancer [292, 293]. Inflammation is thought to incite carcinogenesis by causing cell and genome damage, promoting cellular turnover. In theory, an inflammatory microenvironment may be ideal for the development of tumours, owing to up-regulation of various mitogenic, anti-apoptotic and angiogenic factors. Increased levels of cytokines in response to inflammation may influence cell survival, growth and differentiation. COX-2 is up-regulated by certain mitogens, growth factors and cytokines. The potential chemopreventive effects of various anti-inflammatory agents, i.e. COX-2 inhibitors and NSAIDs, lend credence to these observations. The role of COX-2 in BPH has not been conclusive. However, this study clearly demonstrates that COX-2 overexpression in BPH exists and is associated with a cascade of other events, including increased T-lymphocytes and macrophages. It remains unclear whether or not proinflammatory cytokines induce COX-2 expression, and, if so, the clinical relevance. The role of inflammation in the pathogenesis of BPH and disease progression will be an exciting avenue of research in the near future. The exact cell biological mechanisms may provide novel therapeutic options in the treatment of both BPH and PCa.

COX-2 expression in PIA and the association with increased Bcl-2 level

Both inflammation and apoptosis are of major interest in understanding the etiology of BPH. Bcl-2, an anti-apoptotic factor, is normally detected in the basal cell layer of the prostate glands, while no staining is present in luminal cells[341]. Bcl-2 expression, however, is observed in luminal cells in PIN and in prostate cancer, and its overexpression has been linked to chronic inflammation [314]. COX-2 overexpression and up-regulation of Bcl-2 are associated

with decreased apoptosis [197]. Bcl-2 blocks apoptosis, an important step in the pathogenesis of prostate cancer, responsible for the decreased response to androgen ablation therapy in prostate tumours as compared with normal prostate tissue [44]. Interestingly, treatment of LNCaP cells with a selective COX-2 inhibitor NS398 caused down-regulation of Bcl-2 expression [230]. It suggests that COX-2 might induce Bcl-2 expression.

It is known that inflammation is involved in several kinds of cancers, including prostate. Specifically, free radicals, predominantly oxygen and nitrogen species, and growth factors are assumed to alter protein structure and function, causing lipid peroxidation and gene mutations. Pathological, experimental, and epidemiological data support those relationships. The increased level of Bcl-2 shows indirectly the anti-apoptosis in PIA cells. In fact, relevant pathological evidence has highlighted the role of PIA as a prostate cancer precursor. Moreover, a rat prostate model supplied strong evidence that pathogenesis of prostatic neoplasia proceeds from inflammation to PIA and then to HGPIN [38, 116]. Rats fed with 2-Amino-1-methyl-6-phenylimidazo (4,5-*b*)pyridine (PhIP) showed significant prostate inflammation and atrophy and, HGPIN was also observed to develop directly from the atrophic epithelium [38, 116].

Increased proliferation index in COX-2 positive staining PIA

Normally, luminal epithelial cells have a low proliferating index, as compared with the basal prostate cells, which was assumed to be the place where prostatic stem cells are located. However, in the present study, the COX-2 positive expressing luminal cells showed increased proliferating index, which labelled both for Ki-67 and PCNA. Glands with COX-2 positive staining had higher a PCNA label index than COX-2 negative glands, and the highest PCNA label index appeared in the COX-2 positive cells. Both PAH and SA lesions had higher PCNA and Ki-67 label indices than normal glands.

COX-2 overexpression is assumed to induce the cellular proliferation in various cell lines via related prostaglandin (PG) production. Enhanced PG synthesis may contribute to carcinogenesis in several ways, including direct stimulation of cell growth. Both PGE1 and PGE2 stimulate proliferation of mammary epithelial cells in the presence of EGF [182, 183]. Treatment of cells with a COX-2 inhibitor could reverse this phenotype [197].

Overall, in the study of paper I, we conclude that COX-2 is expressed in benign prostate tissues, especially in atrophic lesions, and is associated with inflammatory cell infiltration. T-lymphocytes and macrophages both appear to play important roles in inducing the luminal

prostate epithelial cells to express COX-2. These COX-2 positive staining epithelial cells have higher proliferation index and increased levels of Bcl-2. This observation, in combination with findings from other studies [342, 343], suggest that chronic inflammation and subsequent up-regulation of COX-2 could be involved in the early pathogenesis of prostate disorders. These COX-2 positive expressing epithelial cells may possibly develop further, for instance, to PIN or prostate cancer, since these cells have both regeneration and anti-apoptosic phenotypes. It is therefore interesting to discuss why COX-2 is up-regulated and how this may influence cellular function in prostate epithelial cells. The COX-2 expression in prostate cancer, just as the chronic inflammation induced the COX-2 expression in BPH, remained the unanswered questions.

COX-2 Expression in Malignant Prostate Tissues and the Relation to Gleason Score and Angiogenesis (Paper II)

In a previous study we have shown that COX-2 is locally up-regulated in prostate atrophic lesions in BPH if T-lymphocytes and macrophages are present. Such COX-2 positive epithelial cells had increased levels of Bcl-2 and a higher proliferation index compared with COX-2 negative cells [344]. In paper II, we tested the hypothesis that COX-2 is associated with local chronic inflammation in prostate cancer, and assessed the relationship between COX-2 and the Gleason score for prostate cancer. In addition, we reported a new finding, that COX-2 was related to angiogenesis in prostate cancer. This finding confirmed the hypothesis that COX-2 contributes to angiogenesis and would support further clinical assessment of anti-angiogenesis therapy and COX-2 inhibitors, which have already shown promise in various trials [345].

COX-2 expression in PCa and relation to Gleason score

In this study, COX-2 positive staining tumour cells were detected in 40 of 43 cancer samples. The quantitative immunostaining data showed that the majority of cases (65%) had weak immunostaining of COX-2, while 28% had the intermediate or strong COX-2 expression. There was a significant association between elevated COX-2 expression and Gleason score: high Gleason score (> 7) patients had stronger COX-2 immunostaining ($P = 0.002$).

Our finding is generally in line with previous observations, which conclude that there is up-regulation of COX-2 in prostate cancer. Furthermore, several studies have shown that COX-2 expression correlated with disease stage, Gleason grade, and progression [163, 164, 166, 167, 346].

Increased COX-2 expression was also seen in lymph node metastases [165, 171]. COX-2 expression has even been regarded as an independent prognostic indicator [225].

However, although most such studies have demonstrated increased COX-2 expression in the prostatic epithelial cells, some investigators have attributed the increased COX-2 expression to the accompanying inflammatory cells found in the neoplastic tissue rather then from the cancer cells themselves [227]. They found that the expression of COX-2 was not elevated in HGPN or in established PCa. In limited cases, when staining for COX-2 was observed in PCa, the extent of positive staining did not correlate with established clinical and/or pathological risk factors - Gleason score or pathological stage. In contrast to the neoplastic tissue, the consistent expression of COX-2 protein in PIA lesions was present [227]. To date, however, there are few quantitative analyses of COX-2 expression in relation to Gleason score. In the present study, the COX-2 positive rate (40/43, 93%) is higher than in any other report. This discrepancy can probably be explained by the fact that there is very heterogeneous expression of COX-2 in prostate cancer tissues. A detailed quantitative analysis, based on the information from each microscopic field instead of the whole slide of each sample, may be the best way to interpret such heterogeneous findings.

COX-2 expression was induced by focal chronic inflammation, especially T-lymphocytes and macrophages infiltration

Although several reports have shown COX-2 overexpression in PCa, none investigated the association between COX-2 expression and the focal inflammation in the PCa areas. It is known that inflammation and the related oxidative stress plays a crucial role in inducing COX-2 expression in inflammatory lesions [121]. Based on our first article, stating that focal chronic inflammatory cell infiltration, especially the T-lymphocytes and macrophages, inducing the COX-2 expression, we hypothesized that the chronic inflammation in the prostate cancer areas may also play a role in COX-2 induction.

COX-2 IHC staining, focally related to chronic inflammation in tumour areas, has heretofore not been noted. Foci of chronic inflammation with accumulation of inflammatory cells were detected in all 43 prostate cancer samples using double-labeling of COX-2/CD3 or COX-2/CD68. Quantification of double labeling showed that T-lymphocytes and macrophages were noted more often in the COX-2-positive than in the negative staining tumor areas. A higher T-lymphocyte density was found in COX-positive than in COX-2-negative fields. Further analysis also showed that T-lymphocyte density was related to COX-2 expression in both the low and high-Gleason score groups. There was also a significant correlation between T-

lymphocyte density and COX-2 expression score. Statistical analysis showed significant correlation between COX-2 IHC expression score and inflammatory cells density. Interestingly, COX-2 expression was significantly related to macrophage density in low but not in high Gleason score specimens.

This observation is in line with our previous finding that COX-2 expression in benign prostate epithelium is correlated to local chronic inflammation. The mechanism behind this is not fully established. It is now known that most pro-inflammatory mediators stimulate COX-2 transcription via different types of signalling [198], including: growth factors, such as IGF, TGFα and EGF [181]; nitric oxide (NO) and reactive oxygen species (ROs) [200]; and several pro-inflammatory cytokines such as IL-1 or IFN-γ [201]. COX-2 protein level is also regulated at prost-transcriptional level via modulation of the stability of its mRNA. Signals from cytokines such as IL-1β, TNF-α or TLR ligands affect COX-2 mRNA stability. Most of above pro-inflammatory cytokines are enriched as inflammatory microenvironment component which is released by macrophages or activated T-lymphocytes [347]. Moreover, in cell co-culture experiments, COX-2 is induced by the presence of inflammatory cells [348], and COX-2 protein levels are increased in normal prostate cells and prostate cancer cells (PC-3, LNCaP, and DU145) after TNF-α stimulation *in vitro* [170, 232]. These studies suggest that local inflammation could up-regulate COX-2 in adjacent tumour epithelial cells.

COX-2 up-regulation contributes to the angiogenesis in prostate cancer

This study provides the first evidence of a direct link between COX-2 and angiogenesis in prostate cancer tissue. CD31 is a myeloid progenitor cell antigen found in endothelial cells. This biomarker can be found in all types of endothelial cells and has been used to correlate angiogenesis in different stages of cancer [188]. The spatial relationship between COX-2-expressing cancer cells and CD31-positive microvessels was investigated using COX-2/CD31 double IHC staining. COX-2 protein expression was not detected in endothelial cells. CD31-positive endothelial cells lining microvessels were noted in all prostate cancers specimens. Microvessel density (MVD) was significantly increased in high Gleason score cancer, compared with those in low Gleason score cancer specimen. MVD was also significantly different between COX-2-negative and COX-2-positive stained areas. This finding confirmed previous studies in which COX-2 expression was associated with aggressive disease in prostate cancer [163, 164, 166-169], and led to the hypothesis that COX-2 drives increased neovascularization. The existence and extent of this relationship is essential in the determination of therapeutic modalities for the prevention and treatment of prostate cancer.

Tumour angiogenesis includes destabilization of pre-existent blood vessels, proliferation of vascular endothelial cells, invasion of endothelial cells into the extracellular matrix and, finally, the migration and positioning of endothelial cells. Recent evidence suggests a role of COX in the process of angiogenesis through the synthesis of angiogenic PGs such as PGE2, which induce MMP and VEGF [121, 184-186]. Numerous studies have shown co-localization of angiogenesis factors, such as VEGF, PDGF, bFGF and TGF-β, with COX-2 by immunohistochemical staining in different cancer types [187]. In some solid tumours such as pancreatic cancer, gastric cancer, and endometrial carcinoma, COX-2 was shown to be involved in the tumour-associated angiogenesis [349-351]. In breast cancer, for instance, the density of microvessels was higher in patients with COX-2 expression than in those without COX-2 expression [188]. Studies of colon cancer cell lines co-cultured with vascular endothelial cells demonstrated that COX-2 supports angiogenesis at multiple steps both directly and indirectly [189, 190]. Through these angiogenesis mediators and their receptors on the endothelial cells, COX-2 and PGs increased vascular permeability and induced endothelial cell proliferation and migration.

Clinical trial of COX-2 inhibitor through inhibiting the angiogenesis

The importance of angiogenesis in tumour metastasis has been well established for over 30 years [352]. In theory, inhibiting angiogenesis might provide a new therapeutic option by targeting cancer growth and spread. Several recent articles report a relationship between mean MVD and advancing disease in prostate cancer [353], while some reports have negative results [354, 355]. Numerous compounds are examined by the National Cancer Institute (NCI) for their potential to prevent or treat cancer. COX-2 inhibitor is currently being tested in both prevention and treatment clinical trials. Epidemiological studies have shown that people who regularly take NSAIDs, such as aspirin and ibuprofen to treat conditions like arthritis, have lower risk of prostate cancer. Celecoxib, a COX-2 inhibitor commonly used to treat arthritis, has been shown to inhibit COX-2, and in turn, to reduce the formation, growth, and metastasis of several types of experimental cancers. Celecoxib has also been shown to effectively decrease tumour angiogenesis and to reduce tumour growth of a variety of experimental primary tumours, including colorectal, prostate, and breast cancers [356, 357].

In summary, the present study raises the possibility that COX-2 may influence tumour progression in prostate cancer through mechanisms of the promotion of angiogenesis. The use of selective COX-2 inhibitors and angiogenesis inhibitors may play a role in the targeted treatment of prostate cancer in the future.

Transcription Factor C/EBPβ Expression in Atrophic Lesions and the Association with COX-2 Expression, and Focal Chronic Inflammation (paper III)

C/EBPβ expression in prostatic atrophic lesions

COX-2 is a highly inducible enzyme triggering diverse actions on cell functions, including proliferation, migration, and DNA damage. COX-2 transcriptional activation by proinflammatory mediators has been extensively characterized. COX-2 could be induced by several extracellular signals, including LPS [199]; nitric oxide (NO) and reactive oxygen species (ROs) [200]; pro-inflammatory cytokines [358]; and growth factors [181]. Activation of C/EBPβ and in turn phosphorylation of cAMP response element-binding protein (CREB) play a major role in the initial stage of COX-2 transcription [359].

CCAAT/enhancer-binding proteins (C/EBPs) comprise a family of transcription factors with at least six members: C/EBPα - C/EBPζ. These transcription factors are known to be involved in the regulation of cell growth and cellular differentiation of several cell types; control of metabolism; inflammatory response; and cellular proliferation [208, 214, 215].

Several cancer models have shown that C/EBPβ may play a role in neoplastic transformation [360, 361]. Some studies of human cancers showed increased expression of C/EBPβ in solid tumours related to poor prognosis [360-364]. Nevertheless, to the best of our knowledge, there is no report dealing with the expression of C/EBPβ in human prostate, either in malignant or benign tissues. To improve the understanding of the role of C/EBPβ in malignant transformation of prostate, we first investigated its expression in benign prostate tissues, especially in the possible cancer precursor PIA [293]. We selected prostatectomy samples of BPH patients to perform this study, since it was known that the same morphologic patterns of atrophy can be seen in any region of the prostate and in and around nodules of BPH [294]. It was also known that the overexpression of COX-2 in benign and malignant prostate tissues is related to local inflammation [344, 365].

In this study, we have identified a novel finding of C/EBPβ expression in benign prostate tissues. C/EBPβ nuclear immunostaining was occasionally observed as focal or scattered among epithelial cells of normal-appearing prostate acini. The appearance of C/EBPβ in these epithelial cells was usually near the area of inflammation. Only 5% (± 7%) of normal-appearing acini showed C/EBPβ positive staining. In contrast, diffused C/EBPβ nuclear immunostaining was present in PIA lesions: 81% (±19%) of examined PIA had C/EBPβ immunostaining.

Normally, C/EBPβ protein is widely expressed in many different tissues, including liver, adipose tissue, ovary, and mammary gland. Several studies have shown that C/EBPβ is a critical mediator of steroid hormone-regulated cell proliferation and differentiation. C/EBPs are involved in the regulation, proliferation and differentiation of the cells in mammary gland. Moreover, both C/EBPα and C/EBPβ play roles in breast cancer development [212]. High C/EBPα and C/EBPσ protein levels correlated significantly with expression of cell-cycle promoters (cyclin D1 and E) and cell-cycle inhibitory proteins (Rb, p27, p16), but with none of the established prognostic parameters. In contrast, C/EBPβ was statistically related to negative estrogen receptor status, high grading, nodal involvement, and high cyclin E and p16 expression in breast cancer. It was suggested that high C/EBPβ expression might be involved in tumour progression and indicative of an unfavourable prognosis [212]. Studies have also shown that C/EBPβ is a critical mediator of steroid hormone-regulated cell proliferation and differentiation in the uterine epithelium and stroma [366]. A study in mice revealed that C/EBPβ is a key mediator of steroid responsiveness in the epithelium and stroma in the mouse uterus [366]. The expression of C/EBPβ is rapidly induced in the pregnant uterus at the time of blastocyst attachment. The expression of C/EBPβ increases further during the decidualization phase of pregnancy. Administration of estrogen or progesterone to ovariectomized females induced C/EBPβ expression in both uterine epithelium and stroma [366].

In addition, C/EBPβ appears to play an important role in promoting cells proliferation, and its levels are increased in a number of tumours. C/EBPβ is involved in antioxidant- or deoxycholic acid-induced apoptosis of colorectal cancer cells. It has been demonstrated that the C/EBPβ protein is essential for lymphocyte differentiation, and is necessary for the antitumour cytotoxicity of murine macrophages. Transfection of C/EBPβ to murine abdominal resident macrophages significantly enhanced their cytotoxicity to tumour cells. Overexpression of exogenous C/EBPβ can induce apoptosis in various malignant cells. Moreover, Fas-induced apoptosis in mouse hepatocytes is dependent on C/EBPβ. Nevertheless, until recently, as we know, there have been no reports about the expression of C/EBPβ in human prostate benign or/and malignant tissues. The role of C/EBPβ in human prostate development and disorders is entirely unknown.

C/EBPβ expression and its association with local chronic inflammation

In the present study, C/EBPβ tended to be present in foci of chronic inflammation. Double IHC staining of C/EBPβ / CD3 and C/EBPβ / CD68 confirmed this finding: C/EBPβ nuclear staining in atrophic epithelial cells was predominantly seen adjacent to the T-lymphocytes and

macrophages infiltration areas. C/EBPβ expression was associated with the severity of inflammation, both in T-lymphocyte and macrophages inflammation.

It is known that C/EBPβ is induced by inflammatory cytokines, such as IL-1, IL-6, and TNF-α [212]. The present results suggest that the local inflammatory cells and its related micro-environment may induce the C/EBPβ over-expression. This hypothesis was supported by additional evidence from the C/EBPβ expression pattern in normal-appearing acini. C/EBPβ positive immunostaining only appeared in a minority (5%) of normal-appearing prostate acini and in most cases this was in areas adjacent to chronic inflammation.

C/EBPβ expression is related to increased COX-2 expression

COX-2 expression has been identified as induced by several proinflammatory cytokines, and it appeared in various inflammatory lesions and tumours, such as PIA and prostate cancer. In this study, we noted that C/EBPβ expression was closely related to COX-2 expression in prostate epithelial cells. Statistical analysis confirmed this observation ($p = 0.001$).

Activation of C/EBPs plays a critical role during the initial stage of COX-2 transcription [359]. COX-2 expression in response to stimulation by proinflammatory mediators is transcriptionally regulated through activation of NF-κB, C/EBPβ, AP-1, and CREB-2.

COX-2 seems to be particularly interesting in PIA, since it was believed to be the potential target gene for chemotherapy or chemo-prevention of prostate cancer [367]**.** COX-2 inhibitors and NSAIDs have been used in the chemoprevention of several cancers. Several laboratory and epidemiological studies suggest that COX-2 inhibitors reduce prostate cancer risk or/and have antitumour activities by inhibiting COX-2 enzyme [221, 367-369]. The present data show the correlation between C/EBPβ and COX-2 in PIA. It is suggested that C/EBPβ may also be involved in the regulation of COX-2 in prostate epithelial cells, just as in other cells, such as fibroblast, macrophage, endothelium, or gastric cancer cells [363, 370, 371]. Recent studies have made it clear that aspirin and sodium salicylate at therapeutic concentrations selectively suppress the expression of COX-2 and inducible nitric oxide synthase (iNOS) induced by LPS and IL-1β through inhibiting C/EBPβ activation in macrophage and fibroblast cells [371, 372]. It may be further speculated that aspirin or NSAIDs and their in vivo metabolites are capable of inhibiting the expression of C/EBPβ and thereby suppressing COX-2 expression and the consequent effects in chemoprevention. Thus, the present study may have clinical implications, since it might be suggested that C/EBPβ could be a target for chemotherapy or chemoprevention for prostate cancer.

Morphological transition from PIA to HGPIN, and prostate cancer (paper I, II, and IV)

Morphological analysis is an important way to investigate the link between prostatic focal atrophy and prostate malignancies. In 1954, Franks considered a specific subtype of PAH as a putative neoplastic precursor, given its close association with PCA [291]. Several recent studies have re-examined the role of focal atrophy as a potential neoplastic precursor and have identified a strong topographic association between atrophy and prostate cancer and/or HGPIN [297, 299, 300].

Recently, a rat prostate model supplied strong evidence that the pathogenesis of prostatic neoplasia proceeds from inflammation to PIA and then to HGPIN. Rats fed 2-Amino-1-methyl-6-phenylimidazo (4,5-*b*)pyridine (PhIP) showed significant inflammation and atrophy of the prostate and, HGPIN was later observed to develop directly from the atrophic epithelium.

It should be noted that not all the pathologists have noted morphologic transition of PIA, HGPIN, and invasive adenocarcinoma [301-305] and that not all HGPIN or small carcinoma lesions are associated with atrophy. It is not clear how these parameters are interrelated and what intermediates are involved. At the WHO Consensus Conference in Stockholm, it was emphasized that no relationship between atrophy and prostate carcinoma or HGPIN has been proven [267]**.** Thus, additional studies are required to more fully understand the relation between focal atrophic lesions and cancer development in the prostate.

In the study with 50 radical prostatectomy specimens of prostate adenocarcinoma, we identified HGPIN and carcinoma lesions and tried to determine how often they merged with PIA.

Presence of PIA-merging HGPIN

Among the total of 1,188 HGPIN lesions from all the 50 cases specimens, 17% (198/1,188) were detected as merging with PIA. According to the subtypes of prostatic atrophy, the majority were simple or mixed atrophy. One interesting observation was that the distribution of PIN patterns in PIA-merging HGPIN lesions was different from the usual histological pattern of HGPIN, tufting HGPIN, in most of the slides: flat pattern was the dominant subtype PIN in PIA-merging HGPIN lesions. The reason may be that the flat pattern of HGPIN is often composed of luminal cells with atypical nuclei in ducts and acini with minor architectural changes and is difficult to identify in H&E stained slides even at high magnification [373], however, it is easy to recognize by means of the CK5 or GSTP1

immunostaining. Another possible interpretation may be that in the PIA-merging HGPIN, the flat subtype is probably the earliest type of HGPIN, since it had somewhat similar architecture as in SA, except for the prominent nucleoli and the absence of CK5 and GSTP1.

CK5 and GSTP1 immunostaining as possible markers for atrophic epithelium

At the beginning of this study, four antibodies, CK5, GSTP1, c-MET, and C/EBPβ, were tested for selecting possible markers in detecting the atrophic epithelium. The results showed that both CK5 and GSTP1 could be used as markers for the atrophic epithelium, where it is difficult to distinguish it from other components on the routine H&E stained slides.

Although a few reports have confirmed the development of PIA to HGPIN [268, 293, 300], there are also numerous contradictory reports [301, 303]. How to recognize the atrophic fraction in such lesions is a crucial question, since it is difficult to detect a tiny atrophic fraction in HGPIN lesions in the ordinary H&E stained slides. The present study showed that by means of CK5 and GSTP1 IHC immunostaining, it was easier to detect the atrophic epithelial cells in PIA-merging HGPIN or PIA-merging PCa lesions, especially in a lesion that has made the transition into malignancy. CK5, a basal cell marker, appeared in the luminal cells of PIA [306, 374], and was absent in both HGPIN and prostate cancer. GSTP1 protein was normally expressed in basal epithelium and was absent in most luminal epithelial cells. In PIA lesions, strong anti-GSTP1 staining was seen in most of the atrophic epithelial cells. Absence of GSTP1 expression is common in HGPIN lesions and prostate cancer cells. These results suggest that both CK5 and GSTP1 are suitable markers for detecting and recognizing the atrophic epithelial cells in various lesions.

Presence of PIA-merging PCa

In morphological studies, atrophic lesions have been noted near early carcinoma lesions [299], and, at times, to have merged with adenocarcinoma [300, 375]. The present work adds evidence that there is a direct transition from PIA to prostate carcinoma. Fourteen (28%) of the samples were found to have small foci of PIA-merging PCa. By means of CK5 or GSTP1 immunostaining, it was easy to recognize the atrophic epithelial cells in these lesions. The contrast image of GSTP1 in the atrophic epithelium and prostate cancer cells demonstrates the GSPT1 hypermethylation in malignant cells. This finding supports the hypothesis that some prostate atrophy lesions may directly give rise to carcinoma, as has previously been suggested [291, 300, 376].

Cluster of atypical epithelial proliferation

One striking finding in this study is that clusters of atypical epithelial proliferation were found in some PIA lesions, where a focal dominant chronic inflammation exists. These cluster cells appeared to grow in a budding pattern in the luminal layer. Cells were variable in size and shape, showed eosinophilic or basophilic cytoplasm, had enlarged nuclear with hyperchromasia, and contained severe nuclear atypia with frequent prominent nucleoli. The latter fulfil the criteria for the diagnosis of HGPIN. Architecturally, such lesions maintain a normal-appearing or atrophic architecture and a continuous basal cell layer. These lesions were usually surrounded by various degrees of chronic inflammation. Like the epithelium in other PIA lesions, such clusters of atypical epithelia also showed increased immunostaining for CK5 and GSTP1, from weak to moderate immunostaining intensity. Since there were no previous data concerning this, we examined these lesions to determine how frequently it appeared. Five cases with 16 clusters of atypical epithelia proliferation lesions were found from 50 prostatectomy specimens.

Such focal atypical epithelial cells, although they were few in isolated PIA lesions, have fulfiled the criteria for HGPIN after routine light microscopic examination. We thereby postulated that a HGPIN lesion might develop from these clusters of atypical epithelial cells, meaning that some PIA lesion may develop directly into HGPIN. It is suggested that these cluster of cells may be a consequence of regenerative proliferation after the activation of "stem cells" or their progeny, the intermediate cells, by chronic stress.

In summary, morphological transition from PIA to HGPIN and carcinoma were occasionally found in 50 prostatectomy specimens. CK5 and GSTP1 are useful markers for detecting and recognizing the atrophic epithelial cells in these lesions. Considering the findings in this study, we hypothesize that the transition from PIA to HGPIN and prostate cancer may go through pathways with and without an intermediate morphological stage of low grade PIN.

CONCLUSIONS

The results from the studies included in the current thesis show that:

- COX-2 is overexpressed in prostate PIA lesions.
- The epithelium of these COX-2 positive immunostained glands has high proliferation index and increased Bcl-2 protein level.
- Transcription factor C/EBPβ is expressed in PIA lesions and is correlated to COX-2.
- The COX-2 expression was positively associated to Gleason score and the angiogenesis in prostate cancer.
- Focal chronic inflammation, predominantly T-lymphocytes and macrophages infiltration, plays an important role in inducing COX-2 expression both in benign and malignant prostate tissues.
- Morphological analysis supported the hypothesis that PIA could develop into HGPIN and/or prostate cancer directly or indirectly.

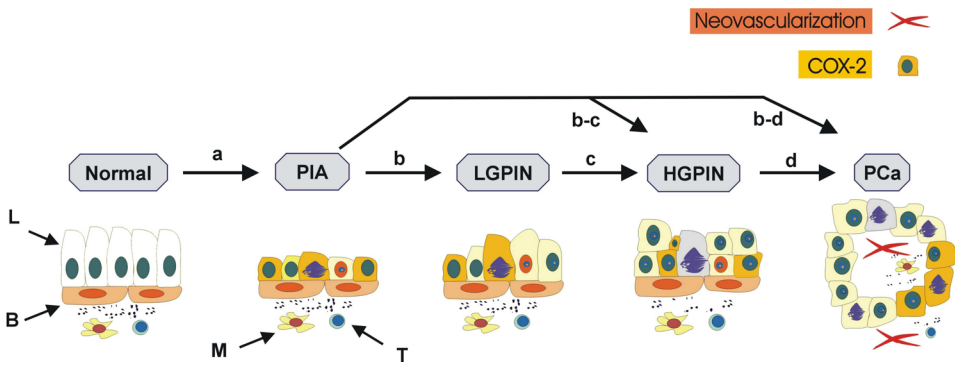

Fig 5. Cellular and molecular model of transition from PIA to HGPIN and prostate cancer.

L, luminal epithelium; B, basal epithelium; M, macrophage; T, T-lymphocyte; PIA, proliferative inflammatory atrophy; LGPIN, low grade prostatic intraepithelium neoplasia; HGPIN, high grade prostatic intraepithelial neoplasia; PCa, prostate cancer. b-c, transition from PIA to HGPIN; b-d, transition from PIA to PCa.

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