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Molecular Endocrinology of Target Enzymes in Androgen Metabolism
-Implications for Prostate Cancer

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Stockholm 2001

STOCKHOLM 2000

ABSTRACT

Some of the same genes that are responsible for variations in drug and hormonal responses will also be associated with the pathophysiology of diseases. Understanding the aetiology of a disease is often essential for the design of effective drugs. Androgens are implicated in the development of prostate cancer and benign prostate hyperplasia (BPH). This study aimed at characterising the therapeutic target enzymes steroid 5α-reductase 1 and 2 and the steroid metabolising enzyme cytochrome P450 1B1 in relation to prostate cancer in order to gain a better view of the prostate tumor biology.

The conversion of testosterone to the more potent metabolite dihydrotestosterone by steroid 5α-reductase 2 is a key mechanism in the action of androgens in the prostate. The 5α-reductase 2-specific messenger RNA (mRNA) levels were measured in 50 biopsies obtained from 31 Caucasian outpatients, using a solution hybridization method. Significant differences were observed between cancerous and noncancerous tissues. The median 5α-reductase 2 mRNA level in noncancerous tissue was 3.4 times higher than in cancerous specimens. 5α-Reductase 2-specific gene expression and enzyme activity was measured in 30 prostatic tissue specimens from 15 Caucasian patients. The enzyme activity at pH 5.5 was significantly correlated to the 5α-reductase 2-specific mRNA expression as measured by reverse-transcription PCR (R_s=0.81). This association makes it possible to predict prostatic 5α-reductase 2 activity using core needle biopsies.

In order to elucidate the role of 5α-reductase 2 polymorphisms, we performed a population based case control study in 176 Caucasian prostate cancer patients and 161 healthy controls of the V89L and A49T polymorphisms on the risk of prostate cancer and in relation to age and tumor characteristics. Carriers of the LL genotype were at increased risk of bone metastases at the time of diagnosis compared to the combined groups of individuals with VL or VV genotypes, OR 5.67 (95 % CI 1.44-22.30), when adjusted for age, differentiation grade, T-stage and PSA. Heterozygous prostate cancer cases carrying the AT genotype were significantly younger than cases harboring the AA genotype (mean age 66 vs 71 years).

In the same patient material, the *CYP1B1* V432L polymorphism was studied in relation to risk of prostate cancer and tumor characteristics. Carriers of the *CYP1B1* 432 LL genotype had a higher risk of metastases at the time of diagnosis compared to individuals with VL or VV genotypes, (OR 2.46, 95 % C.I 1.02-5.93) when adjusted for age, differentiation grade, T-stage and PSA. These findings may contribute to further understanding of the etiology of prostate cancer metastases.

MAIN REFERENCES

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I.   Bjelfman C, Söderstrom TG, Brekkan E, Norlen BJ, Egevad L, Unge T, Andersson S, Rane A. Differential gene expression of steroid 5 α-reductase 2 in core needle biopsies from malignant and benign prostatic tissue. J Clin Endocrinol Metab 1997 Jul;82(7):2210-4

II.   Söderström TG, Bjelfman C, Brekkan E, Ask B,Egevad L, Norlén BJ, Rane A. Messenger Ribonucleic Acid Levels of Steroid 5α-Reductase 2 in Human Prostate Predict the Enzyme Activity. J Clin Endocrinol Metab 2001 86: 855-858.

III.   Söderström TG, Wadelius M, Andersson S-O, Johansson JE, Johansson S, Granath F, Rane A. 5α-Reductase 2 Polymorphisms as Risk Factors in Prostate Cancer. Manuscript.

IV.   Söderström TG, Wadelius M, Lundgren S, Andersson S-O, Johansson JE, Johansson S, Granath F, Rane A. Association of CYP1B1 V432L Polymorphism with Prostate Cancer Metastases. Manuscript.

To my family

"Well, I stand up next to a mountain

and I chop it down with the edge of my hand.

Well, I pick up all the pieces and make an island,
might even raise just a little sand."

/JH

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ABBREVIATIONS

A	alanine
amol	attomoles
bp	base pair
BPH	Benign Prostate Hyperplasia
CaP	cancer prostate
C.I.	confidence interval
CYP1B1	cytochrome P450 1B1
cDNA	complementary deoxyribonucleic acid
cRNA	complementary ribonucleic acid
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
FSH	follicle stimulating hormone
IGF	insulin-like growth factor
L	leucine
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
MAB	maximum androgen blockade
mRNA	messenger ribonucleic acid
OR	odds ratio
PCR	polymerase chain reaction
PSA	prostate specific antigen
T	threonine
totRNA	total ribonucleic acid
V	valine

INTRODUCTION

To guide the reader into the work described in this thesis, an introduction and a background to certain aspects of prostate cancer is given.

Prostate cancer is the most common non-skin malignancy among men and accounts for 27.4 % of all incident cancer cases in Sweden (SOS MARS. Prostatacancer, 2000).

In 1996, 2323 men died from prostate cancer in Sweden (SCB; causes of death, 1996). The number of diagnosed cases of prostate cancer increased from 1554 in 1960, to 5918 in 1997 (Socialstyrelsen. Cancer incidence in Sweden, 1999). During the period 1983-1987, the risk of contracting prostate cancer before the age of 65 and 75 years of age was 1.2 % and 5.8 % respectively (Socialstyrelsen. Cancer incidence in Sweden 1991). Of all diagnosed cases, about 70 % are men 70 years or older (Socialstyrelsen. Cancer incidence in Sweden, 1999). During 1960-1988, the survival in prostate cancer in Sweden increased slowly from 45 % during 1960-1964 to 63 % during 1980-1984 (Helgesen, 1996). This might be interpreted as an increasing diagnosis of non-lethal prostate cancer. Carcinoma of the prostate is also the most common tumor in men in the United States with 180,400 new cases and 31,900 deaths expected in 2000 (Greenlee, 2000). The disease can be found in a histological investigation in as many as 34 % of men in their fifth decade and in up to 70 % of men 80 years of age and older (Holund, 1980; Sakr, 1993). Prostate cancer will be diagnosed in almost one fifth of U.S. men during their lifetime, yet only 3 % of men are expected to die of the disease (Ries, 1998). The high rate of clinically occult prostate cancer in the general population is in deep contrast to the much lower likelihood of death from the disease. This indicates that many of these cancers have low impact on mortality. However, to characterize and find these early after diagnosis in the early stages of disease is currently not possible. Prostate cancer managed by surveillance alone has relatively good survival rates at 5 and 10 years of follow-up (Whitmore, 1991). However, prolonged follow-up over 10 years of moderately and poorly differentiated tumors show a substantial risk of disease progression and death from prostate cancer (Albertsen, 1998). The estimated reduction in life expectancy of men who die of prostate cancer is approximately 9 years (Horm, 1989). Moderately differentiated tumors constitute the majority of tumors now detected (Orozco, 1998).

Treatment options include radical prostatectomy, external-beam radiation therapy, brachytherapy, and surveillance followed by hormonal treatment when symptoms occur. No data demonstrate the clear superiority of any of these forms of treatment for an individual patient (Middleton, 1995). Therefore, any patient with newly diagnosed, localized prostate cancer has to make difficult choices between these treatment options. Problematic issues in

the treatment of prostate cancer include side effects, inability to predict the natural history in the individual case, patient co morbidity that may modulate the patient's risk for morbidity and death in prostate cancer, and a substantial fraction of treatment failures. Because of considerable uncertainty regarding the efficacy of treatment and the difficulty in selecting patients for whom there is a known risk of disease progression, there are many opinions in the medical community regarding the purpose of screening for carcinoma of the prostate. The lack of evidence that screening and treatment affects ultimate population morbidity or mortality may undermine the reasons to screen (Neal, 2000).

The estimated direct medical-care costs for prostate cancer in Sweden in 1993 were 771 million SEK (Holmberg, 1996). The tremendous impact of prostate cancer on the population of western countries, as well as the financial burden of the disease both for patients and society, has led to an increased interest in the treatment and prevention of prostate cancer. There is a critical need to find host and tumor markers that characterize those cancers most likely to progress to metastasis within the human lifespan, but also to evaluate etiologic factors in terms of their potential contributions to risk. It is likely that common polymorphisms of genes involved in metabolic or regulatory pathways will account for a substantial fraction of the inherited prostate cancer burden. Accordingly, further study of genetic factors involved in testosterone biosynthesis, transport to the prostate, activation to dihydrotestosterone (DHT) and transport and degradation within prostate cells is urgent. The functional relevance of any polymorphisms associated with altered prostate cancer risk should be elucidated. It is important to identify markers that alter risk for, and progression of prostate cancer in order to establish polygenic profiles characterizing men at particularly high and low risk. Markers associated with prostate cancer risk have been identified on several genes, for instance the 5-α reductase 2 (Makridakis, 1999), the CYP 17 (Wadelius, 1999; Lunn, 1999; Habuchi, 2000; Gsur, 2000) and the androgen receptor gene (Hsing, 2000; Ingles, 1997), in the androgenic pathway. There are many other candidate genes in the androgen activation pathway, which await their exploration in relation to prostate cancer, see figure 3. The ability to identify these more accurately, and to define prognostic determinants with greater specificity will allow more appropriate selection of targeted drug therapy for localized disease with metastatic potential, as well as more precise management of patients with advanced disease. The broad range of genetic anomalies associated with prostate cancer appears increasingly to be involved in the constitutive heterogeneity of function of prostate cancer, and this, in turn, creates a more complex target for therapeutic endeavors (Beer 2000).

Prostate embryology and histology

The glands of the prostate are derived from the endoderm of the urogenital sinus, while the fibrotic and muscular tissues are derived from the mesoderm (Moore, 1983; Coffey, 1993). The prostate undergoes significant growth during fetal development, puberty, and in most men, during late middle age. Prostate growth and development are dependent on androgen production by the fetal testes, which begins at about the eight week of gestation (Pointis, 1980; Resko, 1978; Siiteri, 1974; Weniger, 1972; Winder, 1981). The differentiation of the urogenital sinus is dependent on DHT, which is essential for the mediation of growth and development of the prostate from the pelvic portion of the urogenital sinus. The Wolffian duct derivatives (seminal vesicle and epididymis), however, are dependent on testosterone for their development (Siiteri, 1974; Cunha, 1972; Wilson, 1981). At the end of puberty, the prostate reaches approximately 26 g and is maintained at that weight unless benign prostatic hyperplasia (BPH) develops. The average weight of the prostate with histological confirmed BPH at the time of autopsy is 33 ± 16 g (Berry et al., 1984).

The largest part of the prostate is the anterior or ventral fibromuscular and non-glandular region, which forms the ventral surface of the gland and which constitutes about one-third of the entire prostate, which can be divided into lobes or zones. The division made by McNeal is currently the one most applied (McNeal, 1988).

The glandular prostate can be subdivided into three zones as follows:

1. A peripheral zone that represents about 70 % of the glandular part of the prostate. This zone forms the lateral and posterior or dorsal part of the organ. The ducts of the peripheral zone open into the distal prostatic urethra.

2. A central zone that comprises about 25 % of the glandular prostate. This zone is edge-shaped and surrounds the ejaculatory ducts. The central zone is surrounded by the peripheral zone, at least in its distal part, and its ducts open into the prostatic part of the urethra, in close proximity to the ejaculatory ducts.

3. A transitional zone that is the smallest glandular part and it comprises only about 5-10 % of the prostate. This zone consists of two independent small lobes whose ducts leave the posterolateral recesses of the urethral wall at a single point, just proximal to the point of urethral angulation and at the lower border of the preprostatic sphincter.

bladder lumen
detrusor
preprostatic sphincter
central zone
urethral lumen
transitional zone

fibromuscular stroma
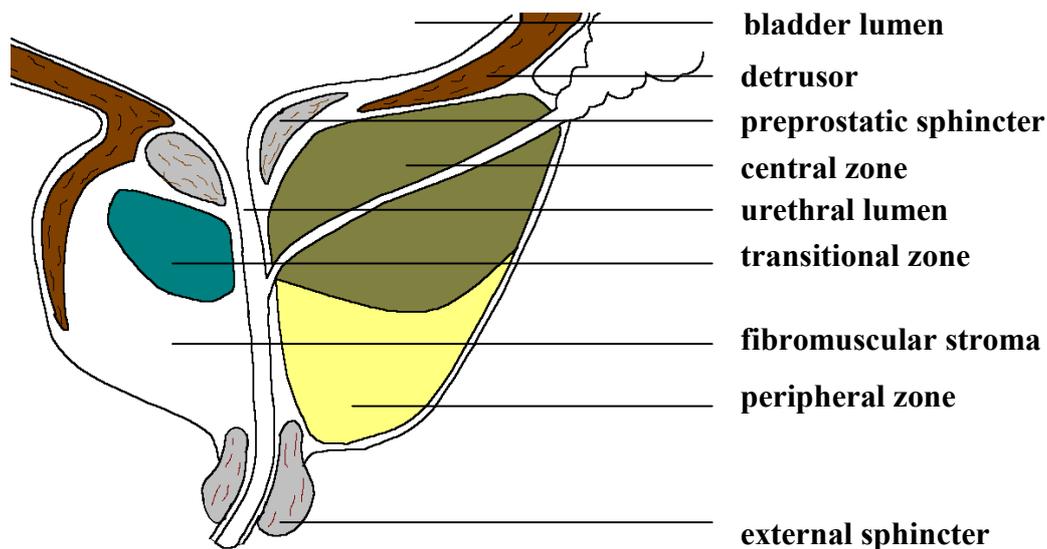peripheral zone

external sphincter

Figure 1. Sagittal view of the glandular prostate which can be subdivided into three zones, the central, the transitional and the peripheral zone.

BPH arises in the transitional and periurethral zones and most cancers in the peripheral zone, although it has been suggested that smaller less aggressive well-differentiated tumors arise in the transitional zone. (McNeal, 1988; Vijayakumar, 1993; Busch, 1993). Prostatic cancer is almost always an adenocarcinoma, although transitional or squamous cell carcinomas have also been described as primary malignancies in the gland (Mostofi, 1993). Neuroendocrine differentiation is present in some prostate cancers, and this has been claimed to be associated with hormone-refractory lesions (Abrahamsson, 1989).

The hormonal chain from hypothalamus to the prostate (and back)

The hypothalamus communicates with the pituitary both by a portal vascular system and by neural pathways. The portal vascular system provides a mechanism for the delivery of releasing hormones from the brain to the pituitary (Wilson, 1998). The medial basal region and the preoptic area of the hypothalamus contain important centers for control of gonadotropin secretion. Neurons in this region secrete luteinizing hormone-releasing hormone (LHRH, GnRH) in a pulsatile fashion (Silverman, 1979). Neurons from other regions of the brain terminate in this area and influence both the frequency and the amplitude of LHRH secretory pulses via catecholaminergic, (Negro-Vilar, 1979) dopaminergic, (Evans, 1980) and endorphin-related mechanisms. The opioid peptide involved in the endorphin-related mechanisms appears to be beta-endorphin, which inhibits gonadotropin secretion (Jenkins, 1993). LHRH is released in pulses, which leads to pulsatile release of luteinizing hormone (LH) and follicle stimulating hormone (FSH), the primary pituitary hormones that regulate the testes. They are glycoproteins composed of two polypeptide chains designated α and β and are secreted by the same basophilic cells in the pituitary (Wilson, 1998). The secretory pulses of LH in adult men occur at a frequency of 8 to 14 pulses/24 h and vary in magnitude (Santen, 1973). Pulsatile secretion of FSH is temporally coupled to that of LH (Veldhuis, 1987). The secretion of LH is controlled by the negative-feedback action of gonadal steroids on the hypothalamus and the pituitary (Wilson, 1998). Testosterone or its metabolites act on the central nervous system to slow the hypothalamic pulse generator and consequently decrease the frequency of LH pulsatile release (Matsumoto, 1984). Acute infusions of estradiol also lower LH levels associated with an increased frequency and decreased amplitude of the LH pulses. DHT, which cannot be converted to estrogen, exerts a negative-feedback control on LH secretion, which indicates that testosterone might not require aromatization to inhibit LH secretion (Santen, 1975). LH interacts with specific high-affinity cell-surface receptors on the plasma membrane of testicular Leydig cells (Dufau, 1978). The Leydig cell is thereby stimulated to activate the synthesis of the enzymes of testosterone biosynthesis (Payne, 1995). In the intact testis and in cultured Leydig cells the number of LH receptors decreases after administration of LH (LaPolt, 1991). This down-regulation of receptor number is associated with a decreased response to subsequent LH administration (Saez, 1978). The decreased response of the Leydig cell to LH after LH administration is important in the intratesticular regulation of testosterone production (Wilson, 1998).

FSH plays an indirect role in steroidogenesis by inducing maturation of Leydig cells during development (Kerr, 1985) and by increasing the number of LH receptors on the Leydig cells (Odell, 1973). Under normal physiologic conditions, the Leydig cells of the testis are the major source of the testicular androgens and the major circulating serum androgen is testosterone, which is to more than 95 % of testicular origin. The rate-limiting reaction in testosterone synthesis under most circumstances is the conversion of cholesterol to pregnenolone. LH regulates the rate of this reaction and thus controls the overall rate of testosterone synthesis (Eik-Nes, 1975). Serum testosterone levels are not extensively related to age between 25 and 70 years, although they do decline gradually. Plasma concentrations of testosterone can vary widely in an individual and may reflect both episodic and diurnal variations in the production rate. Testosterone levels are higher in the morning than at any other time of the day (Campbell, 1998). Although other steroids, such as androstenedione from the adrenals, can be converted by peripheral metabolism to testosterone, they probably account for less than 5 % of the overall production of plasma testosterone. The adrenal steroids include dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEA-S), as well as androstenedione. There are different opinions in the medical society about the significance of the adrenal androgens in the prostate. On one hand, essentially, all of the DHEA in male plasma originates from the adrenal cortex, but less than 1 % of the total testosterone in the plasma is derived from DHEA (Horton, 1976). The effect of normal levels of adrenal androgens on the prostate in noncastrated humans may not be significant because adrenalectomy has very little effect on prostate size or morphology (Oesterling, 1986). Androstenedione cannot be converted directly to DHT and, therefore, is a weak androgen. This has lead to the conclusion that the adrenals do not significantly support growth of prostatic tissue (Campbell, 1998). On the other hand, human adrenals secrete large amounts of the inactive precursor steroids DHEA, its sulfate (DHEA-S), and androstenedione (4-dione), which are converted into potent androgens, and estrogens in peripheral tissues by the enzymes 17β–hydroxysteroid dehydrogenase (17β-HSD) and 3β–hydroxysteroid dehydrogenase (3β-HSD) (Labrie, 1998). Plasma DHEA-S levels in adult men and women are 100 to 500 times higher than those of testosterone, thus providing a large reservoir for conversion into androgens in peripheral intracrine tissues. It is estimated that 30 to 50% of total androgens in men are synthesized in peripheral intracrine tissues from inactive adrenal precursors (Labrie, 1998). The formation of DHEA-S by the adrenals is reduced considerable (5-fold) during aging, which may result in a dramatic fall in the formation of androgens and estrogens in peripheral target tissues (Labrie, 1998). However, no significant inhibition of adrenal

androgen secretion occurs after LHRH agonist treatment (Decensi, 1994), and the prostate tissue concentrations of DHT in pharmacologically castrated subject, compared with untreated patients, are reduced about 90%, and about 75% for testosterone. These results suggest that about 90% of prostatic DHT depend on testicular activity (Salerno, 1988). Nevertheless, low circulating levels of DHEA-S and DHEA have been found in patients with prostate cancer (Stahl, 1992), which is of interest in relation to the results presented in this thesis.
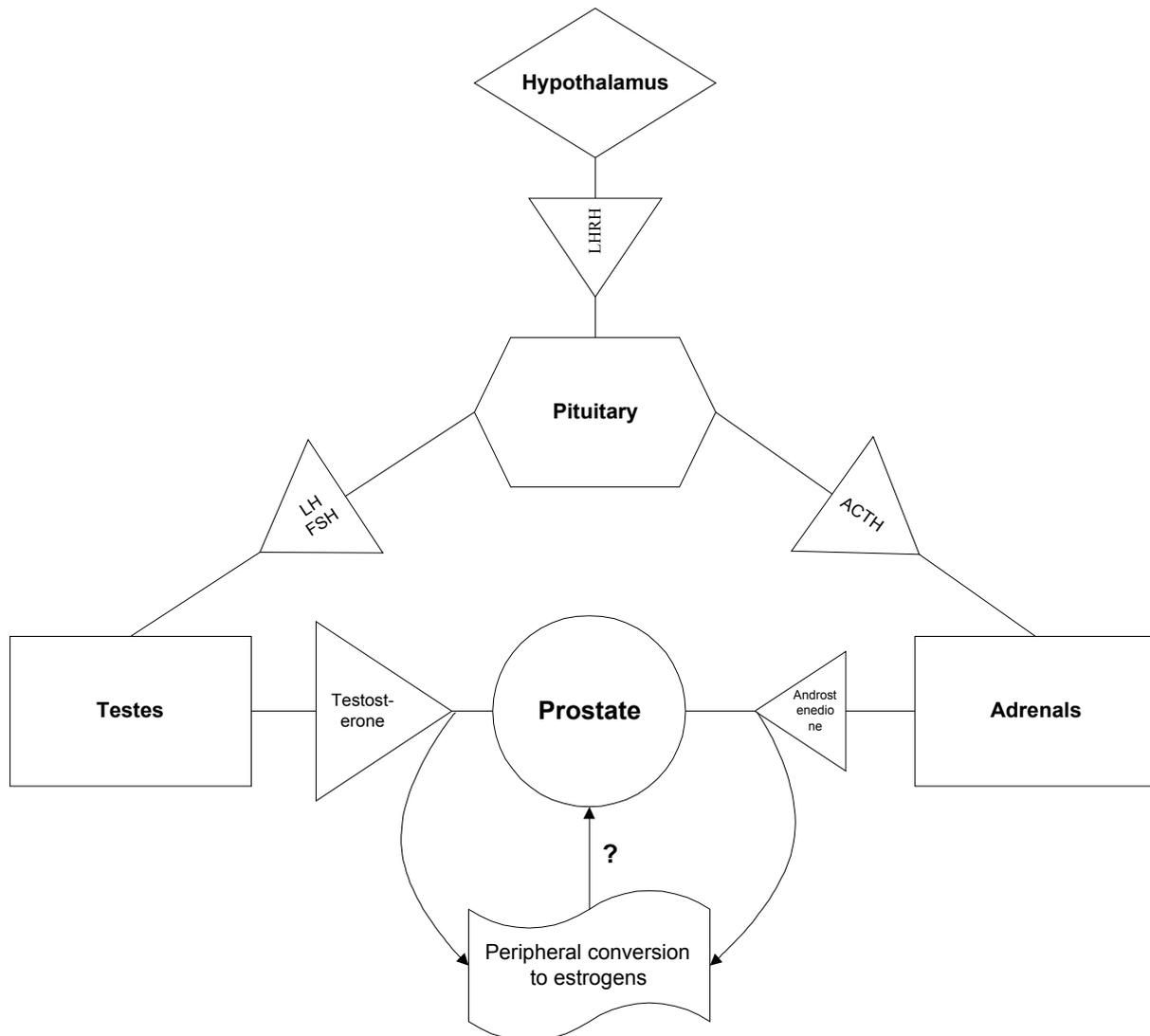

Figure 2.   A schematic view of the hypothalamic-pituitary-testicular-prostatic axis.

The majority of the circulating testosterone is bound to carrier proteins such as the Steroid Hormone Binding Globulin (SHBG) and albumin. In the blood of normal men about 2 % of testosterone is unbound, 44 % is bound to SHBG, and 54 % is bound to albumin and other proteins (Dunn, 1981). Albumin has about a 1000-fold lower affinity for testosterone than does SHBG, but the concentration of albumin is so much higher that the binding capacities

are similar. The proportion of testosterone bound to SHBG in serum is proportional to the SHBG concentration (Wilson, 1998).
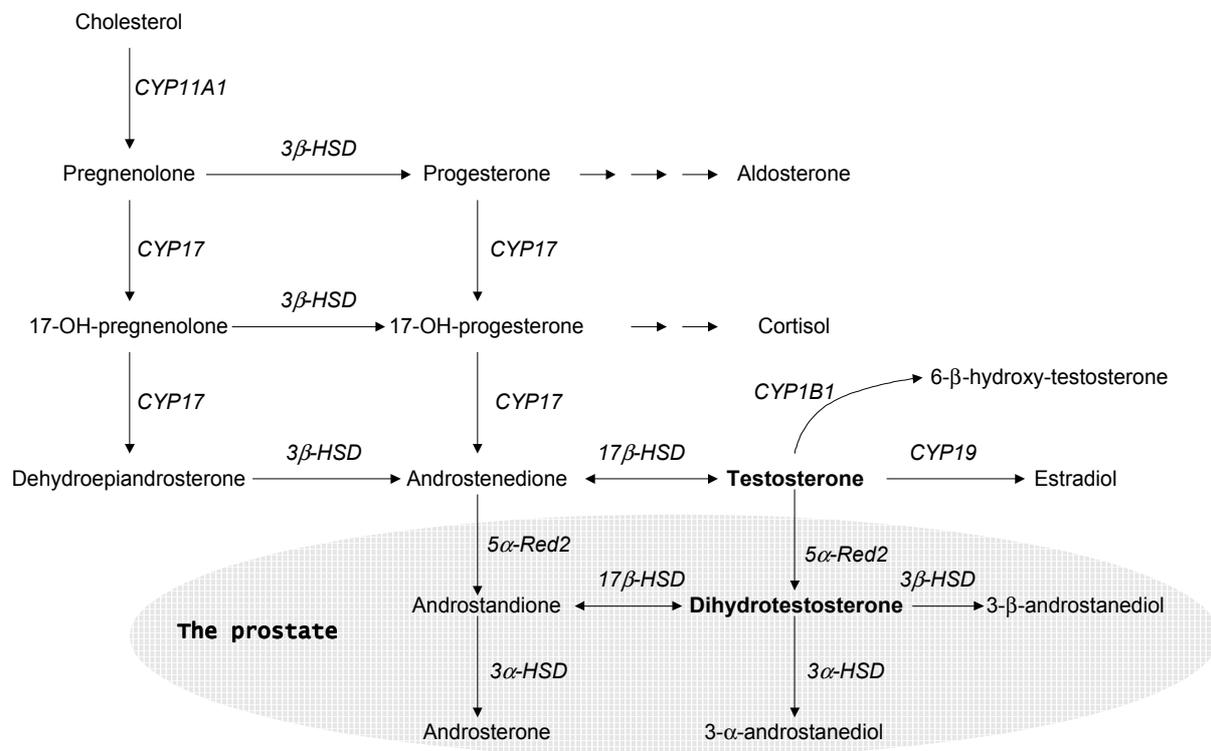
Cholesterol
CYP11A1
Pregnenolone — 3β-HSD → Progesterone → → → Aldosterone
CYP17 ... CYP17
17-OH-pregnenolone — 3β-HSD → 17-OH-progesterone → → Cortisol
CYP17 ... CYP17 ... CYP1B1 → 6-β-hydroxy-testosterone
Dehydroepiandrosterone — 3β-HSD → Androstenedione ← 17β-HSD → Testosterone — CYP19 → Estradiol
5α-Red2 ... 5α-Red2 ... 3β-HSD
Androstandione ← 17β-HSD → Dihydrotestosterone — 3β-HSD → 3-β-androstanediol
The prostate
3α-HSD ... 3α-HSD
Androsterone ... 3-α-androstanediol

Figure 3.   The principal pathways of human androgen synthesis involve several enzymes, including: 17α-hydroxylase/17,20 lyase (CYP17), CYP11A1, 3β-hydroxy steroid dehydrogenase (3β-HSD), 17β-hydroxy steroid dehydrogenase (17β-HSD) and 5α-reductase 2 (5α-Red2). Further metabolism engages the enzymes aromatase (CYP19), 3β-hydroxy steroid dehydrogenase (3β-HSD), CYP1B1 and 3α-hydroxy steroid dehydrogenase (3α-HSD). Adapted from Wadelius 1999.

After testosterone has entered the prostate it is rapidly metabolized to DHT, which is the major form of androgen, present at tenfold higher concentrations than testosterone within the prostate gland (Monti, 1998). The affinity of testosterone to the human androgen receptor is less than that of DHT (Maes, 1979) due to a more rapid dissociation rate for the testosterone-receptor complex. Testosterone-receptor complexes also transform to the DNA-binding state, although less well (Kovacs, 1984). These differences in interaction of the two steroids with the androgen receptor may serve as an amplifying mechanism for androgen action in target tissues that harbor 5α-reductase. In the prostate, DHT binds to the androgen receptor, which transforms to the DNA-binding state (Kovacs, 1983). In the nuclear matrix, the DHT /

androgen receptor complex acts as a transcription factor, increasing the transcription from DNA to messenger RNA (mRNA) which is subsequently translated into proteins by the ribosomes. DHT does, via activation of the androgen receptor, stimulate the production of secretory proteins such as prostate specific antigen (PSA) (Riegman, 1991), and regulate the production of several growth factors that stimulate cell proliferation (Monti, 1997), including insulin-like growth factor 1 (IGF-1) (Huyhn 1998), keratinocyte growth factor (Peehl, 1996), epidermal growth factor (Peehl, 1996; Monti, 1997) and the transforming growth factor beta (TGF-β) receptor (Saez, 1998). This indicates that many signaling pathways that regulate mitogenic activity in the prostate are modulated through activation of the androgen receptor.

5α-REDUCTASE

Testosterone is rapidly metabolized to other steroids by a series of prostatic enzymes (Isaacs, 1981; Isaacs, 1983a; Isaacs, 1983b; Bruchovsky, 1985). Over 90 % of the testosterone is irreversibly converted to the main prostatic androgen DHT through the action of 5α-reductase with NADPH as cofactor. Other substrates of 5α-reductase include 20α-hydroxy-preg-4-en-3-one, 17α-hydroxy-progesterone, epitestosterone, progesterone and androstenedione (Russell, 1994).
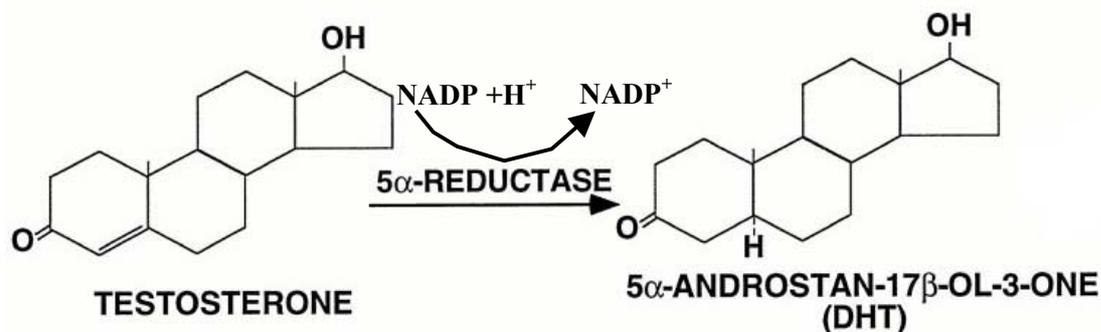

Figure 4.   The enzymatic reaction catalyzed by 5α-reductase. 5α-reductase reduces the unsaturated bond in testosterone between the 4 and 5 positions to form DHT.

The enzyme is located in the endoplasmic reticulum and nuclear membrane. There is a tenfold higher rate of conversion of testosterone to DHT in the stromal tissue as compared to the epithelium (Bruchovsky, 1985). Early observations indicated the presence of different human 5α-reductase enzymes with acidic and alkaline pH optima and that individuals with 5α-reductase deficiency syndrome would have mutations in the acidic pH optimum enzyme (Moore, 1975)

The first cloned and expressed human 5α-reductase (Andersson et al., 1990) was weakly inhibited by finasteride (a 4-aza steroid that is clinically used in the control of BPH). It had an alkaline pH optimum and there were no detectable mutations in 5α-reductase deficient individuals (Jenkins, 1992). That suggested that other human 5α-reductase genes were present. An expression cloning strategy revealed a second human 5α-reductase, which had an acidic pH optimum and was sensitive to inhibition with finasteride (Andersson et al., 1991). This second gene was shown to be mutated in 5α-reductase deficient individuals. The isozymes were numbered in chronological order of their cloning (Russel, 1994).

Human	Type I	Type II
Chromosome	5p15	2p23
Molecular weight	29,000	28,000
Amino acids	259	254
Exons	4	4
Introns	5	5
Homology	49 %	49 %
pH optima	Alkaline (6-8.5)	Acidic (5.0)
Km testosterone (mM)	1.5	0.1-1.0
Ki finasteride (nM)	325	12
Half-life, h	20-30	20-30
5α-reductase deficiency	Normal	Mutated

Table 1.   Properties of human 5-α reductase type 1 and type 2 genes and the respective enzymes (adapted from Wilson, 1998)

The tissue distribution of the 5α-reductase isozymes is different (Thigpen, 1993). The type 1 enzyme is found in the skin and in the adult scalp, and is believed to be involved in hair formation. It is also found in the liver. The type 2 enzyme is predominant in the prostate gland and appears in the basal cells of the epithelium and in the stromal cells but is absent in the secretory epithelial cells. This points to the fact that the stimulation of epithelial cells is derived from DHT that has been formed within the stromal or basal cells and has diffused into the epithelial cells in a paracrine pathway.

Tissue	Isozyme	
	Type 1	**Type 2**
Prostate	(+)	++
Epididymis	-	++
Seminal vesicle	-	++
Genital skin	-	++
Testis	-	-
Ovary	-	-
Adrenals	-	-
Brain	-	-
Kidney	-	-
Liver	+	+
Nongenital skin	++	-

Table 2.   Tissue distribution of the 5α-reductase isozymes in humans, (adapted from Russell 1994).

5α-reductase polymorphisms

There are over 29 known mutations in the 5α-reductase 2 gene that lead to the 5α-reductase deficiency syndrome (Russell, 1994). The rarity of the disease however shows the limited frequency of carriers of these mutations in the population. More frequently appearing functional polymorphisms may be of interest, as they might infer significant health effects in androgen dependent target organs where the enzyme is expressed, such as the prostate.

Makridakis et al (Makridakis, 2000) studied the pharmaco-genetic differences among the most common polymorphisms and mutations and they found a substantial variation in both enzyme activity and sensitivity for the most used inhibitor finasteride, see table 3. Only two of these polymorphisms may be common enough to be of interest in relation to possible significant impact on public health, especially prostate cancer. Those are the V89L and A49T polymorphisms. The development of BPH clearly requires a combination of testicular androgens and aging (Wilson, 1980; Walsh, 1985; Coffey, 1987). Although the role of androgens as the causative factor for human BPH is debated, they undoubtedly have at least a permissive role. Men castrated before puberty do not develop BPH. Furthermore, patients with a variety of genetic diseases that inhibit androgen production, or androgen action, have impaired if not absent prostatic growth (Bartsch, 2000). The best-characterized function of 5α-reductase is in the normal differentiation of the male reproductive tract. Initial male reproductive tract development requires production of testosterone by the fetal testis. The virilization of the external genitalia is dependent on conversion of testosterone to DHT in the

tissues of the urogenital sinus and a deficiency in 5α-reductase 2 activity leads to an incomplete form of male pseudohermaphroditism (Imperato-McGinley 1974, Walsh 1974, Andersson 1991, Thigpen 1992).

Substitution	Frequency (%)	Km	Vmax	Ki (nM finasteride)
Wt	?	0.9	1.9	60
C5R	1.2	0.9	1.8	63
P30L	0.2	2.1	0.5	420
P48R	0.5	2.2	1.2	22
A49T	2	2.7	9.9	180
A51T	0.2	0.7	1.1	49
V89L	32.5	0.6	1.1	113
T187M	0.3	1.1	0.8	70
F194L	0.1	0.7	2.2	7
R227Q	0.5	4.6	0.06	260
F234L	0.3	1.6	1.4	200

Table 3.   Pharmacogenetic variation of steroid 5α-reductase polymorphisms. The differences in Km and Vmax with testosterone as substrate show that the different allelic variants of the enzyme have different metabolic activity and are differentially inhibited by finasteride. (Adapted from Makridakis 2000)

The best-characterized function of 5α-reductase is in the normal differentiation of the male reproductive tract. Initial male reproductive tract development requires production of testosterone by the fetal testis. The virilization of the external genitalia is dependent on conversion of testosterone to DHT in the tissues of the urogenital sinus and a deficiency in 5α-reductase 2 activity leads to an incomplete form of male pseudohermaphroditism (Imperato-McGinley 1974, Walsh 1974, Andersson 1991, Thigpen 1992). This genetic phenotype demonstrates the dependency of the prostate on DHT for its development and suggests a possible role of DHT, and therefore 5α-reductase, in diseases of aberrant prostate growth.

The 5α-reductase inhibitor finasteride is widely used in BPH and suppresses DHT by about 70 % in serum and by as much as 85-90 % in the prostate. The remaining DHT in the prostate is likely to be the result of 5α-reductase 1. Suppression of both 5α-reductase isoenzymes with GI198745 result in greater and more consistent suppression of serum DHT than that observed with a selective inhibitor of 5α-reductase 2 (Bartsch 2000).

A constant increase in adult males in the relative and absolute amounts of stromal component can be shown in spite of the variation in human BPH, i.e. stromal, fibromuscular, muscular fibroadenomatous, and fibromyoadenomatous nodules (Bartsch 1979a, Bartsch 1979b). These data strongly indicate that the enlargement of the gland is mostly due to a relative increase in the stromal tissue and not the glandular part. Stromal overgrowth can, therefore, be viewed as the central feature of human BPH; however glandular tissue plays a relatively large role in larger prostates (Price 1990). Finasteride is however affecting the epithelium. Long-term finasteride treatment (24 to 30 months) results in a marked involution of the prostate epithelium, which continues for many months after stabilization of clinical effects. The epithelium is similar in the peripheral and transitional zones for both morphometric and volumetric changes. Progressive involution of the prostate epithelium appears to constitute the underlying mechanism for sustained action of finasteride (Marks 1999).

DHT promotes mitotic activity in the epithelial cells of the prostate (Hashiba 2000, Coffey 1979) and mitotic activity seems to be an important influence in human cancer (Preston-Martin 1990). Therefore, it has been proposed that testosterone, via the conversion by 5α-reductase 2 to DHT, may be an important cause of prostate cancer (Ross 1992). Three studies have so far tried to elucidate whether polymorphisms in the 5α-reductase 2 gene is modulating the risk of prostate cancer. Makridakis et al (Makridakis 1999) found that the A49T polymorphism was a risk factor for prostate cancer if the subject carried the T allele. In 216 African-American, men, the age-adjusted odds ratio for prostate cancer was 3.28 (95 % C.I. 1.09-11.87). Subdivided into localized and advanced disease, the subjects with advanced disease (n=78) had an age-adjusted odds ratio of 7.2 (95 % C.I 2.17-27.91) whereas subjects with localized disease (n=138) had an age adjusted odds ratio of 1.47 (95 % C.I 0.33-6.83), compared to controls (n=261). They also studied Hispanic men with prostate cancer (n=172) compared to controls (n=200). There was a significant risk for advanced disease among AT/TT genotype carriers. Seven out of 71 subjects with advanced disease carried the risk genotypes, odds ratio 3.60 (1.09-12.27) compared to controls (7 out of 200). The overall risk, odds ratio 2.5 (95 % C.I 0.90-7.40), did however not reach statistical significance in Hispanics. The group of Febbo et al (Febbo 1999) investigated the V89L polymorphism in a nested case-control study within the Physicians Health study, which was a randomized, double blind, placebo-controlled trial of aspirin and beta-carotene in the prevention of heart disease and cancer. As many as 22 071 men were recruited to the original study, and 97 percent were Caucasian. Blood samples were obtained from 14961 men, excluding men not passing inclusion criteria's and those who failed to return a blood sample. Among those

14961 men, 592 men with prostate cancer were identified after a follow up period of 13 years. Seven hundred ninety nine controls matched for age and smoking status were randomly selected. In this study, no significant association between risk for prostate cancer and genotype were detected, odds ratio 0.96 (95 % C.I 0.76-1.20) for VL carriers and 0.84 (95 % C.I 0.57-1.24) for LL carriers, with the VV genotype as reference, compared to controls.

Finally, Lunn et al studied the V89L polymorphism in 108 prostate cancer cases (96 Caucasians and 12 Black) compared to 167 controls (159 Caucasians and 8 Blacks) derived from a urology clinic where they were evaluated and treated for voiding problems (Lunn 1999). Comparison of cases and controls for the L allele did not reveal any association with prostate cancer, Caucasians odds ratio 1.4 (95 % C.I. 0.8-2.4). Stratification by age or BPH classification did not alter the results. No further comparisons were made.

Jaffe et al studied the association of 5α-reductase 2 A49T and V89L polymorphisms in relation to pathological characteristics of prostate tumors (Jaffe 2000). Two hundred sixty five incident prostate cancer cases that underwent prostatectomy and passed the exclusion criteria's were examined. No association between the V89L genotype and stage-related variables were seen. Invasion beyond the prostate capsule occurred significantly more often in the combined group of AT and TT genotypes (χ^2=5.11, p=0.024), compared to AA carriers. They also found that only 46.7 % of men carrying the T allele had organ confined and margins negative disease compared to 73.3 % of AA carriers (χ^2=4.92, p=0.026). This study had the drawback of missing the possibility of evaluating these polymorphisms in relation to metastatic disease.

In summary, 5α-reductase is of great importance because the product DHT is important in the differentiation of the prostate during fetal development and mutations in 5α-reductase give rise to a rare form of pseudohermaphroditism. The 5α-reductase enzyme is also involved in BPH and possibly prostate cancer, but also in male pattern baldness, acne, and hirsutism. The 5α-reductase 2 inhibitor finasteride is being used clinically in the control of BPH. Finasteride has also been tried in prostate cancer, but is less effective (Ornstein, 1996; Presti, 1992). It reduces PSA levels by 50 % in combination therapy and PSA levels has been shown to remain low for longer when finasteride is added (Ekman, 1999a)

CYP1B1

The cytochrome P-450 enzymes are heme-containing enzymes (Casarett, 1996) and function as the terminal oxidase component of an electron transfer system present in the endoplasmatic reticulum. They are responsible for many drug oxidation reactions (Gibson, 1994) and play an important role in both activation of xenobiotics to toxic and/ or carcinogenic metabolites and inactivation of drugs and other xenobiotics (Casarett, 1996).

The cytochrome P450 superfamily is divided into families and subfamilies by their similarities. Sequences with at least 40 % homology are in the same family. Sequences with greater than 70 % homology are gathered in subfamilies (Gibson, 1994). The subfamilies are further subdivided into groups (Gibson, 1994; Nelson, 1996).

The P450 nomenclature system with CYP1B1 as example:

Superfamily	CYP
Family	CYP 1
Subfamily	CYP 1B
Group member	CYP 1B1

CYP1B1 is expressed in many extrahepatic organs such as the kidney, ovary, placenta, testis, endometrium, mammary tissue (Shimada, 1996; Sutter, 1994; Tang, 1996) and the prostate (Williams, 2000; Muskhelishvili, 2001). The enzyme is inducible by adrenocorticotropin, peptide hormones and planar aromatic hydrocarbons like TCDD (2,3,7, 8-tetrachlorodibenzo-p-dioxin) (Sutter, 1994; Tang, 1996). CYP1B1 catalyses testosterone 6β-hydroxylation (Shimada, 1999), which is an inactivation step (Sonderfan, 1989) that might regulate hormonal effect in the prostate. Inherited alterations in the CYP1B1 structure and activity may therefore be of importance in prostate carcinogenesis. The enzyme also metabolizes 17β –estradiol (E2) at position C-4 and to a lesser extent at position C-2 (Hayes, 1996). These hydroxyestrogen metabolites, catechol estrogens (CE), are less potent than E2 but their physiological role remains unclear (Hayes, 1996). The catechol estrogens can directly or indirectly damage DNA, proteins and lipids through generation of reactive free radicals (Hayes, 1996). CYP1B1 is also an activator of a number of environmental carcinogens (Shimada, 1996).

The CYP1B1 gene contains three exons, but it is only exon 2 and 3 that encode the CYP1B1-protein (Bailey, 1998). Exon 3 encodes the heme-binding domain. Because this domain is essential for the catalytic function, polymorphisms in this region may be of functional

importance. Early studies of the enzyme gene revealed that mutations leading to putative functional null alleles were linked to congenital buphthalmos (Stoilov, 1997). One of the polymorphic sites found in exon 3 is a guanine (G) to cytosine (C) transition in nucleotide 1640 which result in a substitution of valine (V) at location 432 to leucine (L) (Bailey, 1998, Stoilov, 1997). The allelic variants have different catalytic properties. When recombinantly expressed, the L variant has been shown to catalyse testosterone 6β-hydroxylation at a V_{max}/K_m ratio which is 3.3-6.5 fold higher than that of the V variant (Shimada, 1999). The frequency of the V allele is approximately 17 % in Chinese (Tang, 2000), 40 % among Caucasians and nearly 70 % among African- Americans (Bailey, 1998). This gradient mirrors the relative prostate cancer incidence among these three ethnic groups. The LL genotype has been associated with an increased breast cancer risk (Zheng, 2000). A small case control study of 50 Caucasian prostate cancer cases and 50 controls, frequency matched for age and residency, revealed an association between the V-allele and increased risk for prostate cancer (OR 3.3 95 % C.I. 1.9-9.0) (Tang, 2000).

RISK FACTORS FOR PROSTATE CANCER

The elucidation of the etiology of prostate cancer is difficult due to the high prevalence of subclinical latent cancer found in autopsy material. Latent cancer has a much lesser geographical variation that the incidence of clinically significant cancer. It is difficult to know whether factors in the environment make the cancer progress from latent to clinical forms or if latent and clinically significant cancers are different entities with different etiology (SOS Mars, 2000). Age, ethnic grouping, geographical residence and family history are confirmed risk factors for prostate cancer (SOS Mars, 2000). The search for risk factors is an increasing area of research and many other risk factors are hypothesized and even supported by data.

Age

While it is very unusual in men before age 50, rates increase exponentially thereafter. In 1997, no cases were detected in men younger than 40. Only 0.3 % of the detected cases were found in the age interval of 40-49 and 5.7 % in the interval of 50-59 years of age. Seventy point two percent were detected among men 70 years of age and older (Socialstyrelsen, 1999). In a study by Wingo et al. the probability of developing prostate cancer was less than 1 in 10,000 in men aged less than 39 years, 1 in 103 for men aged 40 to 59 years, and 1 in 8 for men 60 to 79 years (Wingo, 1995).

Ethnic grouping

The risk of prostate cancer is dramatically higher among African Americans, is of intermediate levels among whites, and is lowest among native Japanese. Survival is also related to ethnicity with 5-year survivals of whites with localized, regional, or metastatic prostate cancer being 94.7 %, 86.6 %, and 29.6 %, respectively, compared to rates of 87.8 %, 69.3 %, and 22.7 %, respectively, for blacks (Ries, 1994).

Geographical residence

Several migrant studies have found that prostate cancer rates shift toward those of the host country. For instance, the rates among Japanese-Americans were found to be intermediate between the very low rates of Japanese men in Japan and the high rates among white males in the US (Haenzel, 1968; Locke, 1980). Similarly, white males in the US were found to have considerably higher prostate cancer rates than Chinese men in China, with Chinese-Americans having intermediate rates (Yu, 1991; Stellman, 1994). This suggests that the underlying cause of disease is partly related to environmental factors.

Family history

In the United States, approximately 15 % of men with a diagnosis of prostate cancer have a first-degree male relative with prostate cancer, compared to approximately 8 % of the general population (Steinberg, 1990). While hereditary prostate cancer may account for a significant proportion of early onset prostate cancer, it is suggested that only about 9 % of prostate cancer in the general population is due to the effects of heredity (Carter, 1992). Segregation analyses has made it possible to separate prostate cancer clusters in families into familial, and into true hereditary prostate cancer (Bastacky, 1995; Carter, 1992). Hereditary prostate cancer has a dominant Mendelian inheritance with high penetrance. The criteria are: more than 3 affected family members, or occurrence in three generations, or that 2 family members have developed the disease before age 55 (Ekman, 1999b). Chromosome 1q24-25 has been identified to contain a gene, *HPC1*, involved in the development of hereditary prostate cancer (Smith, 1996).

In many case–control studies the odds ratio for prostate cancer has been higher for brothers than for fathers of men with prostate cancer (Bratt, 2000). X–linked or recessive inheritance of prostate cancer susceptibility in some families, might explain this. A prostate cancer susceptibility gene has been located to the long arm of the X chromosome (Xq27–28) (Xu, 1998). The gene, named *HPCX*, may account for circa 40 % of Finnish hereditary prostate cancer cases (Xu, 1998), but these findings needs to be confirmed (Bratt, 2000).

Although the large majority of prostate cancers, especially among the elderly, appear to result from environmental factors, genetic predisposition is likely to play a role in the etiology of many sporadic prostate cancer cases. In the Scandinavian countries, the contribution of hereditary factors to the causation of prostate cancer has been estimated to 42 percent (Lichtenstein, 2000).

Hormones

Indirect observations support the hypothesis that androgen levels within the normal range are risk factors for prostate cancer. For instance, subjects with inherited 5α-reductase deficiency neither develops BPH, nor prostate cancer when followed up long-term (NCI, 2001). In men castrated prior to puberty, neither BPH, nor prostate cancer has been reported (Isaacs, 1992). Androgen levels generally parallel prostate cancer risk in various populations of men. Studies have demonstrated that levels of testosterone and, especially DHT, are highest in black males, intermediate in white males, and lowest in native Japanese (Ellis, 1992; Ross, 1992). It is also noted that androgen deprivation therapy leads to involution of the prostate and apoptosis of epithelial cells and clinical response in prostate cancer (Peters, 1987; Kyprianou, 1989).

Furthermore, genetic variation in the androgenic pathway such as polymorphisms in androgen metabolizing enzymes, for instance 5α-reductase 2 (Makridakis, 1999) and CYP17 (Wadelius, 1999; Lunn, 1999; Habuchi, 2000) have been associated with risk for prostate cancer. The lengths of a polymorphic CAG repeat sequence in the androgen receptor gene is inversely correlated with transcriptional activity by the androgen receptor and risk for prostate cancer in some studies (Hsing, 2000; Ingles, 1997) but not all.

Other risk factors

Many other risk factors for prostate cancer are under investigation and many of them have limited support in occasional studies. Examples of risk factors that has been scrutinized are: dietary fat, phytoestrogens, occupation, cadmium exposure, vasectomy, vitamin A, vitamin D, vitamin E, zinc, selenium, lycopene, smoking, energy intake, sexual activity, calcium intake, IGF-1 and physical activity.

DIAGNOSIS, GRADING AND STAGING

Prostate cancer is often symptomless in its initial stages. When symptoms do develop because of significant localized disease, they are frequently indistinguishable from those caused by BPH. Metastatic disease may cause pain, especially bone pain.

Prostatic intraepithelial neoplasia (PIN) is an atypical proliferative disorder of the prostate gland (Haggman, 1997) that can be either high- or low-grade. High-grade PIN, which may be detected on needle biopsy, has been identified as a main precursor to prostate cancer (Haggman, 1997; Bostwick, 1995). There have been suggestions that PIN precedes carcinoma by several years (Kovi, 1988) but its natural history is unknown (Berner, 1996).

Even once carcinoma develops, not all histological prostate cancers become clinically significant during the life of a patient. Prostate cancer is found incidentally in at least 10 % of men undergoing prostatectomy for BPH and in more than 40 % undergoing cystoprostatectomy for bladder cancer (Klotz, 1992). A summary of autopsy series shows that the prevalence of latent histological prostate cancer is approximately 30 % in men over the age of 50 who had no clinical problems during life (Klotz, 1992). Nevertheless, many patients suffer and die from prostate cancer as described in the introduction.

General screening of prostate cancer with PSA is a controversial issue and there is at the moment no documentation in randomized studies of the effects of general screening (SOS Mars, 2000). Men at risk for inherited forms of prostate cancer might however benefit from screening.

The diagnostic tools are digital rectal examination (DRE), transrectal ultrasound (TRUS) and PSA, all which have to be accompanied by a histo-pathological examination of a biopsy from the tumor. A biopsy may be obtained by transrectal ultrasound guided core needle biopsy or by fine needle biopsy. It can also be obtained after transurethral resection of the prostate or after surgery. The extension of the disease may be further examined via bone scintigraphy and in some cases magnetic resonance imaging technique (MRI).

If curative treatment is considered, the grade of differentiation according to the Gleason (Gleason, 1977) and WHO (Mostofi, 1981) index and a clinical staging according to the TNM system (AJCC, 1997) should be obtained as a foundation for decisions about treatment (SOS Mars, 2000). The extent of disease correlates directly with prognosis in men with newly diagnosed prostate cancer.

The TNM staging system is constructed as follows:

Primary tumor (T)

TX: Primary tumor cannot be assessed

T0:   No evidence of primary tumor

T1:   Clinically unapparent tumor not palpable nor visible by imaging

T1a: Tumor incidental histological finding in 5 % or less of tissue resected

T1b: Tumor incidental histologic finding in more than 5 % of tissue resected

T1c: Tumor identified by needle biopsy (e.g., because of elevated PSA)

T2:   Tumor confined within prostate

T2a: Tumor involves 1 lobe

T2b: Tumor involves both lobes

T3:   Tumor extends through the prostatic capsule

T3a: Extracapsular extension (unilateral or bilateral)

T3b: Tumor invades seminal vesicle(s)

T4:   Tumor is fixed or invades adjacent structures other than seminal vesicles:
             bladder neck, external sphincter, rectum, levator muscles and/or pelvic wall

Regional lymph nodes (N)

NX: Regional lymph nodes cannot be assessed

N0:   No regional lymph node metastasis

N1:   Metastasis in regional lymph node or nodes

Distant metastasis (M)

MX: Distant metastasis cannot be assessed

M0:   No distant metastasis

M1:   Distant metastasis

M1a: Nonregional lymph node(s)

M1b: Bone(s)

M1c: Other site(s)

The Gleason grading system is the most commonly used. After microscopic examination of two of the most representative areas of the tumor, the pathologist assigns each a pattern between 1 and 5. The sum of the two patterns, a number between 2 and 10, becomes the total Gleason score.

Gleason pattern:

Pattern 1 single, separate, closely packed acini

Pattern 2 single acini, more loosely arranged, less uniform

Pattern 3 single acini of variable size, cribriform and papillary patterns

Pattern 4 irregular masses of acini and fused epithelium, can show clear cells

Pattern 5 anaplastic carcinoma

Gleason score:

2-4	Well differentiated
5-6	Moderately differentiated
7	Moderately poorly differentiated
8-10	Poorly differentiated

The WHO (Mostofi, 1981) grading system is a three-grade system corresponding to tumors that are well, moderately, and poorly differentiated.

Besides stage and the histological grade of the tumor, the prognosis of prostate cancer patients is also affected by the patient's age, existing co-morbid conditions, and tumor volume (Gittes, 1991; Chodak, 1994). The degree of tumor differentiation expressed as Gleason grade correlates with likelihood of metastatic spread present at diagnosis as well as with patient survival (Gleason, 1974). In general, the more poorly differentiated the tumor, the poorer the prognosis.

TREATMENT OPTION OVERVIEW

Today's best treatment in prostate cancer provides prolonged disease-free survival for many patients with localized disease, but is rarely curative in patients with locally extensive tumor (T3 and T4). It is not determined whether watchful waiting or early intervention is the best choice for localized disease. The prostate cancer intervention-versus-observation trial (Norlen, 1994) may answer this question. Prostatectomy and external beam therapy are curatively intending treatments with similar efficacy (D`Amico, 1997).There are no comparative studies on Brachytherapy (internal radiation plus external beam radiation) (SOS Mars, 2000). Even when the cancer appears clinically localized to the prostate gland, a substantial fraction of patients will develop disseminated tumor after local therapy with surgery or irradiation. This is due to the high incidence of clinical understaging even with current diagnostic techniques. Metastatic prostate cancer is currently not curable.

Prostatectomy is usually reserved for patients in good health, under the age of 70 that have a negative bone scan and tumors confined to the prostate gland and who elect surgical intervention (Catalona, 1990: Corral, 1994: Zincke, 1994). Impotence is a common adverse effect (40-80 %) (Wasson, 1993; Fowler, 1993). Preoperative hormonal therapy is not established at the present time (Witjes, 1997; Fair, 1997).

Hormonal treatment is the cornerstone of therapy for distant metastatic prostate cancer (M1). The principal goal is to prevent the activation of androgen regulated genes (Rane 1998). Cure is rarely, if ever, possible, but responses to treatment occur in the majority of patients. Immediate hormonal treatment with orchidectomy or LHRH analogue may have advantages over deferred treatment, i.e. watchful waiting with hormonal therapy at progression (MRCT, 1997).

Orchidectomy and estrogens yield similar results, and the therapeutic choice depends on patient preference and the morbidity of expected side effects. There is no indication that combined orchidectomy and estrogens are superior to either treatment administered alone (Byar, 1973).

Based on the fact that the adrenal glands continue to produce androgens after surgical or medical castration (Labrie, 1987), studies were performed in which antiandrogen therapy was added to castration. Promising results from clinical trials led to widespread use of the strategy "maximal androgen blockage" (MAB) but superiority over monotherapy has not been proven.

After tumor progression (despite hormonal manipulation) has developed, palliative chemotherapy may be considered. However, to date, no evidence exists that indicates a prolonged survival with chemotherapy (Eisenberger, 1988). Low-dose prednisone may palliate symptoms in about a third of the cases (Tannock, 1989).

Hormonal treatment options:

     a) Orchidectomy.
     b) LHRH agonists
     c) MAB
     d) Estrogens

Orchidectomy

Although orchidectomy as a procedure may be unacceptable to some patients, the advantages are obvious: Huggins and co-workers demonstrated already in 1941 that 15 of 21 patients (71 %) treated with bilateral orchidectomy had either subjective or objective improvement of pain or neurologic symptoms originating from metastatic prostate cancer (Huggins et al, 1941). Very frequent side effects of orchidectomy are almost immediate loss of libido and potency. Those are inherent to any other treatment that eliminates the source or the effect of testicular androgens. However, some patients remain potent under endocrine treatment. Available information clearly states that bilateral orchidectomy produces results that are comparable to pharmacological means of castration (Campbell 1998).

LHRH analogues

Gonadotropin-releasing hormone, which is active in stimulating the pituitary release of FSH and LH, was isolated and structurally described in 1971 (Schally 1971, Matsuo 1971). Whereas endogenous LHRH stimulates LH release and supports its circadian rhythm, synthetic LHRH agonists were shown to lead to suppression of LH and of testosterone production to castrate levels, after an initial phase of stimulation (Auclair et al., 1977). After development of depot formulations, the chemical castration from LHRH agonists is reliable (Debruyne, 1996). LHRH agonists such as leuprolide, goserelin, and buserelin may, similar to orchidectomy and estrogens, cause impotence, hot flushes, sweating and loss of libido. After long use, osteoporosis (Daniell 2000) and decreased mucle mass may develop. During the first 2 to 3 weeks of treatment with LHRH agonists, an initial stimulation of LH and

testosterone secretion occurs. This flare is sometimes followed by clinical symptoms such as increase of bone pain. Acute detrimental progression and even death has also been described (Campbell, 1998). Clinical flare can be prevented by the use of a steroidal or non-steroidal antiandrogen either 1 week before the initiation of the LHRH analogue treatment (Boccon-Gibod, 1986) or simultaneously with the initiation of LHRH treatment (Schulze, 1990). In the first case, if a steroidal antiandrogen is used, the rise in plasma testosterone above pre-treatment levels as well as the biochemical flare phenomenon can be prevented. In the second case, the peak in plasma testosterone is still seen; however, biochemical flare is inhibited by the action of the antiandrogen (Klijn,1985). Antiandrogens are particularly important to use before or at the time of initiation of LHRH analogue treatment in patients with a large metastatic tumor mass. Treatment with LHRH agonists is an expensive alternative to castration (Varenhorst, 1994), but clinically equally effective (Kaisary, 1991).

Antiandrogens

All known antiandrogens interfere with androgen action by binding to the androgen receptor in a competitive fashion. They can be either steroidal or non-steroidal. The non-steroidal group includes for example flutamide (Johansson, 1987), nilutamide (Decensi, 1991) and bicalutamide (Kennealy, 1991). An example of a steroidal antiandrogen is cyproterone acetate (Schulze, 1990). In contrast to the nonsteroidal antiandrogens, the steroidal antiandrogens also block androgen receptors in the area in the brain, the diencephalon, where LHRH production and the feedback mechanism regulating plasma testosterone levels through LH are located. Steroidal antiandrogens thus increase LH, but the effect of the subsequent rise in testosterone is inhibited by the antigonadotropic effect that is associated with the use of these drugs.

Theoretically, antiandrogens may be useful to inhibit androgenic activity from the adrenals, which is still evident after surgical or chemical castration. A clinical feature of nonsteroidal antiandrogen monotherapy is the maintenance of libido and potency. The normal or elevated plasma testosterone levels during therapy might explain this phenomenon. There is a dissociation of the regulation of libido and potency, which is mainly governed by testosterone, with the regulation of growth and function of the prostate, mainly mediated by DHT. It is, however, poorly understood why the nonsteroidal antiandrogen would not inhibit testosterone action at those cerebral androgen receptors regulating libido (Campbell 1998).

The therapeutic effect of antiandrogen treatment seems equal to castration in non-metastasizing disease (Iversen 1998), and equal (Boccon-Gibod 1997) or inferior (Tyrell 1998) in metastasizing disease.

Flutamide, nilutamide and bicalutamide often cause nausea, vomiting, diarrhea and gynecomastia. (FASS 2000) They may all impair liver function and in some cases fatal liver toxic effects have been reported (Wysowski 1993). Nilutamide may also induce impaired speed of dark/light adaptation and interstitial pneumonia. Cyproterone acetate case may exert dose related liver toxicity with potentially fatal outcome. (FASS 2000)

Maximal Androgen Blockade

Maximal Androgen Blockade (MAB) is the inhibition of both testicular and adrenal androgens as endocrine treatment of prostate cancer. (Labrie et al, 1983, 1988). Since surgical hypophysectomy and adrenalectomy in prostate cancer are historical, pharmacological interventions are used. The most common combinations are an antiandrogen plus either castration, an LHRH agonist, or estrogen treatment (Campbell 1998).

The largest positive study that has led to the widespread use of maximal androgen blockade as endocrine treatment showed a significant difference in time to progression and overall survival (Crawford et al, 1989). However, the study has been criticized because the possibility of confounding from treatment induced flare in the control arm (leuprolide unprotected) (Campbell 1998)

A large overview analysis of data from 8275 patients in 27 randomized studies that included MAB showed a statistically nonsignificant increase of 2-3 percent in survival. (Prostate Cancer Trialists Group 2000). This shows that currently available randomized evidence does not demonstrate that maximal androgen blockade results in longer survival than conventional treatment does.

Thirty to 60 percent of patients who progress on maximal androgen blockade with the use of antiandrogens show a remission, biochemically or clinically, after discontinuation of therapy (Scher 1993, Small 1994, Dawson 1995). This is referred to as the antiandrogen withdrawal syndrome. The remission may last more than 1 year. The mechanism is not fully understood but there is a possibility of clonal proliferation of tumor cells with a mutated androgen receptor that by substrate promiscuity is triggered by the antiandrogen (Veldscholte et al, 1992). If progression under maximal androgen blockade occurs, the antiandrogen should be discontinued (Campbell, 1998).

Estrogen treatment

The estrogen receptor alpha is present at low concentrations in the prostatic stroma and there is no clear evidence of a direct effect on prostatic cancer cells by estrogens at physiologic concentrations. Estrogens are effective in humans through negative feedback control at the level of hypothalamus and in this way decreasing LHRH and LH production. For example, a dose of 3 milligrams per day of diethylstilbestrol will achieve castrate levels of testosterone. (Wilson 1998). Treatment with estrogens has been associated with longer time to progression and lower mortality of prostate cancer (Sarosdy, 1990; Johansson, 1991) but also increases morbidity and mortality in cardiovascular diseases. In studies of more than 4 000 patients, a 36 % increase of non-cancer-related mortality in the estrogen groups, as compared to the non-estrogen-treated groups was detected (Mellinger, 1967). The majority of this excessive mortality was cardiovascular, and it over-compensated for the small benefit in terms of prostate cancer mortality (Mellinger, 1967). The presence of previous cardiovascular disease is a risk factor for the development of additional cardiovascular toxicity and death. (de Voogt et al, 1986). It is believed that the cardiovascular side effects seen with the oral application of diethylstilbestrol can be avoided by eliminating the first pass effect through the liver (de Lignieres, 1993).

Other side effects of estrogen treatment are loss of libido and impotence. Gynecomastia is also very common (40 %) but may be prevented by low-dose radiation to the breasts. (Campbell 1998)

Estramustin phosphate

Estramustine is a conjugate of nitrogen mustard (mechlorethamine) and estradiol, originally synthesized as an attempt at targeted drug therapy. Estramustine interferes with mitosis by binding to microtubule-binding proteins (Hudes 1997), which may give synergy when estramustine is combined with other drugs that target microtubule action. Among the side effects, myocardial infarction, cardiac failure and pulmonary embolism are described. In a large Scandinavian study (SPCG-1) the survival in the estramustin group was equal to survival of patients treated with oral estrogen. Nowadays, estramustin phosphate is rarely used as primary treatment in Sweden (SOS Mars, 2000).

Chemotherapy

The proliferation rate of prostate cancer is directly proportional to its growth fraction (Berges et al, 1995). Adenocarcinoma of the prostate has a relatively low growth fraction, compared

with other common tumor types such as adenocarcinoma of the breast. This is most probably an important factor to explain the differences in response rates observed with chemotherapy between these two hormone dependent tumor types. This aspect is particularly important because most chemotherapeutic agents available are more effective in tumors with a high proliferative rate, such as lymphomas, small cell lung carcinomas, and germ cell tumors of the testis (Campbell, 1998).

No single agent or combination therapy has proven to prolong survival in patients with metastatic hormone refractory prostate cancer (Petrylak, 1999) and the true role of chemotherapy for patients with advanced hormone refractory prostate cancer has been a controversial issue for several years (Tannock, 1985; Eisenberger, 1985; Raghavan, 1988). Furthermore, measuring the benefit of therapy for prostate carcinoma patients has been problematic. Between 80 % and 90 % of hormone-refractory prostate carcinoma patients do not have measurable disease, making assessment of efficacy troublesome in clinical trials (Figg, 1996). Osteoblastic bone metastases remain difficult to quantitate accurately, particularly as markers of response to treatment (Sabbattini, 1999). In recent times, the availability of surrogate markers of response such as PSA and quality of life measurements has facilitated the assessment of efficacy of chemotherapy for prostate cancer (Beer 2000). However, the interpretation of therapeutic results on the basis on a decline in PSA levels needs to be undertaken with caution, because various drugs have been shown to reduce PSA secretion without changing tumor growth (Larocca et al, 1991; Steiner et al, 1995). Nevertheless, the availability of PSA monitoring after definitive primary therapy and hormonal manipulation has led to the identification of relapsing patients earlier, resulting in the inclusion of patients with less tumor volume in trials of chemotherapy.

Mitoxantrone, the only agent specifically approved by the Food and Drug Administration for use in hormone-refractory prostate cancer, has been shown in phase II and III trials to improve quality of life in prostate cancer. Two randomized trials have shown that mitoxantrone with low-dose steroids relieves cancer-related symptoms (Tannock, 1996; Kantoff 1999).

Systemic chemotherapy is being investigated at earlier stages of prostate carcinoma at high risk for progression (Oh 2000). At the moment, WHO has classified chemotherapy in prostate cancer as having palliative effect only in metastatic disease (Sikora 1999).

THE PRESENT STUDY

Aims of the study

Understanding the etiology of a disease is often essential to designing effective drug treatments. Therefore, this study aimed at characterizing the therapeutic target enzymes steroid 5alpha reductase 1 and 2 and the enzyme CYP1B1 in relation to prostate cancer, in order to gain a better view of its tumor biology.

The specific aims of the the thesis were:

I. To quantitate the level and variation of the 5α-reductase 2-specific messenger RNA (mRNA) in groups of patients with clinical symptoms of prostate cancer and BPH and to compare the gene expression of this enzyme between cancerous tissue and normal tissue.

II. To determine whether 5α-reductase 2 specific mRNA expression, as assessed by reverse transcription-polymerase chain reaction (RT-PCR), predicts 5α-reductase activity in prostatic tissue.

III. To study the 5α-reductase 2 V89L and A49T polymorphisms in relation to risk of prostate cancer and its disease parameters such as TNM classification, differentiation, heredity and age in a Swedish Caucasian population.

IV. To study the *CYP1B1* V432L polymorphism in relation to risk of prostate cancer, and in relation to disease parameters such as TNM classification, differentiation, heredity and age in a Swedish Caucasian population.

MATERIAL

Patients and tissue samples

I.   Fifty biopsies were obtained from 31 patients (median age 72.2, range 57–88 yr). The patients were referred to the outpatient clinic because of lower urinary tract symptoms and/or elevated serum PSA. Patients consenting to participate underwent transrectal ultrasound (TRUS) and multiple core biopsies (needle diameter 1.2 mm, notch length 15 mm, weight of biopsy 5–10 mg) from the prostate. One or two biopsies were used for determination of 5α-reductase 2 mRNA level. The cancerous biopsies contained 20–100 % (median 94 %) cancerous tissue as determined histopathologically in a parallel biopsy, used for histopathological diagnosis. Three biopsies for which the histopathology was unclear or missing were excluded. Four biopsies containing prostatic intraepithelial neoplasia and 2 containing prostatitis were also excluded from the statistical analysis. Included were 41 biopsies: 27 considered to be noncancer and 14 showing cancer.

II.   Thirty prostatic tissue specimens were obtained at surgery from 15 Caucasian patients age 47-82 years, median 66. Five patients were enucleated due to BPH and 10 underwent total prostatectomy due to bladder or prostate cancer. Five specimens from different patients consisted of cancer, Gleason grade 6 to 7, median 6, while the remaining 25 were derived from BPH. Four lymph node biopsies from 3 patients with prostate cancer were obtained. These nodes did not contain any cancer metastases.

III+IV.   Three hundred thirty seven Caucasians living in the county of Örebro in Sweden agreed to participate in these case/control studies. The cases were 176 patients, age 51 to 79, with clinical and histological prostate cancer, who had been referred to either of three hospitals in the county. They were recruited consecutively between May 1994 and February 1996. Eighty-one percent of those asked agreed to participate. The control subjects were randomly selected every three months from the county population register and frequency matched for age (age intervals 50-59, 60-69, and 70-79 years). They were contacted by mail, and 161 individuals agreed to participate, giving a response rate of 79 %. A blood sample for DNA analysis

was collected. Furthermore, information about heredity for prostate and breast cancer was collected for both cases and controls. There was no screening for prostate cancer among the controls. For the cases, TNM classification, tumor grade according to WHO criteria, PSA levels and medication were recorded. Bone scintigraphy was utilized in the evaluation of bone metastases.

METHODS

Solution hybridization assay for quantitation of 5α-reductase 2 specific mRNA (I)

Figure 5 shows a simplified step-by-step explanation of the solution hybridization assay. The numbers in the figure is corresponding to the steps described here:

Total RNA (2) was extracted from the biopsy (1) using a total RNA kit. A 200 base-pair fragment (3) of the 5α-reductase 2 gene was provided with restriction enzyme cleaving sites in the ends during PCR amplification and subcloned into a transcription vector plasmid (4). The transcription vector was then transfected into *E. coli* cells and amplified by the transfected clones (5). Plasmids were purified from positive clones isolated on selective agar plates using a plasmid kit (6). These plasmids now contained a 200 bp artificial fragmentary 5α-reductase 2 gene with different RNA polymerase promoters (T7 and T3) in each end, making it possible to express the gene fragment from each direction. This made it possible to produce complementary strands. One of the strands was synthesized using radioactive uridine triphosphate, to make up for the probe (7). Using the opposite promoter (T7), the resulting synthetic complementary 5α-reductase 2 specific cRNA was quantitated and used for construction of standard curves. The unknown amount of 5α-reductase 2 specific mRNA from the sample and a series of cRNA standards were then mixed with the known amount of radiolabelled probe for hybridization (8). The hybridized samples were thereafter treated with RNAse that cleaved non-hybridized single stranded RNA and probe. Radioactive RNA-RNA hybrids were protected from RNAse digestion. They were precipitated, collected on a filter and quantitated by scintillation counting (9). The levels of 5α-reductase 2 specific mRNA in the prostate samples were calculated from the standard curve (10). Data represented duplicate samples in each experiment. The limit of detection was 200–500 pg cRNA in the standard curves. The variation between duplicates was less than ± 5 %.
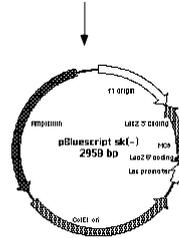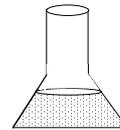
Figure 5:

1. Prostate biopsy
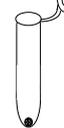
2. Total RNA preparation
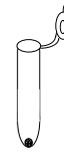
3. 5α-red 2 gene fragment

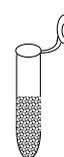4. Subcloning in plasmids

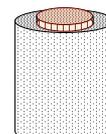5. Amplification in E coli

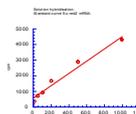6. Extraction of DNA

7. Synthesis of probe ^{35}S-MTP

8. Hybridisation totRNA+probe

9. Counting in β scintillator

10. Calculation and interpretation

Semi quantitative reverse transcription (RT) polymerase chain reaction (PCR) assay for determination of 5α-reductase 1 and 2 specific mRNAs (II)

The fundamental dogma of molecular biology is that DNA produces RNA, which in turn produces protein. Thus the genetic information in the DNA specifying particular functions is converted into an RNA copy. The messenger RNA (mRNA) is single stranded and the nucleotide thymine is replaced by uracil, compared to DNA. mRNA is subsequently translated into protein. The action of the protein then produces the phenotype. In study II, we tried to determine if there was a direct association between the mRNA expression of 5α-reductase genes, and the phenotype assessed as the activity of the corresponding protein.

Total RNA (totRNA) was extracted from frozen pulverized prostatic tissue using a guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski 1987). After isolation of RNA, mRNA was reverse transcribed using a first strand cDNA synthesis kit. After reverse transcription, the samples were analyzed for both 5α-reductase and β-actin in simultaneous parallel PCR reactions. Control samples containing water instead of totRNA were included in each run to detect contamination. The primer pairs were chosen in different exons in order to detect DNA contamination. The PCR temperature cycle was as follows: 95°C for 4 min followed by 35 cycles at 95°C 30 s, 55°C 1 min. and 72° 1 min. and finally a 5 min. extension step after the final cycle. Samples were analyzed for both 5a-reductase and β-actin in simultaneous parallel PCR reactions. Control samples containing water instead of totRNA were included in each run. The linearity of the PCR amplifications was determined by varying the number of cycles and the amount of cDNA in the reaction. The PCR products were analyzed on a 2 % agarose gel and visualized by ethidium bromide. The different primer pairs used yielded a 258 base pair (bp) fragment corresponding to 5α-reductase 2, a 316 bp fragment corresponding to 5α-reductase 1 and a 240 bp fragment corresponding to β-actin. The gels were photographed with a computerized video gel documentation system. The intensity of the bands was measured with image analysis software. The semi-quantitative determination of the 5α-reductase 1 and 2 mRNA levels was made on the basis of the corresponding β-actin mRNA level and was presented as the ratio between the intensity of the respective bands. The β-actin gene was transcribed at a fairly constant level in prostate cells, which was expected due to β-actins vital function as a cytoskeleton.

5α-reductase assay in vitro (II)

Prostate tissues were pulverized in liquid nitrogen using mortar and pestle. The pulverized tissue was thereafter homogenized (Ellsworth 1995) for the metabolic assays. Protein concentration was measured according to Lowry et al (Lowry 1951). The assays were based on a method published by Ellsworth and Harris (Ellsworth 1995).

The 5α-reductase activity at pH 5.5 (5α-reductase 2) was assayed in a mixture of 33 mM succinate, 44 mM imidazol, 33 mM diethylamine, 40 mM potassium phosphate pH 5.5, 0.2 nM ^{14}C- testosterone, 1 mM DTT, and 500 μM NADPH in a final volume of 100 μl. The reaction was started by addition of the enzyme preparation at a final protein concentration of 0.5 mg/ml, and then incubated at 37°C for 10 min.

The 5α-reductase activities at pH 7.0 (5α-reductase 1) was analyzed as described above, except for the use of 40 mM potassium phosphate pH 7.0, 1 nM ^{14}C- testosterone and an incubation time of 5 min. The reactions were stopped by extraction with 300 μl of a mixture of cyclohexane: ethyl acetate (70:30 v/v) containing 12 μg each of DHT and testosterone. Testosterone was separated from DHT using thin layer chromatography (Blanck 1984).

The formation of DHT was analyzed using a Phosphor Imager® (Molecular Dynamics) that analyzed a scan of the radiation emitted by the radioactive testosterone and the formed metabolite DHT. The assay was validated in terms of linearity with protein concentration, incubation time, and substrate concentration.

Taqman® allelic discrimination assay (III + IV)

In papers III and IV, three Taqman® allelic discrimination assays were developed and used. The Taqman® methods were based on a PCR- technique with fluorescence detection, first described by Holland et al., 1991 (Holland, 1991) and reviewed by Livak et al. (Livak, 1999). The system consisted basically of a primer pair, two dye-labeled probes, a PCR and a laser detection device. The probes consisted of an allele specific oligonucleotide sequence with a reporter dye (R) FAM (6-carboxy-fluorescein) or TET (6-carboxy- 4, 7, 2´, 7´-tetrachlorofluorescein) attached in the 5´-end as the detection marker. A quencher (Q), TAMRA (6-carboxy-tetramethyl-rhodamine) was attached to the 3´-end.
Theoretically, the probe annealed only to its complementary allele specific sequence. Therefore, as in theses cases with biallelic single nucleotide polymorphisms, the TET probe bound to one allele and the FAM probe to the other. If the DNA was derived from a

heterozygous subject, both probes could anneal. When the probe was intact, bound or unbound, the reporter fluorescence was suppressed due to proximity of the quencher dye. This phenomenon is called Förster- type energy (electron) transfer. If the probe had annealed to it's complementary DNA strand, it was hydrolyzed by the Taq-polymerase (AmpliTaq Gold) during the extension phase. This was due to the $5´\rightarrow3´$ exonuclease activity of the polymerase. This hydrolysis of the probe separated the quencher from the reporter dye and the subsequent rise in fluorescence, due to interrupted electron transfer between the dye and the quencher, was detected.
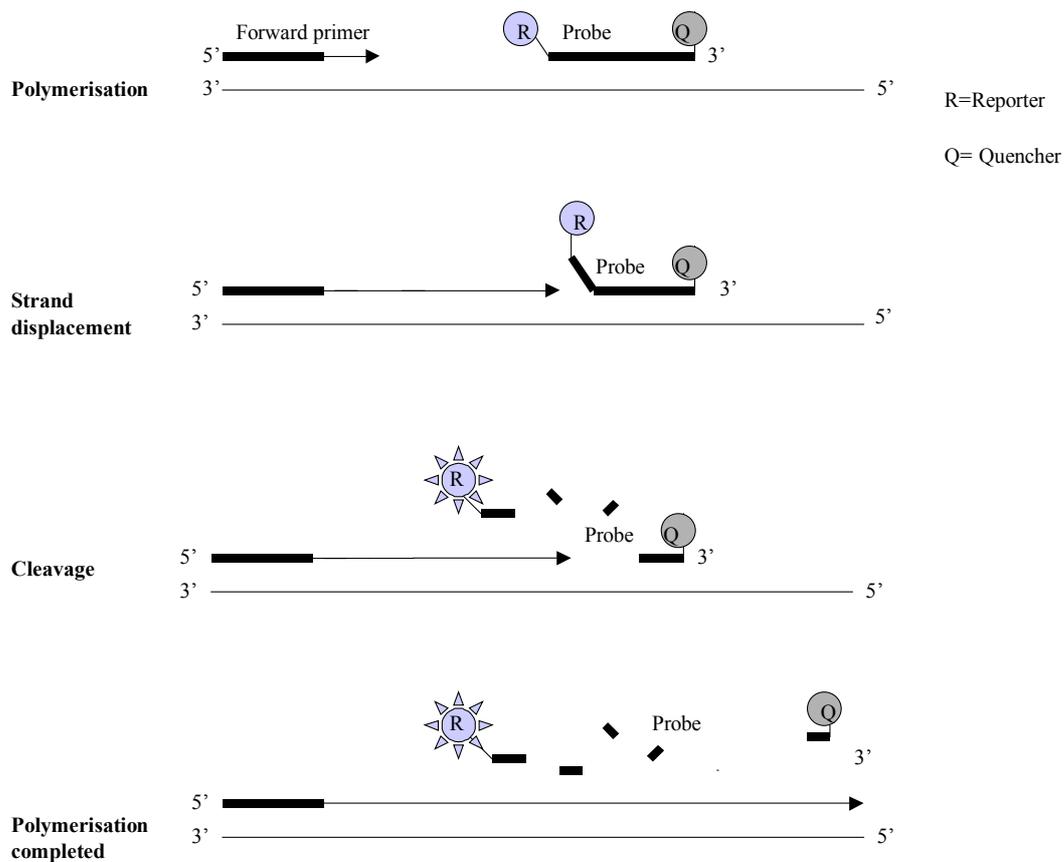
Figure 6.   A stepwise representation of 5'-3' nuclease activity of AmpliTaq Gold™ during each extension phase of PCR. The reporter dye begins to emit light when the probe is cleaved, thus separating the reporter dye from the quencher.

Both primers and probes must hybridize in order to generate a signal, which made this assay reliable and specific. In the ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied biosystems), a multiplexer directed an argon ion laser beam through optic cables that

terminated above each position on the 96-well plate. The light from the laser excited the fluorescent dyes and the emission was collected via the optic cables. A spectrograph measured the intensity of the light in the spectrum between 520 nm and 660 nm using a charge- coupled device camera. This was made every cycle to monitor the PCR reaction.
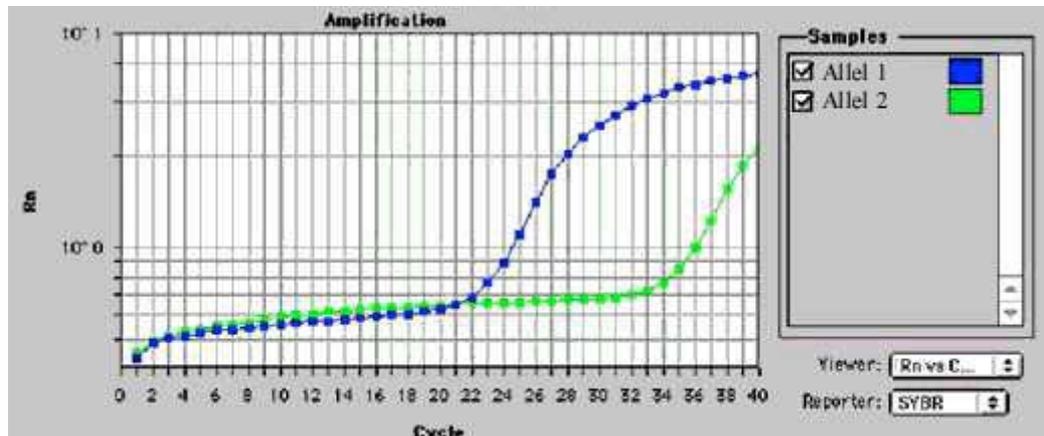

Figure 7. The degradation of the probes and the subsequent raise in fluorescence can be plotted in each cycle. There was a substantial increase in the development of fluorescence during the PCR from the probe that perfectly match the allele (left curve). The mismatching probe never reached the same level of fluorescence. In heterozygous subjects, the rises in fluorescence were similar from both probes.

The primers and probes were partly designed using the Perkin Elmer Primer Express software. The primers were designed to anneal in close proximity to the probes in order to produce short amplicons. The thermal cycling started with 2 minutes at 50 °C, which activated the AmpErase UNG enzyme that acted by hydrolysing uracil-glycosidic bonds at dU-containing DNA-sites. This was done in order to eliminate contamination from PCR products. The template was not affected since it did not contain any dUTP (Longo, 1990). Next step included 95 °C for 10 min, and activated AmpliTaq Gold DNA polymerase. This step also inactivated the AmpErase UNG enzyme. The ensuing 40 cycles started with a 95 °C melting step for 15 seconds, followed by a 1 minute annealing/ extension step at 62.5 °C for 5α-reductase 2 V89L and A49T and 63 °C for CYP 1B1 V432L.
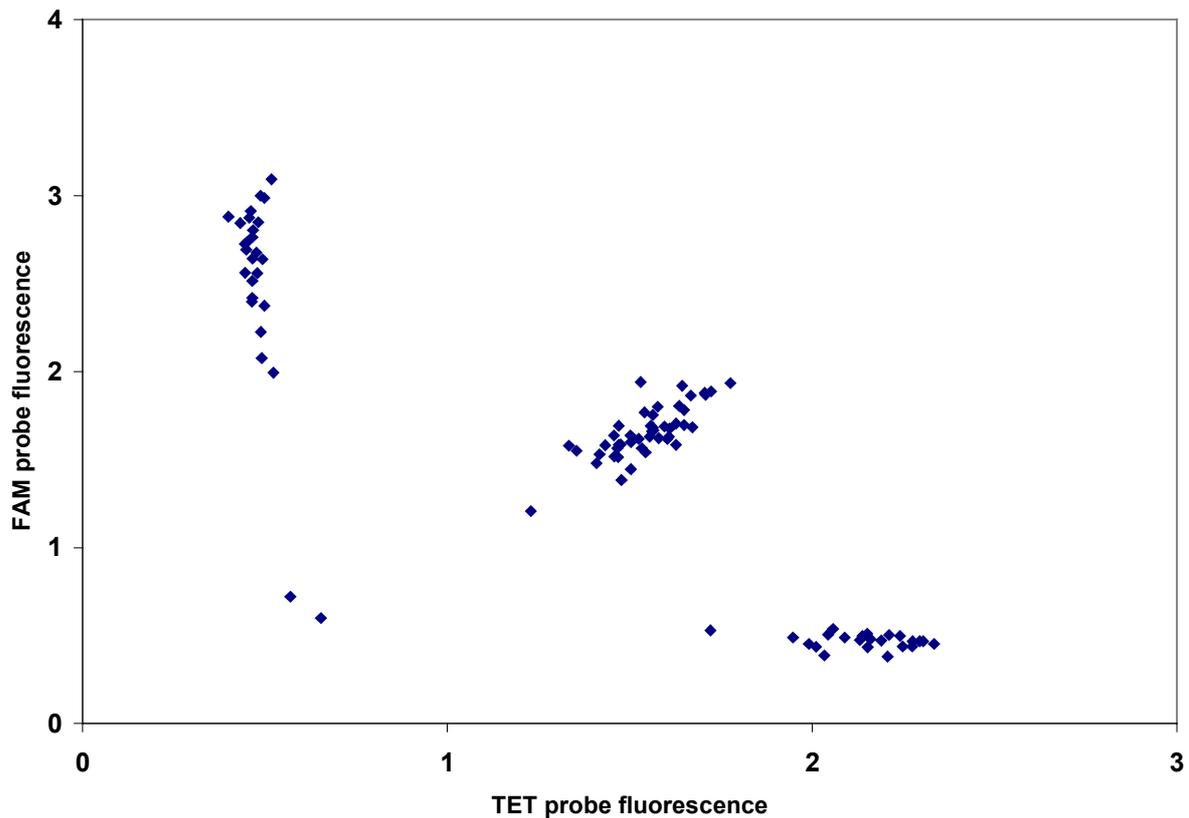
Figure 8.   After completed PCR, the adjusted fluorescence of the probes after the final cycle was plotted in a coordinate system, which enabled allelic discrimination. Allelic discrimination of 94 subjects using the fluorogenic 5' nuclease PCR assay. The cluster in the right lower corner indicates the individuals homozygous for the CYP 1B1 V432L V-allele. The cluster in the left upper corner indicates the individuals homozygous for the L-allele. The cluster in between indicates heterozygous individuals and the two points in the left lower corner are non-template controls.

Optimization of the assays

For economical reasons, the primer concentrations were optimized to determine the minimum primer concentrations giving the maximum efficacy in the PCR. The optimizations were performed using a homozygote template together with the corresponding allele specific probe at 100 nM concentration and primer concentrations ranging from 50 to 900 nM. Every primer concentration was evaluated in triplicate samples. Using the allelic discrimination plot, it was decided that 300 nM forward / 300 nM reverse primer concentration was optimal for all assays used.

The probe concentrations were optimized in order to achieve optimal allelic discrimination. Two approaches were used. For the CYP1B1 assay, the method described in the ABI Prism

7700 Sequence Detection System manual was used successfully. In brief, an array of probe concentrations ranging between 50-200 nM was prepared in standard PCR conditions and the fluorescence from the probes was measured without performing PCR. The concentrations giving the most similar fluorescence intensity were chosen. However, this method was not reliable in achieving optimal allelic discrimination in the 5α-reductase 2 assays. Instead, the probe concentrations giving the best allelic discrimination after PCR were chosen.

Different annealing temperatures were tested during the optimization of the allelic discrimination. A high temperature gave a specific amplification but a decreased yield. A low temperature gave the opposite; a high yield but decreased specificity. A temperature of 63 °C gave the best combination of specificity and sensitivity in the CYP1B1 assay and 62.5°C was optimal in the 5α-reductase 2 V89L and A49T assays.

RESULTS

5α-reductase 2-specific mRNA levels in cancerous and noncancerous prostate tissue (I).

Core needle biopsies from 14 patients clinically diagnosed with BPH and 17 patients with CAP were included in the study. The clinical features of the patients are summarized in table 4.

Clinical diagnosis	n	Age		Prostate volume (ml)		PSA (mg/L)	
		Median	Range	Median	Range	Median	Range
Cancer	17	75.0	57-88	50[a]	30-145	15.0	2.4-1174
BPH	14	73.5	58-87	47[b]	36-200	11.5	4.9-57

[a] Eight values missing. [b] Two values missing.

Table 4. A summary of the clinical features of the participating patients in study I.

The 5α-reductase 2-specific mRNA levels varied from 0–34 amol mRNA/ng totRNA in the 47 biopsies analyzed. The limit of detection was 200–500 pg synthetic mRNA in the standard curves. In 7 out of 47 samples 5α-reductase 2 mRNA levels were undetectable by this method; they are referred to as 0 amol/ng totRNA. The median 5α-reductase 2-specific mRNA levels are presented in table 5 divided into different categories depending on histopathology and clinical diagnosis.

Histopathology of biopsy (n)	Clinical diagnosis of patient	5a-red2 mRNA median (amol/ng totRNA)
Cancer (14)	CaP	3.5
Noncancer (9)	CaP	11.5
Noncancer (18)	BPH	12.5
PIN (2)	CaP	12.0
PIN (2)	BPH	9.5

Noncancer (9), Noncancer (18), PIN (2) grouped as } 12.0

Table 5. The 5α-reductase 2-specific mRNA levels divided into categories depending on histopathology and clinical diagnosis (CaP = cancer prostate).

The median level was lowest in the cancerous biopsies from cancer patients whereas the BPH tissue from noncancer patients had the highest median level. There was no apparent difference

in mRNA level between the noncancer tissues from cancer patients compared to noncancer patients (11.5 vs. 12.5 amol/ng totRNA).

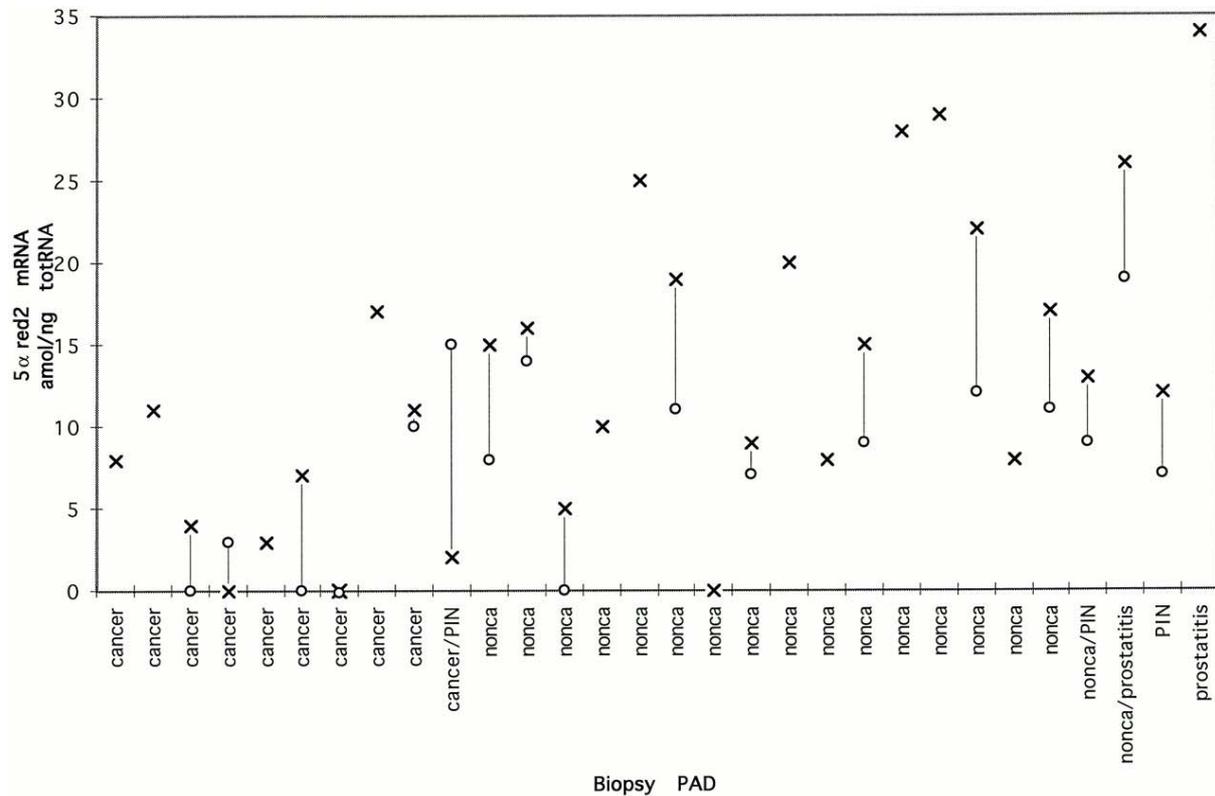The results from the individual samples are plotted in figure 9.

Figure 9.   5α-reductase 2 mRNA levels (y-axis) expressed as amol/ng totRNA in all prostate biopsies included in study I (n = 47). The pathoanatomical diagnosis (PAD) for each specimen as determined by histopathologist is given along the x-axis. A solid line connects duplicate biopsies from same patient.

The biopsies were divided into two groups for comparison of 5α-reductase 2 mRNA levels (noncancer and cancer) depending on the histopathological evaluation of each sample. Significant differences in 5α-reductase 2 mRNA levels between the cancerous and noncancerous groups were observed, as seen in figure 10.
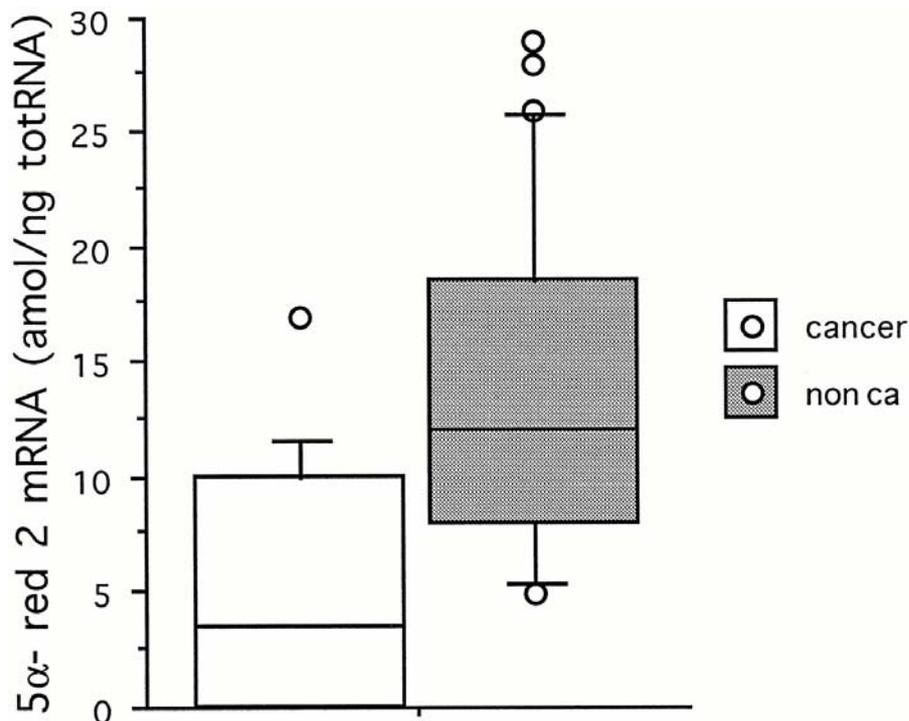
Figure 10. In the 14 biopsies judged to be cancerous, the median 5α-reductase 2 mRNA levels were 3.5 amol/ng totRNA compared with 12.0 amol/ng totRNA in the 27 biopsies showing no cancer (p = 0.0018). The median 5α-reductase 2 mRNA level in noncancerous tissue was thus 3.4 times higher than in cancerous specimens.

5α-reductase 2 specific mRNA expression predicts 5α-reductase activity in prostatic tissue (II).

5α-Reduction of testosterone was assayed in duplicates in 30 prostate specimens from 15 different patients at pH 5.5 and 7.0. The specimen activity values represent means from duplicate samples. The standard deviations of the difference between duplicates were 4.2 and 4.8 (DHT, pmol/mg/min), at pH 5.5 and pH 7.0, respectively. 5α-Reductase 2 specific mRNA was consistently present in 29 of the 30 investigated samples. The ratios between 5α-reductase 2 and β-actin mRNA concentrations varied between 0 (undetectable) and 1.31 with a median of 0.42.

There was a strong association between enzyme activity at pH 5.5 and the 5α-reductase 2 specific mRNA expression, as expressed on the basis of β-actin (r_s=0.81, 95 % confidence interval 0.64 – 0.91, p<0.0001).

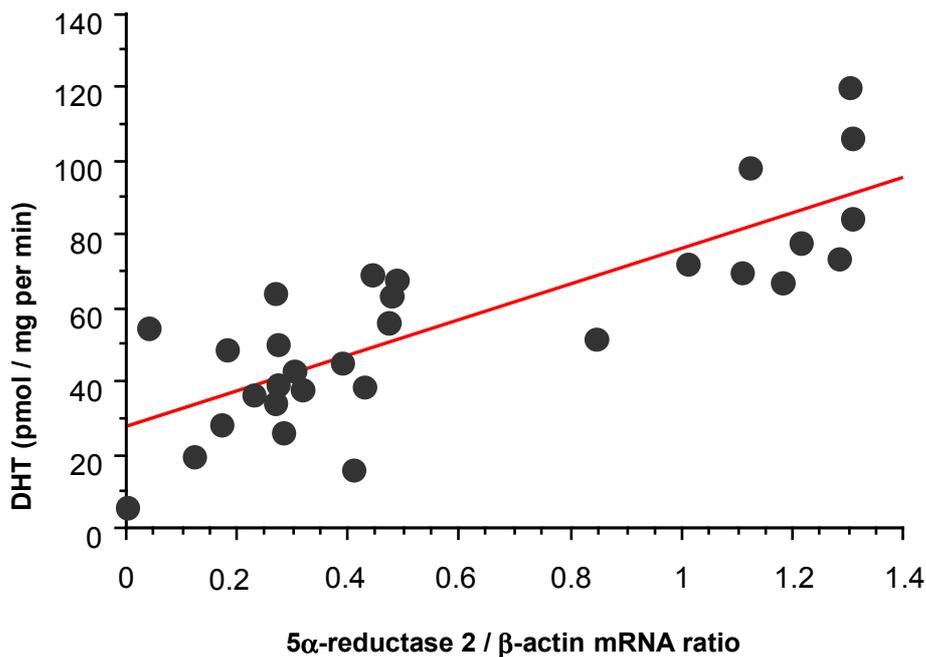Figure 11.　　Scatter plot showing the association between enzyme activity at pH 5.5 and the 5α-reductase 2 specific mRNA as expressed on the basis of β-actin (n=30).

There was also a strong correlation between the enzyme activity at pH 5.5 compared with pH 7.0 (r_s=0.79, p<0.001) indicating that the activity at the latter pH was probably due to the 5α-reductase 2 enzyme. When assessed separately, the 25 BPH specimens showed a strong association between enzyme activity at pH 5.5 and the 5α-reductase 2 specific mRNA expression (r_s=0.84, p<0.0001). The expression of 5α-reductase 2 specific mRNA in the cancer specimens was significantly lower than in the BPH tissue (p=0.03). The enzyme activity in the cancer specimens was also significantly lower than in the BPH specimens when compared at pH 5.5, (p=0.04) which is the optimum pH for 5α-reductase 2 activity in vitro.
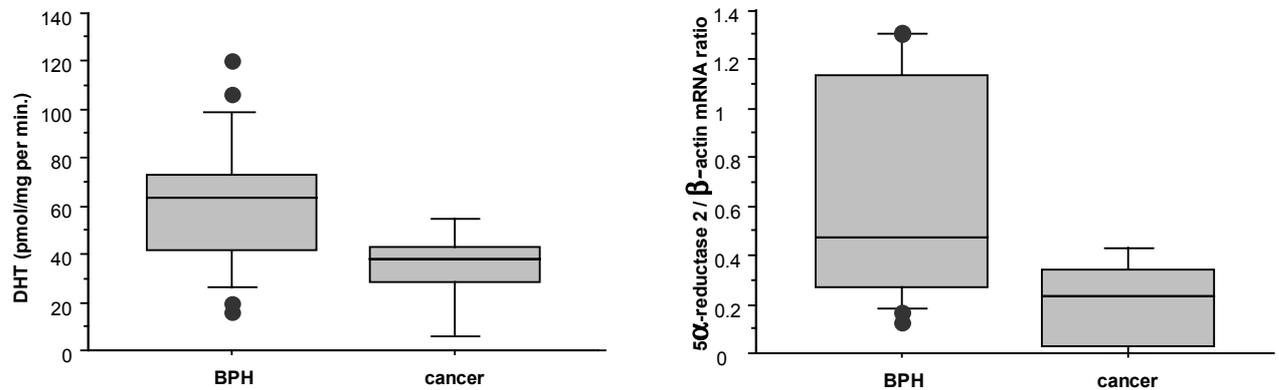
n=25).

Figure 12. Box plots showing 5α-reduction activity with testosterone as substrate at pH 5.5, corresponding to 5α-reductase 2 activity (left panel) and 5α-reductase 2/β-actin mRNA ratios (right panel) measured in prostate cancer tissue (n=5) and tissue with BPH (BPH; n=25)
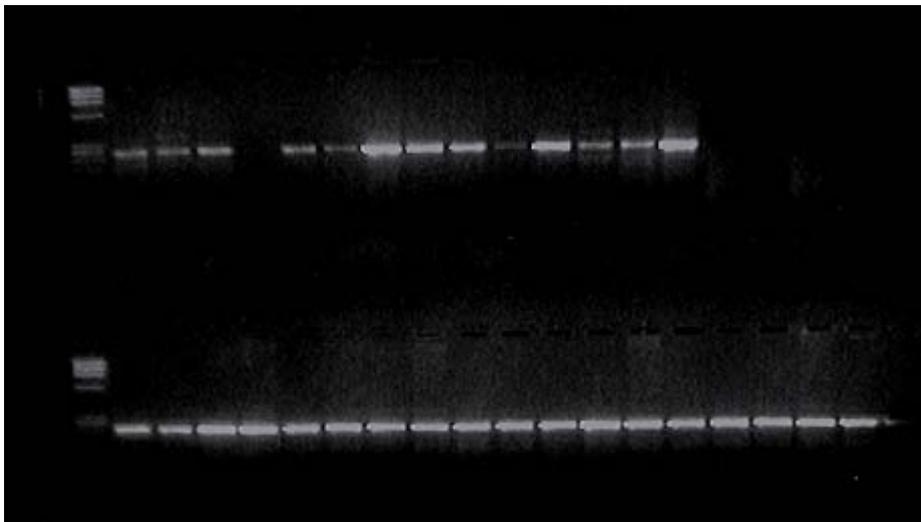

Figure 13. 5α-reductase 2 and β-actin specific mRNA in 14 different prostate specimens from 10 patients, (lanes 1 (left) -14) and 4 lymph node specimens from 3 patients (lanes 15-18). The mRNAs were assessed by RT-PCR and the PCR products were separated in a 2 % agarose gel. Upper row shows the variable expression of 5α-reductase 2 and the lower row shows the less variable constant expression of β-actin. No expression of 5α-reductase 2 mRNA was observed in lymph nodes.

5α-Reductase 1 specific mRNA was detectable in 27 of the 30 investigated samples. The ratios between 5α-reductase 1 mRNA and β-actin mRNA expression varied between 0 and 1.32 with a median of 0.38. There was no difference in the expression of 5α-reductase 1 specific mRNA in the specimens from cancerous tissue compared with BPH, with median ratios of 0.37 and 0.41 respectively (p=0.56). We found no association between 5α-reductase 1 specific mRNA and enzyme activity neither at pH 5.5 (r_s=-0.02, p=0.91), nor pH 7.0 (r_s=0.08, p=0.67). Consequently, there was no association between the gene expression of 5α-reductase 1 and 5α-reductase 2 (r_s=-0.10, p=0.60).

	pH 5.5	pH 7.0	n
BPH	63.6 (16.0-120.3)	16.2 (0-43.2)	25
Cancer	37.9 (6-55)	6.5 (4.4-20.5)	5

Table 6.   The median and range of 5α-reductase activity at acidic and neutral pH in BPH and prostate cancer tissue, expressed as pmol DHT formed per mg protein and min.

The 5α-reductase 2 V89L polymorphism associated with metastatic prostate cancer (III).

In respect of the 5α-reductase 2 V89L polymorphism, neither cases nor controls in our population based patient material deviated from Hardy-Weinberg equilibrium (Chi-sqr: 0.66 and 0.11, respectively). The allele frequency was 31 % for the L allele in the control population, and 34 % in the prostate cancer cases. There were no differences in the distribution of genotypes (chi square 0.96, p=0.62) between prostate cancer cases and controls (table 7).

With LL genotype as reference, the OR for prostate cancer among carriers of VL was 1.28 (95 % C.I: 0.62 – 2.63) compared to controls. The corresponding OR for VV was 1.42 (95 % C.I: 0.70 – 2.89). However, among the 36 patients who presented with metastases at time of diagnosis, carriers of the LL genotype were significantly over-represented (25 %) compared to the control group (10.1 %), OR 2.98 (95 % C.I 1.2-7.4, p=0.024). In a comparison within the group of prostate cancer cases, 25 % (9 out of 36) of the cases that presented with metastases at time of diagnosis carried the LL genotype compared to 10.7 % (14 out of 131) of the patients without metastases, (crude OR 2.79, 95 % C.I 1.1-7.1, p=0.038).

Group, n (%)	Genotype V89L			Genotype A49T	
	VV	VL	LL	AA	AT
controls, total	77 (48,4 %)	66 (41.5 %)	16 (10.1 %)	155 (96.9 %)	5 (3.1 %)
cases, total	78 (44,6 %)	74 (42.3 %)	23 (13.1 %)	168 (96.0 %)	7 (4.0 %)
controls, heredity pos	7 (38.9 %)	10 (55.6 %)	1 (5.5 %)	18 (100.0 %)	0 (0.0 %)
controls, heredity neg	67 (49.3 %)	55 (40.4 %)	14 (10.3 %)	133 (97.1 %)	4 (2.9 %)
cases, heredity pos	6 (28.6 %)	8 (38.1 %)	7 (33.3 %)	20 (95.2 %)	1 (4.8 %)
cases, heredity neg	66 (47.8 %)	57 (41.3 %)	15 (10.9 %)	132 (95.7 %)	6 (4.3 %)
M0	59 (45.0 %)	58 (44.3 %)	14 (10.7 %)	126 (96.2 %)	5 (3.8 %)
M1	15 (41.7 %)	12 (33.3 %)	9 (25.0 %)	35 (97.2 %)	1 (2.8 %)
T1+T2	39 (47.6 %)	32 (39.0 %)	11 (13.4 %)	81 (98.8 %)	1 (1.2 %)
T3+T4	39 (43.3 %)	41 (45.6 %)	10 (11.1 %)	84 (93.3 %)	6 (6.7 %)
poorly differentiated	12 (57.1 %)	6 (28.6 %)	3 (14.3 %)	20 (95.2 %)	1 (4.8 %)
moderately + well differentiated	66 (42.9 %)	68 (44.1 %)	20 (13.0 %)	148 (96.1 %)	6 (3.9 %)
PSA ≤19 (ng/ml)	30 (39.5 %)	33 (43.4 %)	13 (17.1 %)	75 (98.7 %)	1 (1.3 %)
PSA ≥20 (ng/ml)	47 (48.5 %)	40 (41.2 %)	10 (10.3 %)	91 (93.8 %)	6 (6.2 %)

Table 7.　Frequencies, n (%), of the steroid 5α-reductase 2 V89L and A49T genotypes in cases and controls, by heredity, TNM stage, differentiation grade and PSA levels.

This OR was adjusted and stratified for tumor characteristics, table 8. When adjusted for age, PSA level, T stage and differentiation grade, the OR was 5.67 (95 % C.I 1.44-22.30). The differences in OR between strata for T-stage, differentiation grade or PSA levels were not significant when tested for interaction with genotype using likelihood ratio test. The response rate for the question about heredity for prostate cancer among first-degree relatives was 91 % for the cases and 97 % for the controls. The cases with LL genotype more often reported heredity for prostate cancer than carriers of VL or VV genotype (Chi sqr. 8.19, p= 0.017). This difference was not observed in the control group.

Adjusting variable	Stratum	Odds ratio	95 % C.I
crude (no adjustment)		2.79	(1.09-7.10)
age		2.79	(1.09-7.11)
T-stage		3.32	(1.17-9.41)
	T1+T2	8.86	(1.80-43.48)
	T3+T4	1.67	(0.43-6.48)
tumor differentiation		2.95	(1.12-7.78)
	poorly	2.50	(0.19-32.80)
	moderately + well	3.03	(1.07-8.58)
PSA		4.81	(1.56-14.81)
	≥20 ng/ml	3.69	(0.95-14.24)
	≤19 ng/ml	5.80	(1.02-32.89)
total adjustment[a]		5.67	(1.44-22.30)

Table 8.   [a] Adjusted for age, T-stage, tumor differentiation and PSA

Association of the *5α-reductase 2* V89L LL genotype with prostate cancer metastases at time of diagnosis, compared to the combined group of VL and VV carriers, adjusted and stratified for age and tumor characteristics.

The 5α-reductase 2 A49T polymorphism may modulate the risk for early onset of prostate cancer (III).

The A49T polymorphism was found to be relatively rare. No subjects homozygous for the T allele were identified in cases or controls. Seven heterozygous carriers were detected among the cases and 5 among the controls, giving an allelic frequency of 2 % and 1.6 % respectively. Neither cases, nor controls deviated from Hardy-Weinberg equilibrium (Chi sqr=0.073 and 0.043 respectively). There were no differences in the distribution of genotypes (Chi sqr =0.18, p=0.67) between prostate cancer cases and controls. The OR for prostate cancer among carriers of AT was 1.29 (95 % C.I: 0.40 – 4.15) compared to controls, with AA genotype as reference. There was a higher frequency of the AT genotype (6.7 %) among patients with locally invasive disease (T3+T4), compared to the controls (3.1 %), but this difference was not significant, OR 2.21 (95 % C.I 0.66-7.47). If comparing the 1.2 % of cancer cases with T1 or T2 stage that carried the AT genotype, with the 6.7 % of the cases with T3 or T4, this difference was not statistically significant, OR 5.79 (95 % C.I 0.68-49.12, Chi sqr 3.26, p= 0.07). There was no difference in age between AT and AA carriers in the control group. A plot of the cumulative hazard of getting the prostate cancer diagnosis is displaced towards younger ages among carriers of the AT genotype compared with those with the AA genotype (figure 14).

The heterozygous AT cases were significantly younger than the homozygous AA, mean age 66 years compared to 71 (t-test, p=0.038).

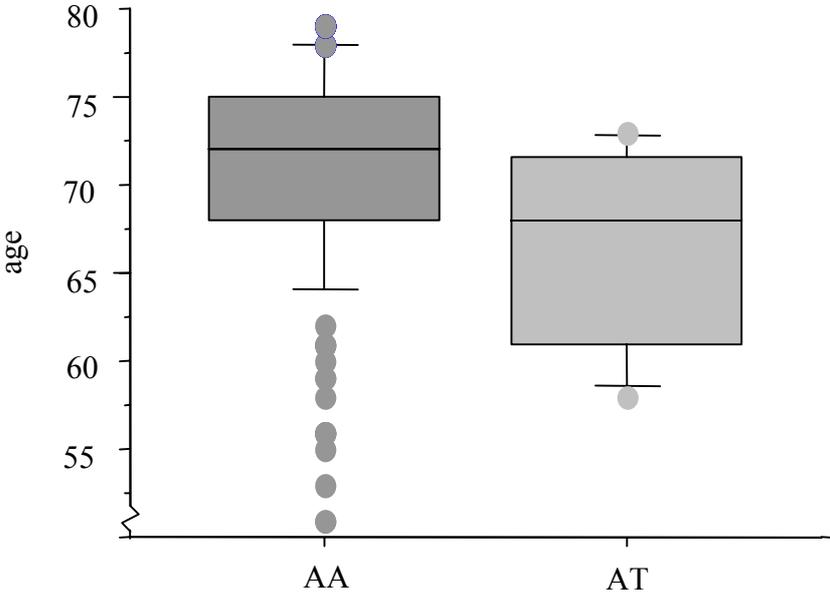Figure 14.   There was a difference in age at diagnosis between the AT carriers (mean age 66 years) compared to the AA carriers (mean age 71 years, p=0.038) among prostate cancer cases.
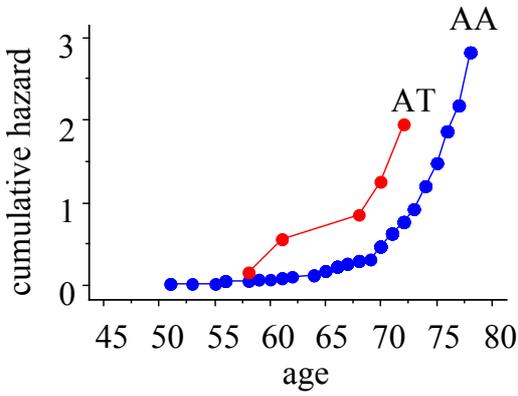

 Figure 15.   A cumulative Kaplan-Meier hazard plot for age at cancer diagnosis, for the 175 cancer cases genotyped for the A49T polymorphism. The cumulative hazard curve is displaced towards younger ages in the 7 patients with AT genotype, compared to the 168 patients with AA genotype.

The CYP1B1 V432L polymorphism associated with metastatic prostate cancer (IV).

In respect of the CYP1B1 V432L polymorphism, neither cases nor controls deviated from Hardy-Weinberg equilibrium (Chi-sqr: 0.206 and 0.068, respectively). Of the 320 alleles assessed in the control population, 43 % were V alleles and 57 % were L alleles. In the prostate cancer cases the allele frequencies for V and L were 46 and 54 % respectively. There were no differences in the distribution of the allelic variants (Chi-sqr: 0.55) or genotypes between prostate cancer cases and controls.

Group, n (%)	Genotype CYP1B1 V432L		
	VV	**VL**	**LL**
controls, total	31 (19.4)	77 (48.1)	52 (32.5)
cases, total	36 (20.6)	90 (51.4)	49 (28.0)
controls, heredity pos	1 (5.6)	11 (61.1)	6 (33.3)
controls, heredity neg	30 (21.9)	64 (46.7)	43 (31.4)
cases, heredity pos	2 (9.5)	13 (61.9)	6 (28.6)
cases, heredity neg	33 (23.9)	66 (47.8)	39 (28.3)
M0	30 (22.9)	70 (53.4)	31 (23.7)
M1	6 (6.7)	15 (41.7)	15 (41.7)
T1+T2	14 (17.1)	42 (51.2)	26 (31.7)
T3+T4	21 (23.3)	46 (51.1)	23 (25.6)
poorly differentiated	4 (19.0)	9 (42.9)	8 (38.1)
moderately + well differentiated	32 (20.8)	81 (52.6)	41 (26.6)
PSA ≤19 (ng/ml)	17 (22.4)	41 (53.9)	18 (23.7)
PSA ≥20 (ng/ml)	18 (18.6)	49 (50.5)	30 (30.9)

Table 9.   Frequencies, n (%), of the *CYP1B1* V432L genotypes in cases and controls, by heredity, TNM status, differentiation grade and PSA level.

With LL as reference, the VV and VL genotypes were not associated with prostate cancer, OR 1.23 (95 % C.I 0.66-2.29) and 1.24 (95 % C.I 0.76-2.03) respectively, compared to controls. Combining the VV and VL groups as reference, carrying the LL genotype was not associated with decreased risk for prostate cancer, OR 0.81 (95 % C.I: 0.51 – 1.29) compared to controls.

There was no statistically significant difference in genotype frequencies between cases with metastases, compared to controls. However, among the 36 prostate cancer cases that presented with metastases at time of diagnosis, 42 % (15 out of 36) carried the LL genotype compared to 24 % (31 out of 131) of the cases not presenting with metastases, (crude OR 2.30, 95 % C.I 1.06-5.00, p=0.035). This OR was adjusted for tumor characteristics (table 11). The OR adjusted for age, PSA level, T stage and differentiation grade was 2.46 (95 % C.I 1.02-5.93).

Adjusting variable	Stratum	Odds ratio	95 % C.I
crude (no adjustment.)		2.30	(1.06-5.00)
age		2.31	(1.06-5.03)
T-stage		2.88	(1.25- 6.60)
	T1+T2	4.71	(1.02-21.78)
	T3+T4	2.31	(0.85-6.27)
tumor differentiation		2.16	(0.97-4.81)
	poorly	23.33	(1.99-273.31)
	moderately + well	1.33	(0.53-3.38)
PSA		2.13	(0.95-4.81)
	≥20 ng/ml	2.80	(1.11-7.04)
	≤19 ng/ml	0.65	(0.07-5.98)
total adjustment.[a]		2.46	(1.02-5.93)

Table 11.   [a] Adjusted for age, T-stage, tumor differentiation and PSA.

Association of the *CYP1B1* LL genotype with cancer metastases at time of diagnosis, with the combined group of cases carrying the VV and VL genotype as reference. The effects of adjustments and stratification for tumor characteristics are shown.

Stratification of data in poorly versus moderately + well differentiated tumors revealed differences in OR (23.33, and 1.33 respectively) that were significant in a likelihood ratio test ($p < 0.05$). This indicates interaction between poor differentiation and LL genotype. The differences in odds ratio between strata for stage T1+T2, compared to T3+T4, and for PSA levels ≥20 ng/ml, compared to PSA levels ≤ 19 ng/ml, were not significant when tested for interaction with genotype.

There were no differences in the reporting of heredity for prostate cancer between cases and controls or between carriers of the different genotypes.

DISCUSSION

New pharmacogenetic approaches in the treatment of prostate cancer are needed and may be possible to achieve today and even more in the future. Important characteristics of drug targets can be exploited with molecular genetics combined with epidemiology and clinical pharmacology. In this study we have found that the important drug target 5α-reductase 2 is differentially expressed in prostate cancer tissue compared to BPH tissue (I, II) and that there is a considerable difference in the mRNA expression between individuals (I). We also found that the 5α-reductase 2 specific mRNA levels are useful as surrogate marker for the 5α-reductase activity in the prostate (II). This might be utilized in core biopsies taken in conjunction with the diagnostic procedures in clinical practice. The effect of different pharmacological treatments on the expression of the 5α-reductase 2 gene can be assessed with this technology. Furthermore, it could contribute to the understanding of drug interactions that influence androgen action in the prostate.

The possibility of up-regulation of androgen activating enzymes as a principle for androgen escape does not seem probable in the case of 5α-reductase 2, as we observed a lower expression in cancer tissue compared to benign (I, II). Other mechanisms are more likely. Mutations in the androgen receptor have been described that would alter the receptor to a form that is activated by non-androgenic steroidal ligands such as cortisol (Zhao 2000), estradiol and progesterone (Taplin 1995).

If the concept that androgens have a crucial role in the development of prostate cancer is valid, the large interindividual differences in the expression of the 5α-reductase 2 enzyme might act as a factor modulating prostate cancer risk in the population. Any factor that would affect this and other enzymes that determine the androgenic load are important to identify. Such factors also include drugs that may inhibit or induce the enzymes. A minimally invasive method, making it possible to measure a surrogate variable for the 5α-reductase 2 enzyme expression in the prostate would be even more useful in the exploration of the impact on variability of enzyme expression as a risk factor for prostate cancer.

The assessment of variations in risk for prostate cancer due to genetic variation meets many difficulties. Prostate cancer is not a homogenous disease, and the differences in incidence of clinically relevant prostate cancer between geographical areas, despite similar incidence in indolent cancer, suggest that genetic and environmental factors play a role. It is in no doubt that patients with risk factors that promote metastasing disease

might benefit from early detection and curatively intending treatment before the development of distant metastases. We investigated genetic polymorphisms in the androgen metabolizing enzymes 5α-reductase 2 and CYP1B1 (II, IV). Even though the OR's were in favor of the established hypothesis, that increased metabolic activation of testosterone to DHT would increase the risk for prostate cancer; we did not observe any statistically significant differences in the frequencies of genotypes between prostate cancer cases and population controls. As different characteristics of prostate cancer tumors are more important for the health of the patient than the diagnosis of cancer *per se*, we also assessed the frequencies of genotypes in relation to the most important tumor characteristics such as stage, grade, PSA and the patient's age. The 5α-reductase 2 V89L and the CYP 1B1 V432L genotypes that functionally reduce the androgenic load in the prostate showed associations with metastatic disease at time for diagnosis. It is not known whether metastatic disease is an entity of its own, or if it is the result of long time progression of an indolent cancer that subsequently acquired aggressive properties. Therefore, comparisons were made both between the cancer cases and the control group and within the group of cancer cases. In study III the differences in genotype frequencies were statistically significant in both comparisons whereas in study IV, only the comparison within the group of cancer cases, with or without metastases, was statistically significant. The interaction between poor tumor differentiation and the CYP1B1 LL genotype may give new insights into the etiology of prostate cancer metastases. The CYP1B1 enzyme has a broad substrate specificity making it difficult to establish which substrates might be responsible for the observed effect. The androgenic metabolism pathways may however be important in both study III and IV. That would considerably strengthen the somewhat controversial hypothesis that a decreased androgenic load during prostate carcinogenesis would more often lead to a metastatic disease. There is suggestive evidence of a mechanism involving the enzyme urokinase (Xing 1999), but this theory warrant further investigation. It is also interesting to note that the pretreatment serum testosterone level is a prognostic factor in androgen deprivation therapy. Patients with low serum testosterone levels have been shown to have a shorter progression-free survival than men whose serum testosterone levels are above normal prior to treatment (Matzkin 1993, Ribeiro 1997).

The 5α-reductase 2 A49T polymorphism was rare with an allele frequency of 1.2-2 %. This was negatively influencing the statistical power of our study. On the other hand, only 4 % of the prostate cancer cases carried the AT genotype. This can be interpreted

that this polymorphism is so rare that it is not likely to be a major threat for the prostate health of the male Swedish population. We also found that the cases with the AT genotype were significantly younger than the AA carriers. Age is an important prognostic factor in prostate cancer due to the fact that younger prostate cancer patients more seldom die from concomitant diseases (Grönberg, 1994). Theoretically, therefore a decrease of life expectancy might be the result for individuals carrying the A49T T allele if they contract prostate cancer.

A logical extension of the work on the case/control material would be to look for interaction between genotypes of importance in prostate cancer, such as the 5α-reductase 2 V89L, A49T, CYP1B1 V432L, Cyp17 A1/A2, the androgen receptor CAG polymorphism and possibly polymorphisms in the vitamin D receptor. As standalone prognostic indicators, these polymorphisms would probably not perform well. However, in a polygenic model including genetic factors that interact with each other and with other known prognostic factors such as grade and PSA, powerful prognostic determinations might be possible, either using conventional statistical methods or pattern recognition techniques including neural networks.

A significant problem (but also a bless) in this research has been the very rapid development of new methods and technologies. The methods of mRNA quantitation van according to vanden Heuvel (vanden Heuvel, 1993) were replaced in the same moment we had evaluated them, and before we had the opportunity to use them. There are no doubts that technical development in the field of molecular genetics will escalate further in the future. Today it is possible to measure the expression of and the genetic variability in thousands of genes on small chips. The demands for information technology and statistical methods that can handle this, and the challenges that scientist are facing in terms of complexity of information, problems, and methods will emphasize the needs for multi-disciplinary research teams with sub-specialized competence. The days of omniscient scientists are gone.

CONCLUSIONS

- The expression of 5α-reductase 2 specific mRNA is significantly lower in cancer specimens than in BPH tissue.

- In prostate tissue, there is a strong association between 5α-reductase activity at pH 5.5 and the 5α-reductase 2 specific mRNA expression, as expressed on the basis of β-actin.

- The sensitivity of the semi-quantitative PCR method makes it possible to use the 5α-reductase 2 specific mRNA expression as a surrogate marker for intraprostatic 5α-reductase activity at pH 5.5 in core needle biopsies commonly used in clinical practice.

- The 5α-reductase 1 specific mRNA expression is not co-regulated with the 5α-reductase 2 specific mRNA expressions in the prostate, indicating different regulatory mechanisms.

- Carriers of the 5α-reductase 2 V89L LL genotype more often presented with metastases at time of diagnosis, compared to carriers of the VV and VL genotypes. This indicates that the LL genotype might be a risk factor for metastatic progression.

- Carriers of the 5α-reductase 2 A49T AT genotype were significantly younger at time of diagnosis than were the AA genotype carriers. This indicates that the AT genotype might be a risk factor for earlier onset of prostate cancer.

- Carriers of the CYP1B1 V432L LL genotype more often presented with metastases at time of diagnosis, compared to carriers of the VV and VL genotypes. This indicates that the LL genotype might be a risk factor for metastatic progression.

- The CYP1B1 V432L LL genotype was significantly interacting with tumor differentiation as risk factors for metastatic progression. The combination of poorly differentiated tumor and the LL genotype constituted the highest risk.

ACKNOWLEDGEMENTS

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APPENDIX

Papers I-IV