DE NOVO ANDROGEN SYNTHESIS AS A MECHANISM CONTRIBUTING TO THE PROGRESSION OF PROSTATE CANCER TO CASTRATION RESISTANCE

by

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Abstract

Prostate cancer (CaP) is the leading cause of cancer in men affecting 24,700 Canadians each year and the third leading cause of cancer mortality with 4,300 deaths each year. CaP cells are derived from the prostate secretory epithelium and depend on androgen ligand activation of androgen receptor (AR) for survival, growth and proliferation. Androgen deprivation therapy (ADT) through pharmacological methods has been the leading form of CaP therapy since Huggin's discovery that castration induced the regression of CaP tumors in 1941. Unfortunately, the cancer often recurs within 2-4 years in what has classically been considered "androgen-independent" (AI) disease. Growing evidence implicates androgens and AR activation in this disease recurrence despite castration, suggesting that this terminology should be more appropriately called "castration-resistant" prostate cancer (CRPC). Firstly, AR is found amplified, overexpressed or mutated in a majority of recurrent cancers as compared to primary cancers and secondly, intratumoral testosterone levels remain the same pre- and post-ADT. Additionally, the measured intratumoral DHT levels are sufficient to activate AR in recurrent CaP cells despite low serum androgen levels suggesting that intratumoral androgens remain important mediators of AR-mediated CaP progression. Previously, we and others discovered that recurrent tumor cells have elevated levels of enzymes in the pathways necessary for androgen synthesis from cholesterol.

The central hypothesis in this thesis is that after ADT, CaP cells adapt to synthesize their own androgens for survival and proliferation. The goal of this PhD dissertation is to decipher the mechanisms whereby prostate tumor cells *de novo* synthesize androgens and how these events contribute to recurrent CaP. We show herein that CRPC tumor cells are capable of producing androgens and that upstream cholesterol and fatty acids are key mediators in this process. Furthermore, CRPC tumor cells adapt quickly to bypass current targeted therapies by utilizing multiple interlinked steroidogenesis pathways to continue to produce androgens necessary for AR activation.

By understanding the mechanisms of intratumoral *de novo* androgen synthesis and how they contribute to CaP progression, more specific and effective therapeutics can be developed to treat this disease.

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ABCA1	ATP binding cassette-A1
ABCG1,5,8	ATP binding casette-G1,5,8
ACAT-1,2	Acyl-CoA cholesterol acyltransferase-1,2
ACBP	Acyl-CoA binding protein
ACN	Acetonitrile
ACOT9	Mitochondrial acyl-CoA thioesterase-9
ACSL-3	Long-chain acyl-CoA synthetase-3
ACTH	Adenocorticotropic hormone
AD	Androgen dependent
ADT	Androgen deprivation therapy
AF1	Activation function 1
AF2	Activation function 2
AI	Androgen-independent
AKR	Aldo-keto reductases
AKR1C1-3	Aldo-keto reductase family 1, member C1-3
AKT	Protein kinase B
AMACR	Alpha-methylacyl-CoA racemase
ANOVA	Analysis of variance
AR	Androgen receptor
ARA55,70	Androgen receptor-associated proteins 55,70
ARE	Androgen response element
BCL2	B cell lymphomal / leukemia gene 2
BF3	Binding function 3
BPH	Benign prostatic hyperplasia
BRCA2	Breast cancer 2 tumor suppressor
C	Cholesterol
CaP	Prostate cancer
Cas	Casodex
CBP	cAMP response element-binding protein
CE	Collision energy
CE	Cholesteryl ester
CID	Collision induced dissociation
Cin	Cinnamic acid
CoA	Coenzyme A
CR	Castration-resistant
CRH	Corticotropin-releasing hormone
CRPC	Castration-resistant prostate cancer
CSS	Charcoal Stripped Serum
CTD	COOH-terminal domain
CV	Cone voltage
CYB5A (1)	Cytochrome b5
CYP	Cytochrome P450
CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1
CYP17A1	17α-hydroxylase/17,20 lyase/17,20 desmolase
Cyt	Cytosol

DBD	DNA-binding domain
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DMEM	Dulbecco's modified eagle's medium
DRE	Digital rectal examination
DTT	Dithiothreitol
EBRT	External beam radiation therapy
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EI+	Electron impact positive
EPCA	Early prostate cancer antigen
ERG	Transforming protein ERG
F	Finasteride
FAME	Fatty acid methyl ester
FAS	Fas ligand (FAS)
FASN	Fatty acid synthase
FBS	Full bovine serum
FDPS	Farnesyl diphosphate synthase
FDPS	Farnesyl diphosphate synthase
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC-MS	Gas chromatography mass spectrometry
GnRH	Gonadotropin releasing hormone
H	Radiolabeled [3H]-progesterone
H + C	Non-radiolabeled (cold) progesterone (H+C)
HCl	Hydrochloric acid
HMG	HMG-CoA reductase
HPG	Hypothalamic-pituitary-gonadal
HPLC-MS	High pressure liquid chromatography mass spectrometry
Hrs	Hours
HSD	Hydroxyl dehydrogenases
HSD17B1-5	Hydroxysteroid (17-β) dehydrogenase 1-5
HSD3B2	Δ-5-steroid dehydrogenase, 3 β- and steroid Δ -isomerase 2
HSL	Hormone sensitive lipase
HSP27,70,90	Heat shock protein 27,70,90
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
IGFBP-2,3,5	IGF-1 binding proteins, insulin growth factor binding protein-2,3,5
IL-6	Interleukin-6
INSIG	Retention protein complex
IPA	Isopropanol
K	Ketoconazole
KGF	Keratinocyte growth factor
KLK	Kallikrein-related peptidase
LBD	Ligand-binding domain
LDL-r	Low density lipoprotein-receptor

LH	Luteinizing hormone
LHRH	Luteinizing hormone-released hormone
LMTK2	Lemur tyrosine kinase 2
LNCaP	lymph node derived prostate cancer
MAB	Maximal androgen blockade
Mit	Mitochondria
MLN64	Metastatic lymph node 64
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MSMB	Microseminoprotein, beta,
MTBE	Methyl tert butyl ether
N	Nadir
NADPH	Nicotinamide adenine dinucleotide phosphate
NCOR	Nuclear receptor co-repressor
NE	Neuroendocrine
NF-kB	Nuclear factor kappa B
NHT	Neoadjuvant hormone therapy
NLS	Nuclear localization signal
NTD	N-terminal domain
P	3H-Progesterone
p300	E1A binding protein
PBR	Peripheral-type benzodiazepine receptor
PCA3	Prostate cancer gene 3
PMSF	Phenylmethanesulphonylfluoride
PR	Progesterone receptor
PSA	Prostate specific antigen
Q-RT-PCR	Quantitative real time polymerase chain reaction
RDH5	Retinol dehydrogenase 5 (11-cis/9-cis)
RIA	Radioimmunoassay
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute
RT	Retention time
RU	RU-486
s.d.	Standard deviation
SCAP	Scaffolding protein SREBP cleavage-activating protein
SEM	Standard error of the mean
SHBG	Sexual hormone binding globulin
shRNA	Small hairpin RNA
SIR	Single ion recording
siRNA	Small interfering RNA
SMRT	Retinoid and thyroid hormone receptors
SR-B1	Scavenger receptor-B1
SRC1-3	Steroid receptor coactivators 1-3
SRD5A1,2	Steroid 5-alpha-reductase-1,2
SRE	Sterol response element
SREBPs	Sterol regulatory element binding proteins

StAR	Steroidogenic acute regulatory protein
STAT-3	Signal transducers and activator of transcription-3
T	Testosterone
TAB	Total androgen blockade
TGF-b	Transforming growth factor beta
TMPRSS2	Transmembrane protease, serine 2
UGM	Urogenital sinus mesenchyme
UGS	Urogenital sinus
VEGF	Vascular endothelial growth factor
YB-1	Y-box factor-1

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This has been one incredible chapter in my life and I wish to extend my deepest gratitude to my mom and dad for their unconditional love and support, to my friends and family for always encouraging me to do something I really enjoy and to John for his undying patience and life-long partnership.

Dedication

To all affected by prostate cancer

Co-authorship statement

The publications presented in this thesis are based on the work that I have carried out for the completion of my PhD program. Following the titles of each chapter I have included citations acknowledging researchers from The University of British Columbia, The Prostate Centre at VGH and other collaborating institutions who have significantly contributed to the production of these publications. I have also specified individual contributions in the carrying out of this research.

CHAPTER 2: Androgen levels increase by intratumoral *de novo* steroidogenesis during progression of castration-resistant prostate cancer: Dr. Colleen Nelson was principal investigator on this manuscript while Dr. Emma Guns was a co-investigator. Amy Lubik performed and trained me in the Q-RT-PCR and Western Blot analysis of tumor samples for the expression of steroidogenesis enzymes. Hans Adomat developed and trained me on the LC-radiometric detection and LC-MS assays for steroid analysis. Stephen Hendy prepared all tissues for *ex vivo* radiotracing assays. Catherine Wood taught me crucial skills for working with mice in the LNCaP xenograft model of prostate cancer progression. Dr. Susan Ettinger and Dr. Martin Gleave provided critical comments and reviewed the work. I performed all of the animal model work, the *ex vivo* raditracing assays and analysis of steroids by LC-radiometric detection and LC-MS. Furthermore, I drafted the first version of this manuscript as well as finalized it for publication.

CHAPTER 3: Steroidogenesis inhibitors alter but do not eliminate androgen synthesis mechanisms during progression to castration-resistance in LNCaP prostate xenografts: Dr. Emma Tomlinson Guns was the principal investigators on this manuscript while Dr. Colleen Nelson was her co-investigator. Hans Adomat supervised me on the LC-radiometric detection assays for steroid analysis. Stephen Hendy and Dr. Martin Gleave provided critical comments and reviewed the work. In this manuscript I conducted all of the *in vitro* and *ex vivo* progesterone metabolism experiments in the presence of inhibitors. I conducted endpoint analyses of both PSA and LC-radiometric detection results. Furthermore, I drafted and prepared this manuscript for publication.

CHAPTER 4: Alterations in cholesterol regulatory processes contribute to *de novo* androgen synthesis in prostate cancer tumors during progression to castration-resistance: Dr. Kishor Wasan collaborated with our lab to amalgamate his expertise in cholesterol processes with our lab's expertise in prostate cancer processes to produce these manuscripts. Under the guidance of Dr. Kishor Wasan, Dr. Carlos Leon founded the HMG-CoA reductase and ACAT activity assays and trained me in these techniques. He also conducted the majority of Western blot experiments and provided extensive insight into the production of the manuscripts in this chapter. Dr. Susan Ettinger, Alexis Twiddy, Rachel Neumann, Dr. Colleen Nelson and Dr. Emma Guns provided critical comments and reviewed the work. Hans Adomat developed the assays for cholesterol and steroid analyses by LC-radiometric detection and LC-MS, respectively. In the first manuscript I performed the cell culture work, the PSA analyses as well as the majority of the HMG-CoA reductase and ACAT activity assays. In the second manuscript I performed the animal model work and the tumor *de novo* cholesterol synthesis and steroid analyses. I also contributed significantly to the drafting and finalization of both manuscripts.

CHAPTER 5: Arachidonic acid activation of intratumoral steroid synthesis during prostate cancer progression to castration-resistance: Dr. Colleen Nelson was the principal investigator on this manuscript. Dr. Katia Margiotti of Dr. Martin Gleave's lab collected patients' samples and prepared the microarray used for RNA analysis. Melanie Lehman and Dr. Susan Ettinger analyzed the human microarray data and contributed to the drafting of the manuscript. Amy Lubik provided the FASN antibody for Western Blot analysis. Dr. Amina Zoubeidi of Dr. Martin Gleave's lab founded the fluorescence localization assays and provided critical comments. Dr. Ladan Fazli (pathologist) of Dr. Martin Gleave's lab analyzed all immunohistochemistry stainings of prostate cancer tissues. Hans Adomat provided guidance in the development of the GC-MS assay for fatty acid analysis. Dr. Kishor Wasan, Dr. Emma Tomlinson Guns and Dr. Martin Gleave provided critical comments and reviewed the work. I conducted all of the cell culture, fractionation steps, Western blot analyses, cholesterol uptake assays, animal model work and fatty acid analyses by GC-MS. I also drafted and finalized this manuscript for submission.

CHAPTER 1: Literature review, hypothesis and specific aims

1.1 Androgen action in the prostate

1.1.1 Biology, origin and function of the prostate

The prostate is an exocrine secretory gland specific to male mammalian genitalia. It surrounds the urethra and is located inferior to the bladder and anterior to the rectum. At birth, the size of the prostate in humans is approximately 1-2 grams and after puberty androgens act to mature the organ to its adult size of approximately 20 grams, or the size of a walnut [1, 2]. An adult human prostate is composed of three zones: transitional, central and peripheral as depicted in **Figure 1.1** [2, 3].

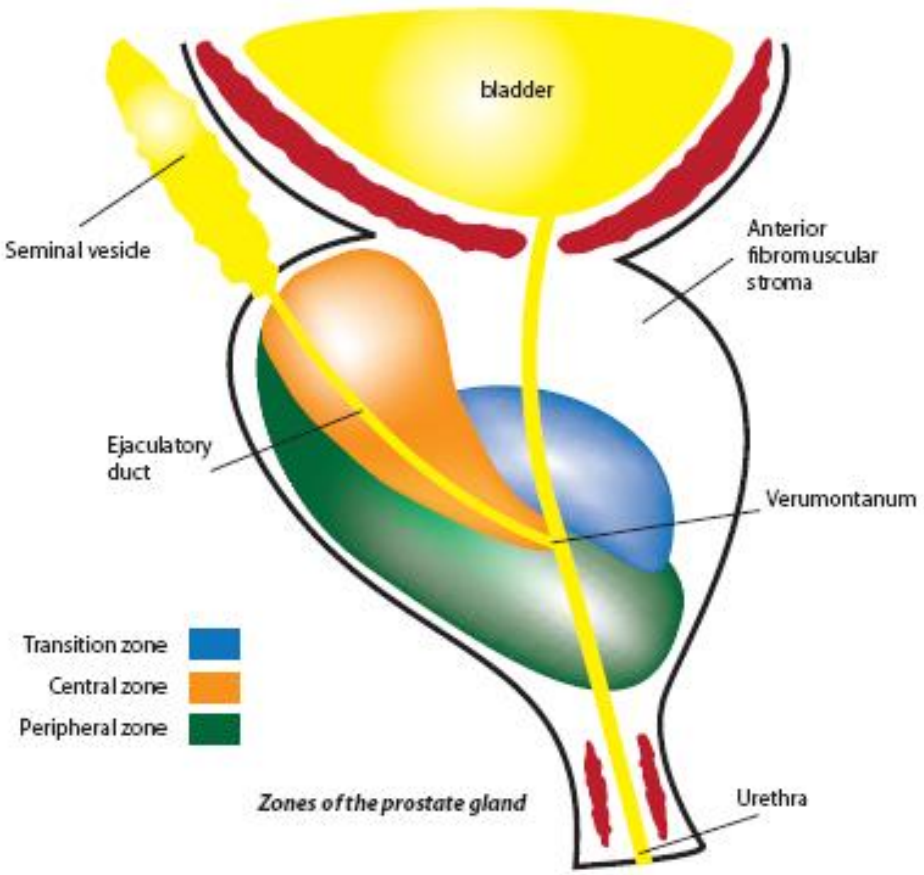

Figure 1.1: Human prostate gland. The prostate gland, located below the bladder surrounding the urethra, is composed of three zones: transitional, central and peripheral.

The transitional zone, immediately surrounding the urethra, is the innermost and smallest component of the prostate [2, 3]. The central zone, making up approximately 25% of the gland, is the next neighboring region and the peripheral zone, making up approximately 70% of the prostate volume, is located closest to the rectum [2, 3].

Development of the prostate before puberty initiates *in utero* from the ambisexual endodermal urogenital sinus (UGS) when androgens (testosterone and dihydrotestosterone) stimulate their associated receptor in the surrounding embryonic connective tissue, urogenital sinus mesenchyme (UGM) [2]. Androgen stimulation of the androgen receptor (AR) in the UGM supports the differentiation of stem cells in the UGS. Stem cells are defined as self-renewing, self-preserving cells that have the ability to undergo a range of differentiation events to become functional cells [4, 5]. AR-induction of various growth factors such as keratinocyte growth factor (KGF) [6] in the UGM trigger UGS stem cells to differentiate into epithelial cells. In turn growth factors such as fibroblast growth factor (FGF) [7] are activated in the UGS to stimulate the differentiation of stem cells in the UGM into mesenchymal cells [8]. Through these reciprocal androgen-mediated epithelial-mesenchymal interactions (**Figure 1.2a**) [9-11] distinct fibroblast, smooth muscle and endothelial mesenchymal stromal cells as well as luminal secretory, basal and neuroendocrine epithelial cells arise making up the adult prostate gland [2, 12-14].

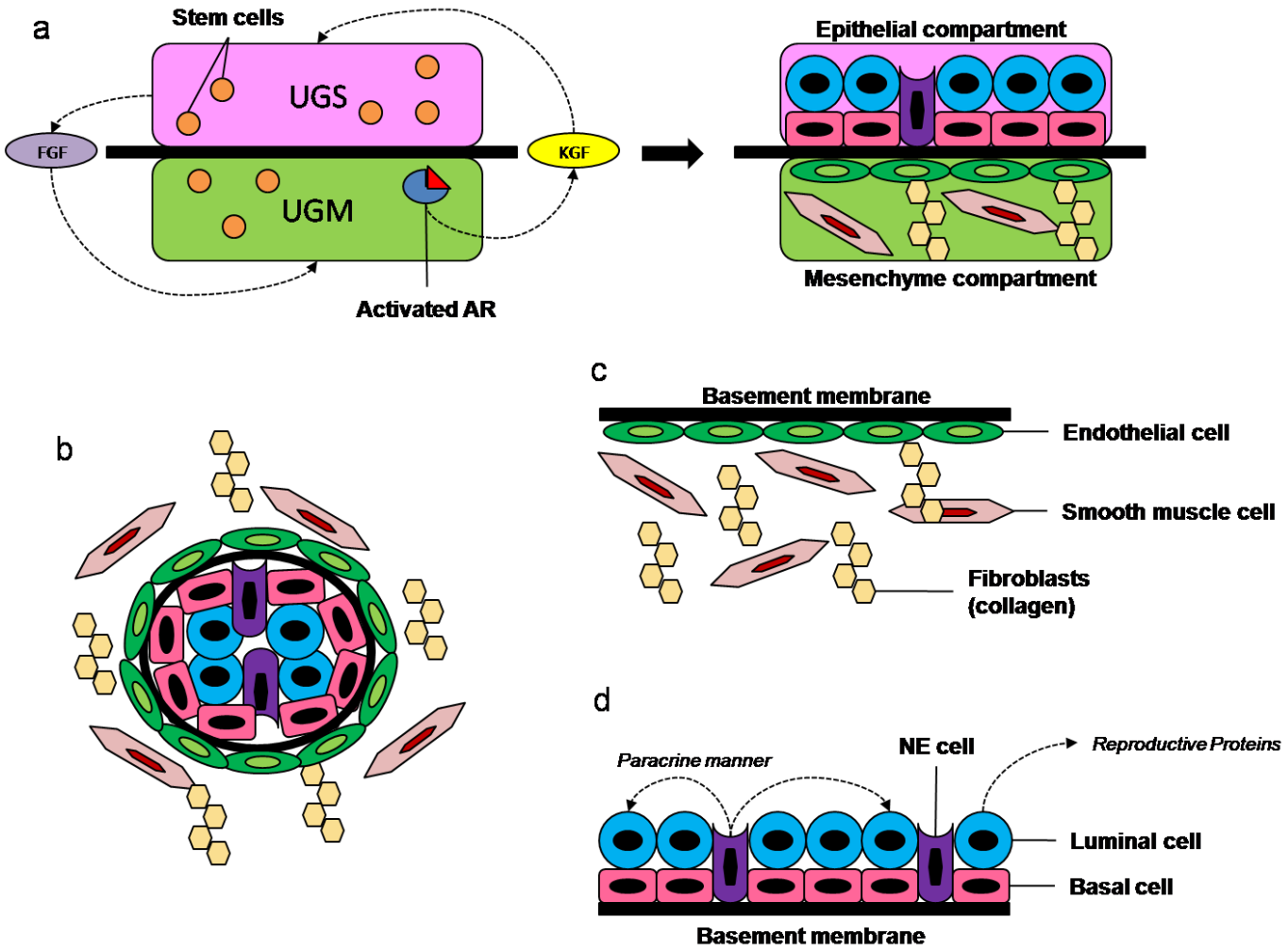

Figure 1.2: Epithelial-mesenchymal interactions in the prostate gland. Depicted action of reciprocal mesenchymal-epithelial interactions during transition from developing endodermal urogenital sinus (UGS) and urogenital sinus mesenchyme (UGM) to adult epithelial and mesenchyme compartments (**a**), cross-section view of a typical adult prostate glandular acinus (b), adult prostate mensenchyme compartment (**c**) and adult prostate epithelial compartment (**d**). Figure modified from [15, 16].

Physiologically, the mature adult prostate consists of a multitude of branching tubuloalveolar glands [8]. Each glandular acinus generally consists of a line of secretory luminal epithelial cells, a discontinuous layer of basal cells and neuroendocrine cells scattered between these two lineages, all surrounded by mesenchymal stroma cells [8, 17] (**Figure 1.2b**). The mesenchymal stroma consists of flibroblast, smooth muscle and endothelial cells [10]; fibroblast cells produce extracellular matrix and collagen tissues for maintenance of the connective tissues of the stroma, smooth muscle cells which are composed of non-striated muscle fibers allow for glandular flexibility and endothelial cells which line the stroma help supply vascularization to the entire gland [10]. These cells combine to make the stroma compartment (**Figure 1.2c**) to provide a nutrient-rich androgen-sensitive microenvironment for embedded epithelial cells to function. In the epithelial compartment (**Figure 1.2d**), androgen-independent basal cells act to initiate growth and proliferation of the compartment while androgen-dependent luminal secretory cells function to secrete proteins necessary for male reproduction [12]. Neuroendocrine (NE) cells also exist in the epithelial compartment, however their exact function remains unknown [12, 18]. It has recently been proposed that NE cells secrete neuroendocrine factors, peptide hormones and pro-hormones that act in a paracrine manner to regulate secretory function [16, 19]. Overall, the basal, luminal secretory and NE cells of the epithelium with the help of the fibroblast, smooth muscle and endothelial cells of the mesenchymal stroma function to maintain glandular formation and more importantly, the secretion of proteins important for reproduction [10].

The functional role of the adult prostate in reproduction is to serve as a secretory gland, primarily to maintain semen gelation, coagulation and liquefaction for successful fertilization [3, 20]. Semen is secreted from the male genitalia during ejaculation and consists of spermatozoa originating from the testis, seminal fluid from the seminal vesicles and a variety of proteins and proteases from the prostate in the form of proteosomes and exosomes [20-22]. Proteomic characterization of human semen content has demonstrated the presence of lipids including phospholipids and sphingomyelin, proteins including enzymes, chaperones and signal transduction molecules and proteases including those of the prostate specific kallikrein-related (KLK) family [23]. Together these molecules assist in the efficient fertilization of the female egg with spermatozoa [21].

1.1.2 Steroids and androgens

Steroids orchestrate crucial endocrine events responsible for the survival and reproduction of living organisms. Androgens are a subclass of steroids that specifically elicit and control the development and maintenance of masculine characteristics [24]. In humans there are many other important subclasses of steroids such as estrogens, the female sex steroid counterpart of androgens, which are responsible for development and maintenance of feminine characteristics and corticosteroids which function to regulate the body's physiological responses to external stimuli [25]. Common to all steroids is their formation from a precursor terpenoid lipid known as cholesterol (**Figure 1.3a**).

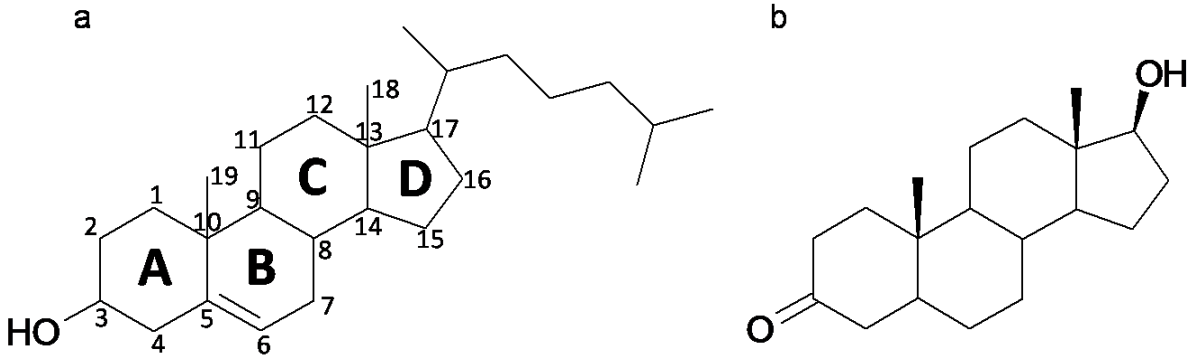

Figure 1.3: Structures of cholesterol and dihydrotestosterone. Depicted molecules are 2-dimensional cholesterol (**a**) and dihydrotestosterone (**b**). Each molecules contain 19-carbon steroid backbone with four interlinked rings designated A-D (three cyclohexanes and one cyclopentane).

Androgens, which include testosterone (T) and dihydrotestosterone (DHT) (**Figure 1.3b**) are formed through numerous step-wise enzymatic bioconversions from cholesterol that will be detailed later. In circulation T is the principal androgen in men as it is found in serum concentrations of 9.2-33.7 nmol/L while DHT, which is found in much lower serum concentrations of 0.47-2.65 nmol/L, is the principal androgen acting in the prostate [26, 27]. This is because in peripheral target tissues including the lung, adipose tissue, blood cells, skin, mammary gland, endometrium, hair and prostate T undergoes preferential conversion to DHT by an enzyme known as SRD5A1,2 [28], while in other tissues lack of SRD5A1,2 ensures that T (and not DHT) is the principal androgen.

1.1.3. Androgen production- the hypothalamic-pituitary-gonadal axis

In the human body endocrine production of steroids is efficiently mediated through the hypothalamic-pituitary-gonadal (HPG) axis. In the female, production of estrogens is regulated by the hypothalamic-pituitary-ovary axis while in the male, production of testosterone is regulated by the hypothalamic-pituitary-testis axis (**Figure 1.4**) [26]. The production of corticosteroids is in turn regulated by the hypothalamic-pituitary-adrenal axis.

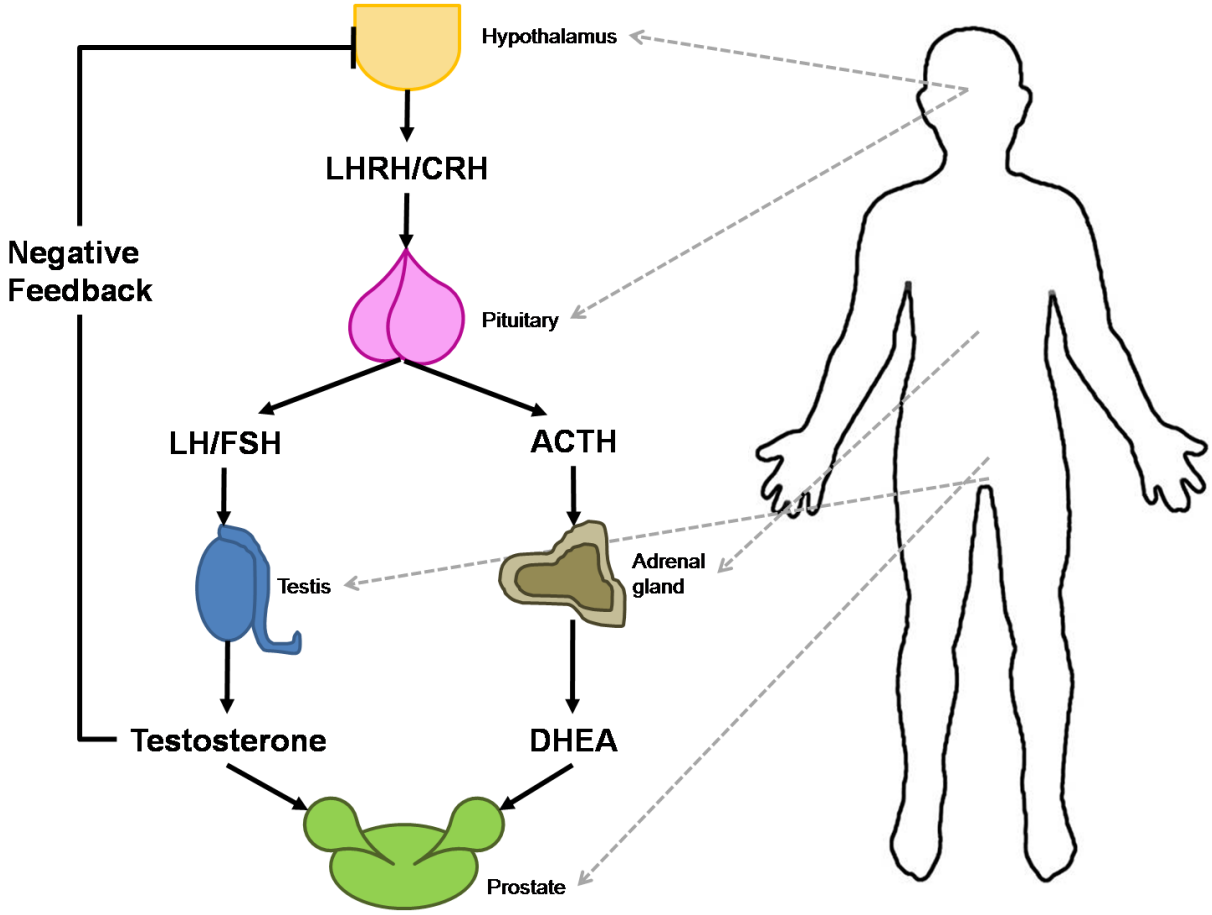

Figure 1.4: Male hypothalamus-pituitary-gonadal axis. Depicted is the male hypothalamus-pituitary-gonadal (testis and adrenal gland) axis in relation to the human body. Figure modified from [26, 29].

In the hypothalamus, peptide hormones: corticotropin-releasing hormone (CRH) and luteinizing hormone-released hormone (LHRH), also known as gonadotropin releasing hormone (GnRH), are produced by the parvicellular neurons and secreted into the hypophyseal portal system [26]. Through this connecting blood vessel system CRH and

LHRH are able to reach their cognate LHRH and CRH receptors in the pituitary [26]. In the pituitary, LHRH acts on the gonadotrope LHRH receptor to release luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the blood stream while CRH induces the release of adenocorticotropic hormone (ACTH) through CRH receptor activation [26]. Through their respective receptors LH, FSH and ACTH then trigger the production of sex hormones in the testis (T), ovary (estrogen) and adrenal glands (deydroepiandrosterone-DHEA) and their subsequent release into circulation [26]. T and DHEA can then be utilized by the prostate to produce principal androgen DHT. In turn, the hypothalamic-pituitary-gonadal pathway is regulated via negative feedback whereby serum T and estrogen inhibit the release of LHRH and LH in the hypothalamus and pituitary [26, 29].

1.1.4 Androgen synthesis in the human

Upon LH and FSH stimulation T is classically synthesized in the testis by a series of step-wise reactions from cholesterol [26]. T can also be synthesized from adrenal gland precursor DHEA [30]. Furthermore, in steroidogenic cells DHT can be synthesized from cholesterol through a mechanism bypassing T as an intermediate [31]. These detailed steroidogenesis mechanisms are outlined below: firstly, in the characterization of important enzymes (**Section 1.1.4.1, Table 1.1**) and secondly, in the detailed mechanistic conversion of cholesterol into androgens using these enzymes (**Section 1.1.4.2, Figure 1.5**).

1.1.4.1 Enzymes

Important enzyme families in androgen synthesis include cytochrome P450s (CYPs), steroid-5-alpha-reductases and hydroxyl dehydrogenases (HSDs), also known as aldo-keto reductases (AKRs). In general, cytochrome P450 enzymes are responsible for hydroxylation reactions (**Figure 1.3a**) and cleavage of carbon-carbon bonds, steroid-5-alpha-reductases are responsible for reducing double bonds between C-4 and C-5 and hydroxyl dehydrogenases regulate the conversion of a given ketosteroid (C-3,17 moieties) and its cognate hydroxysteroid [32]. These reactions are responsible for the bulk of steroidogenesis processes in the cell leading to androgen synthesis. The specific enzymes involved in androgen synthesis are outlined in detail in **Table 1.1.**

Gene Symbol	Name	Localization	Function	Deficiency
CYP11A1	Cytochrome P450, family 11, subfamily A, polypeptide 1	Mitochondria [29, 34]	Side chain cleavage at C-20 [29, 34]	Lipoid congenital adrenal hyperplasia (LCAH) [33]
CYP17A1	17α-hydroxylase/17,20 lyase/17,20 desmolase	Endoplasmic reticulum [29]	Hydroxylation at C-17 and bond cleavage between C-17 and C-20 [29, 34]	1) Hydroxylation deficiency - sexual infantilism with hypokalemic hypertension and lack of body hair development 2) Lyase deficiency - pubertal failure without hypokalemic hypertension [33]
SRD5A1/2	Steroid-5-α -reductase, α-polypeptide 1,2	Nuclear envelope [29]	Reduction of double bond between C-4 and C-5 [29]	Non-life threatening alterations in male genitalia and hair follicle development [35]
HSD3B2	Δ-5-steroid dehydrogenase, 3 β- and steroid Δ -isomerase 2	Cytosol [34]	Isomerisation of double bond from C-5 and C-6 to C-4 and C-5 position [34]	Birth with ambiguous genitalia [44]
HSD17B1-5	Hydroxysteroid (17-β) dehydrogenase 1-5	Nuclear envelope [29]	Reduction of ketone functionality to a hydroxyl group at C-17 [29]	Rare disorder whereby patients are born with female genital but after puberty begin to develop secondary male features [36]
AKR1C1-3	Aldo-keto reductase family 1, member C1-3	Cytosolic [37]	Reduction of ketone functionality to a hydroxyl group at C-3 [37]	Unknown
StAR	Steroidogenic acute regulatory protein	Mitochondria [38-40]	Catalyzes mitochondria cholesterol uptake [38-40]	Same as CYP11A1 deficiency [33]
CYB5A	Cytochrome b5	Endoplasmic reticulum [41-43]	Provides electrons for cytochrome P450 enzyme reactions [41-43]	Same as CYP17A1 deficiency [33]
RDH5	Retinol dehydrogenase 5 (11-cis/9-cis)	Unknown	Oxidation of hydroxyl functionality to ketone group at C-3 [32]	Unknown

Table 1.1 Properties of steroidogenesis enzymes. Depicted in table are the symbol, name, localization of active form in the cell, enzyme function and overall effect of deficiencies of enzymes in humans.

1.1.4.2 Androgen synthesis pathway (Figure 1.5)

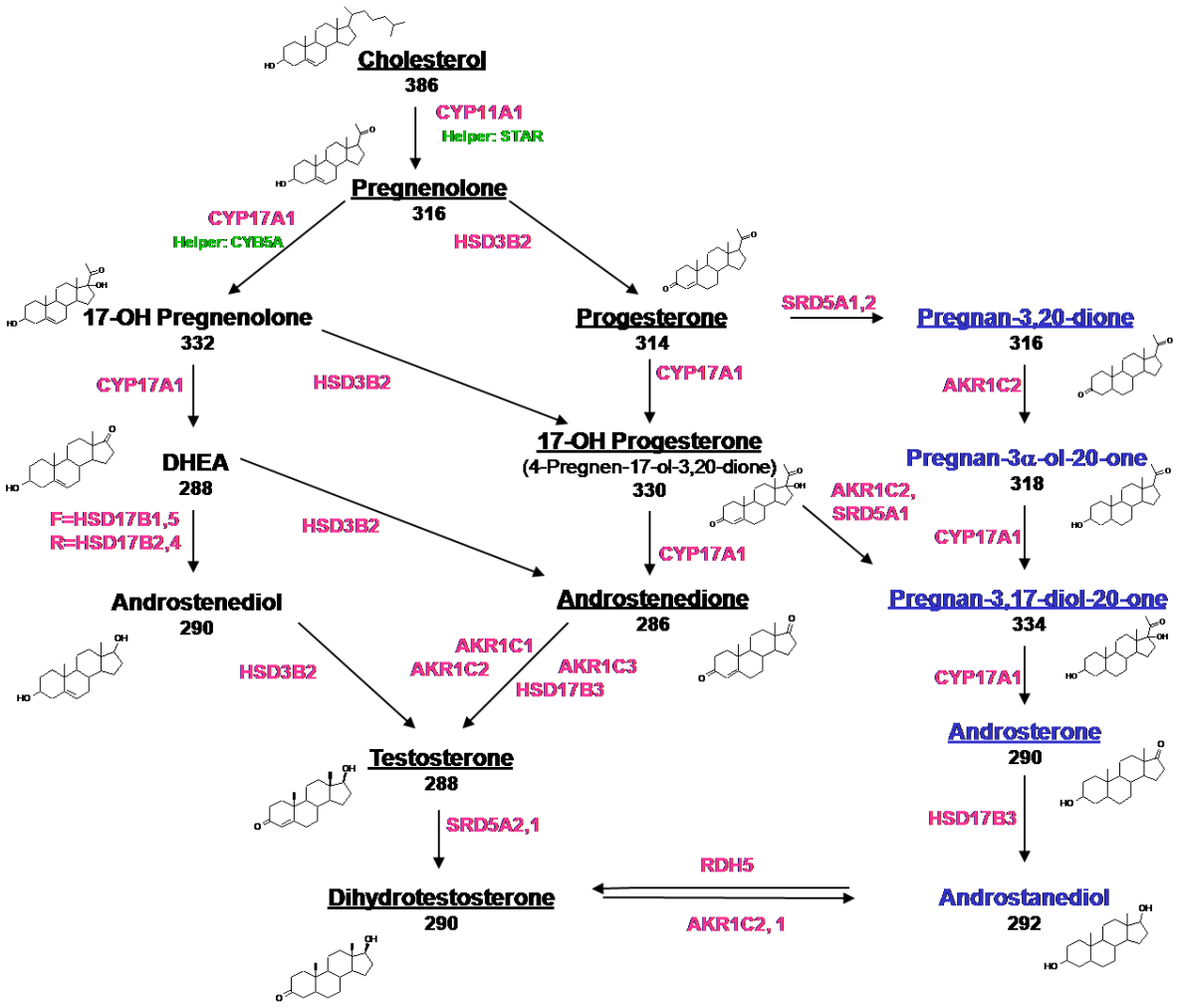

Figure 1.5: Steroidogenesis pathway. Depicted is the steroidogenesis pathway whereby cholesterol is converted to DHT via the pathways involving the steroidal intermediates and interlinked enzymatic reactions indicated. Steroids are portrayed in black (classical steroidogenesis pathway) and **blue** (backdoor steroidogenesis pathway) and enzymes are portrayed in **pink** and **green**. Some of the pathways are reversible while others are irreversible as indicated by direction of arrows. Figure modified from [44].

Androgen synthesis is initiated in the mitochondria of steroidogenic cells in organs such as the ovary, adrenal gland and testis [28, 45]. To begin, cholesterol is shuttled across the outer mitochondrial membrane to the inner mitochondrial membrane by the rate-limiting enzyme, StAR [38]. Within the mitochondrial space, cholesterol undergoes side-chain cleavage by CYP11A1 at the C-20 position, resulting in conversion to the steroid precursor, pregnenolone [33]. Pregnenolone in turn can be converted through a variety of pathways to produce DHT [30, 32]. Classically in the testis, pregnenolone undergoes a 17-hydroxylation

and subsequent 17,20-lyase reaction to produce dehydroepiandrosterone (DHEA). The enzyme responsible for this reaction is CYP17A1 [33] while required helper enzyme CYB5A modulates CYP17A1's lyase activity [40-42, 46]. The formed DHEA can then be converted to androstenediol through reduction of the 17-ketone group to the 17-hydroxy steroid by HSD17B1 or HSD17B5. HSD3B2 then transforms this steroid intermediate into testosterone by isomerizing its double bond from a C-5,6 to C-4,5 position [33].

The upstream precursor pregnenolone can also be converted into progesterone by HSD3B2. Classically, progesterone undergoes 17-hydroxylation and 17,20-lyase activity by CYP17A1 and CYB5A to form androstenedione. Androstenedione is subsequently converted to testosterone using a variety of reductases including ARK1C1, 2, 3 and HSD17B3; however, the most predominant enzyme utilized in this reaction is AKR1C3 [47-49]. Classically, these two biosynthetic pathways involving DHEA and progesterone have been responsible for testosterone synthesis in steroidogenic cells [30] and further conversion of testosterone into DHT takes place in the peripheral tissues [28].

Another steroidogenesis pathway for DHT synthesis was discovered in 2004 whereby progesterone can be converted through alternative steroid intermediates to DHT while bypassing T as an intermediate [31]. In this backdoor pathway, progesterone initially undergoes reduction of its double bond by SRD5A1 to form pregnan-3,20-dione which then undergoes reduction of its 3-ketone group by AKR1C2 to produce 3-hydroxylated pregnan-3α-ol-20-one or alternatively progesterone is converted to 17-OH progesterone by CYP17A1 hydroxylase and then undergoes reaction with SRD5A1 to produce pregnan-3,17-diol-20-one [50]. CYP17A1 then transitions pregnan-3α-ol-20-one via both hydroxylase and lyase action or pregnan-3,17-diol-20-one via only lyase action to androsterone which subsequently reacts with HSD17B3 to produce androstanediol. Androstanediol then undergoes a reversible reaction to form DHT via RDH5, an enzyme specific to the backdoor pathway [32]. The reversible reaction is catalyzed by aldo keto reductase enzymes, AKR1C2 and 1.

Many factors determine the predominant pathway utilized for androgen synthesis within a cell including species differences in enzyme structure and specificity, localization of enzymes within the cell and availability of substrate [37, 51] and therefore these differences can be predictive of a cell's ability to synthesize androgens via the different pathways.

1.1.5 Androgen action in the prostate (Figure 1.6)

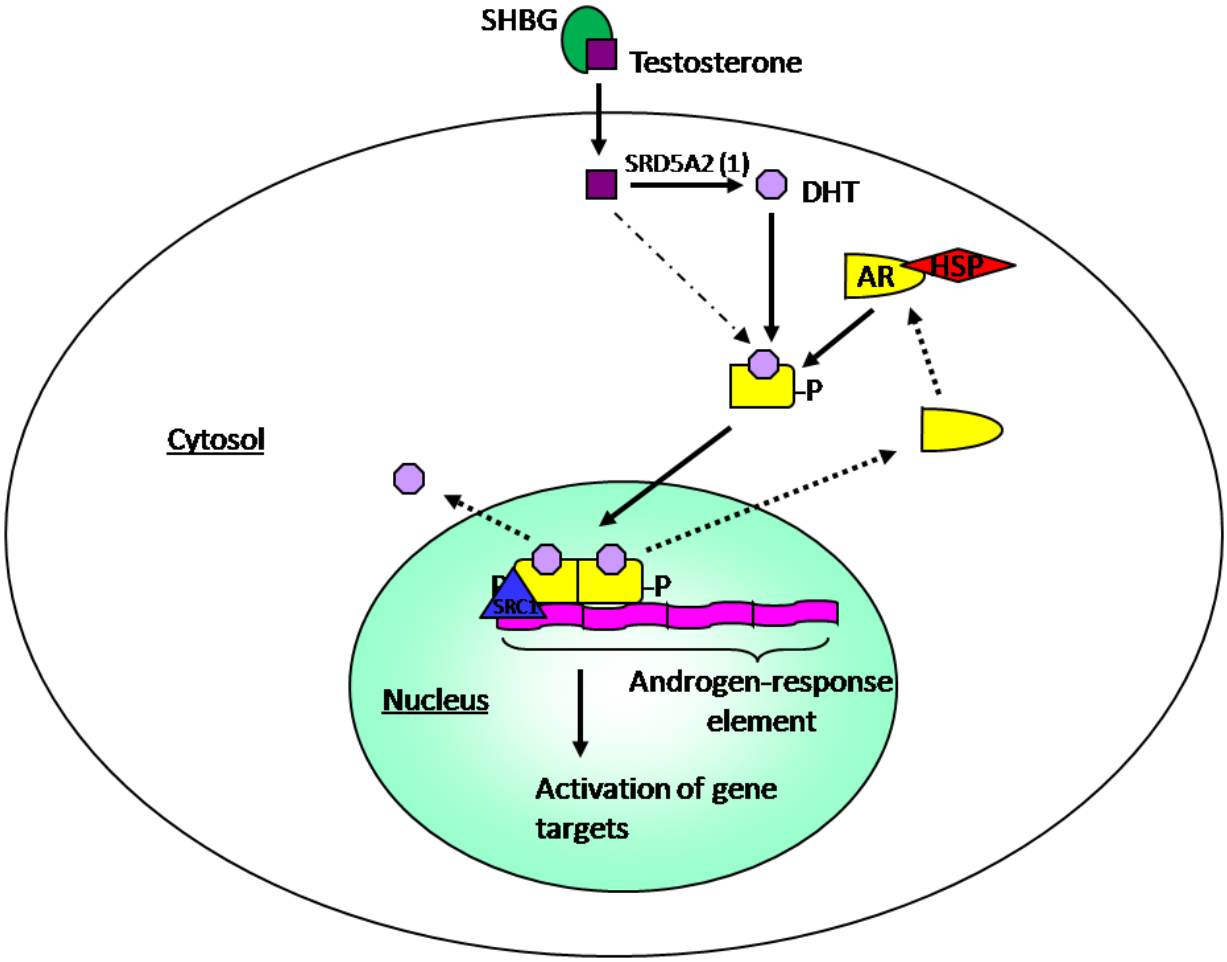

Figure 1.6: Mechanism of androgen action in a prostate cell. Depicted is the mechanism by which circulating testosterone leads to androgen receptor (AR) activation in a prostate cell. Figure modified from [26, 52].

T is normally bound to albumin (54%), sexual hormone binding globulin (SHBG, 44%) or megalin in the blood [26]. SHBG is hypothesized to deliver T to the prostate cell and mediate its transport across the cell membrane where its' free form can then undergo irreversible reaction with SRD5A2 (and SRD5A1 to a lesser extent) to produce DHT [53]. DHT, in turn, mediates its actions primarily through binding to its associated ligand activated nuclear receptor, the androgen receptor (AR), which serves to transactivate downstream target genes (**Figure 1.6**).

Unbound AR initially remains stabilized in the cytosol by chaperone heat-shock proteins such as HSP27, HSP70 and HSP90 [54-56]. Dissociation of heat-shock proteins in

the absence of ligand leads to degradation through an ubiquitine / proteosomal system [57]. However in the presence of DHT it binds AR in the cytosol, causing AR to undergo a conformational change that triggers dissociation of chaperone proteins, then phosphorylation and activation of the nuclear localization signal [26, 52]. These events ultimately contribute to AR translocation into the nucleus where the ligand-bound complex undergoes homo-dimerization and subsequent binding to the androgen response elements (ARE) of target genes [52, 58, 59]. ARE activation by AR in turn triggers the recruitment of coactivator proteins, histone acetylases and the RNA polymerase II complex as well as dissociation of co-repressor proteins, which in combination act to enhance the transcription of hundreds of AR-gene targets involved in regulation of the prostate cell cycle and the production of proteins necessary for survival, growth, proliferation and function of the prostate gland [52, 52, 60].

1.1.6 Androgen receptor

AR is a member of the steroid and nuclear receptor superfamily which also includes the estrogen, progesterone, mineralocorticoid and glucocorticoid receptors [61, 62]. All nuclear receptors regulate specific target genes in a tissue specific manner; the role that differentiates AR from other steroid receptors in humans is that AR-regulated genes are responsible for male sexual differentiation and pubertal changes [61].

The AR gene is located on the human X chromosome between the centromere and q13 and encodes a protein ~919 amino acids in length of about 110 kDa [61, 63]. AR protein consists of three functional domains and a small hinge region (**Figure 1.7a**).

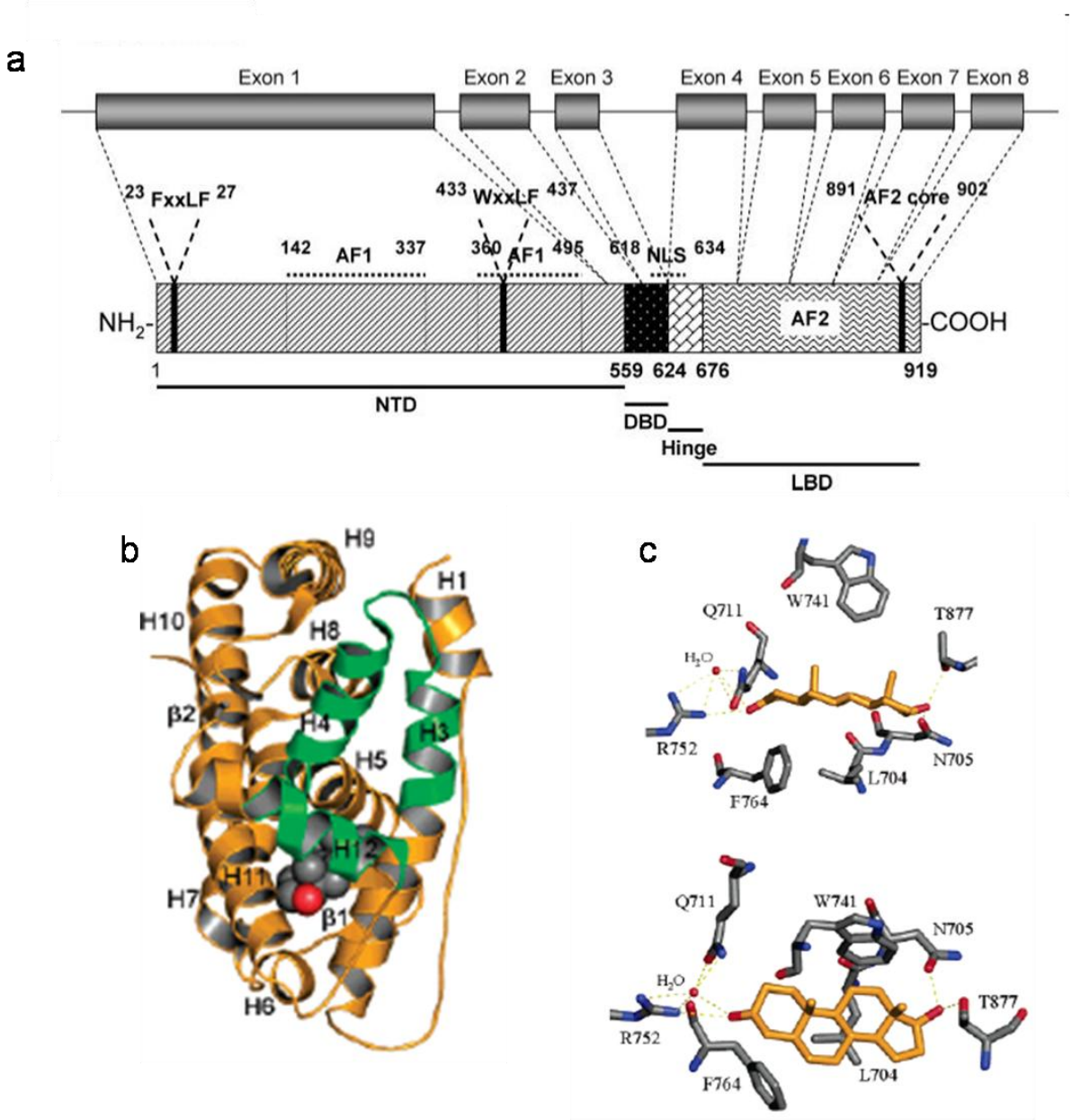

Figure 1.7: Androgen receptor. Depicted is the structure of AR gene (**a**), the structure of AR in its conformation with ligand DHT (**b**) and the interactions of AR LBD binding pocket with DHT from a top down and side point of view (**c**). Figure reproduced from [61] with permission.

The N-terminal domain (NTD), encoded by exon 1, contains activation function 1 (AF1), the conserved binding site of transcriptional regulators that serve to modulate AR activity [64]. The DNA-binding domain (DBD), encoded by exons 2 and 3, functions to bind AREs on target genes to activate or repress transcription [59]. The COOH-terminal domain (CTD) or ligand-binding domain (LBD), encoded by exons 4-8, is the site where androgens bind to the

receptor [59]. A nuclear localization signal (NLS) is present overlapping the DBD and hinge region and its function is to trigger translocation of AR into the nucleus upon ligand binding and phosphorylation [61]. Within the LBD exists an activation function 2 (AF2) site which functions in a ligand-dependent manner to act as a co-regulator binding surface [59] and a site adjacent to AF2 known as binding function 3 (BF3) has recently been identified and shown to allosterically affects AF2's binding to co-regulators [65]. When bound to AR, co-activators such as steroid receptor coactivators 1-3 (SRC1-3), cAMP response element-binding protein (CBP), E1A binding protein (p300) and androgen receptor-associated proteins 55,70 (ARA55,70) act to enhance gene transcription [66] while co-repressors such as silencing mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear receptor co-repressor (NCOR) act to repress gene transcription [67].

The AR's LBD contains eleven α-helices and two short β-turns which are arranged in three layers to form an antiparallel α-helical sandwich. The crystal structure of DHT in the LBD of AR was solved in 2001 by Sack *et al.* [68] (**Figure 1.7b**). In the LBD binding pocket hydrophobic interactions and hydrogen bonding account for ligand specificity [61]. In particular, the LBD allows for close hydrogen bonding of residues Q711 and R752 with the C-3 ketone group on ring A and N705 and T877 with C-17 hydroxyl group on ring C of DHT (**Figure 1.3 and Figure 1.7c**) whereas other steroidal structures like that of testosterone do not exhibit as strong hydrogen bonding in the LBD binding pocket [69].

1.2 Prostate cancer (CaP)

1.2.1 Development of CaP

What differentiates a cancer cell from a normal cell is its ability to grow uncontrollably and invade surrounding tissues, as well as its potential to spread to other distant organs [70]. A combination of events have to occur within a cell in order for it to become malignant; the cell must become self-sufficient in growth signals, insensitive to anti-growth signals, able to evade apoptosis, able to invade surrounding tissues, gain sustained vasculature and acquire limitless replication potential [70].

Within the stromal microenvironment an epithelial stem cell destined for differentiation into a basal or luminal secretory cell can instead be forced to undergo

differentiation into a cancerous cell. A disruption in the mesenchymal-epithelial equilibrium as mediated through changes in growth factors and proteases acting on the mesenchyme, appears to be largely responsible for the development of CaP [5, 71, 72]; however the manner in which these growth factors and proteases become altered remains unknown. Cunha *et al.* has shown that altered growth factors and proteases initially trigger a reduction in smooth muscle content followed by induction of fibroblast content in the stroma and upon AR activation in this abnormal stromal microenvironment paracrine stimulation of epithelial stem cells results in the onset of a malignant phenotype [11, 73]. This overall disequilibrium leads to changes in tissue architecture, tissue boundaries, angiogenesis and metastases; all characteristics of a malignant transition [70, 73]. One single epithelial stem cell can differentiate and metastasize into multiple heterogeneous cancer cells that combine to create a CaP tumor [12].

1.2.2 Epidemiology

Currently CaP is the leading form of cancer affecting Canadian men, resulting in 24,700 diagnoses each year and is the third leading cause of cancer death, with 4,300 deaths each year [74]. Despite improved screening techniques, CaP incidence has remained on the rise in Canada since 2001 and therefore a better understanding of disease development is warranted [74]. There are several predictive factors for CaP onset including age, ethnicity, environment, lifestyle and genetic predisposition [75-77].

Age plays an influential role in the development of CaP. Upon autopsy evaluation of 212 men for microscopic CaP it was determined that 0% of men between the ages of 30 and 39, 5.2% of men between the ages of 50 and 59 and 96.2% of men over the age of 80 had evidence of malignant growth in the prostate [78]. This and other studies demonstrate that the incidence of CaP is significantly proportional to increasing age [75, 78-81].

Ethnically, African-American men have the highest incidence of clinical CaP (137 per 100,000 per year) while Asian men have the lowest (19 per 100,000 per year) [76, 82]. An African-American man with the same stage and grade of disease as a Caucasian male has a significantly reduced chance of survival [76, 83]. The reason for this ethnical discrepancy remains unknown; complex confounding factors exist regarding not only biological

differences (genetic factors) but also lifestyle (diet, environment, culture) differences, challenging our ability to pinpoint the cause [82].

Demographic studies suggest that an African-American man is four times more likely to develop CaP than a native sub-Saharan African man of the same age [76]. Once again this can be attributed to a multitude of factors; possibly under diagnosis and / or lack of screening programs in sub-Saharan Africa, the impact of surrounding environmental factors, and / or perhaps the adoption of a western diet / lifestyle in America which has been previously linked to higher CaP incidence [76]. The latter suggestion is further supported by the statistic that in Asia where the average incidence is 40-fold lower than in North America, countries that are more westernized in terms of diet and lifestyle have a higher CaP incidence (Japan, followed by Singapore and Hong Kong) [76]. Furthermore, in a study conducted by Lee *et al.* it was discovered that a Korean man who migrated to the United States had a four-fold greater chance of developing CaP than if he remained in his native country; further demonstrating a correlation between changes in environmental, lifestyle and dietary factors and CaP onset [84, 85].

Through genetic based studies it has been demonstrated that regardless of other influential variables such as age, demography, diet and lifestyle there exists a strong correlation between CaP incidence and family history [86]; having a first-degree relative with CaP increases the risk of developing the disease two- to three-fold [77]. Since the mapping of the human genome in 2003 several studies have been conducted investigating the role of genetic predisposition in CaP. Emerging from these studies is an association between common germline variants (single nucleotide polymorphisms) on human chromosome 8q24 and CaP risk [87, 88]. Furthermore, several other genes associated with CaP susceptibility have been identified such as BRCA2, MSMB, LMTK2, KLK3, CYP17A1, AR and SRD5A2 [89-91]. Major advances in this growing field linking DNA germline mutations and CaP predisposition are widely anticipated alongside the development and exploitation of these discoveries as diagnostic tools in the future.

CaP incidence can not be attributed solely to one factor but instead a multitude of complex interacting factors. With the increasing number of genome-wide association studies being conducted, screening for genetic susceptibility of CaP may become a reality in the

clinic; however there are several other factors such as age, geography, environment, lifestyle, diet and socio-economic status that contribute to the onset of CaP and will likely complicate the predictive ability of these potential screens in the future.

1.2.3 Diagnosis

In North America, routine screening for the diagnosis of CaP involves a Prostate Specific Antigen blood test, a digital rectal examination and a biopsy [92]. Men over the age of fifty are subjected to a simple blood test in which levels of Prostate Specific Antigen (PSA) are measured. PSA, a member of the prostate specific kallikrein-related family, is produced specifically in the luminal secretory epithelial cells of the gland [16, 22]. PSA normally functions to proteolyse proteins responsible for ejaculate clotting and foster the liquification of fluid upon the emission of sperm [16, 22]. In a diseased prostate transcription of the PSA gene is increased and its protein detection in the serum can then serve as an effective prostate specific biomarker and progression marker of disease. Unfortunately, PSA has an inability to differentiate between an enlarged (benign prostatic hyperplasia- BPH) and malignant (CaP) prostate. However it remains the most useful biomarker and progression marker of CaP to-date. This is because screening of men for this PSA biomarker has been shown to be highly effective in detecting early CaP [22] while progression of CaP is monitored by PSA doubling time (time it takes for the PSA to double calculated as a rate) as this has been shown to predict overall survival of patients with late stage CaP [93].

In order to determine whether the prostate is enlarged or contains any abnormal cancerous nodules, a physician performs a digital rectal examination (DRE) by inserting their finger into the anus of the patient and feeling the general morphology of the prostate. Abnormal lumps are often indicative of CaP nodules [92]. Combining the results of the PSA test and DRE can help predict but not confirm the presence of cancer.

In a further diagnostic step, a biopsy will be carried out to confirm and categorize the grade of the cancer. Unlike the PSA test or DRE, a biopsy is capable of differentially diagnosing CaP from other conditions such as BPH that can also increase PSA levels. During a biopsy, a physician obtains 6-12 core samples of the prostate tissue and upon pathological evaluation stage is assessed using the Gleason grading system [94]. Under a high power microscope, a pathologist is able to assess the architectural pattern of each core

sample of the prostate tumor and assign a corresponding grade [92]. Grading varies from grade 1 (very well differentiated, almost normal) to grade 5 (poorly differentiated, most likely metastasized). After assessment of the two most prominent glandular patterns found in the biopsy specimens, a combined score is determined and defined as the overall Gleason grade (e.g. 4+3=7, or 3+4=7). This system is relatively effective in assessing the aggressiveness of the overall cancer as it takes into account tumor heterogeneity by using more than one core.

Currently, combined results from the PSA test, DRE and biopsy are used in common practice for diagnosing CaP in North America, however improvements in this system are always under investigation. Alternative or complementary biomarkers and progression markers of CaP are being investigated in order to assist in the differential diagnosis of benign versus malignant disease. Emerging potential biomarker candidates include kallikrein-related peptidase 2 (KLK2), early prostate cancer antigen (EPCA), prostate cancer gene 3 (PCA3), hepsin, prostate stem cell antigen, alpha-methylacyl-CoA racemase (AMACR) and TMPRSS2-ERG fusions [95, 96]. Circulating tumor cells have also recently emerged as an independent predictor of CaP progression and overall survival and are currently being evaluated for potential end point use in clinical trials [97-99]. Results from these studies may have significant implications in the accurate diagnosis of CaP as well as in the evaluation of disease progression on current therapeutic regimes.

1.2.4 Role of androgens and the AR in the development of CaP

In 1940, a role for androgens and AR in the pathogenesis of CaP was first alluded to by endocrinologists, Drs. Huggins and Hodges [100]. During initial studies evaluating semen secretion in dogs Huggins observed that estrogen treatment of these dogs led to significant shrinkage of the prostate gland [101]. At the time, Huggins did not know how estrogens induced this shrinkage but it was later discovered that the shrinkage occurred through inhibition of androgen production by the hypothalamus-pituitary-gonadal axis [102]. Further studies in dogs demonstrated that the removal of circulating androgens via castration also led to CaP tumor regression [101]. In transferring this knowledge to humans, Huggins and Hodges conducted bilateral orchiectomies (castration) on advanced CaP patients and observed a remarkable response in the regression of these late stage tumors [100]. Huggins

was awarded a Nobel Prize in Medicine for this work in 1966. Although at the time of Huggins and Hodges' discovery the mechanism by which androgen starvation of CaP cells resulted in tumor regression was unknown, it later became apparent that loss of AR signaling was central to this regression. In the absence of androgens, AR protein normally remains transcriptionally non-functional in the cytosol, resulting in the reduction of AR transcribed genes in the nucleus. Because androgen activation of AR is largely responsible for the induction of several survival, growth and proliferation cell cycle processes, under castration, androgen-sensitive cells undergo rapid apoptosis [103]. The early work conducted by Huggins and Hodges demonstrating a dependency of prostate cells on androgens and a regression in tumor size upon castration was the initial building block to all current knowledge regarding CaP development and progression today.

1.2.5 CaP stages

In North America clinical CaP is often categorized into three phases (androgen-dependent, regression and castration-resistant) as depicted in **Figure 1.8**. When CaP initially develops the majority of malignant cells depend on androgens for growth and survival [104]. This stage of the disease is appropriately termed "pre-castration" (pre-Cx) or "androgen-dependent" (AD) and is often the form detected by initial PSA, DRE and biopsy screening. Removal of testicular testosterone by castration (also known as androgen deprivation therapy- ADT) leads to tumor regression in most men but unfortunately over time (~2-4 years) the cancer recurs in a more aggressive and devastating "castration-resistant" (CRPC) phenotype. CRPC emergence is marked by re-ignition of serum PSA secretion despite low levels of testosterone remaining in circulation.

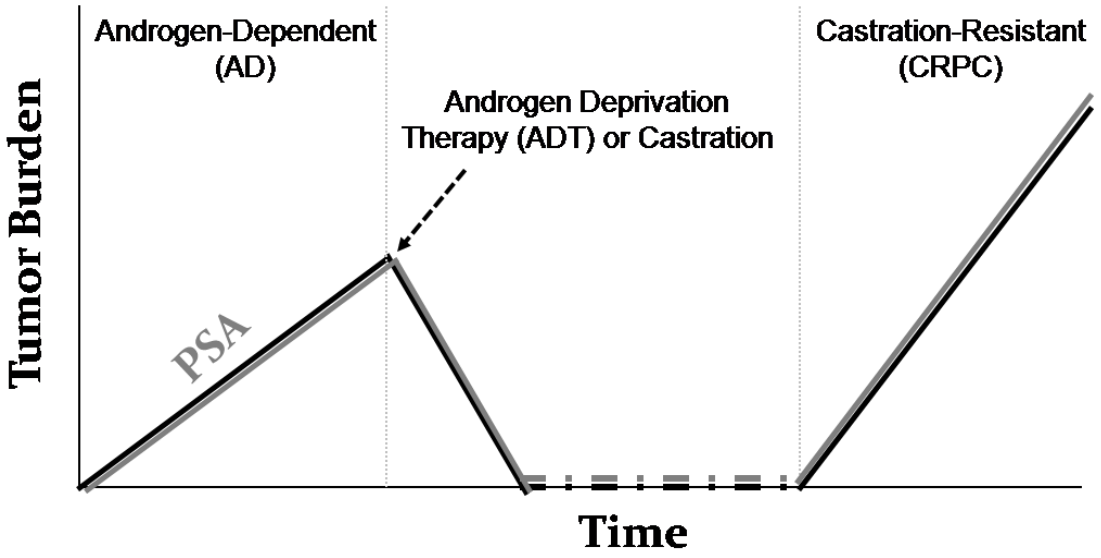

Figure 1.8: Schematic of clinical CaP progression. In CaP, tumor burden is monitored by serum levels of Prostate Specific Antigen.

1.2.6 Current treatments for CaP

There are several different treatment options for CaP patients, depending on the grade and stage of the disease at diagnosis, and the aggressiveness of the tumor after first-line treatment.

1.2.6.1 Localized therapies

Upon diagnosis, if the cancer is locally confined to the gland, then treatments such as radical prostatectomy, radiation therapy or active surveillance may be employed. Radical prostatectomy involves surgically removing the prostate and closely surrounding tissues [105]. Although this procedure has a high success rate of 76%-88% at 15-year progression-free survival in patients with low to immediate-risk disease, [106-108] its invasiveness has numerous associated side effects including immediate post-operative pain, long-term sexual dysfunction, urinary incontinence, gastrointestinal discomfort and emotional distress [109]. Another option for early presenting CaP is radiation therapy, which can be of two forms: external beam radiation therapy (EBRT) or brachytherapy. In patients with low to intermediate-risk disease, radiation therapy is often successful (70-90% at 10-year progression-free survival) [110, 111]. EBRT employs external beam radiation to the area of the prostate in short bursts repeated daily over an extensive time period, while brachytherapy involves the implantation of small radioactive seeds into the prostate that gradually release

radiation into the surrounding area for up to 6 months [112]. Like surgery, there are similar side effects associated with radiation therapy [109]. Active surveillance of the disease in its early stages is also practiced by physicians, often when the patient's age and health status predict that the side effects from surgery or radiation therapy would be more detrimental than the disease itself. Recently, active monitoring of disease progression using serial PSA tests, DRE and repeat biopsies has become more common in the practice of physicians [113]. This is because only 1 out of every 18 men treated for primary CaP have been shown benefit from the treatment and therefore it is hypothesized that by actively monitoring the disease and then treating accordingly, fewer men will undergo the unnecessary side effects of current over-treatments [113]. Nonetheless, surgery and radiation therapy are the only therapeutic modalities that have potential to be curative.

1.2.6.2 Systemic therapies

If the cancer at diagnosis has spread well beyond the gland or is no longer responsive to these localized therapies, then androgen deprivation therapy (ADT) is the most common treatment for patients. Since the efficacy of estrogen treatment and surgical removal of the testes (orchiectomy) was observed by Huggins and Hodges in the early 1940's [100], numerous other chemical methods of castration have been developed based on the same principle. Initially, it was discovered that by competitively binding the LHRH receptor in the pituitary with chemically derived agonists, downregulation of LHRH receptor would suppress release of LH into the bloodstream and the subsequent release of circulating testosterone from the testis (**Figure 1.4**) [114, 115]. Unfortunately, with this agonistic therapy an unanticipated initial surge of LH release caused a short-lived but significant surge in testosterone production [115]. Synthetically derived LHRH antagonists were then developed to avoid this unwanted surge whereby immediate inhibition of LH release was more optimally achieved [115, 116]. Agonists and antagonists based on this principle progressed into clinical trials resulting in the marketing of drugs including Lupron, Suprefact, Synarel, Supprelin, Suprelorin and Zoledex [117]. These less invasive chemical castration methods proved as effective as orchiectomy in treating CaP and have shown to increase the lifespan of a CaP patient by an average of 18 months [118, 119]. Treatment by means of chemical or surgical castration in CaP patients has become universally known as androgen deprivation therapy (ADT) and thus will be referred to as ADT from here on.

Because of the associated side effects with ADT including vasomotor flushing, anemia, metabolic changes, gynecomastia and bone density loss [109, 120] and the re-emergence of CaP after ~18 months, intermittent ADT was investigated as a potential treatment regime. The rationale behind this treatment is by replacing androgens on off cycles of ADT surviving tumor cells can be directed along a path of normal differentiation. In turn, the ability for these cells to undergo apoptosis might be restored, androgen dependence may be prolonged and progression to castration-resistance delayed [121]. This intermittent therapy has shown significant promise in prolonging time to CaP progression and reducing associated side effects; results from clinical trials are widely anticipated [122]. In spite of this, although the survival benefits of continual and intermittent ADT are well-established for non-localized androgen-dependent CaP they are not curative and other forms of treatment are needed.

Antiandrogens such as bicalutamide, flutamide and nilutamide were originally developed with the aim of reducing androgen triggered overgrowth of the prostate gland in both BPH and CaP disease [123-125]. Mechanistically, these nonsteroidal drugs act to competitively inhibit ligand binding to AR and inactivate the receptor in the cytosol [126]. Unfortunately, the binding potentials of bicalutamide, flutamide and nilutamide are much lower than DHT itself; therefore in the presence of high levels of endogenous androgens they are relatively ineffective as AR antagonists. However, when prescribed in combination with ADT (which already acts to reduce systemic androgen levels) the tumoral environment becomes optimal for these antagonists to effectively block residual androgen activation of AR. Combination of ADT with AR antagonists has proven to have an additional 8% reduction in mortality at 5 years as compared to ADT alone as reported in a meta-analysis of approximately 30 clinical trials [126]. Recent investigations have also discovered new antiandrogens with higher androgen competition binding potentials. MDV3100 (Medivation, Inc., San Francisco, CA) and BMS-641988 (Bristol-Myers Squibb, New York, NY) are small molecule antagonists of AR that are currently being investigated in Phase I-II clinical trials. *In vivo* studies investigating MDV3100 have proven it more effective than standard bicalutamide at reducing PSA levels and tumor size and the results of clinical trials are well anticipated [127].

1.2.6.3 Therapies for late-stage castration-resistant CaP

Although combinational ADT and AR antagonist therapies have been shown to increase the survival of patients with CaP by ~18 months, they still do not completely cure the disease. The cancer often recurs in a poorly differentiated, fast growing and metastatic manner. Metastatic CaP involves the spread of the disease from the prostate gland to other distant regions of the body, in particular the bone and lymph nodes [119, 128]. It is very difficult to treat, extremely painful for the patient and is typically associated with mortality from this disease. Currently, bisphosphonates are available to help improve pain associated with bone metastasis of the disease as they act to withstand osteoclast-mediated bone loss through the inhibition of bone resorption and preservation of bone density [129]. However, bisphosphonates do not improve overall survival of this form of the disease. In actual fact, very few chemotherapeutic agents are available for patients with castration-resistant metastatic disease that act to both prolong survival and improve quality of life. After the publication of two Phase III clinical trials demonstrating a 3.5-5.6 month prolonged survival on taxotere as compared to standard therapy it became the only established treatment for patients with castration-resistant metastatic CaP [130, 131]. Taxotere is an anti-mitotic agent and its efficacy works through stabilization of microtubules and inhibition of mitotic cell division [132]. As taxotere is now the only established treatment for patients with castration-resistant metastatic disease it is apparent that other drugs need to be developed. Several other candidates such as systemic satraplatin [133], epothilones (ixobepilane, patupilone) [134, 135], taxanes (XRP-6258) [136] as well as targeted inhibitors of cytoprotective chaperones (OGX-011, geldanamycin, tanespimycin) [137-139], vascular endothelial growth factor (sorafenib, sunitinib, cediranib) [140-142] and AR (abiraterone acetate, SAHA, LBH589) [143-146] are currently entering combinational clinical trials with taxotere. So far many of these candidates have shown promise in terms of prolonged survival of patients with castration-resistant metatastatic CaP however results from Phase III trials are still to come.

Only through understanding the molecular biology underlying disease progression have we been able to develop drugs such as the ones listed previously and by further probing these and other mechanisms by which CaP cells become resistant to castration can we

develop and optimize new, more effective drugs in the treatment of patients with castration-resistant disease.

1.3 Molecular basis for the development of castration-resistant prostate cancer (CRPC)

The re-emergence of disease after ADT remains a common feature of most cancers and is attributed to a variety of complex interrelated changes at the molecular level. Five potential mechanisms contributing to this recurrence were proposed by Feldman and Feldman in 2001 [58]: 1) selected outgrowth of pre-existing androgen-independent lurker CaP cells, 2) upregulation of anti-apoptotic survival pathways, 3) activation of alternative growth factor pathways, 4) increased hypersensitivity of AR to low levels of androgen and 5) increased mutations in AR leading to promiscuous activation by other ligands (**Figure 1.9**). These mechanisms have been built upon extensively in the literature since 2001 and will be discussed in the following sections.

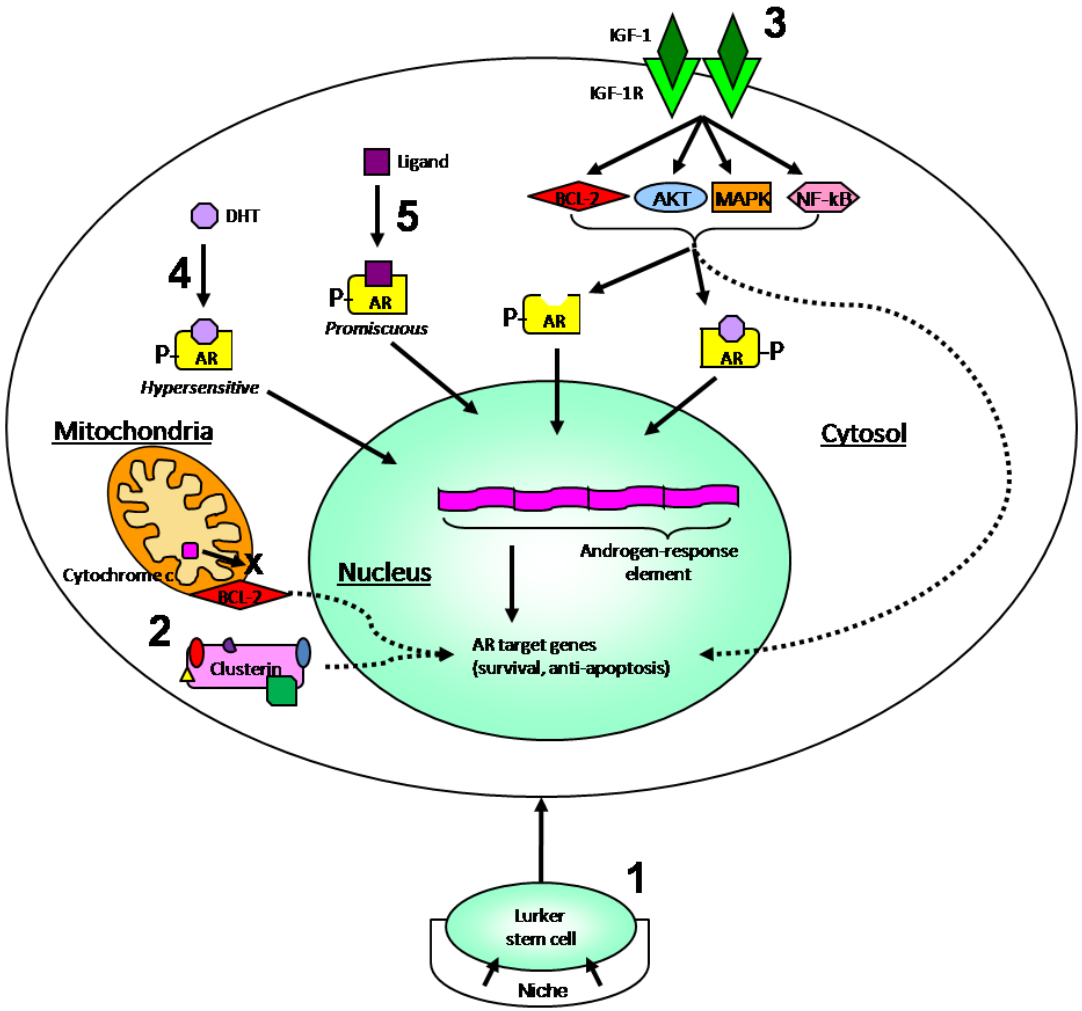

Figure 1.9 Schematic of molecular mechanisms contributing to castration-resistant disease. The detailed mechanisms are outlined below for 1) selected outgrowth of pre-existing androgen-independent lurker CaP cells, 2) upregulation of anti-apoptotic survival pathways, 3) activation of alternative growth factor pathways, 4) increased hypersensitivity of AR to low levels of androgen and 5) increased mutations in AR leading to promiscuous activation by other ligands. Figure modified from [58].

1.3.1 Selected outgrowth of pre-existing androgen-independent lurker CaP cells

The hypothesis of "clonal selection" whereby surviving androgen-independent cells make up the bulk of the resistant tumor was proposed by Isaacs and Coffey [71, 72, 147]. According to Isaacs and Coffey's "prostate stem cell" model, androgen-independent stem cells that remain in the basal epithelial cell layer after ADT-induced apoptosis of androgen-sensitive cells, begin to differentiate into a multitude of different CaP cells through interactions with their surrounding niche [147]. After this model was proposed Collins *et al.* demonstrated that human basal cells of the prostate could be transplanted into an immunodeficient mouse and regenerate into a phenotypically identical tumor [148]. Following this study Gu *et al.* and Patrawala *et al.* verified using different CaP models that human prostate stem cells exist and are capable of regeneration into CaP tumors *in vivo* [149, 150]. Combined these studies demonstrate the potential for a single prostate stem cell remaining after ADT to differentiate into a heterogeneous tumor as Isaacs and Coffey predicted. This mechanism whereby clonal selection allows for the outgrowth of underlying androgen-insensitive lurker stem cells is hypothesized to contribute to CRPC.

1.3.2 Upregulation of anti-apoptotic survival pathways

The cell cycle remains central to both the development and progression of CaP. Upon removal of systemic androgens by ADT, cell cycle regulators immediately become dysregulated leading to decreased proliferation and increased apoptosis. However a variety of anti-apoptotic survival proteins have been hypothesized to compensate for these changes in cell cycle regulation and continue to contribute to the progression of the disease. Several anti-apoptotic survival proteins have been shown to be upregulated after ADT including B cell lymphomal / leukemia gene 2 (BCL-2) [151, 152], clusterin [153, 154], heat shock protein-27 (HSP27) [155], nuclear factor kappa B (NF-kB) [156, 157], protein kinase B (AKT) [158, 159] and Y-box factor-1 (YB-1) [160]. BCL-2, a mitochondrial protein, has been shown to prevent apoptosis after ADT through the selective inhibition of toxic cytochrome c release from the mitochondria into the cytosolic space [161, 162]. Anti-apoptotic proteins, clusterin and HSP27, both function as cytoprotective chaperones binding and stabilizing apoptotic proteins in a time of cell stress (such as ADT) [155, 163, 164]. Knock-down of BCL-2 and clusterin by antisense oligonucleotides have been shown to enhance cytotoxicity of chemotherapeutic agents in numerous cancer cell lines and xenograft

models of progression to CRPC [137, 163, 165-167] while knock-down of HSP27 has been shown to induce apoptosis and enhance radiosensitivity in CaP cells [168, 169]. Furthermore, clinical evaluation of clusterin antisense oligonucleotide therapy is currently undergoing and so far trials have demonstrated promising tolerance and activity profiles in CRPC patients [137]. These reports, amongst other studies, display an ability of CaP cells to alter cell cycle regulation after ADT to induce anti-apoptotic survival pathways and also highlight the necessity for therapeutic targeting of these pathways in CaP progression to CRPC.

1.3.3 Activation of growth factor pathways

Growth factor pathways have also been implicated in treatment resistance. Autocrine, paracrine and endocrine stimulatory cell signaling pathways triggered by epidermal growth factor (EGF) [170, 171], insulin-like growth factor-1 (IGF-1) [172, 173], keratinocyte growth factor (KGF) [170], transforming growth factor beta (TGF-b) [174, 175] and vascular endothelial growth factor (VEGF) [176] are believed to contribute to CRPC progression. Furthermore, the mechanisms triggered by these growth factors are multifaceted including the activation of anti-apoptotic survival pathways independent of AR [58, 173, 177-180] and the enhancement or replacement of androgens for AR activation in an environment of low circulating androgens [170, 181-183]. Androgen-regulated IGF-1 signaling is important in cellular metabolism, differentiation, proliferation, transformation and apoptosis and therefore castration induces significant changes in these processes [173]. The levels of IGF-1 binding proteins insulin growth factor binding protein-2 and 5 (IGFBP-2,-5) increase while IGFBP-3 decrease immediately after castration, creating an environment optimal for IGF-1 to bind and activate its associated receptor (IGF-1R) [177, 184]. In turn, this leads to the activation of various anti-apoptotic survival pathways including AKT, BCL-2, MAPK and NF-kB and these are believed to occur independent of AR activation [173, 185, 186]. Enhancement of AR phosphorylation and migration of AR from the cytosol to the nucleus for gene transcription is also believed to be a repercussion of IGF-1 signaling [187]. Furthermore, knock-down of IGF-1 axis using siRNA, monoclonal antibodies, antisense oligonucleotides and small molecule drug inhibitors has been shown to sensitize CaP cells to conventional anticancer treatments [188, 189]. Increased expression of another growth factor, VEGF has been associated with increased vascularization and angiogenesis within the

tumor microenvironment as well as metastasis during progression of the disease [190, 191]. VEGF is a strong angiogenic cytokine that induces endothelial cell differentiation, migration and proliferation as well as vessel formation [192, 193]. Targeting of VEGF with monoclonal antibodies and small molecules inhibitors has been shown to slow tumor growth and metastasis in CaP models [194, 195] and current evaluation of VEGF inhibitors in clinical trials are demonstrating efficacy in patients with various recurrent cancers [140-142]. As exemplified, both IGF-1 and VEGF mediated mechanisms are complex and interlinked in various survival pathways in CaP cells emphasizing the importance of these growth factor pathways in disease resistance. A plethora of other growth factor mediated events also occur after castration and contribute to the development of the castration-resistant phenotype of the disease.

1.3.4 Increased hypersensitivity of AR to low levels of androgen

Although the expressions of various AR-transcribed genes are decreased by castration several studies have provided evidence of re-emergence of AR expression at CRPC [36, 196-199]. Immunohistochemical staining has shown similar expression of AR in CRPC and benign prostatic hypertrophic (BPH) tissues, verifying that AR is present at the protein level in CRPC tumors [196, 197]. Mostaghel *et al.* analyzed tissues obtained from patients pre- and 1 month post- ADT as well as after 6 months of treatment. This study verified AR's presence after ADT and also showed that ADT does not suppress AR protein expression, as previously hypothesized [200]. Furthermore, approximately 30% of CRPC tumors have an amplified AR gene suggesting that an increase in AR mRNA and protein expression could enhance ligand-occupied receptor and activity could occur in a low androgen environment [36, 197, 201-203]. Furthermore, alterations in AR co-regulators have also been shown to enhance the nuclear localization of AR and increase its overall androgen sensitivity [204]. In turn this increased AR sensitivity has been demonstrated in animal tumor progression models where the amount of DHT required for growth stimulation is four fold lower in CRPC cells as compared to early stage androgen-dependent cells [204, 205]. AR over-expression therefore appears to be a significant mediator of CaP progression to CRPC.

1.3.5 Increased mutations in AR leading to promiscuous activation by other ligands

It is also hypothesized that AR may undergo mutations to become more promiscuous and bind alternative ligands for activation in the absence of circulating androgens [202, 206-214]. Increased somatic AR mutations were discovered in metastatic samples whereas in primary tumors collected prior to localized therapy from the same patient few mutations were observed [206-208]. Particularly, mutations in T877A of AR have been frequent in CRPC and allow for AR to bind and be activated by precursor steroids such as progesterone and pregnenolone [68, 215]. In addition, patients who become resistant to flutamide or bicalutamide AR antagonist therapy often improve clinically when flutamide or bicalutamide are removed from their treatment regime [216]. This is likely because mutations in AR are selected for by these therapies enabling activation of AR agonistically by the antiandrogen treatments. AR promiscuous activity after ADT may also contribute to CRPC progression.

1.3.6 Role of AR in progression to CRPC

Progression after ADT was previously referred to as "androgen-independent" or "hormone-refractory" disease, however more recently this state has been given a new terminology: "castration-resistant prostate cancer" (CRPC). The shift in paradigm from one term to the other reflects the recent advances in the area investigating the underlying mechanisms of CaP progression and how the previous terms represent a basic misunderstanding of this progression. Accumulating evidence suggests that AR remains an important player in CaP progression as its re-emergence is a phenomenon common to most CRPCs. Not only is AR highly expressed in CRPC tumors in both its wild type and mutated forms but the transcription of many AR-regulated genes that are initially suppressed after ADT, become re-expressed in CRPC tumors [36, 217, 218]. Furthermore, knock-down of AR using shRNA, siRNA and various AR antagonists (casodex and flutamide) has been shown to eliminate or delay the progression of CaP after castration in various CaP models [123, 219-221]. Several studies have also documented AR interactions with the upregulated growth factor and anti-apoptotic survival pathways previously discussed (**sections 1.3.2 and 1.3.3**) in CRPC progression [54, 171, 187, 222] emphasizing AR's intricate and highly influencial role in disease progression to CRPC. Further investigation into the mechanisms

mediating AR re-activation after castration is warranted in order to better understand AR's role in disease progression and develop novel drugs targeting these mechanisms.

1.3.7 Potential role of ligand-mediated activation of AR in CRPC progression

Pertaining to this thesis, a role for ligand-dependent activation of AR in a castrate environment contributing to CRPC disease onset will be investigated. When Titus *et al*. measured the levels of T and DHT in tumors from patients with treatment resistant disease (as defined by rising PSA) using HPLC-MS and RIA techniques, they found the levels of T were similar to those in benign prostatic hyperplasia tissues despite observed castrate serum levels. Furthermore, the measured levels of T (3.75 pmol/g tissue) and DHT (1.25 pmol/g tissue) in these recurrent CaP tissues were high enough to activate AR, as determined by *in vitro* studies [223]. Various other groups including Nishiyama *et al.* measured DHT levels in prostate tissues and found similar results [224-227]. Nishiyama *et al.* measured DHT during different stages of ADT in the same patients and discovered that tissue DHT levels remained at ~25% of the amount measured before ADT while serum levels dropped to ~7.5% [225]. Based on these results showing that androgens remain in significant quantities within the tumor despite low levels measured in circulation, ligand-dependent activation of hypersensitive AR in the absence of testicular androgens emerged as a likely mechanism contributing to disease progression.

It was proposed by Labrie *et al.*, Mohler *et al.* and Stanbrough *et al.* that in the absence of testicular androgens an alternate source of native ligands for AR activation may come from precursor steroids from the adrenal gland [36, 116, 196, 223]. These groups hypothesized that precursor steroids such as DHEA formed in large quantities within the adrenal gland could migrate through circulation and be converted to androgens within the prostate when a testicular source of androgens was unavailable [36, 116, 196, 223]. In support of this "intracrinology" hypothesis, RNA, protein and *in situ* expression studies provided verification of conversion enzymes HSD3B2, HSD17B3, HSD17B5, AKR1C2, SRD5A1 and SRD5A2 in the prostate [36, 116, 228]. Metabolism studies in several CaP cell lines provided evidence supporting the ability of these cells to convert adrenal steroids to androgens [229, 230]. Furthermore, as described previously, better clinical responses were observed in patients undergoing combinational androgen blockade (ADT + antiandrogen) as

compared to ADT alone [126, 231]. This result was rationalized by the hypothesis that in the absence of testicular androgens the addition of an antiandrogen such as flutamide could block AR activation by the low levels of androgens produced from adrenal steroid precursors in the prostate [126, 231]. However, studies conducted in the 1970's combining orchiectomy and surgical removal of the adrenal gland (adrenalectomy) in the form of maximal androgen blockade (MAB) did not show significant advantages to orchiectomy alone and in almost all cases the disease relapsed [232, 233]. Moreover, circulating levels of precursor steroids (DHEA and androstenedione) did not appear to be affected by the removal of the adrenal glands in these patients [233]. These observations indicate that either 1) adrenal steroids are not contributing significantly to disease progression, or that 2) another alternative source of steroids are being capitalized on by the CaP cell to produce intracrine androgens.

From these studies investigating the role of intracrinology in disease progression we can postulate that CaP cells which have the appropriate steroidogenesis machinery, are capable of conversion of steroids to downstream androgens in the absence of a testicular androgen source.

1.4 Increased lipogenesis in CRPC progression and its potential role in androgen synthesis

For the past decade, increases in levels of serum PSA (or increases in PSA doubling time) after ADT have been used to monitor disease recurrence. PSA is an androgen-regulated target gene and thus its re-emergence after castration suggests that AR is likely re-activated during CaP progression and that other important androgen-regulated genes could also re-emerge during progression of the disease. In the interest of discovering and characterizing new androgen-regulated target genes involved in cell survival, growth and proliferation pathways Ettinger *et al.* from our lab, as well as others, performed large scale microarray analyses on various CaP tissue samples from patients displaying different stages of the disease. Amongst other important pathways, it was discovered that a subset of androgen-regulated genes involved in lipogenesis, or the production of lipids, were upregulated during progression to CRPC in both human data sets and animal models [60, 197, 217]. Follow-up Q-RT-PCR and immunohistochemistry studies confirmed that androgen-regulated transcription factors, sterol regulatory element binding proteins

(SREBPs), which are responsible for transcribing several lipogenesis enzymes, are increased in expression during progression to CRPC [217, 234]. The exact role of re-emerging SREBP and its corresponding lipogenesis gene targets at CRPC is explored in this thesis.

1.4.1 Sterol regulatory element binding proteins

SREBPs are central to the transcription of many genes involved in lipogenesis including the synthesis and metabolism of cholesterol and fatty acids [235, 236]. The regulation of SREBPs in normal lipogenic cells is depicted in **Figure 1.10**.

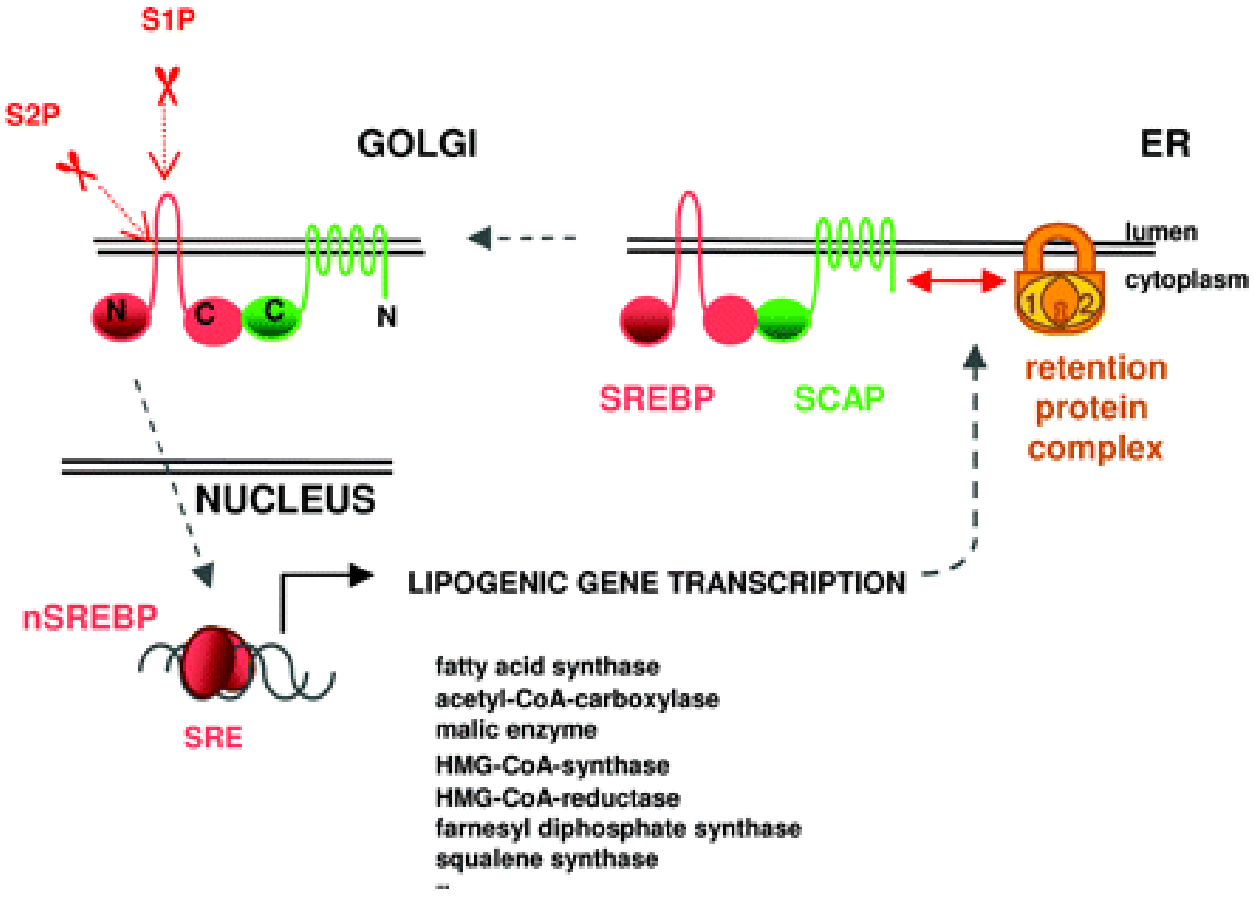

Figure 1.10: Activation mechanism of SREBPs. Schematic diagram portraying regulation of SREBPs by sterols in a normal cell. Figure reproduced from [237] with permission.

Mechanistically, in the presence of sterols SREBP is held inactive in the endoplasmic reticulum with scaffolding protein SREBP cleavage-activating protein (SCAP) and retention protein complex (INSIG). Upon sterol withdrawal the retention protein complex breaks apart and the SREBP-SCAP complex freely migrates to the Golgi and undergoes two protease reactions to produce a free N-terminal fragment of SREBP. This fragment translocates to the nucleus where it binds the sterol response element (SRE) and induces the transcription of numerous genes involved in lipogenesis [235, 237-239].

This process is classically regulated by the availability of sterols. In 2001 Heemers *et al.* documented that androgens, in fact, also play a role in SREBP regulation [240]. Swinnen *et al.* initially discovered that SREBP mediated enzyme acyl-CoA binding protein (ACBP) is actually an androgen-regulated target gene [241, 242] and through follow-up studies investigating the role of androgens in the lipogenesis they deduced that androgens activate SCAP, which in turn leads to the induction of SREBP and increased lipogenesis within the cell [235, 240, 243]. Concurrently, in our microarray investigations of the profiles of androgen-regulated genes during progression to CRPC we discovered that many genes involved in lipogenesis increase in expression at castration-resistance to levels observed prior to castration [217]. We demonstrated that SREBP expression and activity decrease immediately after castration as one would predict in a low androgen environment, but unpredictably SREBP expression and activity actually increase once again when the cancer reaches castration-resistance [217]. Furthermore, expression profiles of key SREBP downstream target genes; acyl Co-A binding protein (ACBP), fatty acid synthase (FASN), farnesyl diphosphate synthase (FDPS) and SREBP cleavage-activating protein (SCAP) followed a similar trend [217]. The observation that these lipogenesis genes, previously shown by Swinnen *et al.* to be androgen-regulated, are increased during the progression of CaP provided further evidence linking re-emergence of AR activation to castration-resistance. Furthermore, many of the genes discovered are known to regulate cholesterol and fatty acid production and therefore the role of these two lipid groups in CaP progression will be addressed in this thesis.

1.4.2 Cholesterol in CaP

Cholesterol, the molecular precursor of androgens, is normally synthesized through a series of step-wise reactions from acetyl-CoA (**Figure 1.11**) [244].

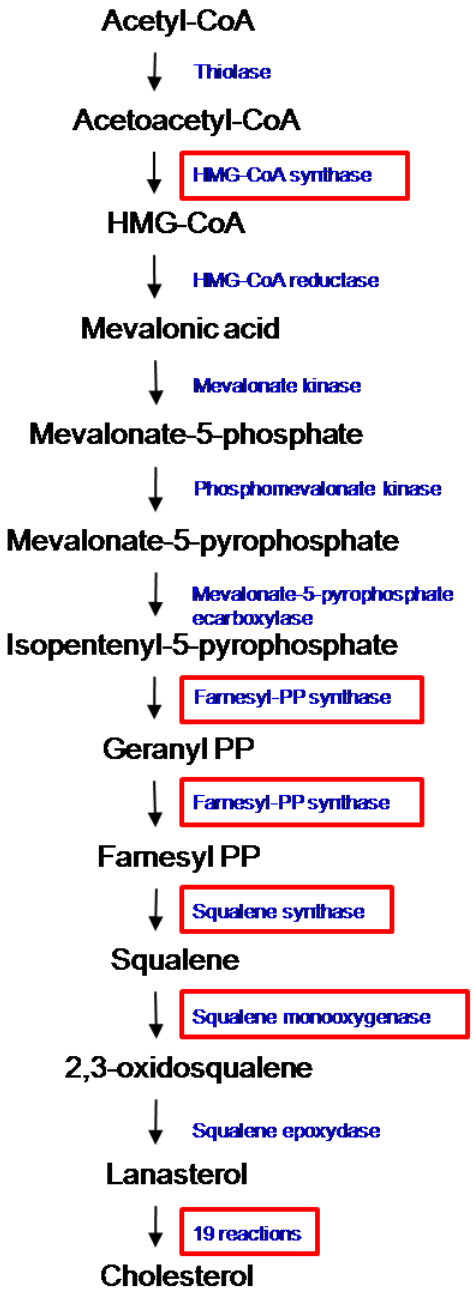

Figure 1.11: Cholesterol synthesis pathway. Depicted is the cholesterol synthesis pathway from an acetyl-CoA precursor. Intermediate molecules are portrayed in **black** while enzymes are portrayed in **blue**. Enzymes that re-emerge in expression during progression to CRPC are highlighted by **red** boxes. Figure modified from [244].

Cholesterol levels are tightly regulated within the cellular environment. Several inter-related enzymes are responsible for cholesterol regulation within the cell (**Figure 1.12**).

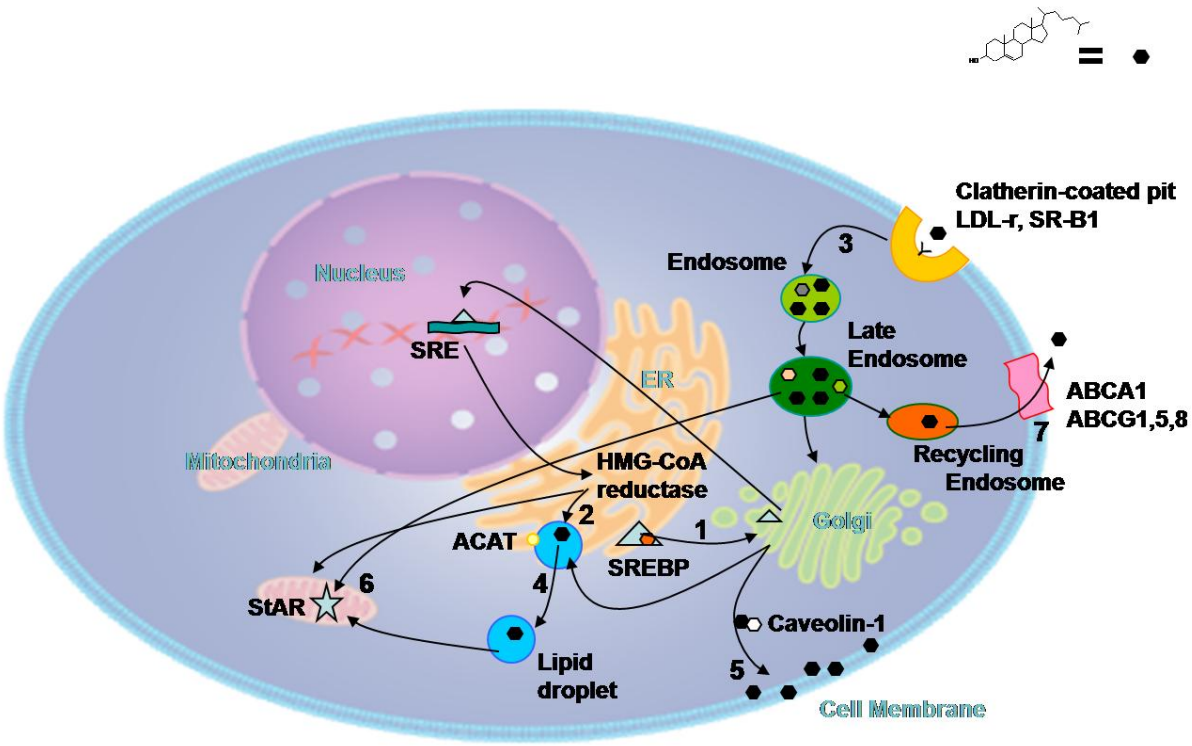

Figure 1.12: Schematic summary of cholesterol regulation processes in the cell. This figure was produced using Ingenuity® Systems, www.ingenuity.com. Briefly, 1) SREBPs are activated through several steps leading to the transcription of HMG-CoA reductase, 2) synthesis of cholesterol is regulated by this rate-limiting enzyme HMG-CoA reductase in the endoplasmic reticulum, 3) exogenous uptake of cholesterol occurs via LDL-r and SR-B1 in clatherin-coated pits, 4) ACAT is responsible for esterifying endogenous cholesterol into the cholesteryl ester form in storage droplets that can then be used by the cell to later: 5) make membrane via caveolin-1, 6) produce steroids in the mitochondria via enzyme steroidogenic acute regulatory protein (StAR) or 7) undergo efflux via ABCA1 and ABCG1,5,8.

Briefly, cholesterol can be obtained by the cell through two counterbalancing mechanisms: exogenous cholesterol can be taken in by the cell by receptor mediated transport (Scavenger receptor-B1 [SR-B1], low density lipoprotein-receptor [LDL-r]) in the form of high density lipoprotein and low density lipoprotein particles, or endogenous

cholesterol can be synthesized in the endoplasmic reticulum from precursors such as acetyl-CoA [245, 246]. Storage of cholesterol occurs in the form of non-toxic cholesteryl esters through a reaction mediated by an enzyme known as acyl-CoA:cholesterol acyltransferase [ACAT] [247]. When the cell requires free cholesterol for membrane formation or steroidogenesis cholesteryl esters can be cleaved by protein hormone sensitive lipase [HSL] [248, 249]. Furthermore, if the cell no longer requires cholesterol it can undergoe efflux from the cell by ATP binding cassette proteins [ABCA1, ABCG1,5,8] [250, 251].

Both the exogenous uptake and endogenous synthesis of cholesterol are controlled by SREBP transcription factors [234, 252, 253]. These counterbalancing processes are altered in many disease states such as heart disease, atherosclerosis and even CaP [234, 246]. In CaP, increased cholesterol has been implicated in many cell functions leading to the survival and proliferation of cells: membrane formation, endoplasmic reticulum stability, initiation of signal transduction events, inhibition of apoptosis and steroid formation [254]. The expression of many enzymes responsible for exogenous and endogenous cholesterol production are increased in CaP cells as compared to non-malignant prostate cells and even more so during progression to castration-resistance [197, 217, 234, 235, 255]. The rate-limiting enzyme involved in cholesterol synthesis is known as HMG-CoA reductase (**Figure 1.11**) and has classically been a target for therapy (using statins) in cholesterol related diseases such as atheroschlerosis and heart disease [256, 257]. Pertaining to CaP, studies evaluating the use of statins targeting HMG-CoA reductase and disease incidence have been conflicting [258, 259]. This may be due to the fact that the evaluated sample populations have abnormally high cholesterol levels relative to the general population, the post-hoc nature of most studies conducted or the potential lack of cholesterol's involvement in the onset of CaP. Few studies have actually investigated the role of cholesterol synthesis specifically in CaP progression. Holzbeierlein *et al.* analyzed samples from men with untreated primary CaP, ADT treated primary CaP, metastatic CaP and ADT resistant metastatic CaP and verified that during progression of the disease many of the enzymes in cholesterol synthesis (HMG-CoA synthase, squalene synthase, squalene monooxygenase and lanosterol synthase) were increased in expression [197]. We have also shown that farnesyl diphosphate synthase is increased in expression during progression to castration-resistance [217] suggesting that *de novo* cholesterol production in CaP cells is fundamental to the

progression of the disease. Only one clinical trial to-date has evaluated statins in CaP patients after castration and in this trial it was demonstrated that statin use was associated with a 50% reduction in the risk of developing CRPC, further predicting an important role for cholesterol in disease progression [260, 261]. Cholesterol is a multi-faceted molecule and therefore its role in disease progression may be complex and intertwined in several pathways. Evidence suggests that cholesterol is the central component of membrane lipid rafts, the site where a plethora of signaling events occur within the cell and therefore this is believed to be one of its functions in CRPC progression [254, 262, 263]. This thesis examines a novel hypothesis whereby during progression to castration-resistance the cells increase their ability to *de novo* synthesize and regulate cholesterol which in turn can be utilized to produce their own intracrine androgens in the absence of a testicular testosterone source.

1.4.3 Fatty acids in CaP

In non-malignant cells fatty acid synthesis is regulated by the availability of exogenous dietary fatty acids. However in diseased cells this regulatory mechanism is turned off and increased fatty acid synthesis occurs regardless of the presence of dietary fatty acids [255, 264]. Fatty acid synthase is the sole enzyme responsible for endogenous fatty acid synthesis in mammals and is composed of six subunits that work together in an assembly line to produce various long chain saturated fatty acids from an acetyl-CoA substrate as well as precursors for unsaturated fatty acids (**Figure 1.13**).

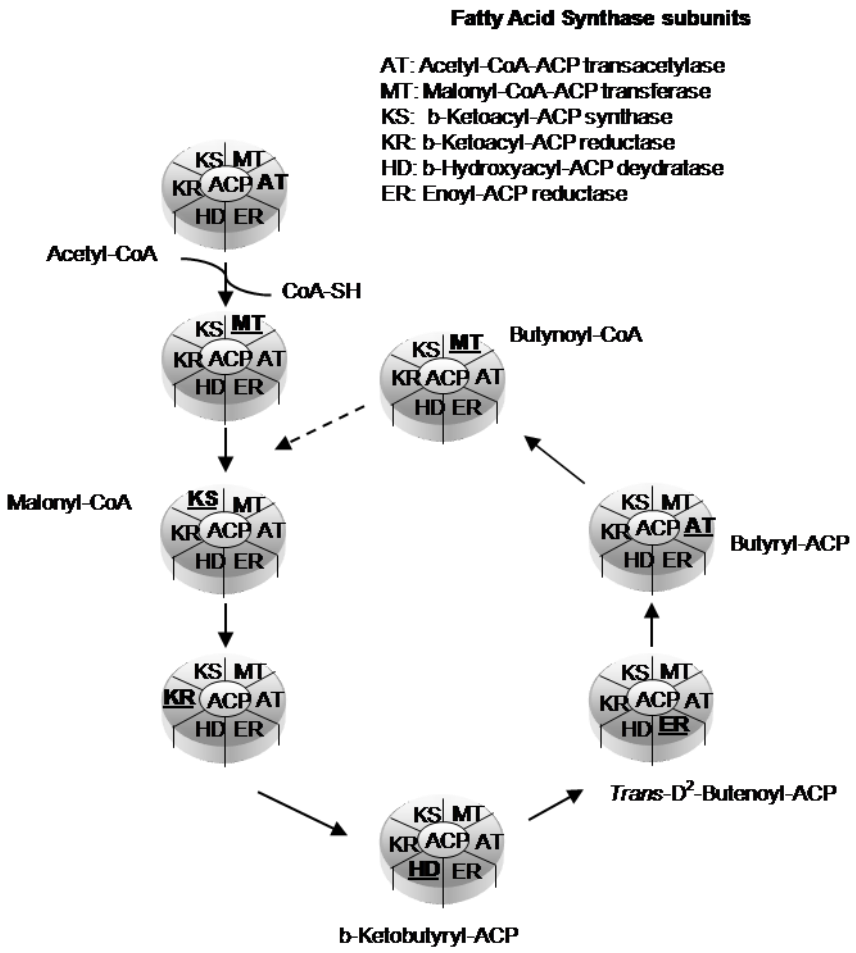

Figure 1.13: Fatty acid synthesis pathway. Depicted is the pathway in which endogenous fatty acids are synthesized by fatty acid synthase (FASN). FASN is composed of six subunits that act sequentially to convert an acetyl-CoA precursor molecule into downstream fatty acids. Figure modified from [265].

In most cancers including that of the prostate, FASN expression is increased during progression of the disease and associated with poor clinical outcome [264, 266-268]. In animal models of CaP progression FASN inhibitors C75 and Cerulenin delay the progression of the disease suggesting that endogenous fatty acid synthesis is crucial for CaP cell survival, growth and proliferation leading to castration-resistance [269-271]. Fatty acids are precursor molecules for several lipids (more complex fatty acids, triglycerides, phospholipids and cholesteryl esters) that have important regulatory functions within the cell and may be important in the development of CRPC disease [272, 273]. For example, fatty acid arachidonic acid has been shown to be metabolized to trigger several anti-apoptotic survival networks after ADT including activation of interleukin-6 (IL-6) and signal transducers and

activator of transcription-3 (STAT-3) as well as induction of AKT phosphorylation [274]. Furthermore, arachidonic acid has also been shown to trigger androgen synthesis within adrenal and testicular steroidogenic cells [275-278]. Inferring from this we suggest that in CaP cells after ADT arachidonic acid may stimulate androgen synthesis for subsequent AR activation and this may be one mechanism contributing to CRPC progression.

1.5 Evidence suggesting *de novo* androgen synthesis as a potential mechanism contributing to CRPC progression

1.5.1 Potential role for fatty acids and cholesterol in *de novo* androgen synthesis in CaP cells

We have shown that farnesyl diphosphate and acyl-CoA binding protein (ACBP), androgen-regulated enzymes known to induce androgen synthesis via cholesterol and fatty acid signaling in steroidogenic cells, respectively, are increased in expression during progression to castration-resistance [217]. Coincidently, through a pathway mediated by fatty acid uptake into the mitochondria via peripheral-type benzodiazepine receptor (PBR) and acyl-CoA binding protein (ACBP), arachidonic acid has been shown to trigger transcription and activation of StAR (section **1.1.4.1**, the rate-limiting step in steroid synthesis) in ovarian, testicular and adrenal steroidogenic cells [275-278]. Inferring from this data we suggest that the observed increase in fatty acid and cholesterol synthesis in CaP cells during progression to castration-resistance may contribute substrate for and trigger activation of *de novo* androgen synthesis within the tumor microenvironment. Work in this thesis describes how this novel mechanism in CaP cells might provide intracellular androgens for AR activation after castration and potentially contribute to the observed pathology and progression of the disease.

1.5.2 Indirect evidence of steroidogenesis enzymes involvement in androgen synthesis during CRPC progression

Further support for *de novo* androgen synthesis in CaP has emerged from studies investigating the hypothesis that adrenal steroids are used by the prostate to produce androgens in an environment deprived of circulating androgens [36, 116, 196, 223]. Stanbrough *et al.* conducted a comparison study of the genes altered in 33 bone metastatic CRPC tissues as compared to 22 laser capture-microdissected primary CaPs [36]. They

discovered that many androgen-regulated genes are in fact decreased in the bone metastatic samples; however they also documented that many androgen-regulated genes in fact increased in the same sample set. In particular, enzymes responsible for steroid synthesis (HSD3B2, AKR1C3, SRD5A1, AKR1C2 and ACR1C1) were in this list of increased gene targets [36]. Recently, Montgomery *et al*. measured T and DHT levels and the expression of enzymes responsible for their synthesis in benign prostatatic tissue, untreated primary CaP and metastases from CRPC patients [266]. In the CRPC samples enzymes FASN, CYP17A1, HSD3B1, HSD17B3 and CYP19A1 were up-regulated in coordination with higher levels of T as compared to the primary CaP samples [266]. The results from these two microarray studies in CaP patient cohorts are quite provocative in that they link increased expression of many enzymes involved in conversion of precursor steroids to downstream androgens at castration-resistance to sufficient amounts of intratumoral androgens for AR activation during progression to CRPC.

Several other *in vitro* mechanistic studies [39, 49, 279-284] as well as drug clinical trials [127, 144, 280, 285, 286] rationalized on this intracrinology adrenal steroid hypothesis have also provided indirect evidence implicating specific steroidogenesis enzymes in the potential *de novo* androgen synthesis mechanism contributing to CaP progression [36, 116, 196, 223].

In many studies the expression of rate-limiting steroidogenesis enzyme CYP17A1 (**section 1.1.4.1, 1.1.4.2**) is increased during progression to castration-resistance and thus it has become a central target for drug development in the field [144, 287, 288]. Stigliano *et al.* measured the RNA expression of CYP17A1 in 60 prostatic tumor samples and showed that this enzyme's expression is correlated with high grade CaP and the appearance of relapse within 24 months [39]. Montgomery *et al.*'s and Stanbrough *et al.*'s microarray studies also confirmed an increase in CYP17A1 RNA expression in late stage metastatic samples as compared to pre-castration samples [36, 266]. The results of these studies spurred the drug industry to produce novel targets of this rate-limiting enzyme in steroidogenesis in an aim to initially reduce the ability of CaP cells to convert adrenal steroid precursors to downstream androgens. Ketoconazole, an azole antifungal agent which exerts its clinical effects through inhibiting cytochrome P450s including CYP17A1 had undergone extensive study in clinical

trials. Small *et al.* showed that ketoconazole treatment in 132 CRPC patients led to a 27% PSA response rate (significant decrease in PSA levels) as compared to 11% in the ketoconazole null group [285]. Furthermore, they verified that downstream steroid products of CYP17A1 action (DHEA and androstenedione) were reduced in the patients' serum by this treatment [285]. However, no improvement in overall survival was observed. A specific CYP17A1 hydroxlase and lyase inhibitor which is twenty times more potent than ketoconazole known as abiraterone acetate was subsequently developed by Cougar Biotechnology Inc. and was recently studied in Phase I and II clinical trials [144]. In these trials abiraterone acetate was better tolerated than the less specific ketoconazole and it triggered declines in PSA of greater than 30%, 50% and 90% in 14, 12 and 6 patients (out of 21 patients total), respectively, with responses lasting up to 578 days [144]. Phase III clinical trials are currently underway in Europe and North America to determine whether this drug will prolong the average lifespan of CRPC patients. Targeting of CYP17A1 inhibition after ADT has undoubtedly been successful through the reduction in levels of downstream steroids produced within the tumor further suggesting a role for *de novo* androgen synthesis in clinical CaP progression.

SRD5A2 is the predominant isoform of 5α-reductase expressed in the normal prostate. However, during transition of normal prostate through precursor prostatic intraepithelial neoplasia (PIN) and finally CaP development, tissue microarray studies have shown that SRD5A2 expression decreases and SRD5A1 expression increases (**section 1.1.4.1, 1.1.4.2**) [279-281]. In cell culture studies Luu-The *et al.* confirmed this phenomenon demonstrating that SRD5A1 was more highly expressed in CRPC cell lines than androgen-dependent cell lines [30]. Interestingly, conversion of steroids through the backdoor pathway described in **section 1.1.4.2** has been shown to predominantly occur via SRD5A1, while conversion of steroid precursors to T and DHT is classically thought to occur via both SRD5A1 and 2 [31], suggesting that the observed increases in SRD5A1 may lead to preferential androgen synthesis via the backdoor pathway. Furthermore, a new isoform SRD5A3 has recently been discovered to be overexpressed in CRPC tissues and its functional role has yet to be deciphered [289].

Inhibitors of 5α-reductase have also been developed and tested in CaP clinical trials. Finasteride, a synthetic antiandrogen and inhibitor of SRD5A2, has proven effective in reducing the incidence of CaP by 25% in a large randomized prevention trial (n=18,882) known as the Prostate Cancer Prevention Trial [127, 280]. Following the development of finasteride, another candidate known as dutasteride which inhibits both SRD5A1 and 2 and is much more potent than finasteride, was introduced by GlaxoSmithKline Inc. and soon after entered clinical trials [280, 286]. By effectively blocking the conversion of residual T to DHT and potentially the backdoor pathway conversion of upstream steroids to androgens as well, these drugs have already shown efficacy in treating CaP and thus also support the role of *de novo* synthesized androgens within the tumor microenvironment.

Changes in many other steroidogenesis enzymes during progression **(section 1.1.4.1, 1.1.4.2)** of the disease have been documented suggesting that during transition to CRPC CaP cells adapt to alter enzymes to potentially begin to make their own androgens. Harkonen *et al.* discovered a significant decrease in the oxidative activity of 17BHSD and increase in reductive activity in a castration-resistant subclone of LNCaP cells as compared to the original androgen-dependent cell line [282]. In particular, HSD17B2 gene expression was decreased signifying that alterations in this enzyme function occur in CaP cells during progression to CRPC ultimately altering their ability to metabolize steroids to androgens [282]. Bauman *et al.* compared the enzymatic profiles of normal prostate, BPH and human prostate adenocarcinoma (or CaP) and discovered remarkable differences in enzymatic expression and activity [49]. Their results suggested a reduction in androgen catabolism from DHT in the adenocarcinoma cells as they had significantly lower levels of AKR1C1,2 and 3 compared to normal epithelial cells. Furthermore, in the adenocarcinoma cells higher expression of RDH5 was observed suggesting a unique ability for these cells to produce large amounts of DHT from androstanediol in the backdoor pathway and enhance its actions through blockade of catabolism (**section 1.1.4.2**). Follow-up studies by Ji *et al.* where they traced the metabolism of radiolabeled DHT in CaP cells, have confirmed that DHT catabolism via AKR1C2 is decreased [283] and further demonstrated a molecular switch between AKR1C1 and AKR1C2 signaling which consequently contributes to androgen signaling within the cell. Ma *et al.* also showed that steroidogenesis enzymes can even undergo changes under selective pressures to function as other enzymes in the pathway

[284]. They showed that AKR1C2, which catalyzes the inactivation of DHT, to be converted to AKR1C1 through the replacement of five loops in the mature steroid binding pocket of the enzymatic structure [284]. This study represents an example of how steroid specificity can be changed at the enzyme level and demonstrates the ability of cells to alter their own androgen synthesis pathways.

Within the field of CaP and androgen synthesis, up until recently, emphasis has been placed on ability of CaP cells to convert adrenal steroids to downstream androgens. The idea that *de novo* androgen synthesis (or synthesis from cholesterol precursors within the tumor itself) might occur in progressing CaP cells and is linked with cholesterol shuttling into the mitochondria has not been rigorously explored. The prostatic expression of StAR, the enzyme responsible for this rate-limiting step involving cholesterol, has never been documented in the literature to our knowledge. Stigliano *et al*. has reported that metastatic lymph node 64 (MLN64), which is a protein with strong homology to StAR, is overexpressed in high grade CaP tumors [39]. StAR accounts for approximately 90% of all steroid synthesis within the body as determined by efficiency of cholesterol uptake assays and MLN64 has been proposed to account for the remaining 10%, however this has yet to be verified [38, 39]. This data suggests a potential role for rate-limiting cholesterol shuttling into the mitochondria of the CaP cells for *de novo* androgen synthesis to occur but to date little investigation on StAR has been conducted in CaP.

Overall, these studies characterizing steroidogenesis enzymes in CaP cells and the evaluation of drugs targeting them in CaP populations have yielded provocative results prompting this investigation of *de novo* androgen synthesis as a central mechanism underlying AR activation in CaP progression to castration-resistance.

1.6 Scope of thesis

1.6.1 Hypotheses

Castration-resistant prostate cancer progression is in part due to the *de novo* synthesis of androgens within the tumor environment,

Endogenous cholesterol metabolism processes contribute substrate for this *de novo* androgen synthesis,

Specific fatty acid production is responsible for triggering *de novo* androgen synthesis from cholesterol.

1.6.2 Rationale and specific aims

Increased lipogenesis is a global phenomenon associated with CaP progression. The androgen-regulated transcription factors (Sterol regulatory element binding proteins-SREBPs) responsible for inducing this lipogenesis have previously been shown to be dysregulated during progression to CRPC. Expression of fatty acid synthase (FASN), HMG-CoA synthase and farnesyl diphosphate synthase, key enzymes in SREBP mediated lipogenesis pathways, are also increased during progression to CRPC. The sole products of FASN, fatty acids, have been shown to induce androgen synthesis from cholesterol in steroidogenic cells and many of the enzymes responsible for cholesterol synthesis and cholesterol conversion to androgens are present, altered and increased in expression in CRPC tumors.

The AR remains an important mediator of disease progression as its activation leads to the induction of several networks involved in cell survival, growth and proliferation. Since intratumoral androgen levels remain high enough to trigger AR activation after castration, it is hypothesized that the observed increase in lipogenesis enzymes may provide the necessary substrate and activation step for *de novo* androgen synthesis within the local environment of the tumor, ultimately contributing to treatment resistance and progression to CRPC.

In the subsequent chapters we aim to test the above hypothesis by following these specific aims:

1) **Determine if CRPC tumors obtained using the LNCaP xenograft model**
    a. **Express the necessary enzymes for androgen synthesis,**
    b. **Are capable of *de novo* androgen synthesis from radioactively labeled precursors.**
2) **Using the LNCaP xenograft model**
    a. **Compare the ability of tumors from different stages of the disease to *de novo* synthesize their own androgens,**
    b. **Investigate the effect of current inhibitors (ketoconazole, finasteride, cinnamic acid, casodex, RU-486) on *de novo* androgen synthesis.**
3) **Using the LNCaP xenograft model characterize processes that can provide cholesterol substrate for *de novo* androgen synthesis**
    a. **Determine if tumor cholesterol levels change during progression of the disease,**
    b. **Decipher profiles of key proteins responsible for upstream cholesterol regulation (SR-B1, LDL-r, HMG-CoA reductase, ACAT1,2, ABCA1) during progression of the disease.**
4) **Investigate the role of fatty acids in triggering *de novo* androgen synthesis**
    a. **Decipher which fatty acids are predominantly produced in CaP cells in an androgen regulated manner,**
    b. **Determine if key proteins involved in fatty acid activation of androgen synthesis (ACBP, ACSL3, HSL, StAR) are altered during progression of the disease so that they trigger cholesterol conversion into downstream androgens in a steroid deprived environment.**

In addressing the *first* aim of this thesis we demonstrate using Q-RT-PCR and Western blot analysis that castration-resistant prostate cancer (CRPC) tumors obtained from the LNCaP xenograft model express all of the necessary enzymes for DHT synthesis (**Chapter 2**). We further demonstrate that these tumor cells are capable of *de novo* androgen synthesis in sufficient quantities to activate AR from a radioactively labeled cholesterol precursor [^{14}C-acetate] as detected by LC-radiometric detection (**Chapter 2**). Using LC-MS we also discovered that progesterone levels are elevated in tumors immediately after castration (but not in the serum) and infer that progesterone is an important steroidal mediator of DHT synthesis. Using radiotracing experiments coupled to LC-radiometric detection / MS we confirmed that progesterone is metabolized by CRPC cells to DHT through both the classical and backdoor pathways (section **1.1.4.2**). This work entitled, "Androgen Levels Increase by Intratumoral *De novo* Steroidogenesis during Progression of Castration-Resistant Prostate Cancer", (Cancer Research 2008 Aug 1; 68:6407-6415), supports the role of ligand mediated AR activation in CaP progression through *de novo* androgen synthesis.

In **Chapter 3** we address the *second* aim of this project and investigate the use of current inhibitors of steroidogenesis (targets SRD5A2, CYP17A1 and AR) on the ability of CRPC tumors to synthesize androgens through the classical and backdoor pathways (section **1.1.4.2**). Using radioactively labeled progesterone as a precursor and LC-radiometric detetion / MS as an endpoint we determine that inhibitors finasteride (SRD5A2) and ketoconazole (CYP17A1) reduce androgen synthesis within the tumoral environment, however do not eliminate it altogether. In the presence of steroidogenesis inhibitors the CaP cells adapt to divert classical steroidogenesis by using alternative pathways to synthesize androgens; thereby continuing to induce AR signaling (which we verify by PSA measurement using a kit). We also compare the ability of tumors at different stages of the disease to convert radioactively labeled progesterone into downstream steroids as determined by LC-radiometric detection / MS and demonstrate that tumors at CRPC are more efficient than tumors immediately after castration at *de novo* synthesizing androgens. In this chapter we demonstrate the ability of CaP cells to avoid castration induced apoptosis as well as apoptosis induced by steroidogenesis inhibitors through the continued synthesis of androgens for AR activation in the absence of circulating exogenous androgens. This work has been

submitted for publication and is entitled, "Steroidogenesis inhibitors alter but do not eliminate androgen synthesis mechanisms during progression to castration-resistance in LNCaP prostate xenografts".

Because CaP cells synthesize androgens even in the presence of steroidogenesis inhibitors we changed our focus to decipher some of the upstream lipogenesis mechanisms of *de novo* androgen synthesis in CRPC progression in order to better understand how the CaP cells initially adapt to ADT or castration to induce *de novo* androgen synthesis as well as address the *third* and *fourth* aims of this project.

In **Chapter 4** of this thesis we aim to investigate some of the mechanisms regulating *de novo* androgen synthesis from the accumulation of cholesterol, as it is the main precursor for androgens. Specifically in the first part, we show using Western blot analysis and radioactive microsomal activity assays that cholesterol synthesis and metabolism are differentially regulated in androgen-sensitive (LNCaP) and androgen-insensitive (PC-3) cell lines. Production of cholesterol stores (cholesteryl esters) by the enzymes HMG-CoA reductase and Acyl-Coenzyme A cholesterol acyltransferase is confirmed to be under the regulation of androgens in LNCaP cells at the RNA, protein and activity levels. It is postulated that the resulting cholesteryl ester stores are an intracellular source of cholesterol sequestered for subsequent steroid formation in the absence of testicular androgens or immediately after ADT. The initial work in this chapter supports the important role of androgen-regulated cholesterol pathways in CaP cells and suggests how they may contribute supply for *de novo* androgen synthesis after castration. In efforts to understand how processes are altered in the tumor to provide cholesterol for *de novo* androgen synthesis we sought to characterize and compare all of the enzymes involved in cholesterol regulation in tumors at different stages of the disease (second part). By Western blot analysis of LNCaP xenograft tumors at different stages of the disease we demonstrate that the protein expressions of key enzymes involved in the cholesterol influx, synthesis and metabolism increase and how this combined process contribute to an overall increase in the accumulation of cholesterol (*de novo* synthesized and in the form of cholesteryl esters) within the tumor cell. Furthermore, using a radiometric tracing approach we observe that *de novo* synthesized cholesterol levels are increased at CRPC and using a cholesterol measurement kit we observe

a decrease in cholesteryl ester levels after castration leading to CRPC suggesting that both break down of this storage product and *de novo* production of cholesterol for subsequent *de novo* androgen synthesis are triggered at castration. We further relate these changes in cholesterol regulation to increasing production of androgens in the tumor at CRPC by LC-MS. Parts of this work entitled, "Alterations in cholesterol regulatory processes contribute to *de novo* androgen synthesis in prostate cancer tumors during progression to castration-resistance" have been submitted for publication, while the remainder has already been published (Prostate 2008 Jan 1; 68:20-33).

In **Chapter 5** of this thesis we aim to explore and combine the concepts of *de novo* synthesized fatty acids and cholesterol in the *de novo* synthesis of androgens in CRPC cells. We show a novel mechanism by which the specific endogenously synthesized fatty acid arachidonic acid is involved in regulating a rate-limiting steroidogenesis enzyme, StAR as determined by Western blot analysis of subcellular fractions of LNCaP cells and fluorescence tagging cell culture experiments. The results suggest that arachidonic acid plays a significant role in mediating *de novo* androgen synthesis from cholesterol in CRPC tumor cells. This work has been submitted for publication entitled, "Arachidonic acid activation of intratumoral steroid synthesis during prostate cancer progression to castration-resistance," and helps demonstrate how upstream pathways involving cholesterol and fatty acid synthesis which were previously investigated in **Chapters 4** of this thesis, combine to initiate androgen synthesis.

In the last chapter (**Chapter 6**) of this thesis we aim to corroborate work shared in previous chapters and provide relevance and future directions that may 1) help better understand how *de novo* androgen synthesis contributes to disease progression to CRPC and 2) develop / integrate the use of drugs that could potentially delay or eliminate progression of CaP to castration-resistance which is mediated through this mechanism.

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CHAPTER 2: Androgen levels increase by intratumoral *de novo* steroidogenesis during progression of castration-resistant prostate cancer[1]

2.1 Introduction

Within the prostate, androgens act to regulate gene networks that promote cell survival through the androgen receptor (AR), a ligand responsive transcription factor. Androgen deprivation therapy (ADT) triggers apoptotic regression of both benign and malignant prostate epithelial cells. Over 80% of patients achieve symptomatic and objective responses following ADT and serum Prostate Specific Antigen (PSA) levels decrease in almost all patients [1]. However, some cancer cells survive and proliferate in this androgen-depleted environment and consequently culminate in an "androgen independent" (AI), or "castrate-resistant" prostate cancer (CRPC) phenotype [2]. Mechanisms underlying this tumor growth during CRPC have been attributed to a complex network of processes including clonal selection [3], adaptive upregulation of anti-apoptotic and survival gene networks [4-6], cytoprotective chaperones [7, 8], and alternative mitogenic growth factor pathways [9-12]. Almost uniformly, progression following an initial response to castration involves the reactivation of androgen regulated processes, as illustrated by sentinel upregulation of PSA, a discretely androgen regulated gene [13]. With the use of genome wide expression profiling, evidence is mounting that in fact most androgen gene networks are reactivated in CRPC progression [14, 15]. Two contending hypotheses stand to explain these observations: AR is aberrantly activated by signaling pathways or upregulation of AR co-activators in the absence of androgens [16-18] or that androgen regulated pathways within CaP cells are activated by alternative sources of androgenic steroids [19-21].

Many reports address the former with a variety of suspect proteins and signaling pathways implicated [2, 22]. The latter has also been investigated, primarily with the suggestion that adrenal androgens provide a systemic source of androgens to be utilized by prostate cancer (CaP) tumors [20, 23]. Mechanisms involving androgen production from an alternative source have been investigated in many trials evaluating maximal androgen

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blockade (MAB), which includes ketoconazole and/or antiandrogens to block adrenal steroidogenesis in addition to medical or surgical castration [24]. Results from these trials suggest that MAB leads to prolonged survival after 5 years but observed adverse side effects and decreased quality of life warrant further investigation of these secondary hormonal therapies [24, 25]. Work in this area pioneered by Liu *et al.* and Labrie was reignited by Titus *et al.* who used liquid chromatography-mass spectrometry (LC-MS) to document levels of androgens, testosterone (T) and dihydrotestosterone (DHT) in prostate tissues obtained from radical prostatectomy and recurrent prostate cancer patients who had "castrate" levels of serum androgens [26-28]. We and others [14, 15, 29, 30] found that androgen-dependent genes become constitutively re-expressed in the absence of testicular androgens during CRPC progression and that down-regulation of AR expression following castration with siRNA can suppress CaP tumor growth [29, 30]. Furthermore, many enzymes responsible for steroid synthesis have been observed to be upregulated leading to the potential reactivation of AR [23]. These data suggest that CRPC progression may not be entirely independent of androgen-driven activity of AR, but in fact non-testicular sources of androgens are being capitalized upon for AR activation. Furthermore, we hypothesize that androgens driving CRPC progression are synthesized *de novo* within the CaP tumors, and in fact may be increased by utilizing a "feed forward" biosynthesis pathway as many of the enzymes are increased by androgens. In the prostate, androgens upregulate the expression of Sterol Response Element-Binding Protein (SREBP), a transcription factor that coordinately regulates cholesterol and fatty acid synthesis [15, 31]. Androgens also stimulate the expression of Acyl-CoA-binding protein (ACBP) in prostate cells which provides a rate limiting step for steroidogenesis by facilitating the transport of cholesterol into the mitochondria for conversion to steroids [32]. We have previously shown that the downstream genes coordinately regulated by SREBP are reactivated during disease progression [15] thus *de novo* synthesis of steroids in CaP cells may provide a plausible rationale for CRPC progression in the absence of testicular androgens.

Following these observations, we tested the hypothesis that this lipogenesis pathway beginning at acetic acid, is converted to cholesterol and further metabolized to androgens in prostate tumor cells through a series of well-characterized step-wise enzymatic events. Androgen synthesis is often described in terms of the classical steroidogenic pathway

through DHEA and T (**Figure 2.1**) however, more recently, a "backdoor pathway" has been described as an alternative synthesis pathway which utilizes progesterone as the primary steroidal precursor of DHT, bypassing T as an intermediate [33].

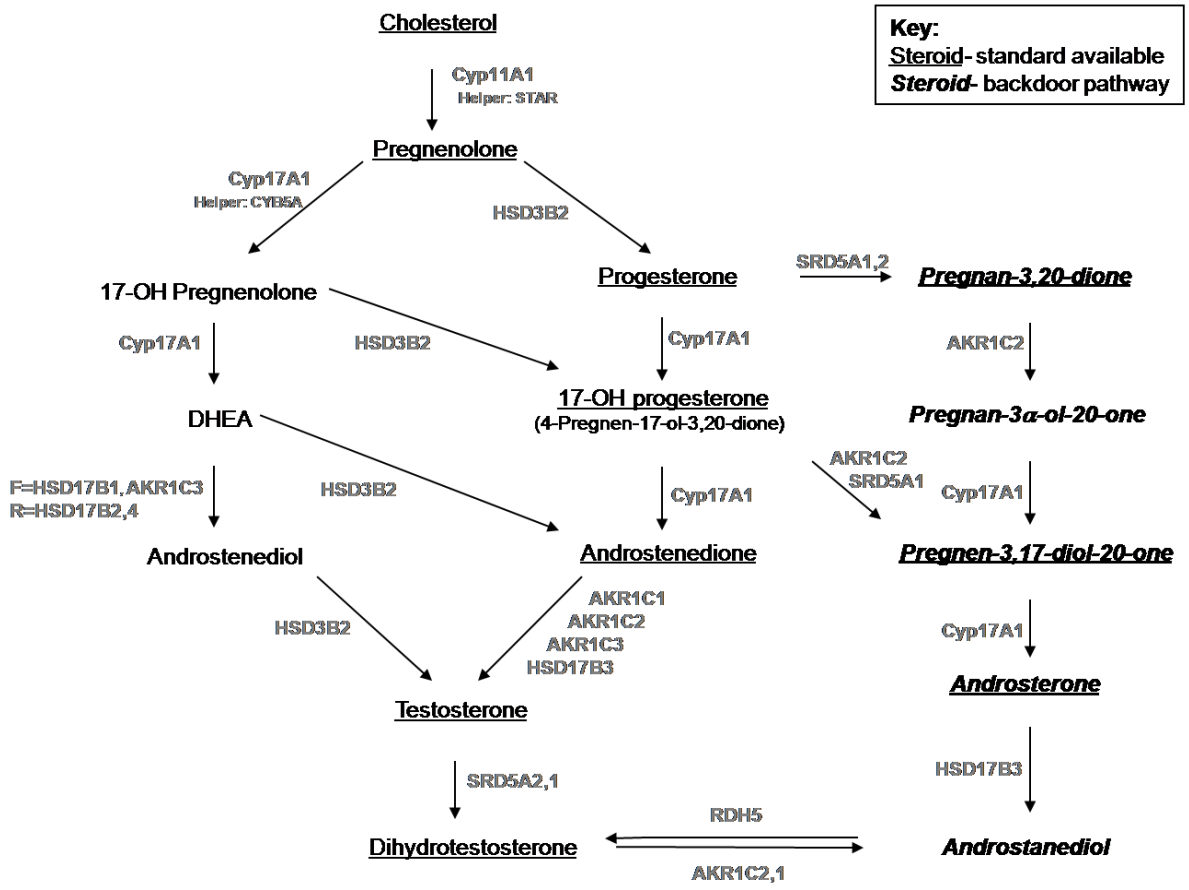


Figure 2.1: Steroidogenesis pathway. Schematic outline of the pathway from cholesterol to DHT, including the "backdoor" pathway (shown in bold italics) [33]. Standards were used for analysis of the underlined steroids.

Following the establishment of LNCaP xenografts in intact male mice, mice were castrated and tumors were followed through CRPC progression as defined by the re-expression of PSA [34]. In this report we demonstrate that androgen levels within CRPC tumors are sufficient for AR activation while corresponding serum androgens remain low in mice after castration [26-28]. These tumors also produce relatively high concentrations of progesterone as compared to downstream androgens, T and DHT. As adrenal cells are unable to synthesize progesterone without exogenous substrate addition [35] and these CPRC tumors express the necessary enzymes for progesterone and androgen synthesis at both the RNA and protein

levels, we conducted further analytical assessments using metabolic radio-tracing combined with tandem LC-MS and confirmed that CRPC tumors are indeed capable of *de novo* synthesis of androgenic steroids.

2.2 Materials and methods

Materials

[1,2,4,5,6,7-^{3}H (N)]-DHT (110.0Ci/mmol, PerkinElmer Life Sciences, Inc., Wellesley, MA), [1,2,6,7-^{3}H (N)]-Progesterone (90.0Ci/mmol, PerkinElmer Life and Analytical Sciences, Wellesley, MA) and [1(2)-^{14}C]-Acetic Acid, sodium salt (55.0 mCi/mmol, Amersham Biosciences, Baie d'Urfe, Quebec, Canada) were used for *in vitro* and *ex vivo* incubations and radiometric standards. Stock solutions of testosterone-16,16,17-d3 (deuterated testosterone) (CDN Isotopes, Pointe-Claire, Quebec, Canada), 4-androstene-3,17-dione (Sigma, Oakville, Ontario, Canada), 4-pregnen-17-ol-3,20-dione (Steraloids, Inc., Newport, Rhode Island), 5α-androstan-17β-ol-3-one (Sigma, Oakville, Ontario, Canada), dihydrotestosterone (DHT) (Sigma, Oakville, Ontario, Canada), 5β-pregnan-3α-27-diol-20-one (Steraloids, Inc., Newport, Rhode Island), 5β-pregnan-3,20-dione (Steraloids, Inc., Newport, Rhode Island), androsterone (Aldrich, Oakville, Ontario, Canada), cortisol (Sigma), pregnenolone (Sigma, Oakville, Ontario, Canada), progesterone (Sigma, Oakville, Ontario, Canada), R1881 (Dupont, Boston, MA) and testosterone (Sigma, Oakville, Ontario, Canada) were prepared in 100% methanol as mass spectrometry standards.

In vitro Model: LNCaP Cells

LNCaP cells (passage 40-48; American Type Culture Collection, Rockville, MD) were cultured in RPMI-1640 (without phenol red) with L-Glutamin, PS and 5% Charcoal Stripped Serum (CSS, Hyclone, Logan, UT).

In vivo Model: LNCaP Tumor Progression to Castrate-resistance

All animal experimentation was conducted in accord with accepted standards of the UBC Committee on Animal Care. LNCaP xenograft tumors were grown in athymic nude mice at four sites as modified from a previously reported method [15]. PSA levels were measured by tail vein sera samples weekly using an immunoassay kit (ClinPro, Union City, CA). At 6 weeks post inoculation mice were castrated. Tumors were harvested from the same mouse (39 mice total) pre-castration (PSA pre-Cx), 8 days post-castration (PSA nadir) and 35 days post-castration (PSA CRPC) as previously conducted [15]. Tumors were

excised, dissected and fragments were either immediately frozen in liquid nitrogen or placed in phenol red-free RPMI-1640 media supplemented with 5% CSS.

Tumor Homogenization

Frozen tumors were homogenized using a PowerGen 125 homogenizer for 35 seconds in buffer (20mM EDTA, 20mM NaCl and 20mM Tris) on ice. 0.1ng of internal standard (deuterated testosterone) was added to each homogenate prior to extraction.

Steroid Extraction

Supernatants, pellets, sera and homogenates were extracted twice with ethyl acetate (EtOAc) (v:v, 1:1) and dried down using a Centrivap™ centrifugal evaporation system (35°C). Samples were then reconstituted in 100µL of 50% methanol.

Steroid Analysis by Liquid Chromatography-Mass Spectrometry (LC-MS)

A Waters 2695 Separations Module coupled to a Waters Quattro Micro was used for LC-MS analysis. All MS data was collected in electrospray ionization positive (ESI+) mode with capillary voltage at 3kV, source and desolvation temperatures of 120ºC and 350ºC respectively and N_2 gas flow of 450 L/hr. Shorter chromatographic separations were carried out using a Waters Exterra 2.1x50mm 3.5µm C18 column equilibrated with 20:80 ACN:H_2O, ramped to 80:20 ACN:H_2O from 0.5-8.0 min, further to 95:5 from 8.0-9.0 min and returned to 80:20 ACN:H_2O from 10.0-10.5 min with a total run time of 15 min. Flow rate was 0.3 mL/min, column temperature 35ºC and 0.05% formic acid was present throughout the run. MS scan data for metabolite identification was collected using both 22V and 35V for CV. Extracted ion chromatograms from extracted samples of radiolabeled alone (H) versus radiolabeled plus non-radiolabeled (cold) progesterone (H+C) spiked incubations were compared and LC retention time alignments were used to identify potential metabolites. Thus precursor ions unique to the H+C sample fractions collected by high performance-LC (HPLC) radiometric detection could be selected for further collision induced dissociation (CID) at both 11V and 22V CE for positive identification.

PCR and Western Blot Analysis of Cells and Tumors

Steroidogenic enzyme RNA quantification in tumor samples was assayed by Q-RT-PCR with the following primers: HSD3B2 5'-cgggcccaactcctacaag-3' (F) and 5'-ttttccagaggctcttcttcgt-3' (R), CYP17A1 5'-gggcggcctcaaatgg-3' (F) and 5'-cagcgaaggcgaaggcgataccctta-3' (R), CYP11A1 5'-agttctcgggacttcgtcagt-3' (F) and 5'-

ggagcccgccttcttga-3' (R), HSD17B2 5'-tttgccggagttttgaatgaa-3' (F) and 5'-gcaggttcttcgcaattcct-3' (R), HSD17B3 5'-tgggacagtgggcagtga-3' (F) and 5'-cgagtacgctttcccaattcc-3' (R), HSD17B5 5'-tgggaggccatggagaag-3' (F) and 5'-tttgacaccccaatggacttg-3' (R), RDH5 5'-gcccgccagcaatgc-3' (F) and 5'-cgcccaaagcctgagtca-3' (R), SRD5A1 5'-acgggcatcggtgcttaat-3' (F) and 5'-ccaacagtggcataggctttc-3' (R), AKR1C2 5'-gggaggccatggagaagtg-3' (F) and 5'-gttggacaccccgatgga-3' (R), AKR1C1 5'-ggaggccgtggagaagtgta-3' (F) and 5'-gttggacaccccgatgga-3' (R), CYB5 5'-cacccgcttcctcaacga-3' (F) and 5'-accagcttgttccagcagaac-3' (R), STAR 5'-gcccatggagaggctctatg-3' (F) and 5'-ttccactcccccattgctt-3' (R). Reactions were conducted with 0.4µL of RT-PCR reaction cDNA, 0.4µL each of forward and reverse primers (10µM), 3,8µL ddH_2O, 5µL Invitrogen Platinum SYBR Green qPCR Supermix-UDG with Rox. Triplicates of samples were run on the default settings of the ABI Real-time PCR machine. Analysis was done using the SDS 2.1 program.

For Western blot analysis, tumor tissue was homogenized in radioimmunoprecipitation assay buffer (RIPA) [PBS, 1% Igepal (Sigma, Oakville, Ontario, Canada), 0.5% deoxycholate, 0.1% SDS, protease inhibitor mixture (Roche, Mississauga, Ontario, Canada)], and protein concentrations were assessed by the BCA method. Relative enzyme levels were quantified using the following antibodies at the indicated dilutions: rabbit polyclonal CYP17A1 (1:1,500) and rabbit polyclonal STAR (1:5,000) were both kindly donated by Dr. Hales (University of Illinois at Chicago) [36], rabbit polyclonal CYP11A1 (1:1,500 Corgen Inc., Guilford, CT), goat monoclonal CYB5A (1:1,000 Abcam, Cambridge, MA), goat polyclonal AKR1C3 (1:500 Abcam, Cambridge, MA), rabbit polyclonal HSD3B2 (1:20,000) kindly donated by Dr. Thomas (University School of Medicine, Macon, GA), goat polyclonal SRD5A1 (1:1,000 Novus, Littleton, CO), rabbit monoclonal HSD17B2 (1:500) kindly donated by Dr. Tremblay (Centre de Recherche de CHUL, Unite de Recherche en Ontogénie et Reproduction, Sainte-Foy, Quebec), mouse monoclonal HSD17B3 (1:250 Abnova, Neihu District, Taipei City, Taiwan) and mouse monoclonal RDH5 (1:250 Abnova, Neihu District, Taipei City, Taiwan).

***Ex-vivo* [^{3}H]-progesterone and [^{14}C]-acetic acid Treatment of Cells and Tumors**

Freshly excised tumor specimens were teased apart in 5 mL phenol red-free RPMI-1640 supplemented with 5% CSS. Excess debris and connective tissue were discarded and

the remaining cells were centrifuged at 500 Xg for 4 min. The supernatant was aspirated and the pellet was resuspended in fresh media, (2mL per test condition) and transferred to six-well tissue culture plates. One hour after plating cells, either 0.001mCi/mL [^{3}H]-progesterone or 0.002mCi/mL [^{14}C]-acetic acid was added to the cultures. Samples for metabolite identification by MS received additional 10µg/mL of unlabelled progesterone. After 4 days of incubation with radioisotope at 37ºC and 5% CO_2, the cells were harvested and centrifuged at 13500 Xg for 10 min. The supernatant (media) was transferred to a fresh tube and both cell pellet and supernatant were stored at -80ºC.

HPLC Separation and Radiometric Detection of [^{3}H]- and [^{14}C]-labeled Steroids

A Waters 2695 Separations Module coupled with a Packard (Perkin-Elmer, Wellesley, MA) Radiomatic™ Model 150TR detector equipped with a 0.5mL flow cell provided chromatographic separation and detection of radiolabeled analytes. Separations of [^{3}H]- labeled steroids were performed using a Waters Exterra 2.1X150mm 5µm, C18 column equilibrated with 10:90 acetonitrile (ACN):H_2O, ramped to 25:75 ACN:H_2O (0.75-1.5 min), further to 35:65 ACN:H_2O (1.5-20 min), then to 45:55 ACN:H_2O (25-30 min). Isopropanol (IPA) was introduced at this time from 45:0:55 ACN:IPA:H_2O to 45:55:0 ACN:IPA:H_2O (30-50 min), retained at 45:55:0 until 55 min and returned to starting conditions at 57 min for re-equilibration up to a 70 min run length. Separations of [^{14}C]- labeled steroids were performed using the same C18 column equilibrated with 10:90 acetonitrile (ACN):H_2O, ramped to 25:75 ACN:H_2O (0.75-1.5 min), further to 60:40 ACN:H_2O (1.5-45 min). Isopropanol (IPA) was introduced at this time from 60:0:40 ACN:IPA:H_2O to 70:30:0 ACN:IPA:H_2O (45-50 min), retained at 60:40:0 until 60 min and returned to starting conditions at 62 min for re-equilibration up to a 70 min run length. LC flow rate was 0.3mL/min, column temperature was 30ºC and Radiomatic™ scintillation fluid (Ultima Flo M, Perkin-Elmer, Wellesley, MA) flow rate was 1mL/min. [^{3}H]-DHT and [^{3}H]-progesterone were used as retention time standards. Radiometric retention times (RT) were observed to lag MS RT by ~1 min and this normalization factor was applied for the additional non-labeled standards. For metabolite identification, [^{3}H]-progesterone radiolabeled (H) versus radiolabeled plus non-radiolabeled (cold) progesterone (H+C) treated extracts were compared and fractionated using the above LC method by setting the Radiomatic™ detector's splitter to 20% with the remaining 80% of LC flow being collected in 1 min

fractions, dried by centrifugal evaporation (Centrivap™, 40ºC for 3hrs) and selected fractions reconstituted in 100µL of 50% MeOH for subsequent MS analysis (above). Addition of excess non-radiolabeled progesterone to radiolabeled progesterone did not appear to alter enzyme activity.

2.3 Results

Steroid levels are increased in LNCaP xenografts obtained from mice exhibiting CRPC progression but remain low in corresponding serum

Progesterone, T and DHT were quantified in LNCaP xenograft tissues obtained from the same mouse (n=5) prior to castration (pre-Cx), 8 days post-castration (N) and 35 days post-castration (CRPC) when PSA is re-expressed and defines CRPC gene expression in this model. Both T and DHT tissue levels appear to be elevated in CRPC tumors as compared to N (**Figure 2.2a**).

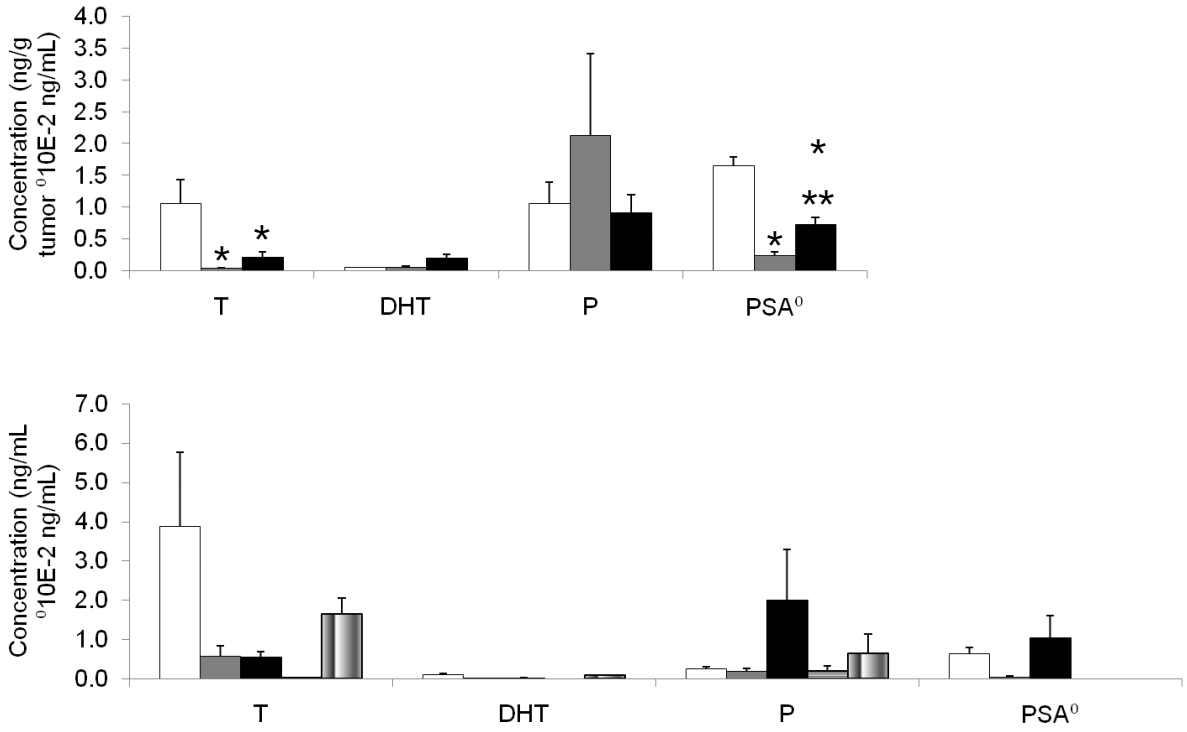


Figure 2.2: Steroid levels in pre-Cx, N and CRPC tumors. Mean concentrations of Testosterone (T), Dihydrotestosterone (DHT) and Progesterone (P) in tumor homogenates obtained from mice pre-castration (pre-Cx, □, n=5), 8 days post-castration (N, ■, n=5) and 35 days post-castration (CRPC, ■, n=8). Serum Prostate Specific Antigen (PSA) was also monitored at time points indicated (**a**). Mean concentrations of T, DHT, P and PSA in serum samples obtained from pre-Cx (□, n=5), N (■, n=5), CRPC (■, n=5), castrate (non-tumor bearing mouse, ▤, n=3) and intact (non-tumor bearing mouse, ▤, n=3) mice (**b**). T, DHT and P concentrations (ng/g tumor + SEM or ng/mL serum + SEM) were normalized to internal standard (deuterated testosterone). PSA concentrations units were *10E-2 ng/mL. Statistically significant differences ($P < 0.05$) from AD and N groups are indicated by * and **, respectively.

In fact, tumor T levels significantly decrease after castration ($p=0.007$; 3.5% of pre-Cx) and then increase slightly once CRPC progression is reached as defined by PSA (20.2% of pre-Cx). DHT concentrations as low as ~10^{-14}M (2.92×10^{-6}ng/g) have been shown to transactivate AR in prostate cancer cell lines [37] and therefore the levels observed in CPRC xenograft tumors ~$6.5 * 10^{-10}$M (0.19 +/-0.07ng/g tissue) would appear sufficient for AR

activation [28]. In agreement with previous studies [38], serum T and DHT concentrations remarkably decrease following castration and remained low (<14 and 12% of pre-Cx, respectively) in mice exhibiting CPRC progression (**Figure 2.2b**). Tumor progesterone levels were initially quite high prior to castration (**Figure 2.2a**) and appear to increase (201% of pre-Cx) post-castration. Furthermore, tumor progesterone levels decrease to 86% of pre-castrate levels during CRPC progression. This trend opposing the characteristic PSA profile suggests that progesterone may be involved in an adaptation mechanism whereby the cancer cell initially adjusts to androgen deprivation by producing more androgen precursor, progesterone. Furthermore, serum progesterone is high (2.0 +/- 1.3 ng/mL serum) in mice exhibiting CRPC progression while serum progesterone from castrated non-tumor bearing mice remains quite low (0.2 +/- 0.1 ng/mL serum) suggesting that the tumor is the source of this progesterone surge (**Figure 2.2b**). Progesterone, like DHT, is known to induce cholesterol synthesis in prostate cancer cells [39]. It is therefore postulated that increased progesterone levels observed in tumors 8 days post-castration and secreted in serum during CRPC progression may be significantly contributing to the underlying mechanisms of progression beyond cholesterol, as an intermediate in androgen synthesis. In addition, we determined that the adrenal glands from mice exhibiting CRPC progression did not contain T, DHT or any of their steroid precursors at detectable levels by HPLC-MS/MS (data not shown) and thus do not appear to be the source of the observed DHT synthesis within our model. This is consistent with other studies as anticipated [40, 41]. Furthermore, as circulating serum T and DHT concentrations remain low after castration the source of the increased androgen levels observed in tumors during progression is most likely from the LNCaP tumor itself.

mRNA and protein of the enzymes required to synthesize DHT from cholesterol are detected in LNCaP xenograft tumor tissues

Using Q-RT-PCR we have verified the presence of mRNA corresponding to each of the enzymes required for DHT synthesis from cholesterol (**Figure 2.1**) in pre-Cx, N and CRPC xenograft tumors (**Figure 2.3a**). The expression of StAR, HSD3B2 and RDH5 at the mRNA level significantly decrease at N relative to pre-Cx (p=0.005, 0.014 and 0.011, respectively) while SRD5A1 and RDH5 significantly increase at CRPC relative to N (p=0.002 and 0.011 respectively). Several other trends appear in the data. For example,

CYP11A1, CYP17A1, CYB5A1, AKR1C1, AKR1C2, AKR1C3 and HSD17B2 appear to increase at CRPC relative to N. However, these trends did not reach statistical significance due to the inter-mouse variability in absolute levels.

Western blot analysis verified that the enzymes necessary for the *de novo* synthesis of steroids from cholesterol are expressed in LNCaP xenografts (**Figure 2.3b**). In this analysis tumors from separate inoculation sites were surgically removed at pre-Cx, N and CRPC stages from seven mice. Trends similar to the mRNA levels support an increase in protein levels of nearly all of the steroidogenic enzymes during transition to the CRPC state.

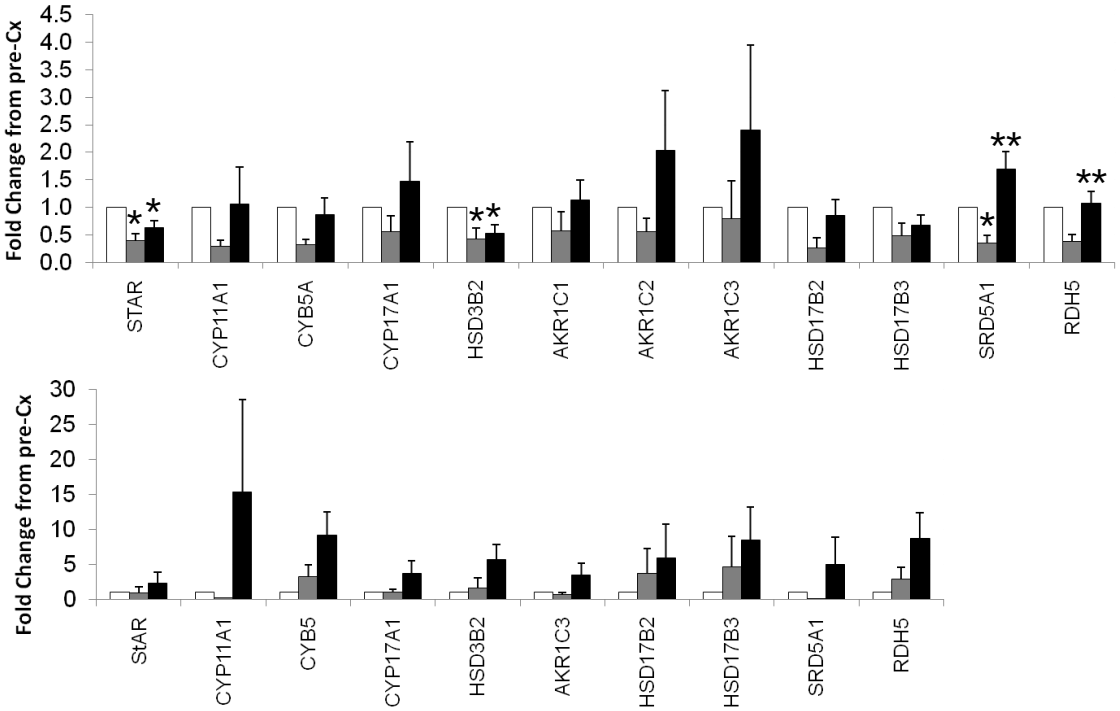
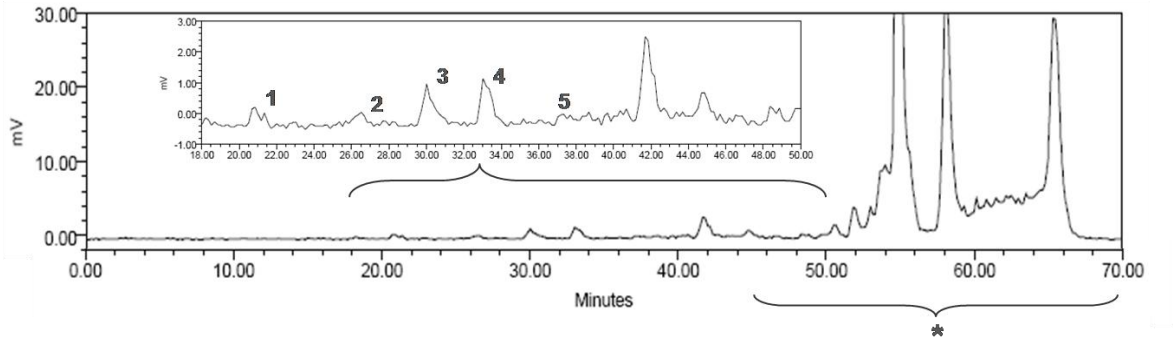


Figure 2.3: Enzyme levels in pre-Cx, N and CRPC tumors. Q-RT-PCR analysis of twelve enzymes involved in the Steroidogenic pathway in pre-Cx (□), N (▒) and CRPC (■) tumor homogenates taken from the same mouse (n=7) (**a**). Mean fold change in fluorescence readings from pre-Cx group for each enzyme are illustrated (+ SEM). Statistically significant differences ($P < 0.05$) from pre-Cx and N groups are indicated by * and ** respectively. Protein analysis of the ten indicated enzymes in pre-Cx, N and CRPC tumors from each mouse (n=7) (**b**). Fluorescence and Western blot readings were normalized to PSA of each mouse at time of tumor excision. Sometimes two tumors were taken from the same mouse at CRPC. Vinculin was used as a loading control.

[^{14}C]-acetic acid is converted to DHT in androgen-starved LNCaP cells and CRPC tumor cells

Initially androgen-starved *in vitro* LNCaP cells were incubated with cholesterol precursor [^{14}C]-acetic acid and *de novo* conversion profiles were characterized using HPLC-radiometric detection. In these androgen-starved conditions, LNCaP cells synthesized large quantities of hydrophobic molecules consistent with cholesterol and a range of lipids (RT= 45-70 min; 91.0% conversion) as well as further minor conversion into steroids: progesterone, 4-pregnen-17-ol-3,20-dione and DHT (0.4% , 0.6% and 1.8% conversion, respectively) as determined by RT agreement with MS standards (**Figure 2.4a and panel a**).

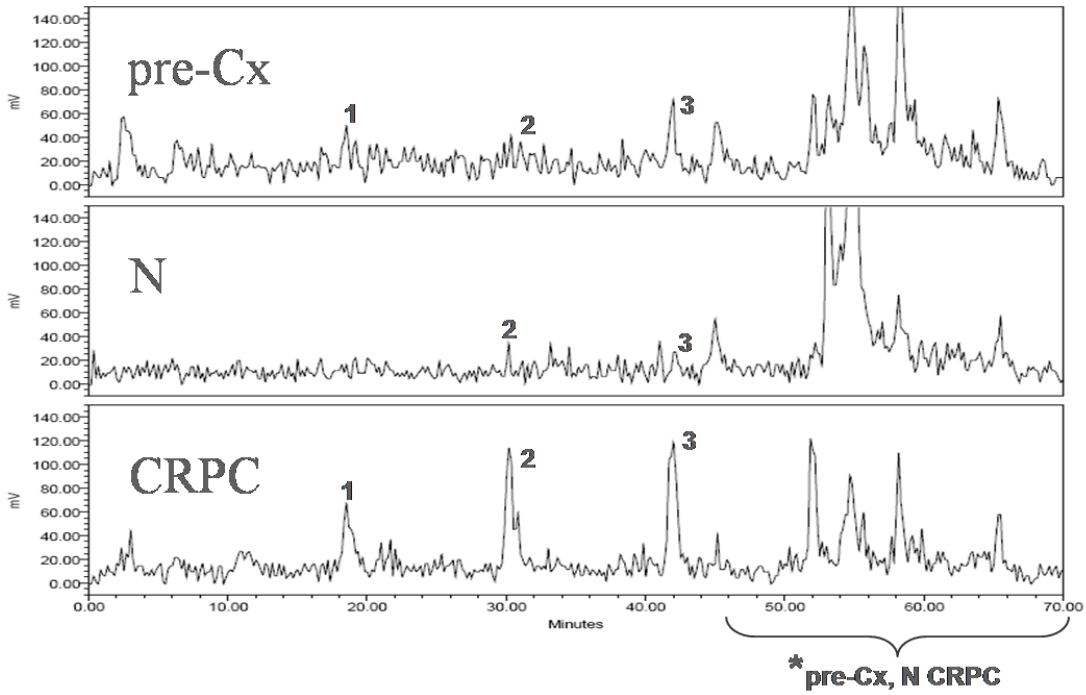
a



Panel a

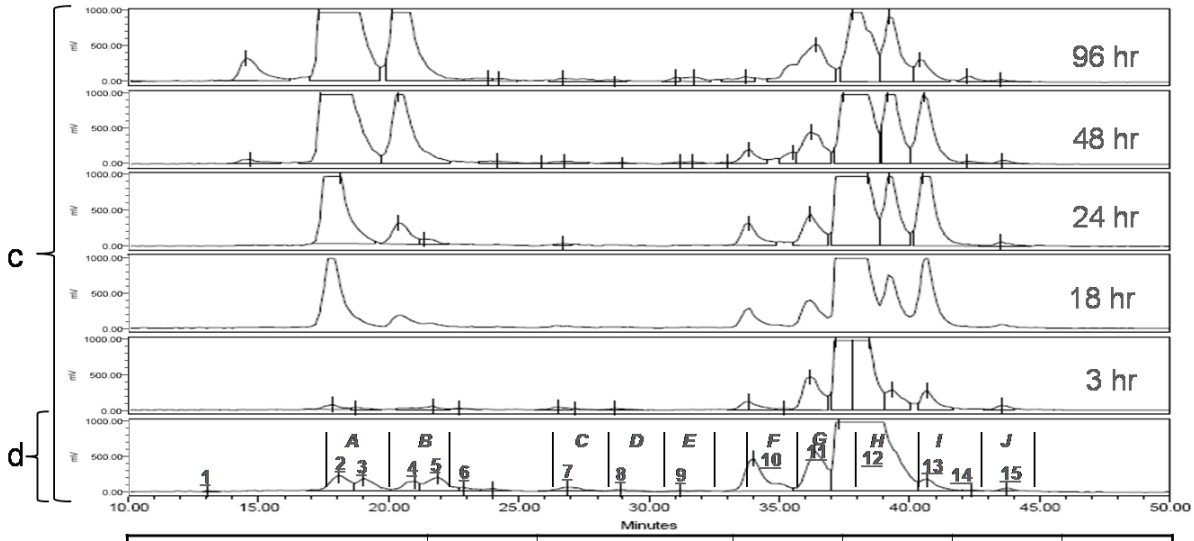
Identification	LC-Radiometric RT (min)	^{14}C peak #	% of total ^{14}C counts	LC-MS Standard RT (min)
Unknown	20.8	1	0.6	
4-Pregnen-17-ol-3,20-dione	26.5	2	0.6	25.4
DHT	30.0	3	1.8	28.9
Unknown	33.0	4	2.0	
Progesterone	37.3	5	0.2	36.4
Cholesterol and cholesterol esters	45.0-70.0	*	94.8	

b



Panel b

Identification	LC-Radiometric RT (min)	^{14}C peak #	% of total ^{14}C counts	LC-MS Standard RT (min)
Unknown	18.5	Int1 CR1	4.2 15.1	
DHT	30.2	Int2 N2 CR2	1.7 1.1 18.6	28.9
Unknown	42.0	Int3 N3 CR3	6.1 2.1 20.3	
Cholesterol and cholesterol esters	45.0-70.0	Int* N* CR*	84.7 92.8 43.8	



Panel c

Identification	Fraction	LC-Radiometric RT (min)	^{3}H peak #	SIR / MRM	Quantity (ng/ml)	LC-MS RT (min)
Unknown 1	A	18-20	2	317		6.4
Unknown 2	A	18-20	3	331		6.6
Unknown 3	B	20-22	4	331		6.8
Unknown 4	B	20-22	5	331		7
Unknown 5	B	20-22	4	317>299		6.8
4-Pregnen-17-ol-3,20-dione	C	26-28	7	331>97	6.7	7.6
Androstenedione	C	26-28	7 S1	287>97	0.1	7.5
4-Pregnen-17-ol-3,20-dione	D	28-30	7	331>97	0.6	7.6
Pregnenolone	F	33-35	10	317>299	104	8.3
Androsterone	F	33-35	10 S1	273>255	7.2	8.3
Pregnen-3,17-diol-20-one	F	33-35	10 S2	335>299	3.9	8.3
Progesterone	G	35-37	12	315>97	622	8.9

Figure 2.4: Radiotracing metabolism profiles. Example chromatographic analysis of extracted supernatants of *in vitro* (n=3) (**a**) and *ex vivo* pre-Cx (n=3), N (n=3) and CRPC (n=3) (**b**) LNCaP cells (from the same mouse) cultured in [^{14}C]-acetic acid for 96 hours *in vitro*. Identification of steroids in underlying LC radiometrically detected peaks (*In vitro* LNCaP 1-5; pre-Cx, N and CRPC 1-3) by retention time (RT) match up to LC-MS standard analysis, along with % of total [^{14}C]-counts are illustrated for *in vitro* and *ex vivo* chromatograms in **Panel a** and **b**, respectively. Both the LC-radiometric and LC-MS runs are 70 min (~1 min lag time between LC-radiometric and LC-MS analysis). Example chromatographic analysis of extracted supernatants of *ex vivo* CRPC LNCaP cells (n=3) cultured in [^{3}H]-progesterone for 3, 18, 24, 48 and 96 hours *in vivo* (**c**). Example chromatographic analysis of extracted supernatant from *ex vivo* CRPC LNCaP tumor cells (n=3) cultured in [^{3}H] + 10µg/mL progesterone for 96 hours *in vivo* (**d**). 1 – 15 are peaks detected by radiometric detection and A – J are LC fractions collected for further LC-MS analysis (**Panel c**). The LC-MS run is 15 min. compared with the 70 min. radiometric run. Correct combined MRM/RT parameters are the basis for all identifications. S1 and S2 designations indicate presence of analyte in shoulder of peak. Quantitation is via 2-point calibration. No steroids were observed in fractions E and J.

Following this experiment, mice (n=3) each bearing three tumors were serially excised at the time points of pre-Cx, N and CRPC, disaggregated and cultured *ex vivo* with [^{14}C]-acetic acid. The majority of *de novo* synthesis activity in pre-Cx and N tumors (representative

example **Figure 2.4b and panel b**), like i*n vitro* LNCaP cells, appears in later eluting cholesterol and lipid peaks (RT=45-70 min; 84.7 and 92.8% conversion) and not in the steroid eluting region (12.0 and 3.2% conversion) from RT= 18-45 min. However, in CRPC tumors, *de novo* synthesis of more hydrophilic species with shorter RTs including steroids (54.0% conversion) was observed. In fact, overall CRPC tumors (n=3) were capable of *de novo* synthesis of analytes with matching RTs to standards 4-pregnen-17-ol-3,20-dione, progesterone and DHT (4.0%, 1.5% and 8.3% conversion, respectively) by MS.

[^{3}H]-progesterone is converted to steroid precursors of DHT in both the classical and backdoor pathways in CRPC tumor cells

As tumor progesterone levels appear high post-castration and enzymes necessary for progesterone synthesis from cholesterol (CYP11A1and StAR) and metabolism (CYP17A1 and SRD5A1) appear to be increased in CRPC tumors, it was hypothesized that progesterone is an important mediator of *de novo* synthesis of DHT in prostate cancer progression. In order to investigate the pathway (backdoor or classical) whereby progesterone leads to DHT synthesis, androgen-starved LNCaP cells were incubated with [^{3}H]-progesterone and *de novo* conversion profiles were obtained (data not shown). These cells were capable of *de novo* synthesis of analytes with matching RT to that of our testosterone, 4-pregnene-17-ol-3,20-dione, 5-pregnan-3,17-diol, androsterone, pregnan-3,20-dione and DHT standards by MS. We further incubated excised CRPC tumors *ex vivo* with [^{3}H]-progesterone in a time-course experiment in order to explore the kinetics of this conversion to downstream steroids. Media was sampled from radiolabeled tumors at 3, 18, 24, 48 and 96 hrs with corresponding radiometric LC profiles of extracts shown in **Figure 2.4c and panel c**. Major steroid products are evident at 18, 21, 34, 36, 39, 41 and 44 min. in addition to intact progesterone, which remained after 96 hrs. This data reflects a complex series of steroidogenic metabolic steps on-going in this CRPC tumor model system over time.

In order to conclusively identify the *de novo* synthesized steroids in either the classical or backdoor pathway from [^{3}H]-progesterone to DHT, extracts from supernatants of both [^{3}H]-progesterone (H) and [^{3}H] plus non-labeled progesterone (H+C) treatment also underwent radiometric HPLC analysis (**Figure 2.4d**) with a fraction diverted and collected for further MS analysis. Multiple-reaction monitoring (MRM) analysis, ESI+ MS scans and fragment ion scans using the masses selected from scan data were performed. Equivalent

fractions from (H) and (H+C) labeled samples from the same tumor were compared in order to identify unique masses (**Figure 2.4e**). Analysis of *ex vivo* cells from CRPC tumors incubated with (H+C) progesterone, illustrated steroids predominant in both the classical and backdoor pathways to DHT synthesis (pregnenolone, 4-pregnen-17-ol-3,20-dione, pregnan-3,20-dione, pregnen-3,17-diol-20-one, androsterone and androstenedione) were identified and semi-quantitatively assessed by MRM data using a 2 point calibration versus standard. In addition, some peaks were evident with RT which differed from those determined for known standards, representing different steroids with a common transition (**Figure 2.5**). Precursor mass and fragmentation patterns for these unknowns are consistent with a steroidal entity; however their precise identities are currently being investigated.

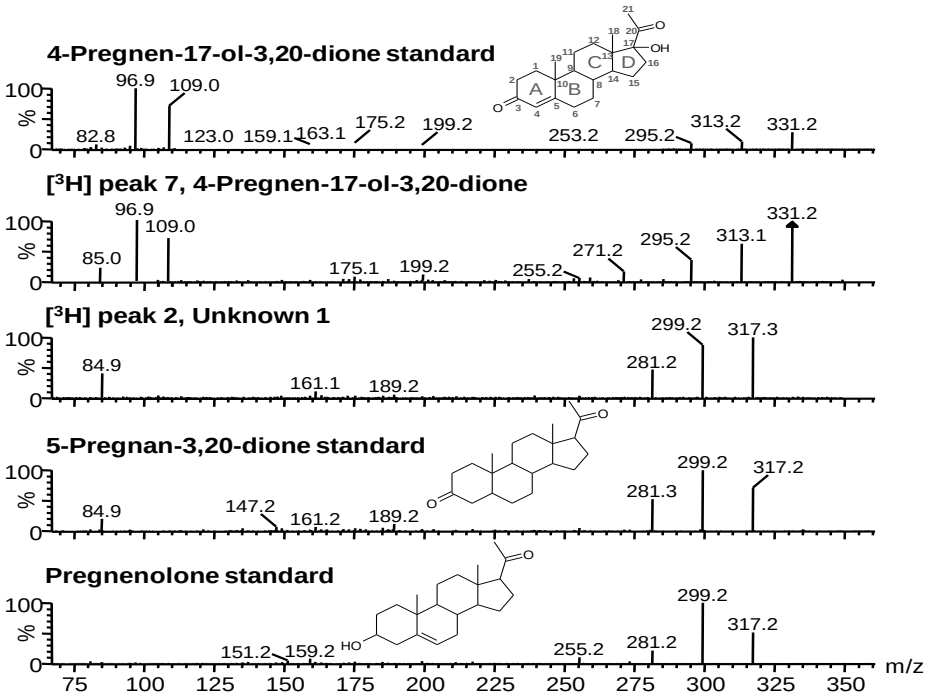


Figure 2.5: Mass spectrometry fragmentation patterns of steroids. Fragment ion scans of positively identified steroid 4-Pregnen-17-ol-3,20-dione (peak 7) as well as unknown 1 (peak 2) from the extract of the [^{3}H] + 10µg/ml progesterone treated *ex vivo* LNCaP cells (n=3) supernatant as compared to steroids standards. Unknown 1 shows high similarity to 5-pregnan-3,20-dione and less to pregnenolone however retention times differ. Steroid structures also depicted.

2.4 Discussion

'Castration-resistant' disease is defined by rising PSA following ADT and by many lines of evidence that suggest reactivation of the AR [13, 14]. In attempting to account for this observation some researchers hypothesize that adrenal androgens are synthesized during CRPC progression and supply the prostate tumors with androgen following ADT

administered either through LHRH agonists action or physical castration [20, 23]. Consequently, attempts have been made clinically to induce total suppression androgen blockade using ketoconazole to block adrenal steroidogenesis and/or antiandrogens to directly antagonize the AR in addition to castration [24]. However, the basic premise of this hypothesis has recently been met with skepticism. Research carried out by Liu *et al.* and Labrie, and more recently Titus *et al.* demonstrates that substantial amounts of androgens, T and DHT are present in human prostate tissues in levels capable of activation of AR despite "castrate" circulating androgen levels in serum [26-28]. We show that CRPC tumors develop compensatory mechanisms during androgen deprivation, tailored to the synthesis of intra-tumoral androgens, triggering AR activation and disease progression. The increased T and DHT levels observed in CRPC LNCaP tumors reinforces the observations in patients, with corresponding low levels circulating T and DHT is sera. In addition, the reported intra-tumoral concentrations of T and DHT appear to be sufficient for AR transactivation and the further observed induction of androgen-regulated genes [37]. This suggests that androgens are not supplied via circulation from an external source such as the mouse adrenal glands (which were shown to lack steroid precursors of DHT as well; data not shown) to the prostate. Furthermore, using LC-MS we demonstrate that tumor progesterone levels are high immediately after castration when circulating progesterone remains low. Therefore we postulate that progesterone produced through extension of the cholesterol synthesis pathway may be crucial for the *de novo* synthesis of DHT within the local environment of the tumor. In keeping with this, in 2004, Payne *et al.* reviewed past studies investigating the *de novo* synthesis of hormones in diseased forms of cardiac tissues and nerves as a potential compensatory mechanism for cell survival [42].

We observed that LNCaP tumors contain all enzymes necessary for DHT synthesis. Furthermore, SRD5A1 and RDH5, enzymes responsible for progesterone metabolism to DHT *via* the backdoor pathway, were upregulated in mRNA during transition to CRPC state as defined by reactivation of PSA. In agreement with Stanbrough *et al.*'s findings, upregulation ARK1C1, AKR1C2 and AKR1C3 also appear during CRPC progression. We are however, limited in our ability to determine statistically conclusive trends in enzyme expression at both the mRNA and protein level due to inter-animal variability in time to progression observed in our xenograft mouse model. Nonetheless, the LNCaP tumors were

confirmed to express steroidogenic enzymes with a trend of increase in CRPC and this was validated at both the mRNA and protein level.

We also show [^{14}C]-acetic acid conversion to steroids and verify that CRPC tumors are capable of *de novo* androgen synthesis, albeit the downstream androgenic metabolites are detectable in moderate proportions. Acetic acid is a precursor to many molecules including cholesterol, lipids (triacylglycerides and phospholipids) and fatty acids and therefore a significant dilution of signal to steroids is expected [39]. Furthermore, rate-limiting enzymatic reactions involving CYP11A1 and helper enzyme, StAR, recognized for their ability to shuttle cholesterol into the cell and convert it to upstream steroid pregnenolone, are known to regulate androgen biosynthesis [43, 44] and may be the rate limiting enzymes in our acetic acid conversion to steroids. Previously Acevedo *et al.* also reported low conversion rates of steroidogenic metabolite precursors to androgens [45]. Nonetheless, we have definitively shown conversion of [^{14}C]-acetic acid to DHT in CRPC LNCaP xenograft cells using LC-radiometric detection. T did not appear to be synthesized in [^{14}C]-acetic acid conversion assays while the formation of two upstream steroids, progesterone and 4-pregnen-17-ol-3,20-dione, were. Lack of T synthesis in tumor cells suggests that the backdoor pathway to DHT may be utilized. Furthermore mRNA upregulation of backdoor pathway specific enzyme, RDH5 alongside SRD5A1, an enzyme responsible for backdoor pathway entry from progesterone was observed in CRPC tumors; thus further supporting the utilization of the backdoor pathway in CRPC progression.

We found progesterone to be a predominant steroid detected in significant quantities in pre-Cx, N and CRPC LNCaP tumors and, as an upstream steroidogenic precursor to androgen *via* both the classical and backdoor pathways, we also incorporated progesterone into assays to determine metabolic activity in CRPC tumor cells. Upon tandem-MS analysis of samples from HPLC-radiometric fractionation, the identities of most DHT precursors (with the exception of T) in both the classical and backdoor pathways were confirmed as being synthesized in LNCaP cells and CRPC tumors. The lack of T observed suggests that prostate cancer cells utilize the backdoor pathway to DHT synthesis and we further propose that this may result from inefficient CYP17A1 lyase activity as suggested by Auchus *et al.* [33]. Further investigations of this potential mechanism to backdoor synthesis of DHT as well as the identification of steroidal unknowns are currently underway. Our ability to

identify steroids synthesized (as determined by MRMs) has been restricted by the availability of commercially available steroid standards. We understand that progesterone metabolite, unknown 1, has previously been documented but not identified in the literature [45] and is thought to be a hydroxylated steroid with a similar structure to pregnan-3,20-dione.

In summary, we report evidence that CRPC tumors 1) contain sufficient levels of T and DHT for AR transactivation, 2) express all necessary enzymes for *de novo* synthesis of DHT, 3) are capable of intratumoral conversion of precursor [^{14}C]-acetic acid to DHT and 4) utilize progesterone to synthesize DHT through various steroid intermediates in both the classical and backdoor steroidogenic pathways.

This research attempts to provide rational explanation for consistent reports in the literature of the concerted upregulation of androgen-regulated pathways characteristic of CRPC as well as the presence of high tissue levels of androgens in AI human prostate tissues relative to circulating androgen levels [14, 15, 46]. CRPC progression remains a major obstacle to treatment of advanced CaP. Improving our understanding of the underlying mechanisms of CRPC progression will provide a valuable insight for identifying new therapeutic targets and strategies.

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CHAPTER 3: Steroidogenesis inhibitors alter but do not eliminate androgen synthesis mechanisms during progression to castration-resistance in LNCaP prostate xenografts[2]

3.1 Introduction

Progression of prostate cancer (CaP) emerging after therapeutic approaches to block testicular androgen synthesis leads to "Androgen-Independent" or "Castration Resistant" Prostate Cancer (CRPC) which is the lethal component of this disease [1]. Several treatments targeting hormone synthesis and androgen receptor activation have been used both individually and in combinations in patients displaying CRPC disease [2-6]. These agents have been shown to alleviate symptoms of the disease and evoke prostate specific antigen (PSA) responses but have not yet proven to prolong survival [2-6]. In order to effectively treat CRPC patients and improve survival, a better understanding of the underlying mechanisms of CaP progression to CRPC (and how drugs affect these mechanisms) is necessary in order to strategically identify key targets, develop drugs inhibiting these targets and administer effective therapeutic interventions.

Analysis of CaP tumors from patients as well as human derived-xenograft CRPC progression models such as LNCaP have shown that many androgen-regulated genes, including the steroid metabolizing enzymes HSD3B2, SRD5A1, CYP17A1, AKR1C1, AKR1C2, AKR1C3 and SREBPs become re-expressed in CRPC tumors [7-9]. Recently, Titus *et al.* used liquid chromatography-mass spectrometry (LC-MS) to show that tumors obtained from recurrent CaP patients contain testosterone and dihydrotestosterone (DHT) in high enough levels to activate the androgen receptor (AR) in CRPC cells, despite observed low levels of circulating androgen in the serum [10, 11]. Labrie *et al.* and others have shown evidence suggesting that after castration steroid precursors obtained through circulation from the adrenals can be captured and utilized by CaP tumors to make these androgens [10, 12, 13]. We and others have demonstrated using radiotracing techniques that CRPC tumor cells can in fact *de novo* synthesize their own androgens from cholesterol and upstream precursors

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of cholesterol [14-16]. Combined, these studies suggest that tumor cells can develop an ability to evade castration induced steroid starvation by utilizing upregulated androgen synthesis enzymes to produce their own androgens for AR activation and progression to CRPC.

In concordance with these discoveries several drug candidates targeting the androgen axis were being developed and evaluated in their ability to treat CRPC patients. Ketoconazole, an azole antifungal agent which exerts its clinical effects through inhibiting CYP17A1 (and other cytochrome P450's) can induce reasonable PSA response rates in CRPC patients but no studies have demonstrated improvement in overall survival [17, 18]. A new more specific CYP17A1 hydroxlase and lyase inhibitor, abiraterone acetate, has triggered declines in PSA of greater than 30%, 50% and 90% in 14, 12 and 6 CRPC patients (out of 21 patients total), respectively, in Phase I and II clinical trials [6]. SRD5A1/2 inhibitors are used in the treatment of hair loss, benign prostatic hyperplasia and prevention of CaP [19-22]. Clinical trials evaluating the use of these drugs, finasteride and dutasteride, in treating CRPC disease are currently underway [23-25]. These steroidogenesis inhibitor drugs and other developing candidates targeting androgen synthesis pathways show significant promise in treating CaP advancing to CRPC through androgen synthesis mechanisms.

In this study we evaluated the effects of various steroidogenesis inhibitors and anti-androgens on androgen synthesis pathways in steroid-starved LNCaP cells and CRPC xenografts. Better understanding of how these agents alter androgen synthesis in CaP tumors will optimize their use therapeutically. Using LC-MS, we previously observed that LNCaP tumors excised shortly after castration, as compared to tumors from intact (pre-castration) mice, contain elevated levels of progesterone relative to testosterone and DHT, despite low serum levels [14]. Furthermore, mRNA levels of enzymes responsible for progesterone synthesis (CYP11A1, StAR and HSD3B2) and metabolism (CYP17A1, AKR1C1 and SRD5A2) increased during progression to CRPC [8, 14, 16], suggesting that high progesterone levels may be involved in androgen synthesis under androgen-deprived conditions. We therefore chose to study progesterone as a key steroidal precursor and investigate its downstream androgen synthesis mechanisms. Classically progesterone is converted to 17-OH progesterone and androstenedione by CYP17A1 [26], subsequently

converted to testosterone by AKR1C3 [27, 28] / HSD17B3 [29] and finally DHT by SRD5A1/2 (**Figure 3.1**).

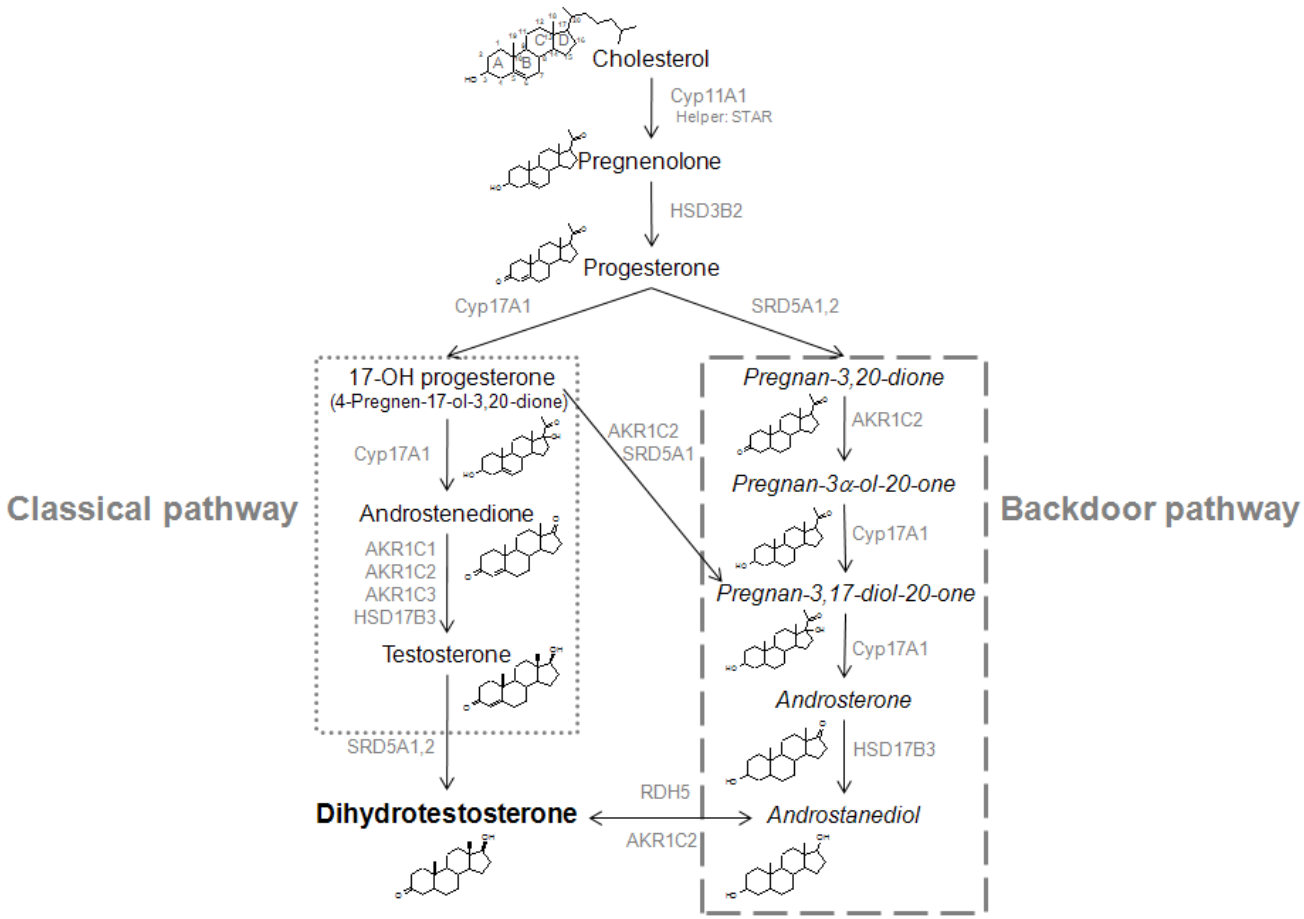


Figure 3.1: DHT synthesis pathway from cholesterol. Characteristic enzymes and intermediates displayed. Progesterone can be metabolized via the classical pathway or the backdoor pathway to DHT.

Auchus *et al.* recently described a second "backdoor" pathway to DHT synthesis that bypasses testosterone as an intermediate (**Figure 3.1**) [30-32]. In this pathway progesterone is initially converted to pregnan-3,20-dione by SRD5A1/2 before undergoing conversion to pregnan-3α-ol-20-one by AKR1C2. This intermediate is then converted by CYP17A1 to androsterone and further bioconversion by HSD17B3 to androstanediol. Androstanediol is then likely reversibly converted to DHT by RDH5.

We aim herein to explore the effects of steroidogenesis inhibitors on androgen production in this dynamic steroid synthesis system in *ex vivo* CRPC tumors using progesterone as a steroidal precursor. Further to these studies we aim to evaluate and compare the ability of *ex vivo* LNCaP xenograft tumors from different stages of the disease to synthesize androgens from progesterone.

3.2 Materials and methods

Materials

[1,2,4,5,6,7-^{3}H (N)]-DHT (110.0Ci/mmol, PerkinElmer Life Sciences, Inc., Wellesley, MA) and [1,2,6,7-^{3}H (N)]-Progesterone (90.0Ci/mmol, PerkinElmer Life and Analytical Sciences, Wellesley, MA) were used for *in vitro* incubations and radiometric standards. Stock solutions of testosterone-16,16,17-d3 (deuterated testosterone) (CDN Isotopes, Pointe-Claire, Quebec, Canada); 4-pregnen-17-ol-3,20-dione, 5β-pregnan-3α-27-diol-20-one and 5β-pregnan-3,20-dione (Steraloids, Inc., Newport, Rhode Island); androsterone, 4-androstene-3,17-dione, 5α-androstan-17β-ol-3-one, pregnenolone, progesterone, testosterone and dihydrotestosterone (Sigma-Aldrich, Oakville, Ontario, Canada); and R1881 (Dupont, Boston, MA) were prepared in 100% methanol for use as standards for mass spectrometry and ethanol for *in vitro* incubations. Inhibitors: ketoconazole, finasteride, cinnamic acid, RU-486 and casodex (Sigma-Aldrich, Oakville, Ontario, Canada) were prepared in ethanol.

In vitro models: LNCaP cells

LNCaP cells (passage 40-48; American Type Culture Collection, Rockville, MD) were cultured in RPMI-1640 (without phenol red) with L-Glutamine, penicillin streptomycin (PS) and 5% Fetal Bovine Serum (FBS, Hyclone, Logan, UT) or 5% Charcoal Stripped Serum (CSS, Hyclone, Logan, UT). Cells were maintained and grown in FBS; however, prior to all treatments cells were cultured in 5% CSS for 48 hours.

***In vivo* model: LNCaP tumor progression to castration-resistance**

All animal experimentation was conducted in accord with accepted standards of the UBC Committee on Animal Care. LNCaP xenograft tumors were grown in athymic nude mice at four sites as modified from a previously reported method [9]. Also as done before [14], PSA levels were measured by tail vein sera samples weekly using an immunoassay kit (ClinPro, Union City, CA). At 6 weeks post inoculation mice were castrated. Tumors were harvested from the same mouse (16 mice total) pre-castration (PSA androgen-dependent-AD), 8 days post-castration (PSA nadir-N) and 35 days post-castration (PSA castration-resistant-CRPC) (see supplementary data section for PSA and tumor volume profiles). Tumors were excised and immediately placed in phenol red-free RPMI-1640 media supplemented with 5% CSS.

Treatments of LNCaP cells and castration-resistant xenograft tumors

In every ex vivo assay cells were teased apart from a fixed weight of xenograft tumor section and debris was removed prior to plating and treatment in CSS supplemented media. Steroid starved (CSS) cells and AD, N and CRPC xenograft tumor cells were treated with 1μCi/mL [^{3}H]-progesterone for 48 hours. Steroid starved cells and CRPC tumor cells were treated with an additional 1nM R1881 and inhibitors of CYP17A1, SRD5A2, AKR1C3, steroid receptors and AR: 20μM ketoconazole, 25μM finasteride [33, 34], 50μM cinnamic acid [35], 10μM RU-486 [36] and 25 μM casodex [37], respectively for 48 hours to determine the effect of inhibitors on steroidogenesis downstream of progesterone. Dose titrations of steroid starved LNCaP cells with 10μM progesterone and 0, 0.1, 1, 10, 20 / 25, 50, 100 and 1000μM ketoconazole / finasteride, were conducted in order to verify optimal doses for metabolism studies.

Cell viability determination

Cell viability was determined by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (Promega, Madison, WI). 20μL of reagent was added to each well (96-well plate) and left to incubate at 37ºC in the dark for 1 hr. The viability of the cells was determined based on measuring spectrometer absorbancies at 490 nm wavelength of samples as compared to EtOH treated cell.

PSA determination

Secreted PSA levels were determined from 10μL of media diluted in 40uL of H_2O using an immunoassay kit (ClinPro, Union City, CA). Concentration was determined using a standard

curve (0-120ng/L). The intra-assay and inter-assay coefficients of variation for this assay were measured to be 5.5% and 5.4%, respectively.

Steroid extraction for Liquid Chromatography-Mass Spectrometry (LC-MS) and radiometric analysis

Pellets underwent a rigorous freeze thaw protocol with liquid nitrogen and boiling water three times. Then supernatants and pellets were extracted twice with ethyl acetate (EtOAc) (v:v, 1:1), washed with H_2O (v:v, 1:1) once and dried down using a Centrivap™ centrifugal evaporation system (35°C). Samples were then reconstituted in 100µL of 50% methanol.

Steroid Analysis by LC-MS

LC-MS protocols were carried out as developed previously [14]. A Waters 2695 Separations Module coupled to a Waters Quattro Micro was used for LC-MS analysis. All MS data was collected in electrospray ionization positive (ESI+) mode with capillary voltage at 3kV, source and desolvation temperatures of 120ºC and 350ºC respectively and N_2 gas flow of 450 L/hr. Chromatographic separations were carried out using a Waters Exterra 2.1x50mm 3.5µm C18 column equilibrated with 20:80 ACN:H_2O, ramped to 80:20 ACN:H_2O from 0.5-8.0 min, further to 95:5 from 8.0-9.0 min and returned to 80:20 ACN:H_2O from 10.0-10.5 min with a total run time of 15 min. Flow rate was 0.3 mL/min, column temperature 35ºC and 0.05% formic acid was present throughout the run. Extracted ion chromatograms from extracted samples of radiolabeled alone (Hot) versus radiolabeled plus non-radiolabeled (Cold) progesterone (H+C) spiked incubations were compared and LC retention time alignments were used to identify potential metabolites as conducted previously [14]. Precursor ions unique to the H+C sample fractions collected by high performance liquid chromatography (HPLC) radiometric detection were selected for further collision induced dissociation (CID) at both 11V and 22V CE resulting in positive identification of progesterone, 17-OH progesterone, pregnan-3,17-diol-20-one, androsterone and testosterone.

HPLC separation and Radiometric Detection of [^{3}H]-labeled steroids

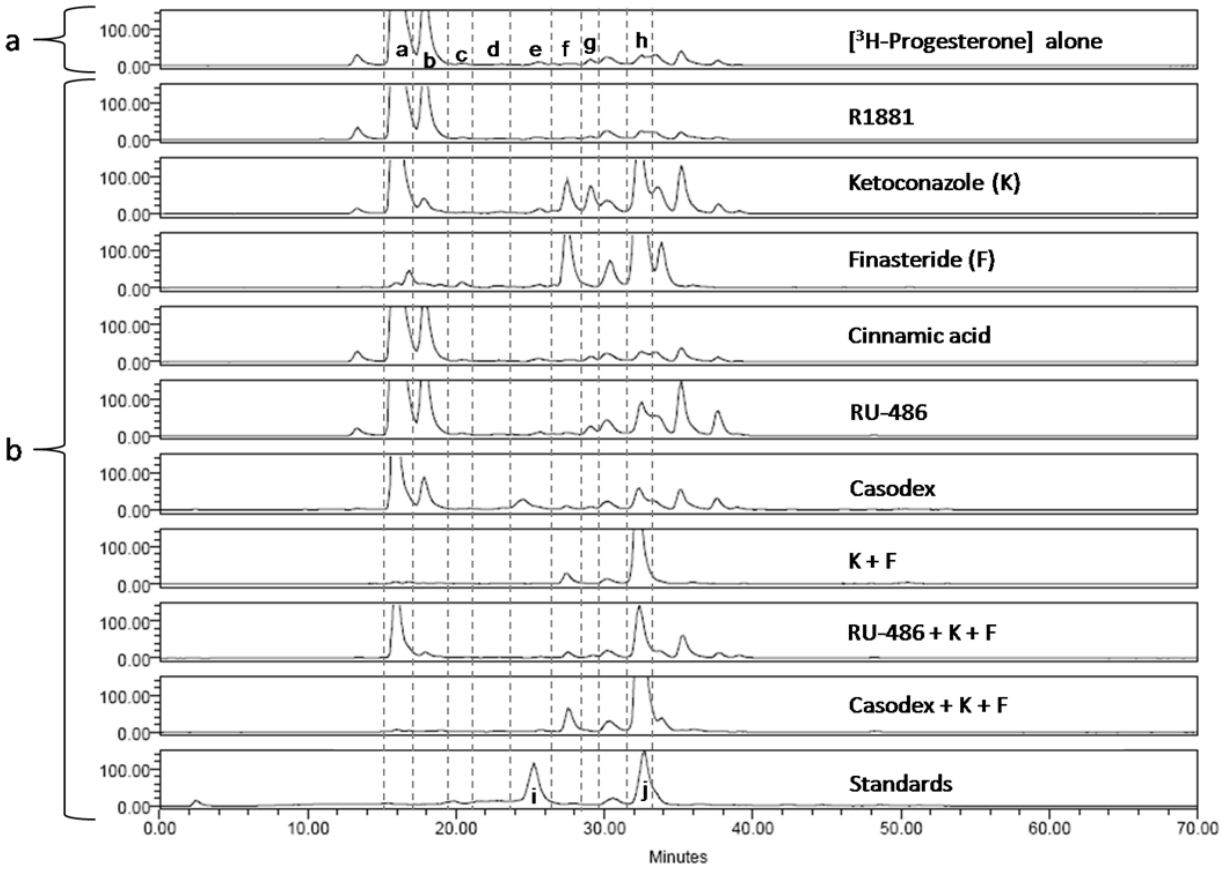
HPLC-radiometric detection methods were developed previously [14]. A Waters 2695 Separations Module coupled with a Packard (Perkin-Elmer, Wellesley, MA) Radiomatic™ Model 150TR detector equipped with a 0.5mL flow cell provided chromatographic separation and detection of radiolabeled analytes. Separations of [^{3}H]- labeled steroids were performed using a Waters Exterra 2.1X150mm C18 column equilibrated with 10:90 acetonitrile

(ACN):H_2O, ramped to 25:75 ACN:H_2O (0.75-1.5 min), further to 35:65 ACN:H_2O (1.5-20 min), then to 45:55 ACN:H_2O (25-30 min). Isopropanol (IPA) was introduced at this time from 45:0:55 ACN:IPA:H_2O to 45:55:0 ACN:IPA:H_2O (30-50 min), retained at 45:55:0 until 55 min and returned to starting conditions at 57 min for re-equilibration up to a 70 min run length. LC flow rate was 0.3mL/min, column temperature was 30ºC and Radiomatic^{TM} scintillation fluid (Ultima Flo M, Perkin-Elmer, Wellesley, MA) flow rate was 1mL/min. DHT identification was evidenced based on RT match-up to available radiolabeled and non-labeled steroid standards on the same LC gradient (Figure 2 table). Radiometric retention times (RT) were observed to lag MS RT by ~1 min when using this LC setup with the Quattro Micro and this normalization factor was applied for the additional non-labeled standards. Statistics on intra run variation in the retention time (RT) of both [^{3}H-DHT] and [^{3}H-Progesterone] standards were conducted by LC-radiometric detection to ensure consistency in peak identification and RT match up to steroidal standards by LC-MS. RT shift was found to be +/- 0.1min SEM from run to run confirming the reproducibility of this assay.

3.3 Results

Steroidogenesis enzyme inhibitors alter the *de novo* conversion of [^{3}H]-Progesterone to DHT in LNCaP cells and CRPC tumors

We initially investigated the ability of steroidogenesis inhibitors and antiandrogens to alter androgen synthesis pathways from radioactively labeled progesterone in both steroid starved LNCaP cells and CRPC tumor cells (and media). Both ketoconazole and finasteride but not R1881 or cinnamic acid appeared to alter progesterone metabolism to DHT in serum starved LNCaP cells and CRPC xenograft tumors (**Figures 3.2a, b, 3.3**). Ketoconazole significantly inhibited progesterone conversion to downstream metabolites (P=0.003) and also altered the relative amounts of progesterone metabolites that were still able to form (**Figure 3.2c**).



Table

^{3}H Peak	Steroid Standard	Radiometric RT (min)	SIR/MRM
a	Metabolite 1	15.9	317
b	Metabolite 2	17.9	331
c	Testosterone	20.4	289 > 97
d	17-OH Progesterone	22.7	331 > 97
e	DHT	25.2	287>97
f	Pregnan-3,17-diol-20-one	27.5	331 > 97
g	Androsterone	29.4	273 > 255
h	Progesterone	32.5	315 > 97
i	[^{3}H]-DHT Standard	25.1 +/- 0.1	_
j	[^{3}H]-Progesterone Standard	32.6 +/- 0.1	_

Figure 3.2a: Progesterone metabolism profile in LNCaP cells. Example chromatographic profile of metabolites from steroid starved LNCaP + [^{3}H]-Progesterone by HPLC-radiometric detection. **Figure 3.2b**: Example chromatographic profile of metabolites from LNCaP + [^{3}H]-Progesterone and 1nM R1881 or inhibitors 20μM ketoconazole (K) (CYP17A1), 25μM finasteride (F) (SRD5A2), 50μM cinnamic acid (AKR1C3), 10μM RU-486 (PR and AR), 25μM casodex (AR), 20μM ketoconazole + 25μM finasteride in combination, 10μM RU-486 + 20μM ketoconazole (K) + 25μM finasteride (F) in combination and 25μM casodex + 20μM ketoconazole (K) + 25μM finasteride (F) in combination by HPLC-radiometric detection. **Table 3.1: Radiometric standards information.** [^{3}H-DHT] and [^{3}H-Progesterone] were analyzed for retention time (RT) match up to LC-MS standards. HPLC-radiometric detection identified peaks (a-j) matched up to RT of steroidal standard as determined by LC-MS. SIR/MRM precursor masses and fragment masses were used to identify and quantify steroids listed. All experiments were conducted in triplicate.

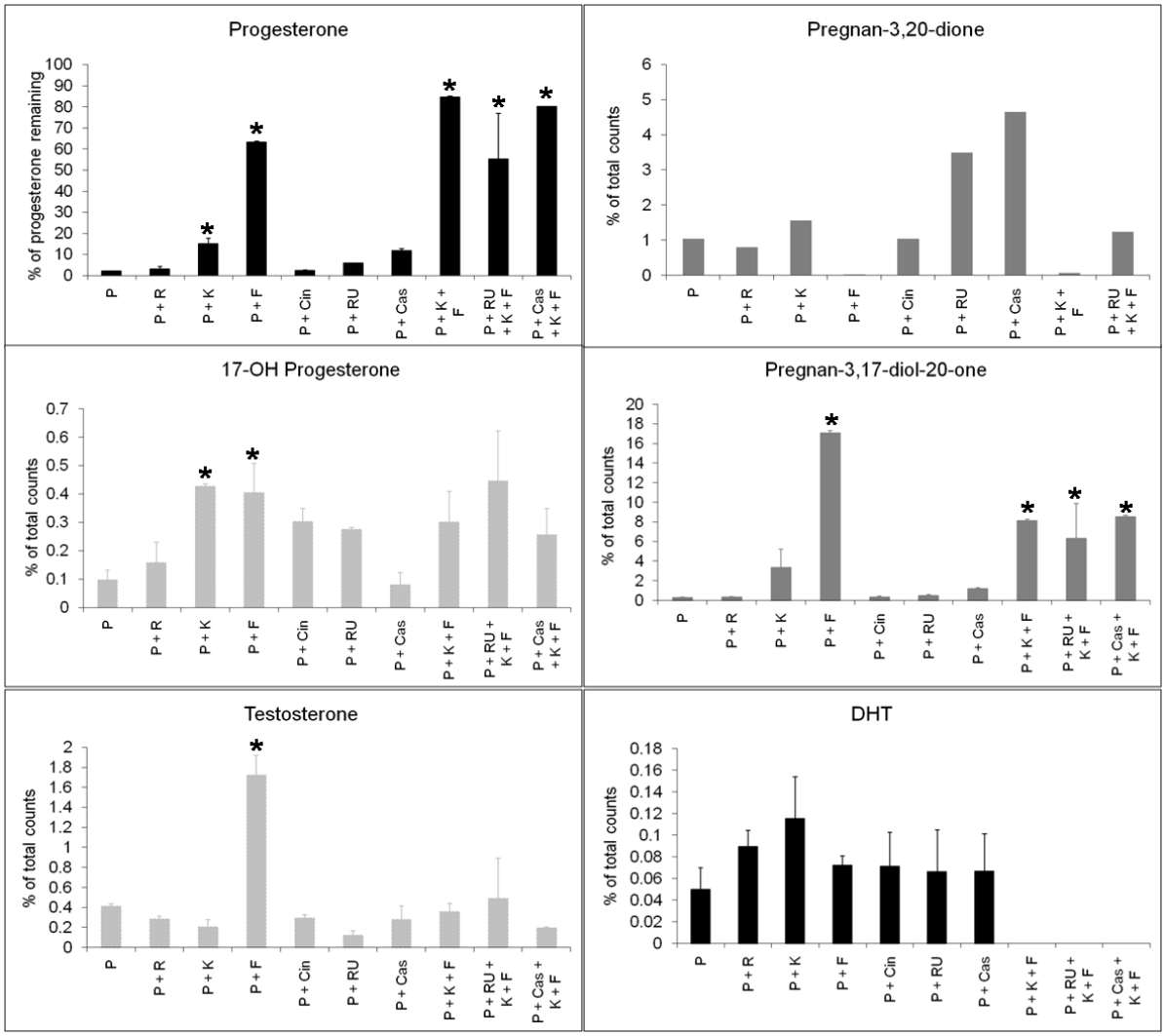


Figure 3.2c: Effect of inhibitors on progesterone production of steroids. Effect of 1nM R1881 and inhibitors: 20µM ketoconazole (CYP17A1), 25µM finasteride (SRD5A2), 20µM ketoconazole and 25µM finasteride in combination, 50µM cinnamic acid (AKR1C3), 10µM RU-486 (RU) (PR and AR) and 25µM casodex (AR) on the conversion of progesterone to downstream steroids in the classical pathway and backdoor pathway. Graph displayed of each metabolite steroid as % of total counts in [^{3}H-Progesterone] (P), P + ketoconazole (P +K), P + finasteride (P + F), P + cinnamic acid (P + Cin), P + RU-486 (P + RU), P + casodex (P + Cas), P + ketoconazole + finasteride (P + K + F), P + RU-486 + ketoconazole+ finasteride (P + RU + K + F), P +casodex + ketoconazole + finasteride (P + Cas + K + F) treated LNCaP cells (Mean + SEM). * indicates statistically different from LNCaP cells with no treatment ($p<0.01$). All experiments were conducted in triplicate.

Metabolite 1 was formed in a similar manner to that observed when cells undergo progesterone treatment alone; however much less of metabolite 2 formed (**Figures 3.2a, b**). Furthermore, 17-OH progesterone formation was not inhibited by ketoconazole as predicted by CYP17A1

inhibition (**Figure 3.2c**), in fact it was increased (P=0.001), as was pregnan-3,17-diol-20-one (not statistically significant). The rate-limiting step of dual enzyme CYP17A1 is believed to be its lyase action [31]. Formation of 17-OH progesterone and pregnan-3,17-diol-20-one via hydroxylation of progesterone and other progesterone-derived steroids upstream of CYP17A1 lyase action or perhaps the existence of another enzyme that is capable of hydroxylation at the 17-C site may therefore account for the increased levels of these steroids (**Figure 3.2c**). Nonetheless, this data suggests that ketoconazole affects conversion of progesterone to DHT via both the classical and backdoor pathways.

Finasteride inhibition of progesterone conversion (P=0.001) appeared to affect formation of DHT by both the classical and backdoor pathways (**Figure 3.2c**). Formation of Metabolites 1 and 2 were both dramatically inhibited (**Figures 3.2a, b**). As expected, testosterone levels were significantly increased by finasteride treatment (P=0.001) [23] (**Figure 3.1**; **Figure 3.2c**). Both 17-OH progesterone and pregnan-3,17-diol-20-one were also significantly increased by finasteride treatment and this inhibition profile was similar to that observed with ketoconazole (P=0.049, P<0.001 respectively). These results suggest that finasteride inhibits the conversion of progesterone via both the classical and backdoor pathways and upon inhibition of SRD5A2 activity, CYP17A1 conversion of progesterone to 17-OH progesterone in the classical pathway and downstream conversion pregnan-3,17-diol-20-one (via SRD5A1) in the backdoor pathway are increased in a compensatory manner.

Combined finasteride + ketoconazole treatment inhibited progesterone conversion to a greater extent than finasteride or ketoconazole monotherapy (P=0.002) (**Figures 3.2a,b, c**). In fact, the formation of metabolites 1 and 2 was significantly blocked upon combination treatment with ketoconazole + finasteride (P<0.001) (**Figures 3.2a, b**). Levels of pregnan-3,17-diol-20-one, albeit much lower than in finasteride only treated cells, were still significantly higher than those produced in the progesterone alone treated cells (P<0.001) (**Figure 3.2c**). Furthermore, upon combination treatment of cells with finasteride and ketoconazle DHT levels became undetectable (**Figure 3.2c**).

Neither R1881 nor cinnamic acid significantly affected the *in vitro* conversion of [^{3}H-progesterone] by steroid starved LNCaP cells in the presence of exogenous progesterone treatment (**Figure 3.2b**). The lack of effect observed by cinnamic acid treatment suggests that either an alternative enzyme is capable of metabolizing steroids similarly to AKR1C3 (such as

HSD17B3) or that this compound is not effective in inhibiting AKR1C3 in steroid starved LNCaP cells at the dose previously reported by Brozic *et al.* [35].

In CRPC xenograft tumors [^{3}H]-Progesterone also appeared to be metabolized to DHT (**Figure 3.3**) and inhibitors ketoconazole, finasteride and ketoconazole + finasteride combination treatment appeared to significantly inhibit this metabolism. This result demonstrates that these inhibitors effect androgen synthesis intratumorally at CRPC by altering steroid production via both the classical and backdoor pathways.

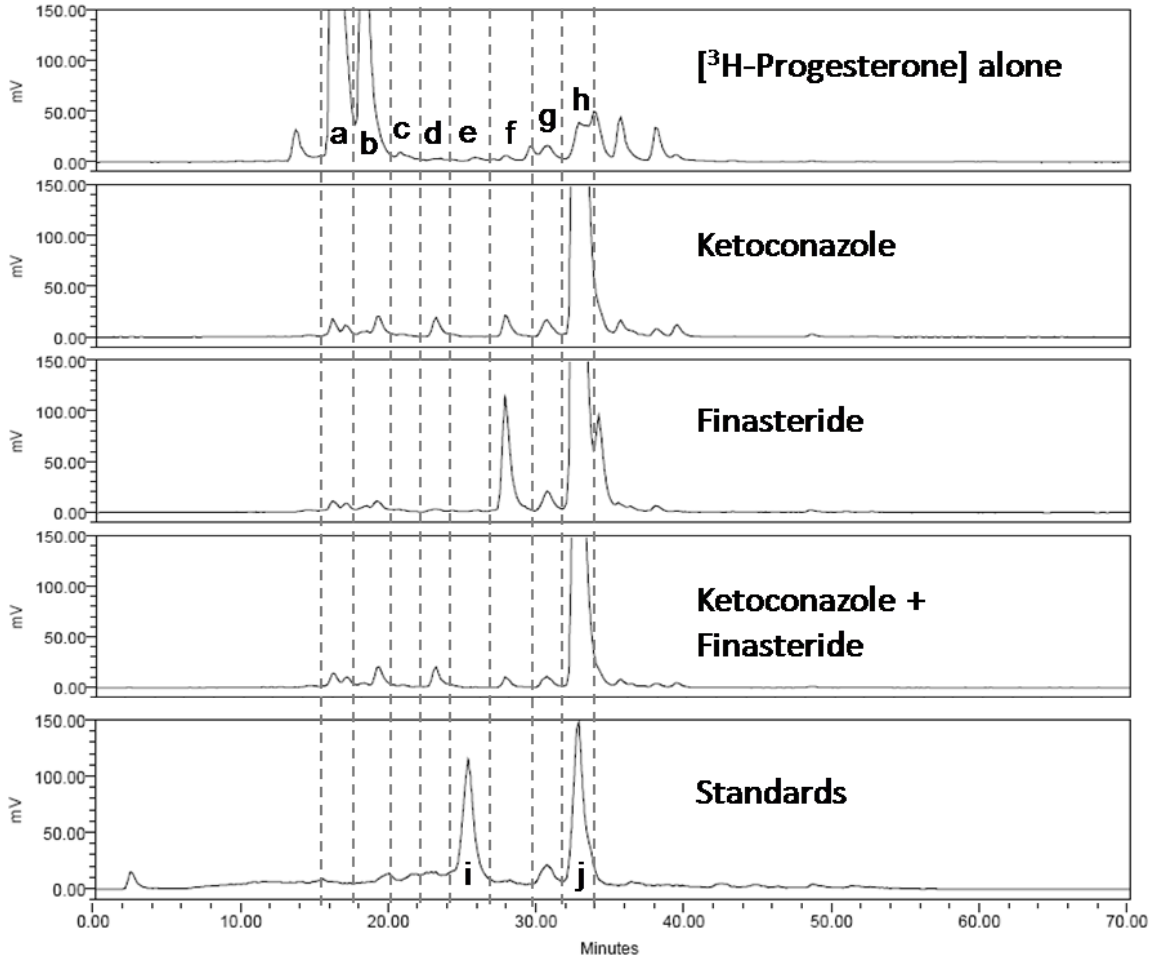


Figure 3.3: Progesterone metabolism profile in CRPC tumor cells. Example chromatographic profile of metabolites from CRPC xenograft tumor *ex vivo* cells + [^{3}H]-Progesterone and inhibitors 20μM ketoconazole, 25μM finasteride, and 20μM ketoconazole + 25μM finasteride in combination by HPLC-radiometric detection.

In conclusion, conversion of progesterone to downstream steroids is significantly and differentially altered by ketoconazole and finasteride treatments in both steroid starved LNCaP and CRPC xenograft tumors. When finasteride + ketoconazole were used in combination, progesterone metabolism was inhibited to a much larger extent. These results suggest that inhibition of enzymes in either the classical or backdoor pathway may lead to a compensatory increase in the steroid levels of other respective pathways which in turn can provide the cells with alternative androgen synthesis mechanisms to AR activation.

Receptor antagonists in combinational treatments with steroidogenesis inhibitors alter the *de novo* conversion of [^{3}H]-Progesterone to DHT in steroid starved LNCaP cells

Steroid receptor antagonists RU-486 (inhibits PR and AR) and casodex (inhibits AR) [36, 37] dosed individually did not appear to alter progesterone metabolism to DHT synthesis via either pathway, however they did appear to enhance the production of more hydrophobic metabolites (longer RT) (**Figures 3.2a, b**). Furthermore, while we saw large variation in results, combination treatment with RU-486 + finasteride + ketoconazole appeared to significantly inhibit progesterone metabolism ($P<0.001$) but not to the same extent as finasteride + ketoconazole alone (**Figure 3.2c**). Casodex + finasteride + ketoconazole also inhibited progesterone metabolism ($P<0.001$) more so than finasteride + ketoconazole alone (**Figure 3.2c**). All combinational treatments increased the amount of pregnan-3,17-diol-20-one produced in the backdoor pathway ($P_{finasteride+ketoconazole}<0.001$, $P_{RU-486+finasteride+ketoconazole}<0.001$, $P_{casodex+finasteride+ketoconazole}<0.001$) (**Figure 3.3c**).

Steroidogenesis inhibitors and receptor antagonists significantly decrease but do not eliminate progesterone-induced secretion of PSA in steroid starved LNCaP cells

We and others have previously hypothesized that cancer cells synthesize DHT at levels high enough to activate AR leading to a cascade of events linked to tumor growth and proliferation [10, 12-16]. Thus we deemed that the effect of steroidogenesis inhibitors and receptor antagonists on PSA secretion is appropriate to verify AR activation since PSA is androgen regulated target gene [38]. Progesterone treatment of LNCaP cells led to a significant increase in PSA secretion into media as compared to ethanol treatment ($P<0.001$) (**Figure 3.4a,b**).

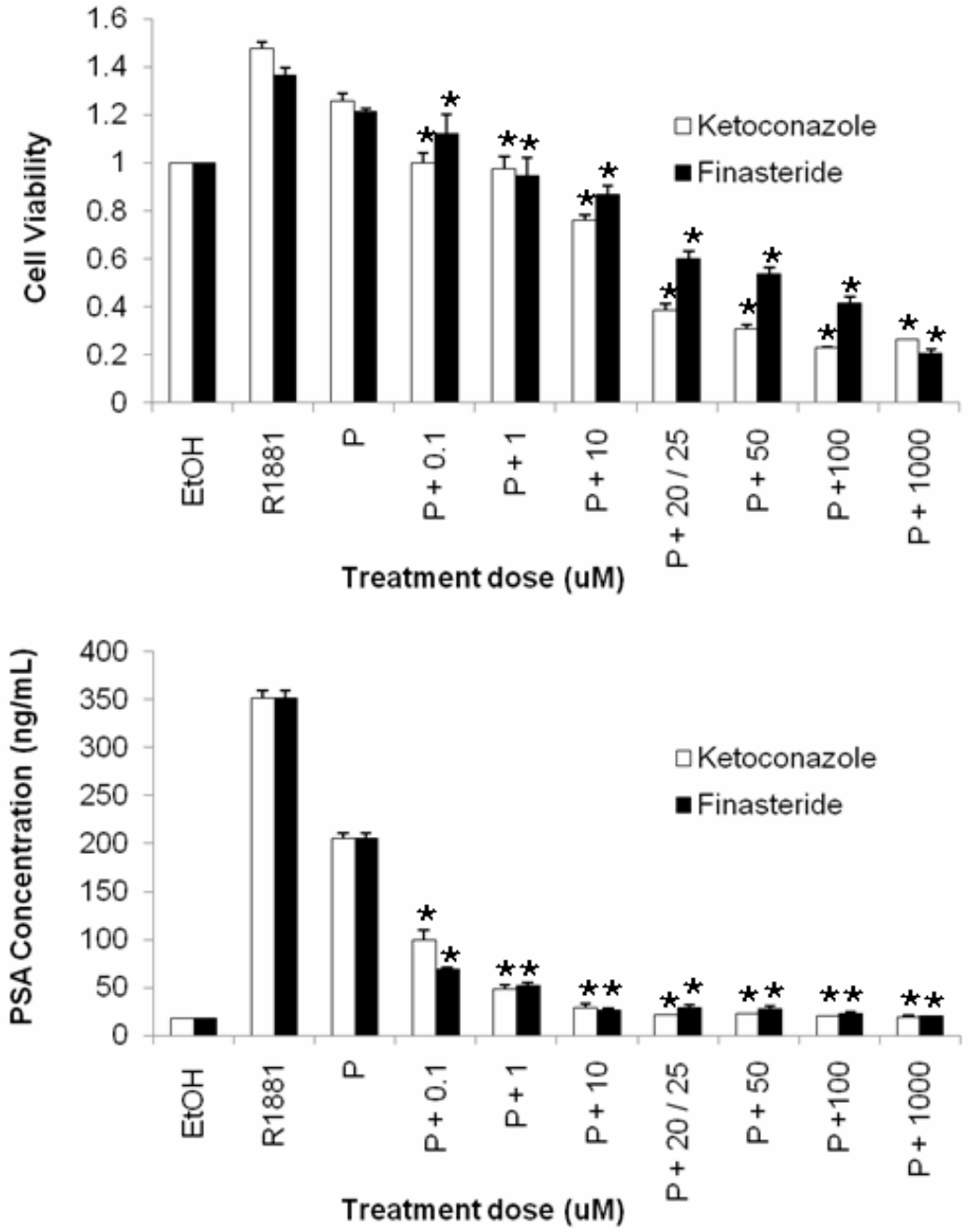


Figure 3.4a: Effect of 0, 0.1, 1, 10, 20 / 25, 50, 100 and 1000µM ketoconazole and finasteride on LNCaP cell viability and progesterone-induced secretion of PSA. * indicates statistically different from progesterone treated LNCaP cells ($p<0.01$). All experiments were conducted in triplicate.

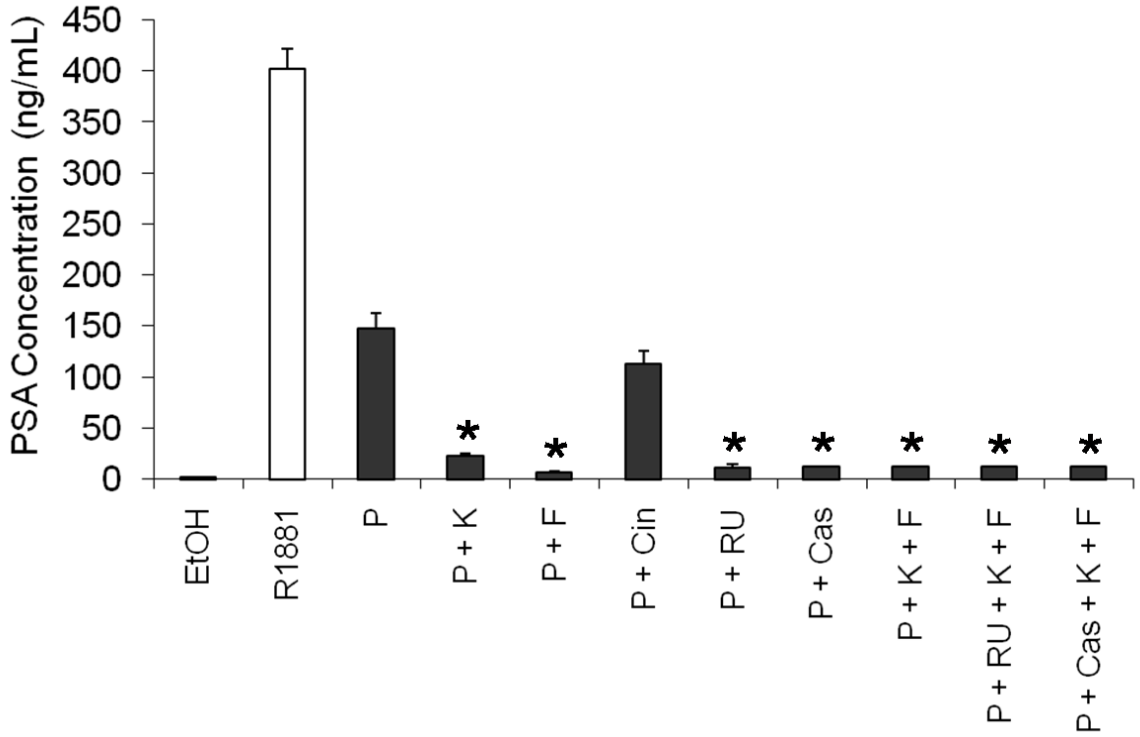


Figure 3.4b: Effect of inhibitors on progesterone induced PSA secretion. Effect of 1nM R1881 and inhibitors: 20μM ketoconazole, 25μM finasteride, 50μM cinnamic acid, 10μM RU-486 (PR and AR) and 25μM casodex (AR) on progesterone-induced secretion of PSA. Graph displayed as EtOH, R1881, Progesterone (P), P + ketoconazole (P +K), P + finasteride (P + F), P + cinnamic acid (P + Cin), P + RU-486 (P + RU) and P + casodex (P + Cas), P + ketoconazole + finasteride (P + K + F), P + RU-486 + ketoconazole + finasteride (P + RU + K + F) and P + casodex + ketoconazole + finasteride (P + Cas + K + F). * indicates statistically different from [^{3}H-Progesterone] treated LNCaP cells ($p<0.01$). All experiments were conducted in triplicate.

Initially we evaluated the effect of increasing doses of ketoconazole and finasteride on cell viability and progesterone mediated PSA secretion. As demonstrated in **Figure 3.4a** at doses of 20μM ketoconazole and 25μM finasteride cell viability was reduced to 38.6 +/- 0.03% and 60.0 +/- 0.03%, respectively. Progesterone induced PSA secretion was also affected by increasing doses of both drugs. Both ketoconazole and finasteride treatment led to decreases in measured PSA levels even at a dose of 0.1μM and at 1000μM progesterone mediated PSA secretion was completely inhibited as compared to EtOH treated cells (**Figure 3.4a**).

In **Figure 3.4b** ketoconazole, finasteride, RU-486, casodex, finasteride + ketoconazole, RU-486 + finasteride + ketoconazole and casodex + finasteride + ketoconazole treatments significantly inhibited progesterone-induced PSA secretion into the media ($P_{Ketoconazole}<0.001$,

$P_{Finasteride}<0.001$, $P_{RU-486}<0.001$, $P_{Casodex}<0.001$, $P_{Finasteride+ketoconazole}<0.001$, $P_{RU-486+finasteride+ketoconazole}<0.001$, $P_{Casodex+finasteride+ketoconazole}<0.001$) but do not completely abrogate AR activation at the doses evaluated. The observed decrease in PSA secretion upon treatment with ketoconazole, finasteride, RU-486 and casodex suggests that progesterone in part induces PSA secretion through downstream conversion to androgens, and not only through direct binding to PR or AR in steroid starved LNCaP cells as suggested by Grigoryev *et al*. [39]. Grigoryev *et al*. previously showed that AR found in LNCaP cells contains a mutation in the form of T877A and with this mutation can bind and be activated by ligands such as progesterone in high concentrations [39, 40]. Our result does not necessarily demonstrate that progesterone is mediating its effects solely through metabolism to DHT prior to AR activation but does show that at least some of the effect on PSA secretion is mediated through this mechanism. Furthermore, cinnamic acid did not significantly inhibit progesterone-induced PSA secretion suggesting also that this particular compound does not affect steroidogenesis leading to AR activation at the dose used (10µM).

From this experiment it was determined that progesterone mediated PSA secretion (via AR activation) was decreased but not completed inhibited by the presence of steroidogenesis inhibitors ketoconazole and finasteride and antiandrogens RU-486 and casodex at the doses evaluated.

[^{3}H]-Progesterone metabolism in AD, N and CRPC LNCaP xenograft tumors cells occurs via different enzymatic reactions and steroidal intermediates

In order to determine whether tumors growing at different stages of progression to CRPC have differential abilities to synthesize androgens we evaluated the progesterone metabolism profiles in AD (pre-castration; n=3), N (8 days post-castration, n=3) and CRPC (upon PSA relapse or 35 days post-castration; n=3) tumors obtained using the LNCaP xenograft model (see supplementary data section for PSA and tumor volume profiles). When we compare the chromatographic profiles of AD, N and CRPC tumors shown in **Figure 3.5a** the AD and CRPC tumor metabolism of progesterone appears to be similar with only very subtle differences.

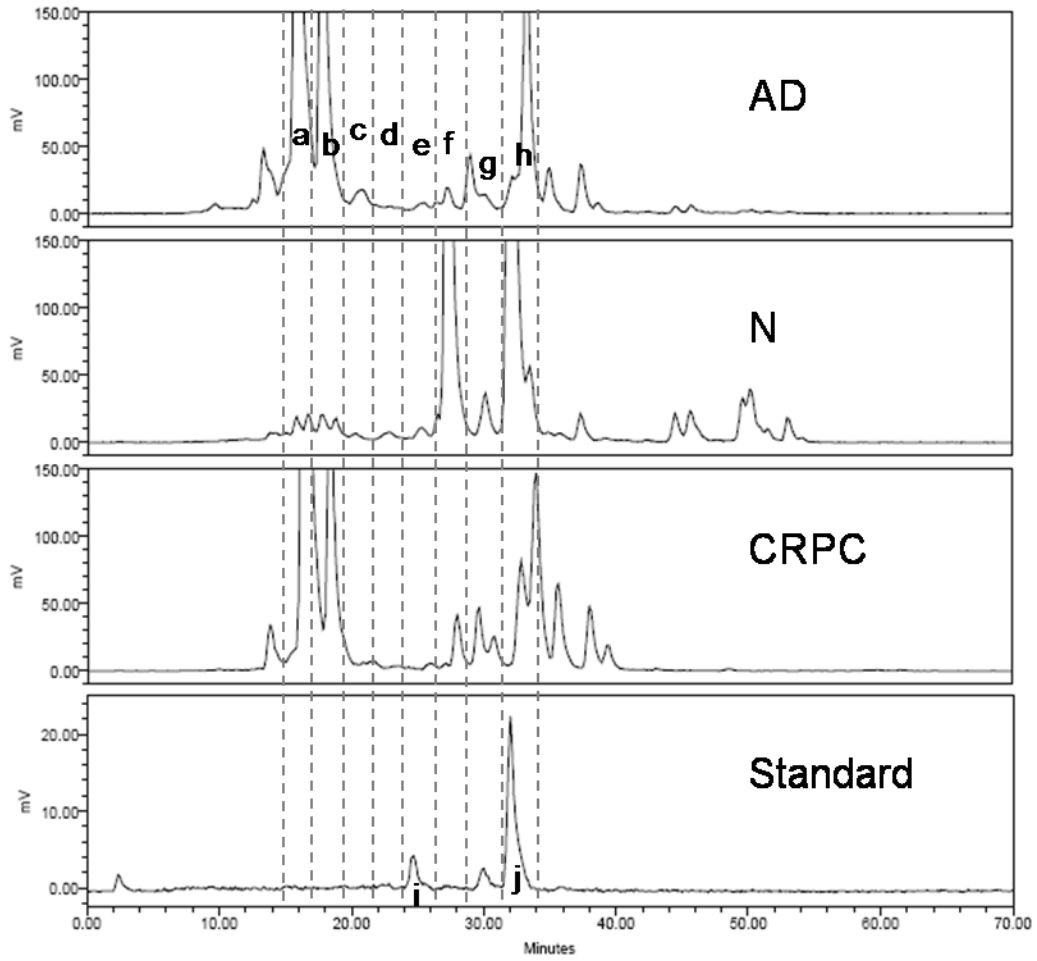


Figure 3.5a: Progesterone metabolism profile in AD, N and CRPC tumor cells. Example chromatographic profile of metabolites from LNCaP xenograft androgen-dependent (AD), nadir (N) and castration-resistant (CR) tumor cells + [^{3}H]-Progesterone by HPLC-radiometric detection.

In contrast, the N tumor metabolism of progesterone is significantly less extensive (~22% of AD or CRPC) (P=0.04) and yields more hydrophobic metabolites (later retention times) (**Figure 3.5b**).

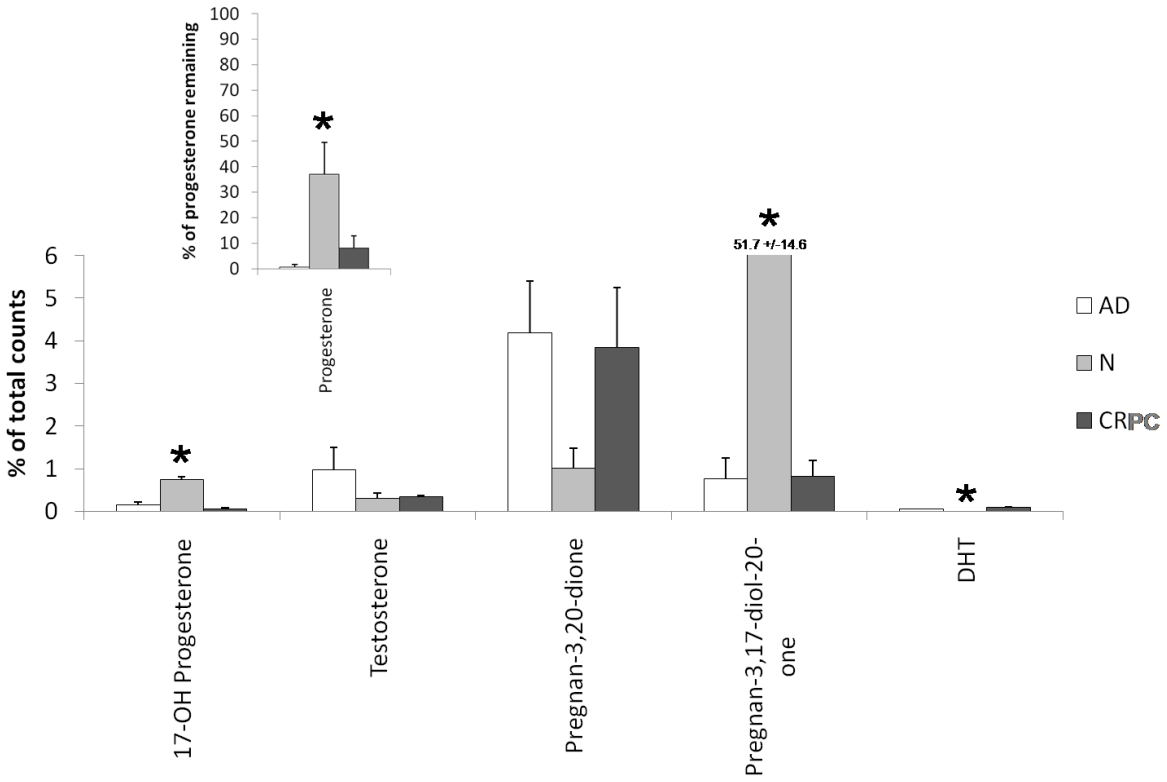


Figure 3.5b: Progesterone metabolites in AD, N and CR tumors. Levels of steroidal intermediates measured in androgen-dependent (AD), nadir (N) and castration-resistant (CR) tumor progesterone metabolism profiles as a % of total counts (Mean + SEM). * indicates statistically different from AD tumor ($p<0.01$). All experiments were conducted in triplicate.

Furthermore, N tumors produce significantly more 17-OH progesterone (~5-fold, $P<0.001$) and pregnan-3,17-diol-20-one (~62-fold, $P=0.008$) and significantly less DHT ($P=0.002$) than both AD and CRPC tumors. In fact, in N tumors there was no evidence of DHT formation while pregnan-3-17-diol-20-one was formed in such large quantities that it is likely to be the main end product of progesterone metabolism in these tumors. In contrast, metabolites 1 and 2 are likely to be the final end products of progesterone metabolism in AD and CRPC tumors. As demonstrated by this study N tumors obtained immediately after castration have a significantly hampered capacity to *de novo* metabolize progesterone as compared to AD tumors obtained prior to castration and CRPC tumors obtained once PSA had relapsed which we have previously shown have the potential ability to *de novo* synthesize androgens themselves [14]. In fact N tumors exhibit several different steroidal intermediates likely undergoing alternative enzymatic biotransformation than those seen in both AD and CRPC tumors.

Upon comparison of the AD and CRPC tumor progesterone metabolism profiles (**Figure 3.2a**) they appear relatively similar with perhaps more testosterone produced by AD tumors than CRPC tumors (not statistically different) (**Figure 3.2b**). This suggests that the enzymatic systems utilized by AD and CRPC tumors are similar.

In summary, it appears that steroid intermediates and enzymatic reactions in both classical and backdoor steroidogenesis pathways are utilized by AD, N and CRPC tumors. However, because more testosterone (classical pathway) was produced by the AD tumors than the N and CRPC tumors, prior to castration tumors utilize the classical pathway more predominantly. The predicted steroidal end product (pregnan-3,17-diol-20-one) in N tumor progesterone metabolism is principally observed in the backdoor pathway and because it forms in such a large amount it appears to act as a sink. This may indicate an inability of N tumor cells to utilize CYP17A1 lyase to produce downstream steroids. Furthermore, both AD and CRPC tumors were able to *de novo* produce DHT (albeit in small amounts compared to other steroid intermediates). Likely this low production reflects the cell's need for only minimal androgen for AR activation.

This work uniquely demonstrates using the LNCaP CRPC progression model that tumors of different stages of classical disease progression possess differential abilities to synthesize androgens and do so using different steroidal intermediates and enzymatic reactions.

3.4 Discussion

Increasing lines of evidence indicate that androgens remain important mediators of CRPC progression despite the low levels of androgens observed in serum after castration therapy [10, 12, 16, 41-43]. It has recently been shown that DHT synthesis can occur intratumorally from both adrenal steroid precursors and *de novo* from cholesterol [10, 12-16]. We identified progesterone as an important intermediate steroid that can be metabolized by CRPC LNCaP tumors through both the classical and backdoor pathways [14].

We further demonstrate here that inhibitors targeting the androgen synthesis axis alter the metabolism of progesterone to downstream androgens in steroid starved LNCaP cells and CRPC LNCaP xenograft tumors. Using progesterone as a steroidal precursor we demonstrate that inhibitors of enzymes CYP17A1 (ketoconazole) and SRD5A2 (finasteride), alter the levels of given intermediates in these two pathways and thereby the steroidogenesis profile observed in CRPC cells. In contrast, antiandrogens targeting AR (casodex and RU-486) did not alter

progesterone metabolism profiles significantly. Furthermore, the steroidogenesis inhibitors used did not completely eliminate progesterone induced PSA secretion suggesting that DHT synthesis from progesterone is not completely inhibited and can occur via these alternative pathways in a compensatory manner at the doses evaluated. Survival and proliferation of these evading tumor cells is therefore a likely event and we propose that inhibition of steroidogenesis enzymes in patient's displaying CRPC disease might result in disease relapse through mechanisms such as these described. Using the LNCaP progression model we also compared the ability of tumors at different stages of disease progression to synthesize steroids from progesterone and found that immediately after castration tumor cells utilize different enzymatic reactions to produce different steroid metabolites compared to progressing CRPC tumors. Because of these dramatic differences observed immediately post-castration as compared to when they have become CRPC, targeting the CaP tumors in patients prior to PSA relapse with steroidogenesis inhibitors may offer a more effective method in prolonging the progression of the disease and improving overall survival of the patients.

Steroidogenesis drugs such as ketoconazole and aminoglutethimide and antiandrogens such as flutamide, nilutamide and casodex have been widely used in treating patients with CRPC disease because of their demonstrated PSA responses even after androgen deprivation therapies has become exhausted [2, 17, 44-47]. The development and evaluation of several other steroidogenesis inhibitors such as statins [48], abiraterone acetate [6], VN/124-1 [49, 50], cinnamic acid [35], finasteride and dutasteride [23] as well as antiandrogens such as MDV3100 (Medivation, Inc., San Francisco, CA) and BMS-641988 (Bristol-Myers Squibb, New York, NY) [51-53] and AR chaperone proteins like Hsp27 [54] are on the rise.

Therapeutic responses demonstrated in this study using the LNCaP progression model for CaP suggest that CRPC tumors that respond initially to steroidogenesis inhibitors are likely to develop resistance and the disease will ultimately progress. We demonstrate that inhibitors targeting CYP17A1 (ketoconazole) and SRD5A2 (finasteride) do indeed alter the metabolism of progesterone to downstream androgens but do not completely inhibit it as other alternative steroidal pathways to DHT synthesis become utilized. Furthermore, progesterone induced PSA response, although decreased by these inhibitors, is not completely eliminated even at very high doses. Previously, it has been shown that CRPC patients who initially respond to ketoconazole display reduced amounts of CYP17A1 produced steroids in their serum and when they develop

resistance to ketoconazole these steroids once again increase in the serum [49, 55, 56]. In this manuscript we provide mechanistic rationale as to how some CRPC patients on ketoconazole treatment might become resistant to therapy as tumor cells develop an ability to produce these androgens by alternative mechanisms. Upon ketoconazole treatment CRPC LNCaP tumor cells produce more 17-OH progesterone and pregnan-3,17-diol-20-one than in the absence of this CYP17A1 targeting inhibitor. In humans 17-OH progesterone is a poorer substrate for CYP17A1 lyase activity than pregnan-3,17-diol-20-one [57] and in the presence of ketoconazole it appears that the CRPC cells convert progesterone via 17-OH progesterone to pregnan-3,17-diol-20-one for further bioconversion to DHT, thus demonstrating a logical escape mechanism after ketoconazole treatment. Furthermore, although finasteride has not yet been evaluated in a large population of CRPC patients as a potential treatment, according to our metabolism study CRPC cells will likely develop a steroid synthesis escape mechanism similar to that observed with ketoconazole treatment. In fact, we have found that significantly greater amounts of pregnan-3,17-diol-20-one and testosterone are produced in the presence of finasteride in steroid starved LNCaP cells and CRPC LNCaP xenograft tumors as compared to EtOH control or ketoconazole treatment. Interestingly, DHT levels do not appear altered by finasteride treatment suggesting that 17-OH progesterone is converted to pregnan-3,17-diol-20-one and through step-wise reactions in the backdoor pathway to DHT for AR activation, even in the presence of inhibitors blocking the SRD5A2 conversion of testosterone to DHT in the classical pathway. Conversion of 17-OH progesterone to pregnan-3,17-diol-20-one is predominantly mediated by SRD5A1 [58] while finasteride is known to predominantly target SRD5A2 [23, 59] suggesting that CRPC cells find alternative pathways to produce DHT by utilizing more readily available steroid substrates such as pregnan-3,17-diol-20-one and enzymatic reactions such as those mediated by SRD5A1 rather than SRD5A2. SRD5A1 and SRD5A2 are both known to be expressed in LNCaP cells [59], however SRD5A2 to a much lower degree suggesting that the backdoor pathway may be the predominant route utilized in LNCaP cells. Furthermore, although SRD5A2 expression is the predominant isoform found in the prostate, SRD5A1 tissue expression has been shown to increase and surpass SRD5A2 expression during progression of the disease to CRPC [60, 61]. Clinical trials evaluating treatment inhibition of SRD5A2 with finasteride in CRPC patients may yield further insight into whether this mechanistic hypothesis is valid in human CaP disease progression.

Androgen signaling may be eliminated by the development of more potent steroidogenesis inhibitors such as abiraterone acetate. Interestingly CRPC patients who developed resistance following initial response to abiraterone acetate treatment did not have increased levels of CYP17A1 mediated steroid production in their serum as previously observed in the ketoconazole relapsing patients [49]. Furthermore in a Phase I clinical trial reported by Ryan *et al.* 52% of patients (total 19) who previously became resistant to ketoconazole treatment displayed further PSA response (>50% decline) to abiraterone acetate treatment despite the fact that both drugs target the same enzyme [62]. Abiraterone acetate is a 20 times more potent inhibitor of CYP17A1 than ketoconazole [56] which is a broad spectrum inhibitor of steroid drug metabolism and this may explain why ketoconazole resistance can occur through alternative synthesis mechanisms while abiraterone acetate may completely block all androgen synthesis pathways downstream of CYP17A1. Furthermore, as we demonstrated a potential resistant mechanism of CRPC tumors to finasteride whereby the cells potentially divert to SRD5A1 driven metabolism upon SRD5A2 inhibition, dutasteride (targets both SRD5A1 and 2) may be able to eliminate androgen synthesis by blocking both classical and backdoor metabolism to DHT [23, 24, 59, 63]. In brief, assessment of more potent and targeted steroidogenesis inhibitors and antiandrogens may provide more effective "maximal androgen blockade" than the drugs studied here. These detailed metabolism studies also provide rationale for the use of combination therapies targeting steroidogenesis enzymes and AR in CRPC patients. Ketoconazole combined with finasteride treatment as well as in both/either drug combined with antiandrogens RU-486 and casodex was observed to alter androgen synthesis through progesterone metabolism to a much greater extent than ketoconazole or finasteride alone. Perhaps by utilizing these inhibitors or other inhibitors targeting rate-limiting CYP17A1, SRD5A1/2 and AR in combination "maximal androgen blockade" can be facilitated [64]. In support of this, a Phase II clinical trial in 57 CRPC patients investigating ketoconazole and dutasteride combination treatment versus ketoconazole alone previously demonstrated a prolonged time to relapse in the combination treated patients (13.7 months) as compared to ketoconazole alone treated patients (8.6 months) [65].

Lastly, we propose that the timing of treatment with steroidogenesis inhibitors and antiandrogens might be better optimized and further evaluation of the timely emergence of *de novo* steroidogenesis mechanisms is warranted during disease progression in humans. Our data

demonstrating a significant difference in the ability of tumors at different stages of the disease to synthesize androgens suggests that targeting the androgen axis with potent inhibitors such as abiraterone acetate or combinations of these inhibitors may be more optimally administered in patients prior to PSA relapse than when they have already reached CRPC. Increased PSA levels after castration is a measure of AR activation [7, 66] and patients displaying PSA relapse likely exhibit tumors that are already capable of androgen synthesis. Since we demonstrate that immediately after castration tumors are significantly worse at producing androgens (testosterone and DHT) from progesterone than tumors that are already castration-resistant perhaps targeting the androgen axis immediately after castration with steroidogenesis inhibitors and antiandrogens will prevent acquired mechanisms of *de novo* steroidogenesis from developing.

In summary we demonstrate that current steroidogenesis inhibitors do alter androgen synthesis mechanisms in CRPC tumor cells. However, we also identify potential mechanisms by which tumor cells can evade these drug treatments. Based on these results we suggest that targeting the androgen axis with combination treatments before cells develop the ability to make their own androgens may be optimal for improving CaP patient survival rather than waiting to treat patients with these inhibitors individually once the cancer has relapsed. While future clinical trials evaluating these combination therapies and their effect on overall survival should also consider drug-drug interactions and their resulting side effects this research provides rationale for the evaluation of combined steroidogenesis inhibitors concomitant to androgen deprivation therapy with a goal to preventing the emergence CRPC.

3.5 References

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CHAPTER 4: Alterations in cholesterol regulatory processes contribute to *de novo* androgen synthesis in prostate cancer tumors during progression to castration-resistance[3]

4.1 Introduction

Androgens are known to play a central role in malignant prostate cancer (CaP) growth and therefore androgen deprivation therapy (ADT) or surgical castration are front line treatments of this disease [1, 2]. Most cancer cells are terminated by these treatments, however, over time, the tumor can recur in a more aggressive and devastating phenotype termed "hormone refractory" or "castration-resistant" (CRPC) disease [3, 4]. The androgen receptor (AR) is known to be a central mediator in this progression to CRPC as re-ignition of AR expression and activity has been observed via numerous mechanisms in human CRPC tumors [5-9, 9-16]. One contending hypothesis to explain the role of AR in the emergence of the CRPC phenotype is that it becomes hypersensitive to low levels of androgens following castration [17, 18]. We and others have provided evidence that enzymes in upstream pathways of androgen synthesis such as cholesterol synthesis, lipid metabolism and fatty acid synthesis are altered during CaP progression [19-24]. The potential for altered lipogenesis in providing cholesterol precursor for androgen synthesis and subsequent AR activation in CaP progression is therefore provocative.

The regulation of cholesterol involves a multitude of enzymes and complex pathways within each cell, ultimately leading to the uptake, storage, synthesis, transport and excretion of this important survival molecule (**Figure 1.12**). Exogenous cholesterol is brought into the cell through scavenger receptor-B1 (SR-BI) and low density lipoprotein-receptor (LDL-r) while intracellular synthesis of cholesterol is controlled by a cascade of enzymes including rate-limiting HMG-CoA reductase [25-27]. Intracellular storage and transport of cholesterol in the form of cholesteryl esters (CEs) is regulated by acyl-CoA cholesterol acyltransferase-1 and -2 (ACAT1,2) enzymes while excretion of cholesterol occurs through ATP binding cassette-A1, -G1, and -G8 (ABCA1, ABCG5 and ABCG8) [25-27].

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[3] Sections of this chapter have been published. Locke, J.A. Wasan, K.M. Nelson, C.C. Guns, E.S. and Leon, C.G. (2008) Androgen-Mediated Cholesterol Metabolism in LNCaP and PC-3 Cell Lines is Regulated Through Two Different Isoforms of Acyl-Coenzyme A: Cholesterol Aceyltransferase (ACAT). The Prostate. 68:20-33.

The remaining sections of this chapter have been submitted for publication. Locke, J.A. Leon, C.G. Adomat, H.A. Ettinger, S.L. Twiddy, A.L. Neumann, R.D. Nelson, C.C. Guns, E.S. and Wasan, K.M. Alterations in cholesterol regulatory processes contribute to *de novo* androgen synthesis in prostate cancer tumors during progression to castration-resistance.

Most cholesterol processes are regulated by a group of transcription factors known as the sterol regulatory element binding proteins (SREBPs) which are in turn regulated by cholesterol levels [28-30]. Mechanistically, SREBPs remain in the ER with scaffolding proteins, SREBP cleavage activating protein, SCAP and retention protein, Insig [28]. Low cholesterol levels trigger the cleavage of Insig allowing SCAP-SREBP to travel to the Golgi. In the Golgi, SREBP is cleaved by two proteases called S1P and S2P, and it is then activated for nuclear transcription of lipogenesis factors [26]. Swinnen *et al.* proposed that the conformational change that leads to the cleavage of this complex relies on the androgen regulation of the escort protein, SCAP [31]. This was verified by Heemers *et al.* who demonstrated that synthetic androgen (R1881) stimulation of cells induced an increase in nuclear SREBP expression [31, 32]. This increase in expression did not occur in androgen-unresponsive cells and thus this process is believed to be androgen dependent in nature [33].

Androgens appear to be master regulators of cholesterol processes via the induction of SREBPs [19, 31], however the exact role of androgens in the induction of rate-limiting cholesterol regulation enzymes HMG-CoA reductase and ACAT has not been investigated in CaP cells. HMG-CoA reductase is an important enzyme in the biosynthesis of cholesterol as it catalyzes the conversion of HMG-CoA to mevalonate, a precursor of cholesterol [25, 34]. ACAT is an integral membrane protein localized in the endoplasmic reticulum and is important in catalyzing the transfer of cholesterol to CEs [35], which are further stored in lipid droplets of the cell. Two ACAT genes are found in mammals, ACAT1 and ACAT2. ACAT1 controls CE formation in the brain, adrenal glands, macrophages and kidneys while ACAT2 controls the CE formation in the liver and intestine [26]. Due to the functional importance of these enzymes (HMG-CoA reductase and ACAT), the role of androgens in their regulation will be investigated in this chapter. Furthermore, the potential for these regulation enzymes to become altered in order to provide cholesterol substrate for androgen synthesis and activation of AR during progression of the disease has only recently been suggested [19, 23, 24].

In this chapter, we first explore the effect of synthetic androgen on important cholesterol synthesis and esterification enzymes, HMG-CoA reductase and ACAT, respectively, in an androgen-sensitive LNCaP cell model as well as an androgen-insensitive PC-3 cell model. In the second part of this chapter using the LNCaP xenograft model of CaP progression we investigate

the collective roles of key cholesterol regulation enzymes (SR-B1, LDL-r, HMG-CoA reductase, ACAT-1,2, ABCA1) in providing precursor cholesterol for androgen synthesis after ADT.

4.2 Materials and methods

Materials

[1,2,6,7-^{3}H (N)]-Progesterone (90.0Ci/mmol, PerkinElmer Life and Analytical Sciences, Wellesley, MA) and [1(2)-^{14}C]-Acetic Acid, sodium salt (55.0 mCi/mmol, Amersham Biosciences, Baie d'Urfe, Quebec, Canada) were used for radiometric standards and *ex vivo* incubations, respectively. Cholesterol and cholesterol oleate (Sigma, Oakville, Ontario, Canada) were prepared in methanol:chloroform (70:30) for mass spectrometry standards. Steroids including internal standard testosterone-16,16,17-d3 (deuterated testosterone) (CDN Isotopes, Pointe-Claire, Quebec, Canada) as well as reference standards progesterone, pregnenolone, testosterone and dihydrotestosterone (DHT) (Sigma, Oakville, Ontario, Canada), were prepared in 50% methanol as mass spectrometry analyses.

Cell culture and androgen treatment

PC-3 cells were cultured in DMEM with L-Glutamine, Penicillin Streptomycin (PS) and either 10% Full Bovine Serum (FBS) or Charcoal Stripped Serum (CSS; Hyclone, Logan, UT). LNCaP cells were cultured in RPMI-1640 (without phenol red) with L-Glutamine without sodium bicarbonate and with PS and either 10% FBS or CSS. PC-3 and LNCaP cells were purchased from the American Type Culture Collection (Rockville, MD) and were used within passages 10-25 and 40-47, respectively. When the cells reached 40% confluency, the media was changed from full (FBS) to stripped (CSS) serum. After two days, cells were treated with 1 nM R1881 (Dupont, Boston MA) synthetic androgen. After 4 days of treatment, cells were pelleted and stored at -80°C.

In vivo model of prostate cancer progression

All animal experimentation was conducted in accord with accepted standards of the UBC Committee on Animal Care. CaP progression was mimicked using the LNCaP xenograft model. Briefly, tumors were grown in athymic nude mice at four sites as modified from previously reported methods [19]. Progression of the disease was monitored by tumor volume and serum levels of prostate specific antigen (PSA). Tumors were harvested from the same mouse (n=10) pre-castration (androgen-dependent; AD), 8 days post-castration (nadir; N) and 35 days post-

castration (castration-resistant; CRPC) and placed in warmed media for *ex vivo* assay or frozen immediately and stored at -80ºC.

Prostate specific antigen (PSA) Assay

Media was obtained during each cell culture treatment step. Media fractions of 50µl and mouse serum samples of 10µl in 40µl water were analyzed using a PSA ELISA kit from ClinPro (Union City, CA).

Microsome isolation

Cell pellets were resuspended in isolation buffer (50mM Tris pH 7.8, 1mM EDTA, 1mM fresh PMSF plus protease inhibitors) (Sigma, St Louis, MO), homogenized by incubating on ice for 30 minutes and by disrupting them by stroking using a microcentrifuge tube homogenizer. Then lysates were centrifuged in polycarbonate centrifuge bottles at 16,000 g for 15 min at 4ºC. The supernatant of this was centrifuged at 40,000 g for 60 min at 4ºC. The pellet from this was resuspended in isolation buffer and centrifuged at 40,000 g for another 45 min at 4ºC. This pellet was resuspended in Buffer A (50mM Tris pH 7.8, 1mM EDTA, 1mM PMSF) containing protease inhibitors (Sigma, St Louis, MO). Total protein content in this mixture was quantified and this amount was used in the following activity analyses.

HMG-CoA reductase activity assay

Fifty micrograms of sample was placed in 27 µl homogenization buffer (30 mM EDTA, 250 mM NaCl, 1 mM DTT, 50 mM potassium phosphate pH 7.4) and water up to a total volume of 125 µl. This mixture was incubated for 5 min in a 37ºC water bath. Treatment with 50 µl Cofactor-substrate solution containing radioactive substrate for 3 min was conducted and stopped using 25 µl 12 N HCl. An internal standard ^{3}H-Mevalonic acid was then added in a 20 µl volume. This sample was incubated for 30 min in the 37ºC water bath. After incubation, 50µl of each sample was blotted on a silica gel thin layer chromatography (TLC) sheet 7 µl at a time. Cholesterol was developed for 60 min in 50 ml of (1:1 v/v) benzene-acetone and visualized using iodine crystals. Bands were cut from the TLC sheet and analyzed for ^{3}H and ^{14}C activity using a scintillation counter. Enzyme activities are expressed as picomoles of mevalonic acid synthesized per min per mg protein (pmoles/min per mg).

ACAT activity assay

Fifty micrograms of sample was mixed with 6.7 µl of 50 mg/ml BSA and ACAT buffer (0.1 M Tris HCl, 0.25 M sucrose, and 1 mM EDTA, pH 7.5) to a final volume of 200 µl. This

mixture was incubated for 5 min in the 37ºC water bath. After incubation, the sample was treated with 2.5 µl (56.0 mCi/mmol in 200µl) ^{14}C-oleyl coenzyme A for 3 min. This reaction was stopped using 1 ml of chloroform:methanol (1:1 v:v). After the reaction was stopped 2 ml more of chloroform:methanol (2:1), 20 µl ^{3}H cholesterol, 1 ml 0.9% NaCl, 50 µl of 1 mM cold cholesteryl oleate and 10 µl cold cholesterol were added to the mixture. This was incubated for 60 min in the 37ºC water bath. Samples were centrifuged at 1000 g for 3 min for phase separation and then the top layer was aspirated away. The nitrogen blower was then used to dry down the samples. Residues were treated and analyzed by the same method outlined in the radioactive cholesterol and cholesteryl ester quantification experiment above. ACAT activities are expressed as picomoles of cholesteryl oleate synthesized per min per mg protein. Our ACAT assay uses oleyl coenzyme A as substrate which is preferentially used by ACAT1 [36].

Cell and tumor cholesterol and cholesteryl ester analysis

Cells and tumors were lysed in 400µL of modified RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40 plus protease inhibitors) for 30 min. Tumors were thawed on ice then homogenized using a PowerGen 125 homogenizer for 35 seconds in 3X (volume per weight) water on ice. Both cell and tumor homogenates were extracted with chloroform:methanol (2:1) and 0.9% NaCl at 37ºC for 1 hour. Samples were centrifuged at 2000 RPM for 1 min in order to separate phases. The top phase was discarded and the bottom phase containing the lipids was dried down under nitrogen gas flow. Samples were reconstituted in 100 µL isopropanol for cholesterol quantification. Ten microliters of sample in triplicate were analyzed for total cholesterol content (492 nm) (Stanbio, Boerne, TX) as well as for free cholesterol content (650 nm) (Wako, Richmond, VI). Cholesteryl ester concentration was determined by subtracting the free cholesterol from the total cholesterol.

Cells incubated with [1(2)-^{14}C]-Acetic Acid (55.0 mCi/mmol in 20µl, Amersham Biosciences, Baie d'Urfe, Quebec, Canada), a precursor of cholesterol, for 96 hrs were lysed and extracted following method above. Remaining lipid residue were reconstituted in 75 µL chloroform and sealed with Parafilm^{TM}. Samples were blotted onto a silical gel TLC plate and developed in 80 ml of hexane:ethyl acetate (9:1, vol:vol). After the samples had separated in the TLC chamber for 90 min. they were visualized using iodine crystals. The cholesterol and cholesteryl oleate bands were analyzed using a scintillation counter.

Lipid content was corrected by protein concentration which was measured using a BIO-RAD protein determination kit (Hercules, CA) as per manufacturer instructions. Absorbencies were read at 590 nm on the spectrometer and compared to a BSA standard curve in order to determine total protein concentration in each sample.

Western blot analysis

Cell lysates, microsomes and tumors were separated by SDS-PAGE, electroblotted into nitrocellulose membrane and probed with different antibodies: HMG-CoA reductase (Fitzgerald Industries, Concord MA), ACAT1 and actin (Santa Cruz Biotechnology, Santa Cruz, CA), LDL-r, SR-B1 and ABCA1 (1:1,000 Novus Biologicals, Littleton, CO) and ACAT2 (kindly provided by Dr L. Rudel, Wake Forest University, Winston-Salem, NC). Actin was used as a protein loading control.

Tumor *de novo* synthesized cholesterol analysis

Tumor cells were radiolabeled with [^{14}C-acetate] as described previously in **Chapter 2**. Briefly, excised tumor specimens were teased apart in 5 mL of media and excess debris was removed. After centrifuging remaining fraction at 500 Xg for 4 min the supernatant was aspirated and the pellet was resuspended in fresh media, (2mL per test condition) and transferred to six-well tissue culture plates. One hour after plating cells 0.002mCi/mL [^{14}C]-acetic acid was added to the cultures. After 4 days of incubation with radioisotope at 37ºC and 5% CO_2, the cells were harvested and centrifuged at 13500 Xg for 10 min. The supernatant (media) was transferred to a fresh tube and both cell pellet and supernatant were stored at -80ºC.

Lipids were extracted using MTBE. Briefly, *ex vivo* cell pellets (and tumor homogenates) were reconstituted in 450uL MeOH, 50uL 1M NaOH and 1500uL MTBE with rotation for 30 min. at room temperature. This extraction was repeated and lipid layer was pooled and dried down with previous extract. [^{3}H-progesterone] was utilized as an internal standard.

For LC-radiometric detection quantification of cholesterol, samples were redissolved initially in 40uL of 70:30 chloroform:MeOH and then 160uL of MeOH was added to this mixture. 10uL was injected for analysis on a Waters 2695 Separations Module coupled with a Packard (Perkin-Elmer, Wellesley, MA) Radiomatic^{TM} Model 150TR detector. Separations of lipids was performed using a Waters Exterra 2.1X150mm 5µm, C18 column equilibrated with 10:90 acetonitrile (ACN):H_2O, ramped to 25:75 ACN:H_2O (0.75-2 min), then to 80:20

ACN:H_2O (2-7 min). Isopropanol (IPA) was introduced at this time from 80:0:20 ACN:IPA:H_2O to 70:30:0 ACN:IPA:H_2O (7-30 min), retained at 25:0:75 until 31 min and returned to starting conditions at 35 min for re-equilibration up to a 35 min run length. LC flow rate was 0.3mL/min, column temperature was 30ºC and Radiomatic^{TM} scintillation fluid (Ultima Flo M, Perkin-Elmer, Wellesley, MA) flow rate was 1mL/min.

Tumor steroids analysis

Tumors were quickly thawed and pulse homogenized using a PowerGen125 instrument for 35 seconds in 3X (volume per weight) water on ice. Homogenates were stored at 80^0C until further steroid analysis. Extraction of steroids was carried out using the same assay as done for the analysis of *de novo* synthesized cholesterol in tumors.

Tumor homogenate extracts were reconstituted in 50% MeOH and derivatized in 0.2M hydroxylamine-HCl at 65^0C for 1hr for steroid analyses. Mass transitions specific to the individual steroids were identified to create mrm's (multiple reaction monitoring) similar to those outlined in **Chapter 2**. A 7-point calibration curve was used to quantify levels of pregnenolone, progesterone, testosterone and dihydrotestosterone using deuterated testosterone as an internal standard with detection limits of approximately 100, 25, 5 and 25pg/mL, respectively. Levels of steroids are normalized to initial tumor weight.

Statistical analysis

Normalized protein expressions were compared between treatment groups by an unpaired t-test (INSTAT; GraphPad) or among AD, N and CRPC tumors by a one-way ANOVA. Critical differences were assessed by Tukey post hoc tests. A difference was considered significant if the probability of chance explaining the results was reduced to less than 5% ($P<0.05$).

4.3 Results

Part 1:

Androgen effect on PSA, LDL-r and HMG-CoA reductase expression in PC-3 and LNCaP cells

At a functional level the response of CaP cells to R1881 was assessed by measuring AR-induced PSA secretion. LNCaP cells (n=4) treated with R1881 showed a marked increase in PSA secretion ($p<0.001$), while its expression was unchanged in PC-3 cells (n=4) (**Figure 4.1a**).

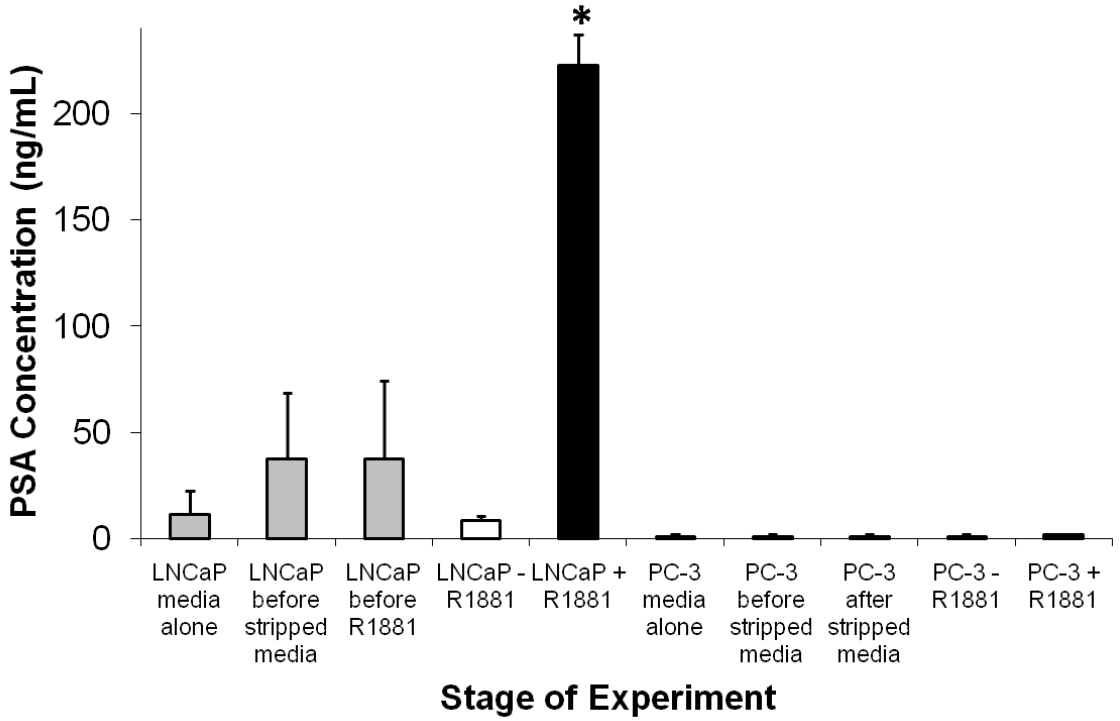

Figure 4.1a: Effect of R1881 treatment on PSA secretion in PC-3 and LNCaP cells. Serum PSA protein concentrations in LNCaP and PC-3 cells undergoing treatment with ethanol [control] (☐) and R1881 (■) (n=5 per group). Bars represent the mean value + s.d. * indicates a significant (p<0.01) increase compared to ethanol control.

Additionally we assessed the expression of the LDL-r and HMG-CoA reductase in PC-3 cells (**Figure 4.1b**).

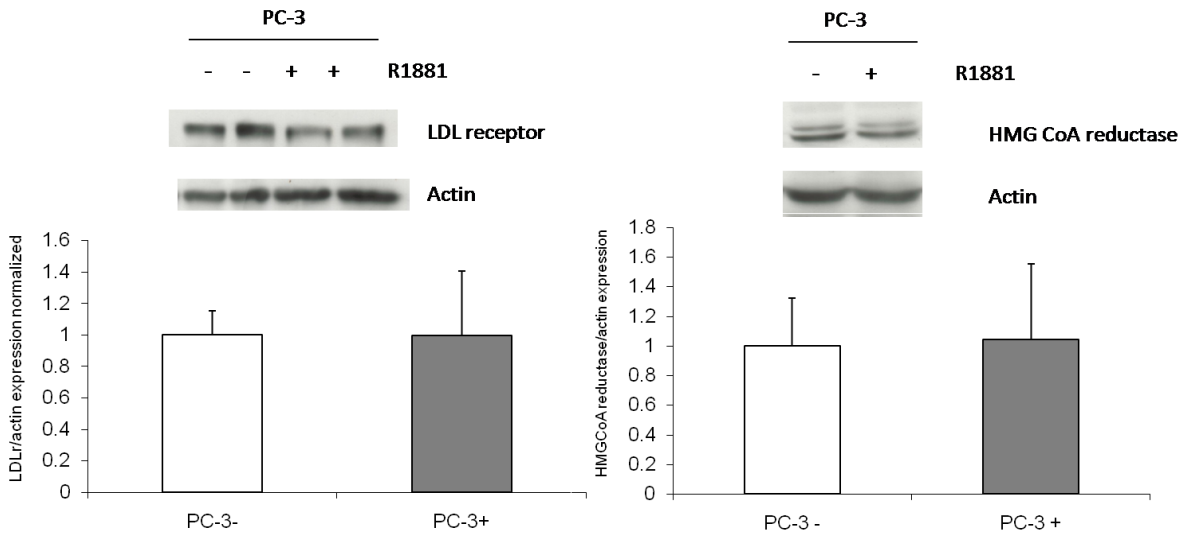

Figure 4.1b: Effect of R1881 on LDL-r and HMG-CoA reductase in PC-3 whole cell lysates. LDL receptor and HMG-CoA reductase protein expressions in lysates from PC-3 cells from control (□) and R1881 treated (■) cells (n=7 per group). The bottom panel represents densitometric scanning of the results normalized by actin levels. Bars represent the mean value + s.d.

The LDL-r was highly expressed in PC-3 cells, though its expression did not change significantly upon R1881 treatment (n=7). LNCaP cells presented a low expression of the LDL-r (data not shown). The HMG-CoA reductase protein levels were also higher in PC-3 cells (**Figure 4.1b**) compared to LNCaP cells (data not shown), however there was no significant change in expression in untreated and R1881-treated groups in either cell line.

Androgen effect on cholesterol and cholesteryl ester levels in PC-3 and LNCaP cells

The levels of free cholesterol and CEs were similar in PC-3 cells (n=3) treated with R1881 compared to the controls (**Figure 4.2a**). LNCaP cells (n=3) treated with R1881 showed a decrease in free cholesterol ($p<0.001$) and an increase in CEs ($p<0.001$) (**Figure 4.2b**). Overall, PC-3 cells contain higher levels of free cholesterol and CEs than LNCaP cells.

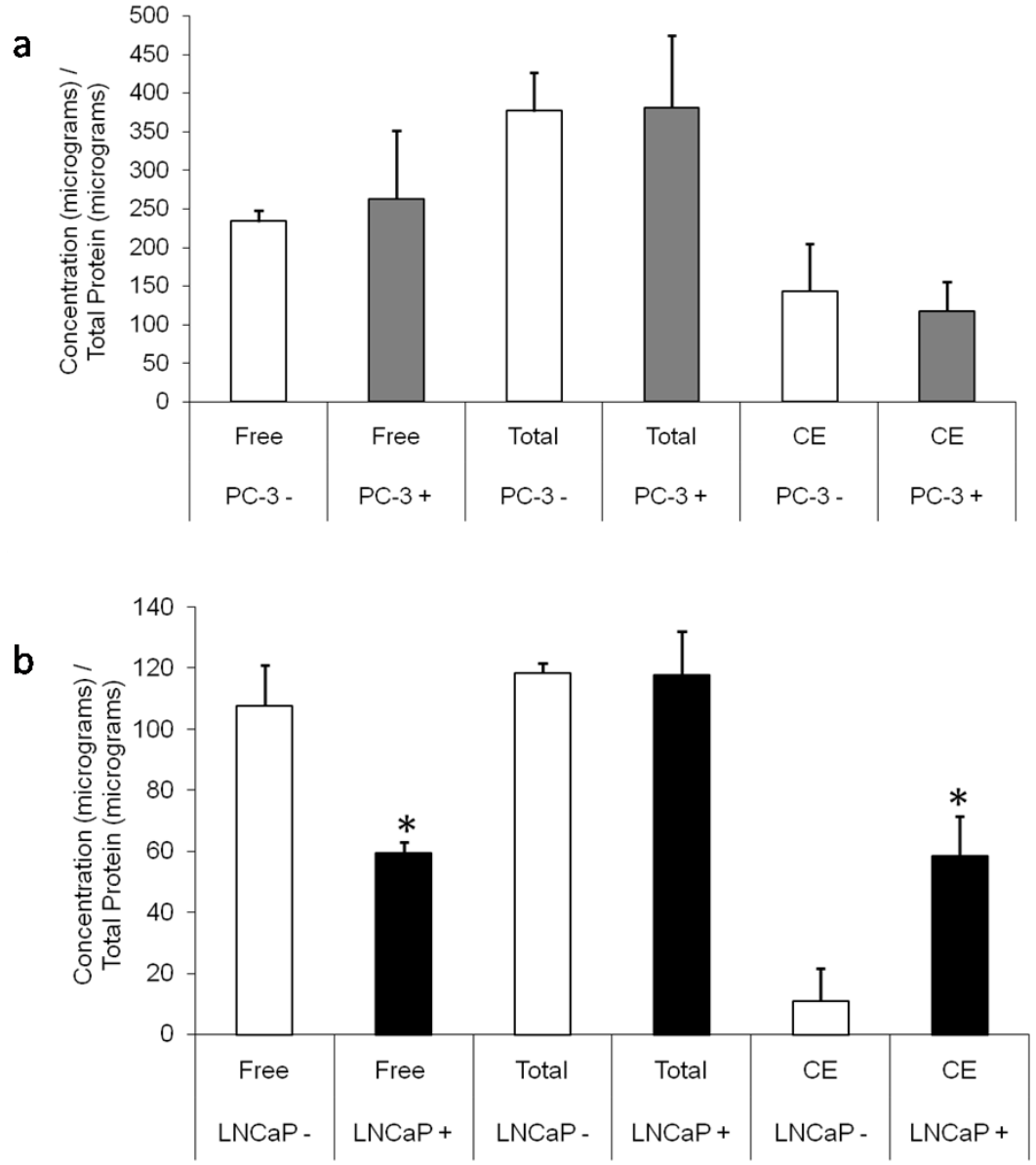

Figure 4.2a,b: Effect of R1881 on cholesterol and cholesteryl ester levels. Free cholesterol, total cholesterol, and cholesteryl ester concentrations as depicted in micrograms normalized to total protein content in control (□) and R1881 treated (■) PC-3 (**a**) and (■) LNCaP (**b**) cells (n=3 per group). Bars represent the mean value + s.d. * indicates a significant ($p<0.001$) increase compared to control.

Microsomal HMG-CoA reductase activity and expression in PC-3 and LNCaP cells

Results shown in **Figure 4.3a** indicate a significantly higher HMG-CoA reductase activity associated with the R1881-treated PC-3 cells compared to the untreated cells (n=4,

P=0.0329). Similarly LNCaP cells treated with R1881 (**Figure 4.3a**) showed a higher HMG-CoA reductase activity compared to untreated cells, albeit not significantly different (n=4, P=0.1266). HMG-CoA reductase activity in PC-3 cells treated with R1881 was higher than any of the other groups.

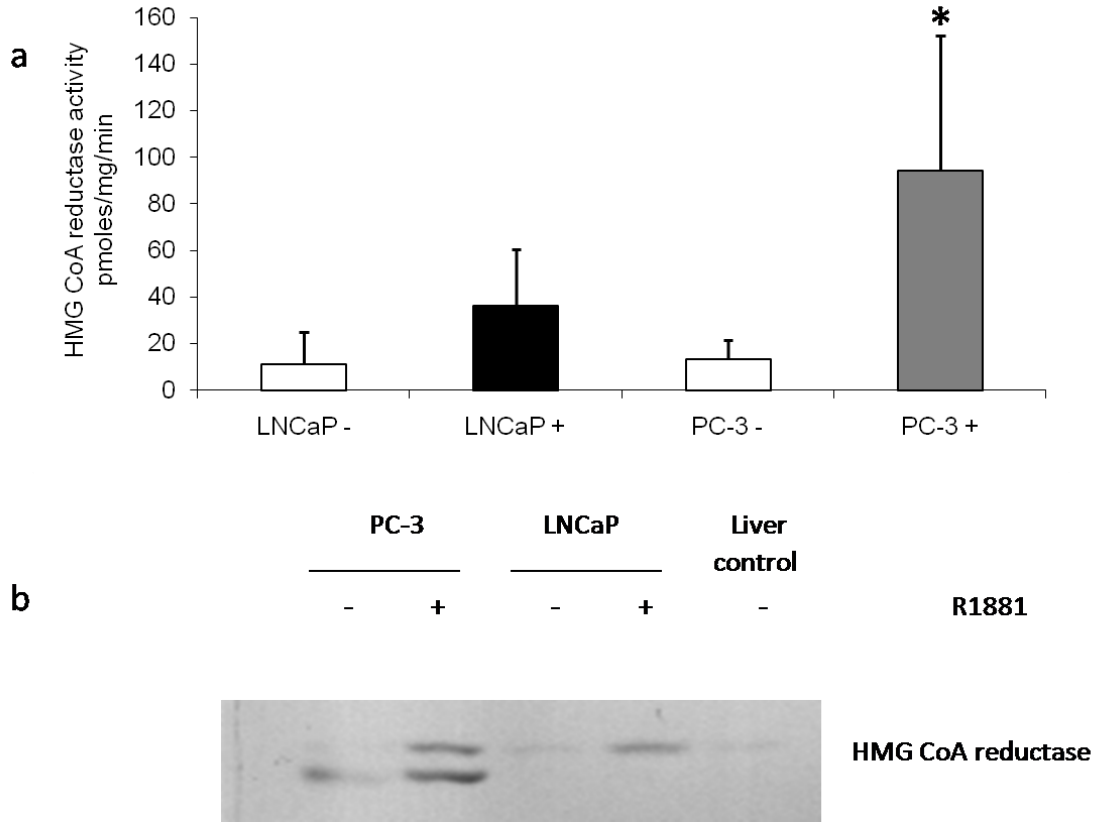

Figure 4.3a,b: Effect of R1881 on HMG-CoA reductase. HMG-CoA reductase activity (pmoles/mg/min) in microsomes obtained from control (☐) and R1881 treated (■) LNCaP and (■) PC-3 cells (n=5 per group) (**a**). Bars represent the mean value ± s.d. * indicates a significant (p<0.01) increase compared to control. HMG-CoA reductase protein expression in microsomes from control (-) and R1881 treated (+) LNCaP and PC-3 cells (n=3 per group) (**b**). The results correspond to one representative experiment and liver isolates were used as a positive control.

There was an increase in HMG-CoA reductase protein expression in microsomes in R1881-treated PC-3 and LNCaP cells as compared to untreated cells (**Figure 4.3b**). LNCaP microsomes showed a lower overall expression of HMG-CoA reductase compared to PC-3 cells (**Figure 4.3b)**.

ACAT1 and 2 activity and expression in PC-3 and LNCaP cells

In **Figure 4.4** there is a higher *in vitro* ACAT1 activity associated with the PC-3 (n=4) treated with R1881 than the untreated cells (P= 0.045). LNCaP cells (n=6) treated with R1881 exhibited a reduced ACAT1 activity (**Figure 4.4**) which was not significantly different to control cells (P=0.180).

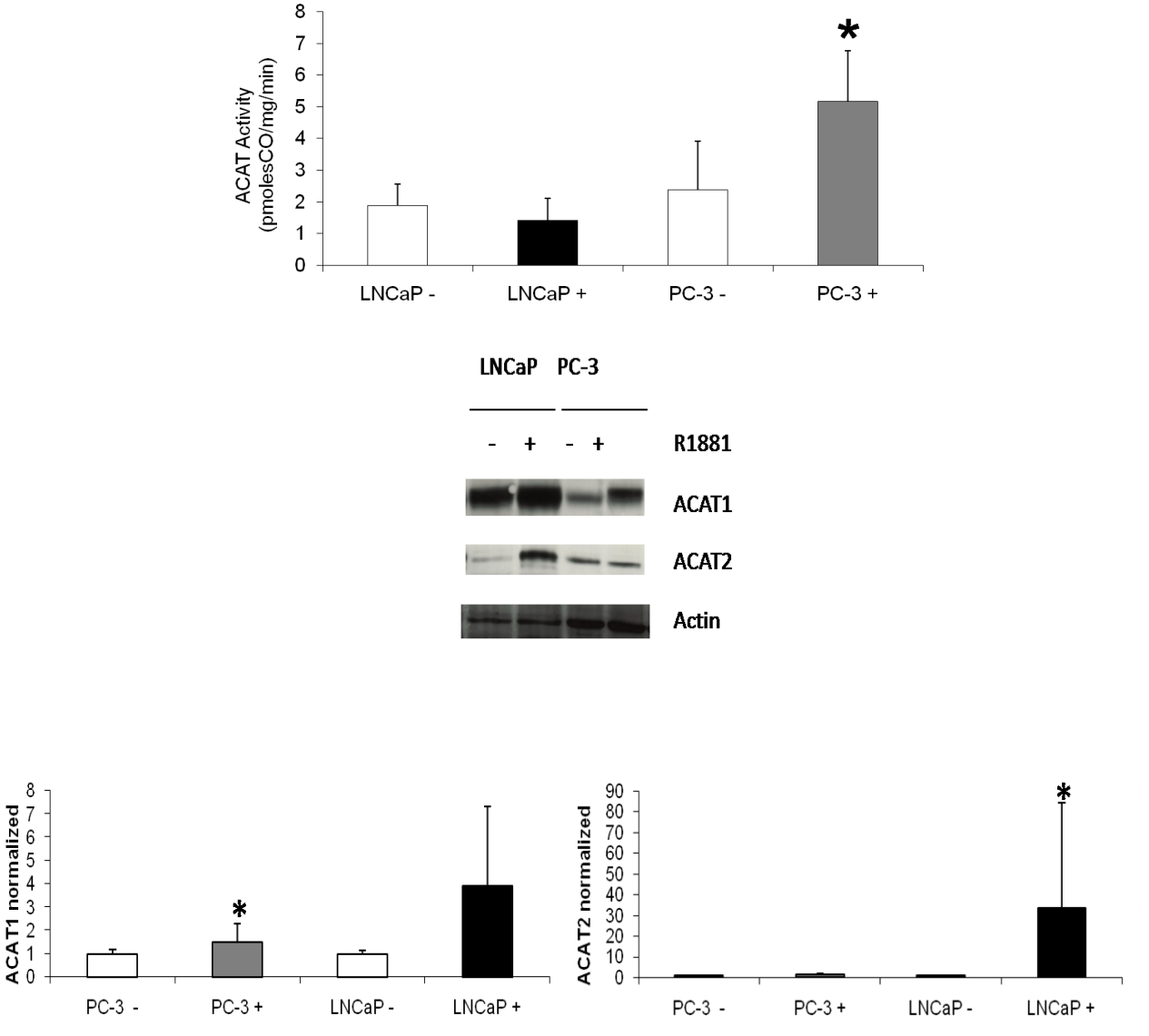

Figure 4.4: Effect of R1881 on ACAT. ACAT activity (pmoles/mg/min) in microsomes obtained from control (□) and R1881 treated (■) LNCaP and (■) PC-3 cells (n=6 per group) (bottom). Bars represent the mean value + s.d. * indicates a significant (p<0.01) increase compared to control. ACAT1 and ACAT2 protein expression in lysates from control (-) and R1881 treated (+) LNCaP and PC-3 cells (n=3 per group) (middle). The results correspond to one representative experiment for protein expression (bottom).

To investigate the molecular mechanisms accounting for the increased *in vitro* ACAT activity found in the PC-3 cells treated with R1881, we analyzed the expression of the two known ACAT isoforms, ACAT1 and ACAT2. ACAT1 expression was induced in PC-3 cells (n=5) treated with R1881 (P=0.0023) (**Figure 4.4**), which is consistent with the increased ACAT1 activity that we detected previously. ACAT2 expression was not significantly affected in PC-3 cells. In the LNCaP cells, we found an increase in ACAT2 expression (**Figure 4.4**) ($p<0.05$) but not ACAT1. The lack of induction of ACAT1 expression in LNCaP cells treated with the agonists, explains why we did not observe an increased microsomal ACAT1 activity.

Part 2:

Total, free, *de novo* synthesized and esterified cholesterol levels in AD, N and CRPC LNCaP xenograft tumors

The tumor levels of total and free cholesterol increase gradually from AD (n=10) to N (n=10) to CRPC (n=10) (not statistically significant) (**Figure 4.5a,b**) while CEs significantly increase in N (n=10) tumors as compared to AD (n=10) tumors ($p<0.05$) (**Figure 4.5d**). Furthermore, the cholesteryl esters appear to decrease in CRPC (n=10) tumors to levels previously observed in AD (n=10) tumors (not statistically significant). The *de novo* synthesized cholesterol levels decrease from AD (n=3) to N (n=3) (not significantly) and then increase at CRPC (n=3) ($P<0.05$) (**Figure 4.5c**).

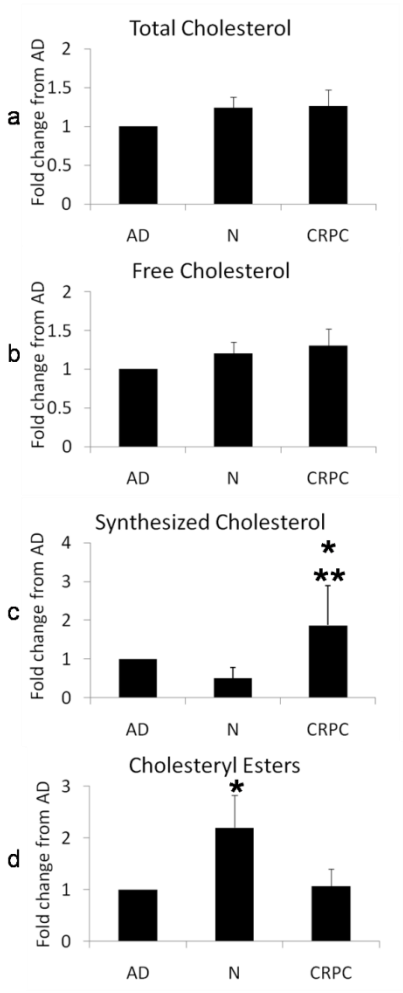

Figure 4.5a-d: Total, free, *de novo* synthesized and esterified cholesterol levels in AD, N and CRPC tumors. Total cholesterol (**a**), free cholesterol (**b**) and cholesteryl ester (**d**) concentrations as depicted in micrograms normalized to total protein content and AD value of the same mouse in AD (n=10), N (n=10) and CRPC (n=10) tumors. *De novo* synthesized cholesterol (**c**) concentrations as depicted as radiometric counts normalized to internal standard and AD value of the same mouse in AD (n=3), N (n=3) and CRPC (n=3) tumors. Bars represent the mean value + standard error of the mean. *,** indicate a significant (p<0.05) increase compared to AD and N, respectively.

Protein expression of the enzymes required for cholesterol influx, synthesis, metabolism and efflux in AD, N and CRPC LNCaP xenograft tumors

The protein expression of uptake enzyme SR-B1 decreases from AD (n=10) to N (n=10) (not statistically significant) and then re-emerges at CRPC (n=10) (p<0.05) (**Figure 4.6a**). Although much lower, LDL-r expression follows a similar trend to that of SR-B1; however it is not statistically significant (**Figure 4.6b**). HMG-CoA reductase expression appears to increase in N (n=10) tumors as compared to AD (n=10), however this is not statistically significant (**Figure 4.6c**). Both ACAT1 and 2 appear to decrease in protein expression from N (n=10) to CRPC (n=10) (p<0.05 for ACAT-2) (**Figure 4.6d,e**). ABCA1 expression is significantly increased at N (n=10) and remains high at CRPC (n=10) (p<0.05) (**Figure 4.6f**).

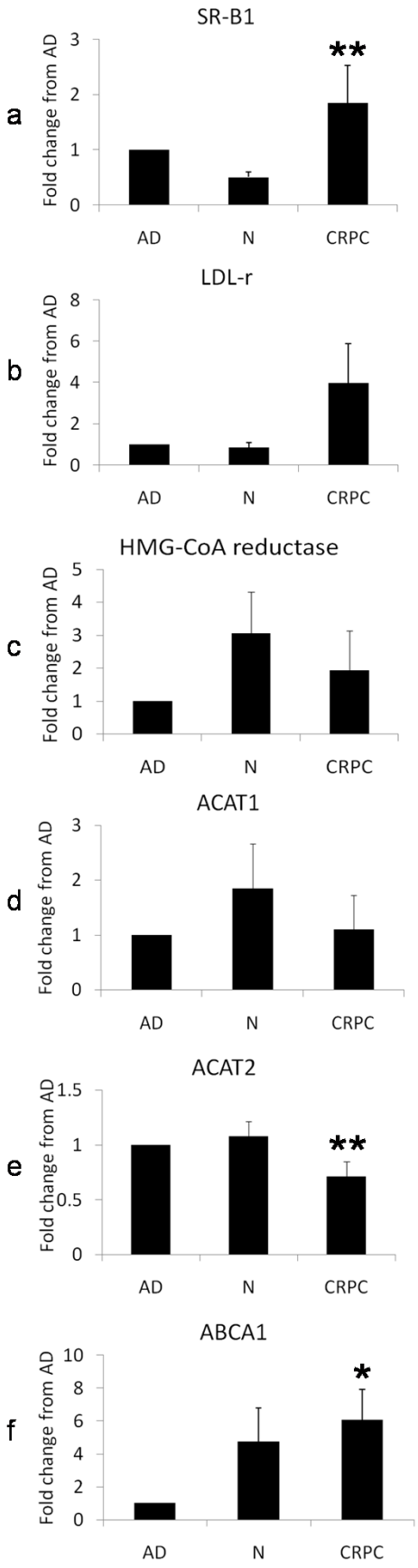

Figure 4.6a-f: Enzyme expressions in AD, N and CRPC tumors. SR-B1 (**a**), LDL-r (**b**), HMG-CoA reductase (**c**), ACAT1 (**d**), ACAT2 (**e**) and ABCA1 (**f**) protein expressions in AD (n=10), N (n=10) and CRPC (n=10) tumors. Bars represent the mean value + standard error of the mean. *, ** indicates a significant (p<0.05) difference compared to AD and N, respectively.

Tumor androgen levels in AD, N and CRPC LNCaP xenograft tumors

Levels of testosterone significantly decrease from AD (n=6) to N (n=6) 98.4% ($p<0.001$) and then increase slightly from N (n=6) to CRPC (n=8) (93.5% of AD) ($P<0.05$) (**Figure 4.7a**). DHT levels increase approximately 2-fold from AD (n=6) to N (n=6) to CRPC (n=8), although this increase is not statistically significant (**Figure 4.7a**). Precursor steroid progesterone appears to increase slightly from AD (n=6) to N (n=6) and remain at this level at CRPC (n=8) (not statistically significant) (Figure 5a). The results for testosterone, DHT and progesterone are consistent with our previously findings (**Chapter 2**). We also determined the levels of pregnenolone, the most upstream precursor steroid of testosterone and DHT; it appears to decrease from AD to N and then increase once again at CRPC (not statistically significant) (**Figure 4.7a**).

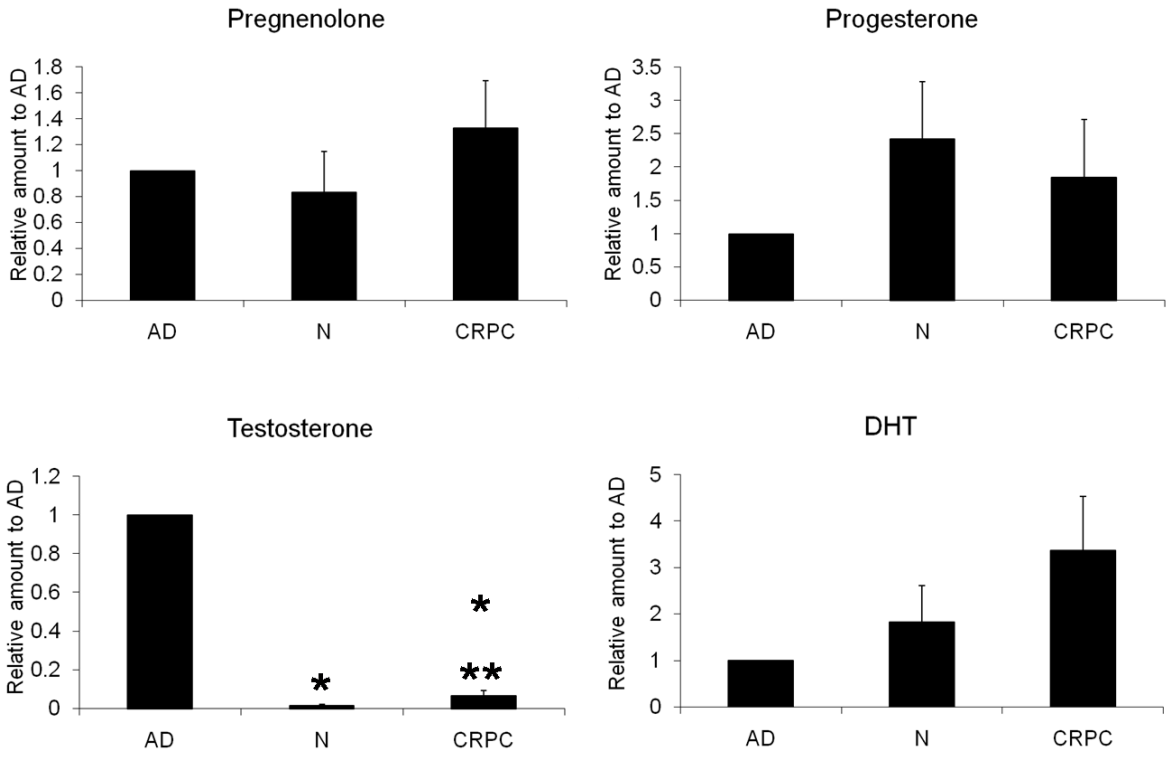

Figure 4.7a: Steroid and androgen levels in AD, N and CRPC tumors. Depicted are the mean levels of pregnenolone, progesterone, testosterone and DHT in AD (n=6), N (n=6) and CRPC (n=8) tumors. Amounts normalized to tumor weight and depicted as fold change from AD tumor in the same mouse. *, ** indicate statistically different from AD and N, respectively ($P<0.05$).

4.4 Discussion

In the **part 1** of the study the activity of cholesterol metabolism enzymes, HMG-CoA reductase and ACAT were investigated in PC-3 and LNCaP cells in the absence and presence of synthetic androgen, R1881.

The HMG-CoA reductase activity was higher overall in PC-3 cells as compared to LNCaP cells and this result is consistent with a study by Sivaprasad *et al*., [37] which showed that PC-3 cells required higher levels of pravastatin (an HMG-CoA reductase inhibitor) than LNCaP cells to inhibit cholesterol synthesis. Androgen treatment led to an increase in HMG-CoA reductase activity in both PC-3 and LNCaP cells. In LNCaP cells this induction likely occurred through AR activation however, it was unexpected that the PC-3 cells responded to androgen treatment as this cell line is believed to lack appreciable AR expression [38] and thereby not normally responsive to androgen stimulation. Our PSA expression results also indicate the latter as PSA is an androgen-inducible target gene [39, 40]. However, the induction of microsomal HMG-CoA reductase *in vitro* activity in R1881-treated PC-3 cells was associated with an increase in microsomal HMG-CoA reductase expression (**Figure 4.3b**) not observed in whole cell lysates (**Figure 4.1b**) suggesting an enhanced translocation or reduced turnover of the enzyme in the microsomal compartment of R1881-treated cells perhaps through an AR independent mechanism. An alternative explanation is the triggering of post-translational modifications that could modulate HMG-CoA reductase activity [41, 42]. These mechanisms may explain how HMG-CoA reductase activity is induced in R1881-treated PC-3 cells. Contrary to the HMG-CoA reductase activity results in **Figures 4.2a** it was demonstrated that there is no difference in the levels of cholesterol upon R1881 treatment in PC-3 cells. Combined these results suggest that there are other mechanisms that mediate the efflux of this lipid that may be upregulated in PC-3 cells treated with R1881 that work to balance the overall cholesterol levels within the cell.

In agreement with results in **Figure 4.2b**, the induction of CE levels by R1881 have been reported by Carpten *et al*., [43] in LNCaP cells. This functional endpoint result indicates that either one or both of the ACAT isoform(s) is (are) expressed in these cells OR there is an induction in CE uptake (or a reduction of CE secretion) upon R1881 treatment AND/OR there is a decrease in cholesteryl hydrolase activity. We demonstrate that ACAT2 expression but not activity was increased upon androgen stimulation in LNCaP cells (**Figure**

4.4) while both microsomal *in vitro* ACAT1 expression and activity were increased upon androgen stimulation in PC-3 cells (**Figure 4.4**). Lack of ACAT activity observed in androgen stimulated LNCaP cells may have occurred in this experiment because ACAT2, the enzyme induced in LNCaP cells utilizes preferentially linolenoyl CoA as substrate [36] and not oleyl CoA as in the case of ACAT1. This would explain why we could only detect *in vitro* ACAT activity in PC-3 where ACAT1 may play a more important role than ACAT2 which shows no change in expression in that model.

In the first part of the study it was verified that both HMG-CoA reductase and ACAT activity are regulated by androgens in CaP cells. In the future, the enzymes' activity profiles may contribute to deciphering the mechanism of change from androgen-dependent to castration-resistant disease. However, it is difficult to determine this in the current explored incongruent CaP cell models. Additionally, a growing theme from this *in vitro* study is that there are possible compensatory mechanisms within the cell which can ultimately culminate in the tight regulation of intracellular cholesterol and CEs at different times during disease progression. In order to address this issue in **part 2** of this chapter we explored the expressions of these enzymes in the context of all-encompassing cholesterol regulation as well as global cholesterol levels during CaP progression as mimicked by the LNCaP xenograft model. No significant differences in the tumor levels of total and free cholesterol were observed. This result is expected as free cholesterol in its native form is toxic to the cell [26, 35]. The interlinked influx, efflux, synthesis and metabolism mechanisms balance each other out and minimize the cellular toxicity associated with free cholesterol; thereby contributing to an overall constant cholesterol level within the tumor. However, changes in cholesteryl ester levels and *de novo* synthesized cholesterol levels were observed during the course of the disease to CRPC in this model. The observed increase in cholesteryl ester levels immediately after castration (N) may reflect the tumor's ability to store and then provide cholesterol in the absence of testicular androgens for steroid synthesis or membrane formation during progression to CRPC. Furthermore, *de novo* synthesized cholesterol levels appear to increase during progression from N to CRPC suggesting that increased cholesterol production via both *de novo* cholesterol synthesis and cholesteryl ester breakdown occurs in tumors at CRPC. The increased *de novo* synthesized cholesterol at CRPC is supported by the increased protein expression of HMG CoA reductase, the rate-limiting enzyme responsible

for endogenous cholesterol synthesis [26, 25] at N. Furthermore, the increased cholesteryl ester formation at N is supported by the high protein expression of ACAT1 and 2, enzymes responsible for cholesteryl ester formation from cholesterol [26], at N. In support of cholesteryl ester breakdown at CRPC in another report we demonstrated that the expression and activity of hormone sensitive lipase (HSL), which is crucial for cholesteryl ester cleavage in steroidogenic cells [44, 45], is increased at N and remains high at CRPC, coincidently at the same time cholesteryl ester cleavage would be necessary for providing precursor for *de novo* androgen synthesis or membrane formation (**Chapter 5**). These results demonstrate that changes in the enzymatic profiles of cholesterol synthesis and metabolism are altered after castration likely to produce larger amounts of cholesterol for *de novo* androgen synthesis in an environment deprived of testicular androgens during progression to CRPC (while maintaining overall free and total cholesterol levels within the cell).

The levels of cholesterol uptake enzymes, LDL-r and SR-B1 decrease in expression at N and then increase once again at CRPC suggesting that exogenous cholesterol uptake may also provide precursor for intratumoral androgen synthesis at CRPC. SR-B1 has been shown to mediate both the influx and efflux of cholesterol [46, 47]. We suggest that it is an influx protein in CaP cells because they are steroidogenic in nature and SR-B1 has previously been verified to be the main HDL-cholesteryl ester uptake transporter in steroidogenic cells [44,48-50]. Furthermore, overexpression of SR-B1 has previously been shown to induce steroidogenesis (progesterone synthesis) in adrenal cells and therefore may provide a similar function in CaP cells after castration [51]. LDL-r, an alternate route for LDL cholesterol internalization [44], is also increased in protein expression from N to CRPC, however this difference was not significant. Although the main mechanism for exogenous cholesterol internalization occurs via SR-B1, LDL-r may also be contributing to the increased amounts of intratumoral cholesterol for *de novo* steroidogenesis at CRPC.

The observed increase in efflux protein, ABCA1 at CRPC was not initially anticipated. Upon investigation, this result may account for the decreased cholesteryl ester content at CRPC or ABCA1 may act through a compensation mechanism for the hugely increased influx, synthesis and de-esterification of cholesterol within the tumor to maintain overall free and total cholesterol levels.

Several groups have demonstrated that after castration, or in an androgen deprived environment, intracellular cholesterol is important for mediating membrane cell signaling events linked to survival, proliferation and metastasis of the CaP cell [20,52,53]. In this report we describe a new mechanism that may contribute to the survival, proliferation and metastasis of these cells. In another report we demonstrated that the expression and activity of steroidogenesis rate-limiting mitochondrial cholesterol transport enzyme, steroidogenic acute regulatory protein (StAR), is increased in a castrate environment (**Chapter 5**). Combining these previously reported results (**Chapter 5**) with those suggesting increased cholesterol levels at CRPC in this paper we suggest that changes in cholesterol regulation enzymes provide precursor for *de novo* androgen synthesis and that this is an important event during progression of the disease.

Upon further investigation into this potential mechanism we also measured the tumor levels of progesterone, testosterone and DHT. Our results are consistent with our previously reported findings (**Chapter 2**) in this LNCaP xenograft model, that both testosterone and DHT increase during progression from N to CRPC to levels sufficient for AR activation (greater than 10^{-14}M ($2.92x10^{-6}$ng/g)) [8]. This result further supports that the observed changes in cholesterol regulation processes are related to increased production of androgens for subsequent AR reactivation after castration within the tumor. Herein, we demonstrate that in coordination with a decrease in levels of cholesteryl ester stores, increased *de novo* cholesterol synthesis and increased expression of enzymes responsible for mitochondrial cholesterol uptake (HSL, StAR, CYP11A1) (**Chapters 2 and 5**), an increase in testosterone and DHT production from cholesterol also occurs at CRPC. This finding for the first time provides evidence that increased intratumoral cholesterol may impact *de novo* steroidogenesis in a castrate environment during progression of CaP to CRPC. In this study we also evaluated the levels of pregnenolone, the most upstream precursor steroid of androgens [54-56]. The rate-limiting step in androgen synthesis stems from cholesterol conversion to pregnenolone in the mitochondria by enzymes CYP11A1 and StAR [56, 57]. Our data suggests that CRPC tumors do in fact utilize cholesterol to produce large quantities of pregnenolone (14.4X DHT levels). Furthermore, this dilution in total androgen production from pregnenolone is in agreement with the literature as pregnenolone is a precursor for

several other biologically important steroids (corticosteroids, progesterone, estrogen, etc.) [56-58].

There are however, two limitations to this study 1) is our chosen time point for nadir tumors may not reflect a truly nadir point for cholesterol regulation processes. Initially, 8-days post-castration was chosen for nadir tumor excision because this time point most ideally reflected a nadir PSA measurement in the serum of the corresponding mouse (**Chapter 2** and **APPENDICES**) [59]. Because alterations in cholesterol regulation processes are likely a fast adapting event in the castrate tumor environment as compared to downstream PSA secretion, our time point 8-days post castration may be too late to capture the nature of cholesterol regulation processes in a truly nadir state. Nonetheless, at our chosen time the data reflects an increasing rise in cholesterol synthesis and esterification enzymes alongside an increase in overall cholesteryl ester levels. Furthermore, the trend in enzymes appears to continue to favor cholesterol accumulation within the cell at CRPC via cholesterol influx enzymes and *de novo* synthesis. These results show that cholesterol regulation processes are altered initially from AD to N and then once again in a different manner from N to CRPC and demonstrates the truly complex events that occur in a tumor during progression of the disease. 2) it is difficult to decipher if the increased production of cholesterol can be attributed to *de novo* androgen synthesis or purely membrane formation in the ever proliferating cells. Future radiotracing studies are aimed to delineate these mechanisms.

Many reports have previously demonstrated increased expressions of several enzymes important in cholesterol synthesis in clinical progression [24, 19, 23] but few have investigated cholesterol influx, metabolism and efflux processes. Our work highlights the importance of not only cholesterol synthesis via HMG CoA reductase but also the influx via SR-B1 and the formation of cholesteryl ester stores via ACAT1 and 2. In addition, this work also provides new information for therapeutic intervention during progression of the disease to CRPC. Inhibitors targeting HMG-CoA reductase (statins) have been used for over 30 years in order to reduce hypercholesterolemia, a common disorder in men over the age of 50 [25, 60]. Coincidently, men over the age of 50 are also more likely to develop CaP [61] and so numerous retrospective studies have been conducted investigating the use of these statins and CaP incidence. Unfortunately, these studies have led to conflicting results; some suggesting that the use of statins leads to a lower prevalence of CaP incidence while

others show no correlation between statin use and CaP onset [62, 63]. These conflicting results may be attributed to the post-hoc nature of the studies, the inability to control variables in retrospective analyses and the sample populations likely having abnormally high cholesterol levels relative to the general population. Most meta-analysis reports corroborating these retrospective studies conclude that the use of cholesterol synthesis inhibiting agents (statins) can not be correlated with decreased CaP incidence [62, 63]. Herein, we introduce a novel perspective in analyzing the patient cohorts previously used in these retrospective studies and that is in the use of statins in CaP patients after diagnosis and how they effect the time to progression to CRPC. If our *in vivo* data suggesting that cholesterol processes are altered during progression of the disease leading to cholesterol accumulation for *de novo* androgen synthesis and AR activation is transferable to clinical CaP progression then statins blocking these mechanisms may contribute to increased time to progression of CRPC in humans. Few retrospective studies have been conducted investigating statin use and time to disease progression but results from them have been in agreement. To date, two large prospective cohort studies involving men who used statins, showed that patients had half the risk of developing advanced CRPC compared with control untreated patients [64, 65]. The results from these studies are not only supported by the data presented in this paper but also provide evidence to continue the evaluation of statins clinically during progression of the disease.

The work in **part 2** of this chapter for the first time demonstrates 1) that cellular cholesterol regulation processes are altered during progression to CRPC, 2) that levels of endogenously synthesized cholesterol are increased while levels of cholesteryl esters are decreased at CRPC and that both are likely being used as precursor for intratumoral *de novo* androgen synthesis and subsequent AR activation as well as membrane formation and 3) pre-clinical proof-of-principle data for the evaluation of cholesterol regulation inhibitors such as those targeting HMG-CoA reductase during progression of the disease to CRPC.

4.5 References

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CHAPTER 5: Arachidonic acid activation of intratumoral steroid synthesis during prostate cancer progression to castration-resistance[4]

5.1 Introduction

Most men with metastatic prostate cancer (CaP) initially respond to androgen deprivation therapy (ADT) but unfortunately, castration-resistant prostate cancer (CRPC) ultimately prevails. Despite reduced levels of circulating androgens in these patients after ADT, androgen receptor (AR) activation remains a central mechanism in CRPC progression [1-6]. We and others have recently demonstrated that tumors adapt to an environment deprived of circulating androgens to synthesize their own androgens for AR activation [7-9]. In clinical trials evaluating androgen synthesis inhibitors in patients with CRPC, AR-mediated PSA responses have been achieved in more than 50% of the patients supporting the role of these intratumoral androgens in disease progression [10, 11]. Future exploration into the mechanisms underlying intratumoral androgen synthesis is therefore of central importance to developing and improving treatments for this disease.

Many enzymes responsible for androgen synthesis are under the control of AR-regulated transcription factors, sterol regulatory element binding proteins (SREBPs) and are increased in expression during CRPC progression including those responsible for the regulation of endogenous fatty acids (FAs) (fatty acid synthase-FASN) and cholesterol, the central precursor of androgens [2, 9, 12-16]. FAs and cholesterol have many regulatory functions in the cell [17-21] and are the main precursors for several lipids that have been implicated in CaP development and progression [19, 22, 23]. Additionally, in steroidogenic ovarian, adrenal and testicular cells FAs have been shown to trigger steroid synthesis from cholesterol [24, 25]. We hypothesize that FAs play a significant role in mediating intratumoral androgen synthesis in CaP cells in a castrate environment.

In this study, increased AR-mediated synthesis of arachidonic acid (AA) was observed in CaP cells and interestingly, AA is known to be the most efficient FA to induce

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steroid synthesis [25]. Several groups have explored the detailed mechanisms involved in AA activation of steroid synthesis from cholesterol in other organs [24-26]. Herein, we have characterized the key proteins involved in AA activation of steroidogenesis (**Figure 5.1**, **Table 5.1**) in CaP cells and how this mechanism may contribute to the progression of CaP to CRPC (**Figure 5.1**).

Symbol	Full name	Function
ACAT2	acetyl-Coenzyme A acetyltransferase 2	catalyzes formation of cholesteryl esters from free cholesterol and free fatty acids
ACBP	acyl-Coenzyme A binding protein	involved in transport of activated fatty acid into the mitochondria by PBR
ACOT9	acyl-Coenzyme A thioesterase - 9	catalyzes reaction which converts activated fatty acid into a free fatty acid in the mitochondria
ACSL3	long-chain acyl-CoA synthetase -3	catalyzes activation of fatty acid in the cytosol and initiates transfer of activated fatty acid across the mitochondrial membrane
FASN	fatty acid synthase	responsible for endogenous fatty acid synthesis
HSL	hormone sensitive lipase	catalyzes the breakdown of cholesteryl esters into free cholesterol and free fatty acid; carries free cholesterol to outer mitochondrial membrane
PBR	peripheral benzodiazepine receptor	catalyzes the transport of activated fatty acid from the outer to inner mitochondria membrane
StAR	steroidogenic acute regulatory protein	catalyzes the transport of free cholesterol into the mitochondria for steroid synthesis

Table 5.1: Enzymes predicted to be involved in fatty acid activation of steroid synthesis. Symbols, names and functions pertaining to this mechanism are depicted.

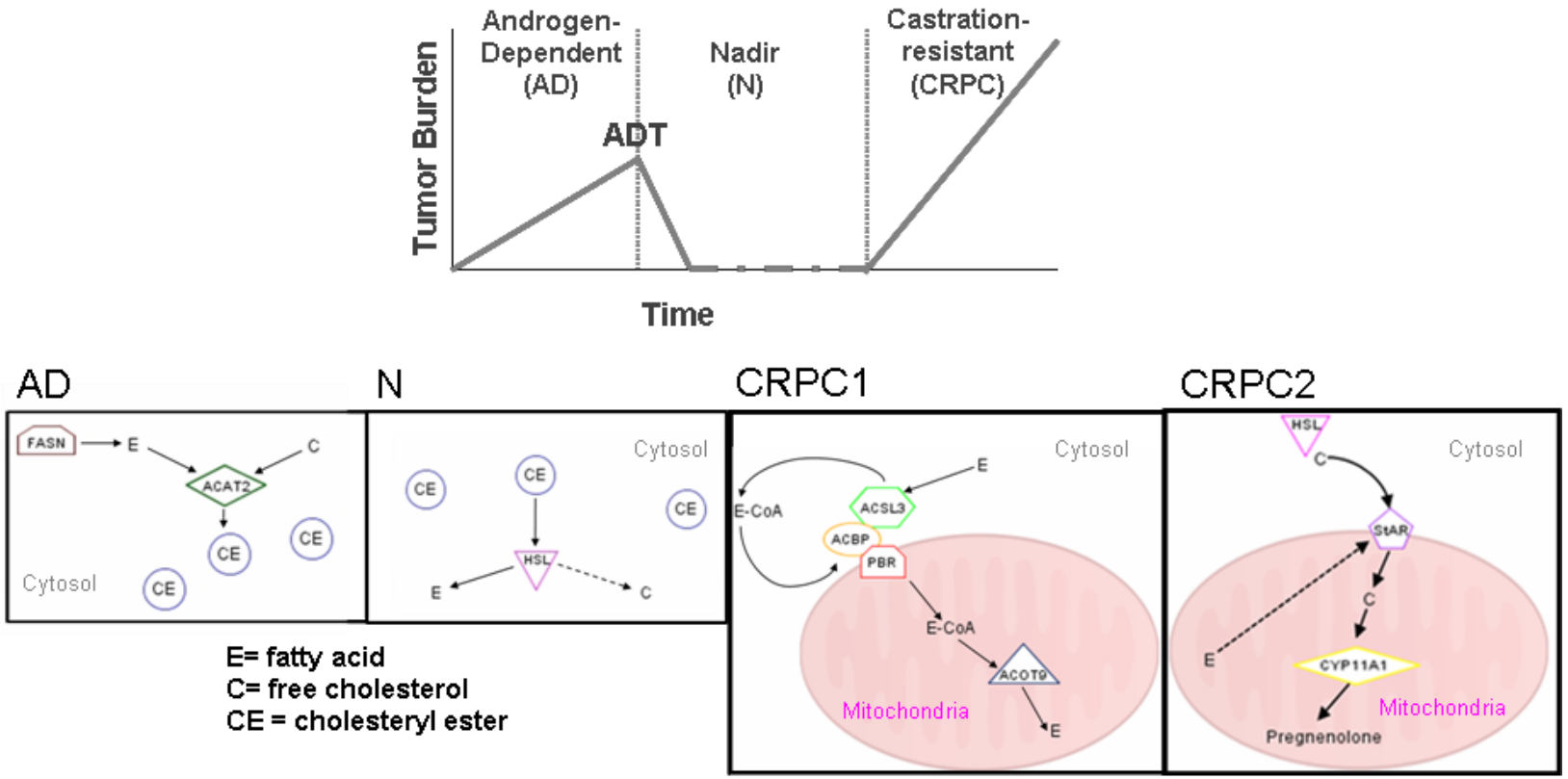


Figure 5.1: Overall schematic diagram of the proposed mechanism in CaP cells during progression to CRPC. Schematic of clinical CaP progression: initial tumor is androgen-dependent (**AD**) and after androgen deprivation therapy (ADT) the tumor enters a nadir (**N**) state until it progresses to a castration-resistant (**CRPC**) form. In the presence of androgens (**AD**) increased FASN production of arachidonic acid (E) leads to ACAT-2 induced accumulation of storage CE's. Upon ADT the tumor is deprived of androgens (**N**) and HSL cleaves CE's to form free C and free E. Free E is activated by ACSL3 in the cytosol to produce activated arachidonic acid (E-CoA) which then migrates to the outer mitochondrial membrane for transfer to the inner mitochondria via an ACSL3-ACBP-PBR complex (**CRPC1**). In the mitochondria E-CoA undergoes thioesterase reaction to produce free E which is then metabolized and activates transcription of StAR gene leading to increased accumulation of StAR protein at the mitochondrial membrane. Free C is then transported into the mitochondria by newly synthesized StAR where it undergoes side chain cleavage by CYP11A1 to form pregnenolone for subsequent conversion to downstream androgens thereby contributing to AR-mediated disease progression (**CRPC2**).

Mechanistically, in the presence of exogenous androgens *in vitro* or before castration *in vivo* CaP cells preferentially produce large endogenous stores of non-toxic cholesteryl esters (CEs) from cholesterol and FAs by an enzyme known as acyl-CoA:cholesterol acyltransferase-2 (ACAT2) [27] (**Figure 5.1-AD**). After castration *in vivo* or in the absence of exogenous androgens *in vitro*, CEs are cleaved by an enzyme known as hormone sensitive lipase (HSL) to produce free cholesterol and free FAs [28] (**Figure 5.1-N**). In turn, these FAs including AA are known to be activated by long-chain acyl-CoA synthetase (ACSL) [29] (**Figure 5.1-CRPC1**). The isoform that is highly expressed in the human prostate, ACSL3, is preferentially responsible for AA activation [30, 31]. Activated AA-CoA can be transported through a series of reactions with acyl-CoA binding protein (ACBP) and peripheral-type-benzodiazepine-receptor (PBR) to ultimately cross the mitochondrial membrane [25, 29]. In the mitochondria, AA-CoA undergoes another reaction with mitochondrial acyl-CoA thioesterase-9 (ACOT9) to once again produce free AA which can then undergo metabolism and activate the transcription of steroid acute regulatory protein (StAR) in the nucleus [24-26] (**Figure 5.1-CRPC2**). New StAR protein accumulates at the mitochondrial membrane and catalyzes the rate-limiting step involved in steroid synthesis [24-26, 32-35]; through an interaction between mitochondrial StAR and cytosolic HSL free cholesterol is shuttled into the mitochondria and subsequently converted by CYP11A1 into downstream steroid pregnenolone [24-26, 32-34, 36, 37]. This in turn triggers a cascade of reactions to produce androgens [38] which previously have been shown to be synthesized in CRPC tumors in levels sufficient for AR activation [7-9].

We propose that this characterized arachidonic acid induced steroidogenesis mechanism significantly contributes to CRPC progression and may therefore be of significance in developing and improving therapies targeting this disease.

5.2 Materials and methods

Materials

Fatty acid methyl ester (FAMEs) standards were reconstituted in hexane (Sigma Aldrich, Oakville, ON). R1881 (Dupont, Boston, MA) and casodex (Sigma-Aldrich, Oakville, Ontario, Canada) were prepared in 95% EtOH. Radioactively labeled ^{14}C-cholesterol was obtained from GE Healthcare Bio-Sciences (Piscataway, NJ).

***In vitro* Model: LNCaP Cells**

LNCaP cells (passage 40-48; American Type Culture Collection, Rockville, MD) were cultured in RPMI-1640 (without phenol red) with L-Glutamin, PS and 5% Fetal Bovine Serum (FBS, Hyclone Logan, UT) or 5% Charcoal Stripped Serum (CSS, Hyclone, Logan, UT). LNCaP cells were maintained in 5% FBS, however, 48hrs prior to treatment with R1881 cells were cultured in 5% CSS. After 72hrs of treatment with 0, 0.1, 1 and 10nM R1881 in the presence/absence of 25μM casodex, media was removed, cells were washed with PBS, scraped, pelleted at 2,000 g for 5min. and stored at -80°C.

***In vivo* model: LNCaP tumor progression to castration-resistance**

All animal experimentation was conducted in accordance with accepted standards of the UBC Committee on Animal Care. LNCaP xenograft tumors were grown in athymic nude mice at four sites; progression of the disease was monitored by tumor volume and PSA measurements [2]. Tumors were obtained at androgen-dependence (AD, pre-castration), nadir (N, 8 days post-castration) and castration-resistance (CRPC, 35 days post-castration) and processed as reported [7].

Human Prostate Samples

Prostastic tissues included 14 primary CaPs from patients undergoing radical prostatectomy with no therapy before surgery, 12 primary CaPs after 1-3 months of Neoadjuvant Hormone Therapy (NHT), 5 primary CaPs after 5-6 months NHT, 4 primary CaPs after 8-9 months NHT, and 3 CRPCs. Tissues obtained were flash frozen in OCT Compound (Tissue-Tek) until processing.

Fatty acid derivatization and extraction

Fatty acids in cell pellets and xenograft tumor homogenates were derivatized with 2% H_2SO_4 in MeOH for 2hr at 37°C in glass tubes. Hexane was added to tubes for extraction of FAMEs and phases were separated by centrifugation at 1,000 g for 5 min. 100uL of the top layer was transferred to a GC-MS vial for analysis.

FAME Analysis by Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS method for FAME analysis was adapted from [39]on a Varian 210 ion trap mass spectrometer. All MS data was collected in electron impact positive (EI+) mode with capillary voltage at 3kV, source and desolvation temperatures of 120ºC and 350ºC, respectively and N_2 gas flow of 450L/hr. Chromatographic separations were carried out

using a temperature gradient of 80ºC held at for 1min, then increased at a rate of 20ºC/min to 250ºC final temperature which was held at for another 6min; total run time of 15.50min. Flow rate was 0.3mL/min, column temperature 35ºC and 0.05% formic acid was present throughout the run. MS scan data was compared to NCIS Library for metabolite identification. Peak area comparison to a 6-point calibration curve was used to determine overall FAME yield.

Laser Capture Microdissection and Microarray Analysis

Laser capture microdissection (LCM) was performed on cancer cells using the PALM Microlaser system (P.A.L.M. Microlaser Technologies, Germany). Samples were catapulted into sterile caps of 0.5ml eppendorf tubes (RNAse-free) containing 40uL extraction buffer and total RNA was isolated according to manufacturers' instructions (PicoPure RNA Isolation Kit, KIT0204, ARCTURUS) and subjected to DNase treatment using Qiagen RNase-Free DNase kit (Qiagen, Inc). The RiboAmp HS RNA Ampification kit (KIT0215, ARCTURUS) was used to generate amplified amino allyl-modified antisense RNA according to manufacturer's instructions; amplified aRNA was quantified by spectrophotomer and the quality was assessed by running a 1% denaturing agarose gel. Amino allyl-modified aRNA was labeled using the Amino Allyl Message Amp IIa RNA Amplicfication Kit (Ambion), and the labeled aRNA was fragmented with RNA Fragmentation Reagents (Ambion) prior to hybridization. Microarrays of 34,580 (70-mer) human oligos representing 24,650 genes and 37,123 gene transcripts (Human Operon V3.0, Operon Technologies, Huntsville, Al) were printed on slides (Matrix Technologies, Hudson, NH). Microarrays were competitively hybridized with 2 ug amplified aRNA from the microdissected samples labeled with Cy5 and 2ug of amplified Universal Human Reference RNA (Stratagene) labeled with Cy3 fluors (Amersham Bioscience). Following overnight hybridization and washing, arrays were scanned on a Scan Array Express Microarray Scanner (Perkin Elmer). Signal quality and quantity were assessed using ImaGene 8.0 software (BioDiscovery, San Diego, CA). Feature data extracted by ImaGene were subjected to background correction, print-tip lowess within-array normalization and quantile between-array normalization using the LIMMA Bioconductor package [40]. A moderated t-statistic (corrected for a false discovery rate of 10%) from linear models built using LIMMA was used to determine differential expression between the treatment groups.

Immunohistochemistry

Immunohistochemical staining was conducted by Ventana autostainer model Discover XT ™ (Vantana Medical System, Tuscan, AZ) using enzyme labeled biotin streptavidin system and solvent resistant DAB Map kit. Antibodies: mouse monoclonal ACBP (1:100, Abcam, Cambridge, MA), rabbit polyclonal ACSL3 (1:1000, Abgent, San Diego, CA), rabbit polyclonal FASN (1:1000, Santa Cruz Biotechnologies, Santa Cruz CA), rabbit polyclonal HSL (1:1000, Abcam, Cambridge, MA) and polyclonal rabbit StAR (1:100; kindly donated from Dr. D. Hales, Chicago, IL) were used for immunohistochemical staining.

Mitochondrial fractionation

Mitochondrial fractionation of LNCaP cells was conducted using a Mitosciences cell fractionation kit (Eugene, OR). Verification of mitochondrial isolation was conducted by Western blot analysis of cytosolic specific protein GAPDH (1:5000, Abcam, Cambridge, MA) and mitochondrial specific protein cytochrome c (1:100; BD Biosciences, San Jose, CA) (data not shown).

Western blot analysis

Cell pellets were reconstituted in 100uL of RIPA buffer + protease inhibitor, sheared and spun down at 13,000 g for 5min. Supernatants from pellet extractions and mitochondrial isolations were analyzed for total protein content using the BCA protein determination kit (Sigma, Oakville, Ontario, Canada). 15ug of total protein was loaded for each sample onto a 9% acrylamide gel. Antibodies previously mentioned in immunohistochemistry section were used to identify and quantify respective proteins.

Fluorescence microscopy

Cells were fixed in ice-cold methanol completed with 3% acetone for 10min at 4°C, then washed with PBS and incubated with 0.2% Triton/PBS for 10min, followed by washing and 30min blocking in 3% nonfat milk before the addition of antibodies overnight to detect cytochrome c, DAPI (1:100; Vector laboratories, Inc, Burlingame, CA) and StAR. Antigens were visualized using anti-rabbit or anti-mouse antibodies coupled to FITC or rhodamine (1:500; 30min). Photomicrographs were taken at 20x magnification using Zeiss Axioplan II fluorescence microscope, followed by analysis with imaging software (Northern Eclipse, Empix Imaging, Inc., Mississauga, ON).

Mitochondrial ^{14}C-cholesterol uptake assay in LNCaP cells

Steroid deprived LNCaP cells were treated with +/-1nM R1881 in combination with 10μCi of ^{14}C-cholesterol for 2 and 48hrs. As previously documented, cells were pelleted and mitochondria were isolated. Fractions of mitochondria, cytosol and entire cell media were analyzed by a scintillation counter.

Statistical Analysis

Normalized protein expressions were compared between AD, N and CRPC groups by a one-way ANOVA test. Critical differences were assessed by Tukey post hoc tests; a significance threshold of 5% was used ($P<0.05$). All data are expressed as mean + standard error.

5.3 Results

Fatty acid levels increase during progression to CRPC

To expand on the observation that increased FASN expression occurs during CaP progression [41-43], we analyzed tumors obtained using the LNCaP xenograft model at various stages of the disease for FA levels by GC-MS. Results indicate that tumor levels of the 9 FAs analyzed in **Figure 2a** slightly decrease after castration (N (n=5) compared to AD (n=5)) and then increase in CRPC tumors (n=6).

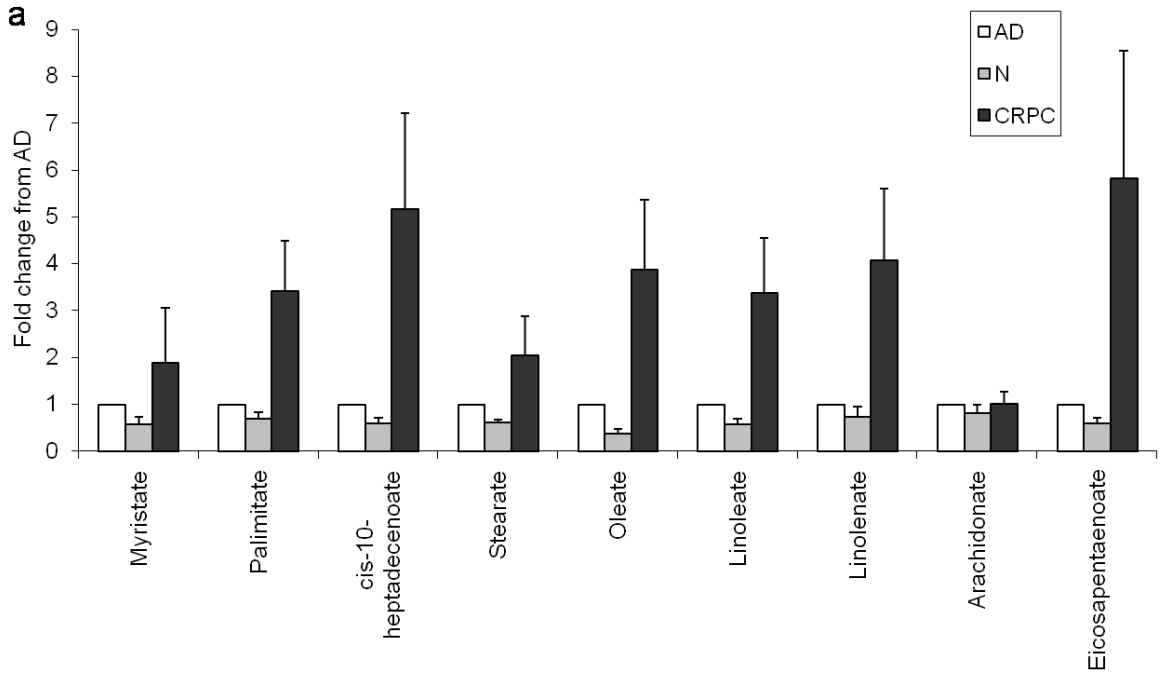


Figure 5.2a: Fatty acid methyl ester levels in AD, N and CRPC tumors. GC-MS analysis for fatty acid content in LNCaP xenograft tumors at androgen-dependence (AD, n=5), nadir (N, n=5) and castration-resistant (CRPC, n=6) stages of the disease. Mean fatty acid levels depicted as normalized initially to tumor weight (g) and then fold change in AD levels of the same mouse (+SEM).

Specific fatty acids are produced in an androgen-dependent and androgen-receptor mediated mechanism

To investigate the specific FAs that are endogenously synthesized in an androgen-dependent manner, we conducted dose-titration studies with synthetic androgen, R1881, in steroid starved LNCaP cells. Results indicate that three particular FAs are produced by LNCaP cells in a dose-dependent androgen-mediated manner: myristic acid ($r=0.899$, $p=0.101$), arachidonic acid ($r=0.910$, $p=0.089$) and eicosapentaenoic acid ($r=0.950$, $p=0.050$) (**Figure 5.2b**). In the presence of antiandrogen, casodex, which inhibits AR signaling, the androgen-mediated production of myristic acid, arachidonic acid (AA) and eicosapentanoic acid are depleted (**Figure 5.2c**).

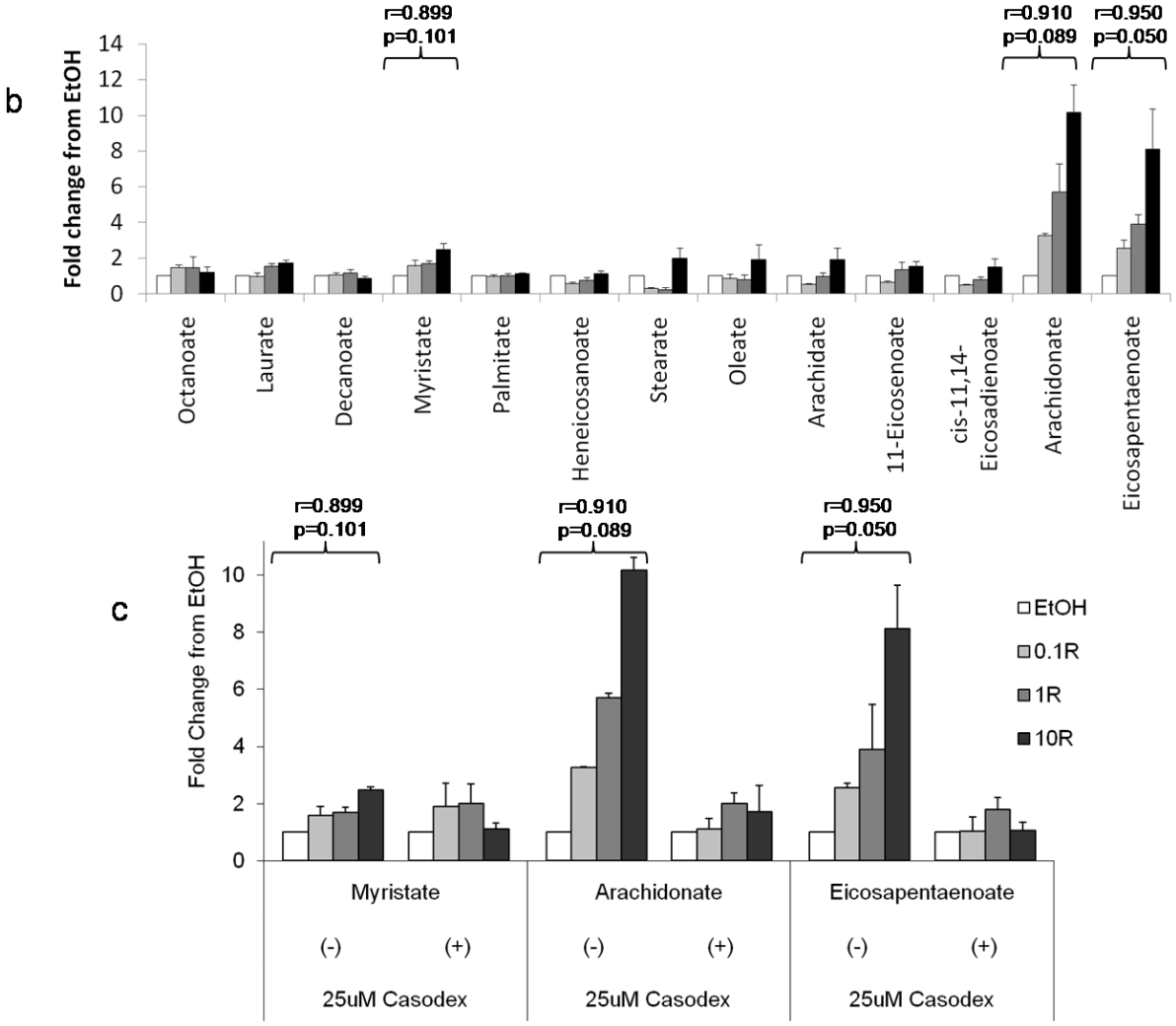


Figure 5.2b: Effect of R1881 on the production of fatty acids. GC-MS analysis for fatty acid content in steroid starved LNCaP cells in the presence of 0 (EtOH) (n=3), 0.1 (n=3), 1 (n=3) and 10nM (n=3) R1881. Mean fatty acid levels depicted as normalized initially to pellet weight (g) and then fold change in EtOH alone levels of the same experiment (+/- SEM). Linear regression statistics were employed to identify dose-dependent trends in fatty acids levels and are depicted as r-values and respective p-values. **Figure 4.2c:** GC-MS analysis for myristate, arachidonate and eicosapentanoate content in steroid starved LNCaP cells in the presence of 0 (EtOH) (n=3), 0.1 (n=3), 1 (n=3) and 10nM (n=3) R1881 in the absence or presence of 25uM antiandrogen, Casodex. Mean fatty acid levels depicted as normalized initially to pellet weight (g) and then fold change in EtOH alone levels of the same experiment (+/- SEM). Linear regression statistics were employed to identify dose-dependent trends in myristate, arachidonate and eicosapentanoate levels and are depicted by r-values and respective p-values.

These results indicate that select FAs are produced by CaP cells in an AR-mediated manner. AA has previously been shown to be the most efficient FA to induce steroid synthesis from cholesterol [25].

Proteins responsible for arachidonic acid activation of steroid synthesis are expressed in human CaP tissues

Drawing from findings in other steroidogenic cell lines (**Figure 5.1, Table 5.1**), we have identified five key proteins responsible in mediating AA activation of steroid synthesis: ACBP, ACSL3, FASN, HSL and StAR. To determine if this mechanism could occur within CaP tumor cells, we initially analyzed human tissues obtained from CaP patients by immunohistochemistry for these proteins (**Figure 5.3**).

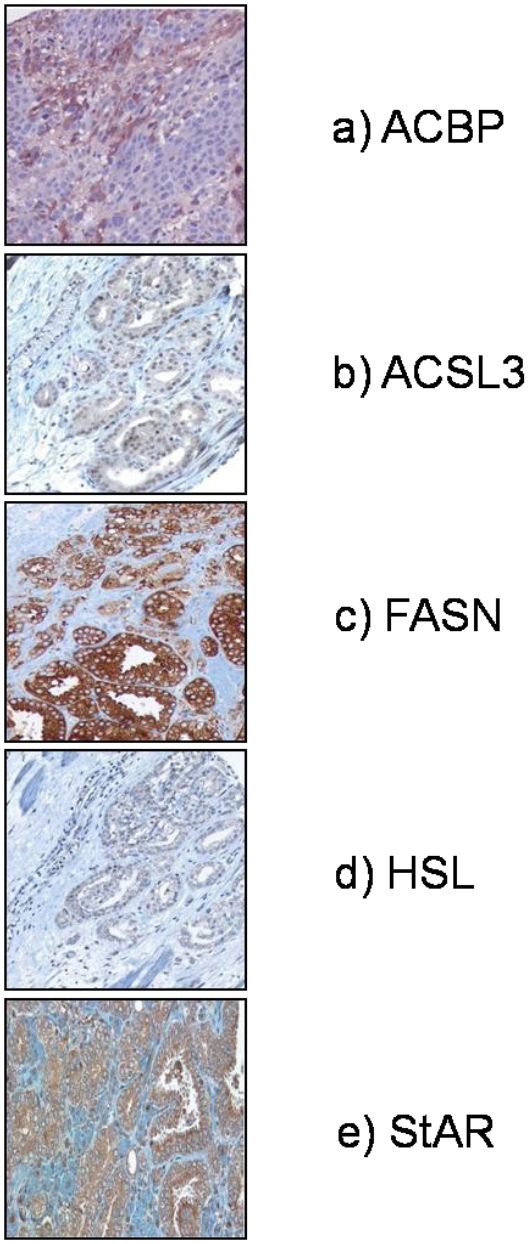


Figure 5.3: Staining of various proteins involved in fatty acid activation of steroidogenesis in human prostate tissue. Immunohistochemistry analysis for the presence of ACBP (**a**), ACSL3 (**b**), FASN (**c**), HSL (**d**) and StAR (**e**) in CaP tumor specimens collected from patients undergoing radical prostatectomy.

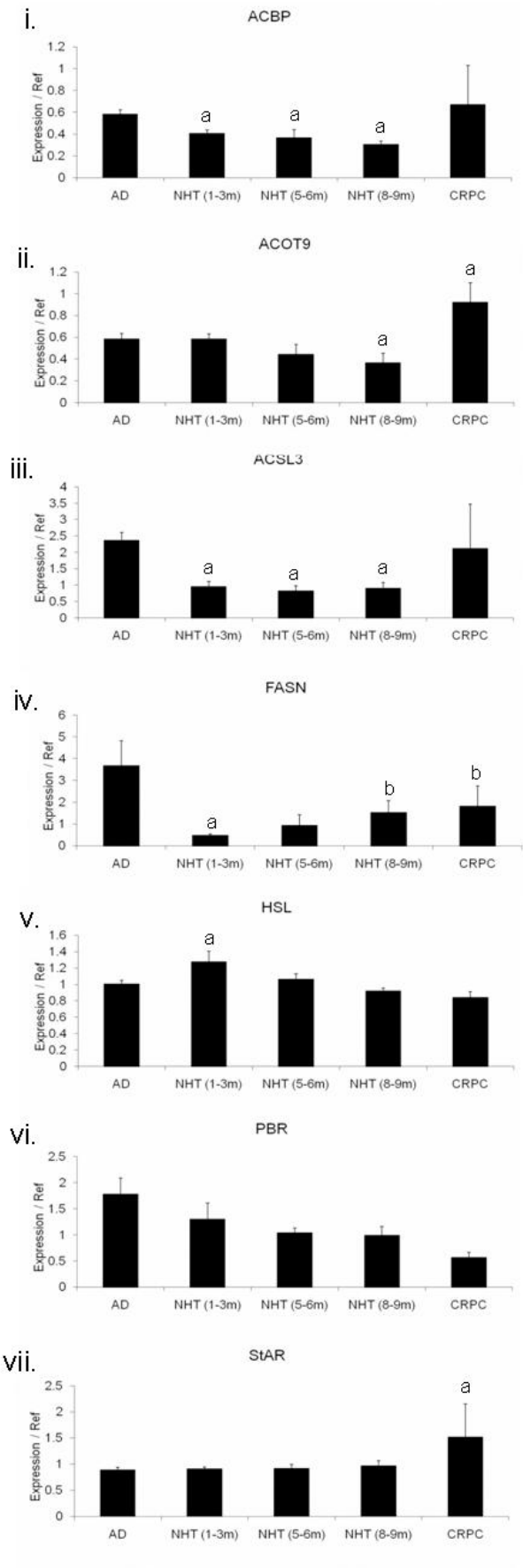
We confirm our previously reported result of ACBP expression in human CaP (**Figure 5.3a**) [2]. ACSL3 is found to be present in human CaP tumors and expressed in a nuclear manner while HSL is expressed in human CaP tissues in a cytoplasmic pattern (**Figure 5.3b, d**). Furthermore, StAR exhibits immunoreactivity in both benign and cancer cells (**Figure 5.3e**) while FASN appears to be over-expressed in cancer cells as compared to benign cells

(**Figure 5.3c**). These results verify that ACBP, ACSL3, FASN, HSL and StAR are expressed in human CaP tissues.

Expressions of proteins responsible for arachidonic acid activation of steroid synthesis are increased during progression to CRPC

We assessed the transcriptional expression of the indicated enzymes needed for AA activation (**Figure 5.1, Table 5.1**) during progression to CRPC in patient tissue samples in relation to ADT treatment response (untreated androgen-dependent (AD) cancer, patients undergoing neoadjuvant hormone therapy (NHT) for 1-3 months (1-3m), 5-6 months (5-6m), 7-8 months (7-8m) and at castration-resistance (CRPC) as defined by recurring PSA). We confirm that in human CaP tumors ACBP mRNA levels significantly decrease after castration (neoadjuvant hormone therapy) ($p=0.002$) and appear to increase in tumors from patients at CRPC (**Figure 5.4a-i**) [2].

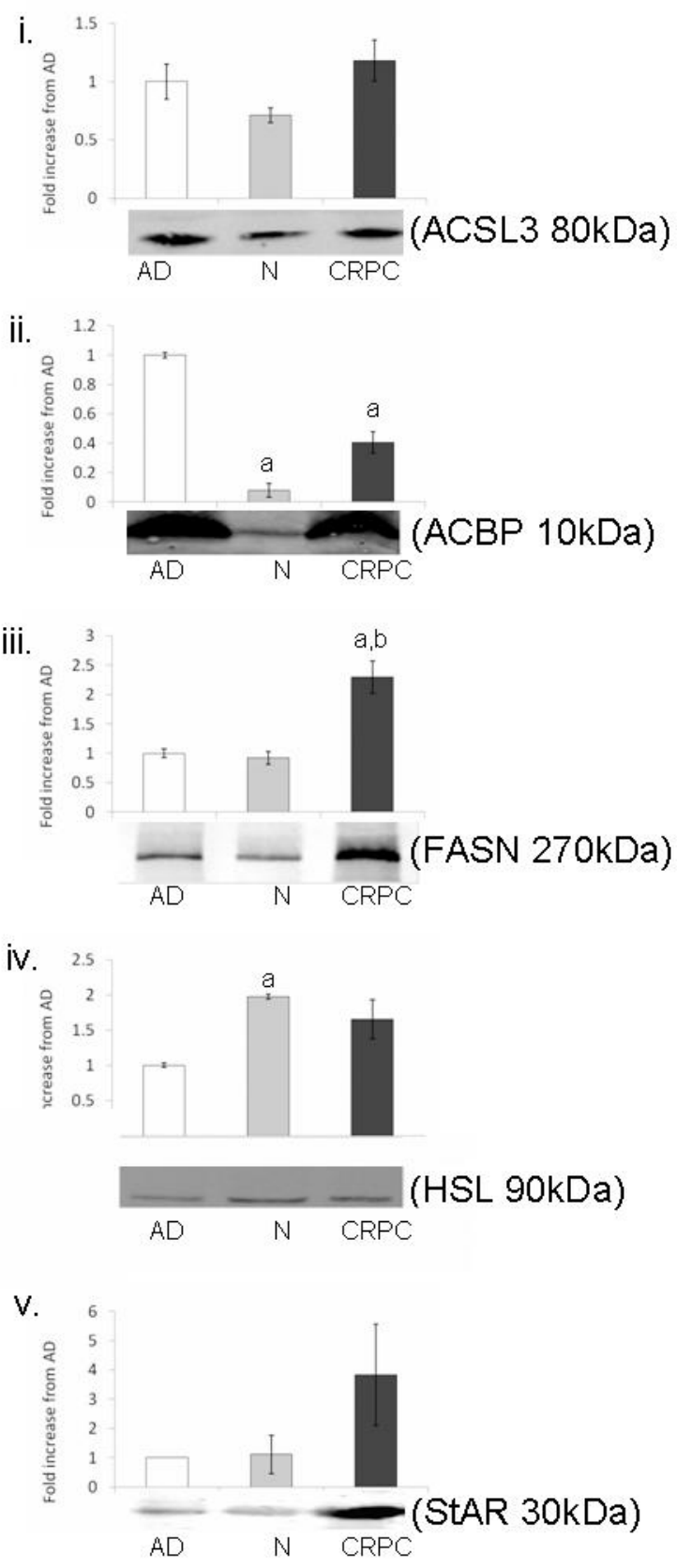
Figure 5.4a: Profiles of important proteins in human CaP progression. Microarray analysis for mean mRNA levels of ACBP (**i**), ACOT9 (**ii**), ACSL3 (**iii**), FASN (**iv**), HSL (**v**), PBR (**vi**) and StAR (**vii**) in tumors from untreated androgen-dependent (AD) patients, patients treated with neoadjuvant hormone therapy (NHT) for 1-3 months (1-3m), 5-6 months (5-6m), 8-9 months (8-9m) and at castration-resistance (CRPC) as defined by recurring PSA (+SEM). a indicates statistically significant from AD ($p<0.05$) while b indicates statistically significant from 1-3 months NHT ($p<0.05$).


i.
ACBP
Expression / Ref
1.2
1
0.8
0.6
0.4
0.2
0
a
a
a
AD
NHT (1-3m)
NHT (5-6m)
NHT (8-9m)
CRPC
ii.
ACOT9
Expression / Ref
a
a
AD
NHT (1-3m)
NHT (5-6m)
NHT (8-9m)
CRPC
iii.
ACSL3
Expression / Ref
4
3.5
3
2.5
2
1.5
1
0.5
0
a
a
a
AD
NHT (1-3m)
NHT (5-6m)
NHT (8-9m)
CRPC
iv.
FASN
Expression / Ref
6
5
4
3
2
1
0
a
b
b
AD
NHT (1-3m)
NHT (5-6m)
NHT (8-9m)
CRPC
v.
HSL
Expression / Ref
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
AD
NHT (1-3m)
NHT (5-6m)
NHT (8-9m)
CRPC
vi.
PBR
Expression / Ref
2.5
2
1.5
1
0.5
0
AD
NHT (1-3m)
NHT (5-6m)
NHT (8-9m)
CRPC
vii.
StAR
Expression / Ref
2.5
2
1.5
1
0.5
0
a
AD
NHT (1-3m)
NHT (5-6m)
NHT (8-9m)
CRPC

Furthermore, human tumor ACSL3 mRNA levels significantly decrease immediately after castration ($p<0.001$), remain low up to 9 months (NHT) ($p=0.003$) and then increase in tumors from CRPC patients (**Figure 5.4a-iii**). Human tumor HSL mRNA expression increases immediately post-castration (1-3 months) ($p=0.048$) and then gradually decrease in time leading to CRPC (**Figure 5.4a-v**) while after castration human tumor PBR mRNA levels decrease gradually to CRPC (**Figure 5.4a-vi**). Human tumor ACOT9 mRNA expression appears to decrease gradually after castration to 8-9 months NHT ($p=0.037$) and then increase once again at CRPC ($p=0.022$) (**Figure 5.4a-ii**) while tumor StAR mRNA levels remain the same immediately after castration and then increase at CRPC ($p=0.040$) (**Figure 5.4a-vii**). As predicted by previous studies, FASN tumor mRNA expression decreases after 1-3 months of NHT ($p=0.017$) and then FASN's expression re-appears during progression of the disease to CRPC ($p=0.008$) (**Figure 5.4a-iv**).

Due to the limited availability of human tissues to analyze protein levels of these enzymes, we utilized tumors obtained at various stages of the disease from the LNCaP xenograft model. Previously, ACBP and StAR protein levels at AD, N and CRPC in the LNCaP xenograft model have been reported [2, 7]. In replication of these results, we show that ACBP protein levels significantly decrease at N ($p<0.001$) and increase once again during progression of the disease to CRPC ($p<0.001$) (**Figure 5.4b-i**) and StAR protein levels appear to increase from AD to N to CRPC (**Figure 5.4b-v**).

Figure 5.4b: Profiles of important proteins in AD, N and CRPC tumors. Western blot analysis for mean protein levels of ACBP (**i**), ACSL3 (**ii**), FASN (**iii**), HSL (**iv**) and StAR (**v**) in AD (n=3), N (n=3) and CRPC (n=3) tumors from the LNCaP xenograft model normalized to GAPDH of the same blot and AD of the same mouse (+ SEM) as well as representative Western blot depicted below. a indicates statistically significant from AD ($p<0.05$) while b indicates statistically significant from N ($p<0.05$).


i.
Fold increase from AD
1.5
1
0.5
0
(ACSL3 80kDa)
AD
N
CRPC
ii.
Fold increase from AD
1.2
1
0.8
0.6
0.4
0.2
0
a
a
(ACBP 10kDa)
AD
N
CRPC
iii.
Fold increase from AD
3
2.5
2
1.5
1
0.5
0
a,b
(FASN 270kDa)
AD
N
CRPC
iv.
2.5
2
1.5
1
0.5
a
(HSL 90kDa)
AD
N
CRPC
v.
Fold increase from AD
6
5
4
3
2
1
0
(StAR 30kDa)
AD
N
CRPC

In this study ACSL3 protein levels appear to decrease immediately after castration and significantly increase once again at CRPC (P=0.005) (**Figure 5.4b-ii**). HSL protein levels increase in N tumors as compared to AD (P<0.001) and remain relatively high in CRPC tumors (**Figure 5.4b-iv**). Finally, FASN expression is high at AD, decreases at N and then once again emerges at CRPC (P=0.002) (**Figure 5.4b-iii**).

The mRNA and protein profiles of these enzymes determined from CaP tissues obtained from men undergoing various NHT treatment time courses and tumors obtained using the LNCaP xenograft model of CaP progression are consistent with each other.

Proteins responsible for arachidonic acid activation of steroid synthesis are localized in their active site in androgen deprived CaP cells

We evaluated the localization of these proteins within the CaP cell's mitochondria in the absence and presence of androgens in order to infer their active / inactive involvement in this AA mediated steroidogenesis mechanism. Initially, in order to verify that the 1nM R1881 treatment was effective, the expression of these proteins were analyzed in whole cell lysates by Western blot analysis; all (except StAR) are significantly induced by 1nM R1881 treatment in steroid starved LNCaP cell lysates (**Figure 5.5a-i-vi**).

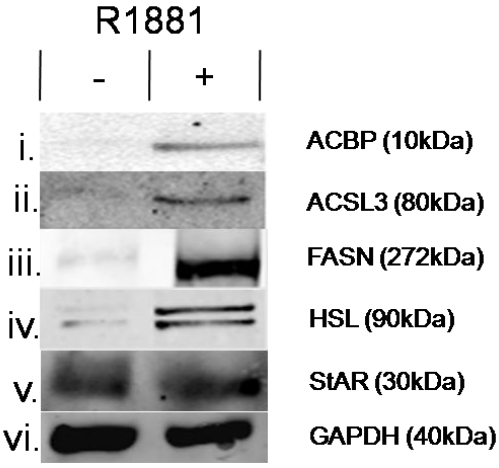
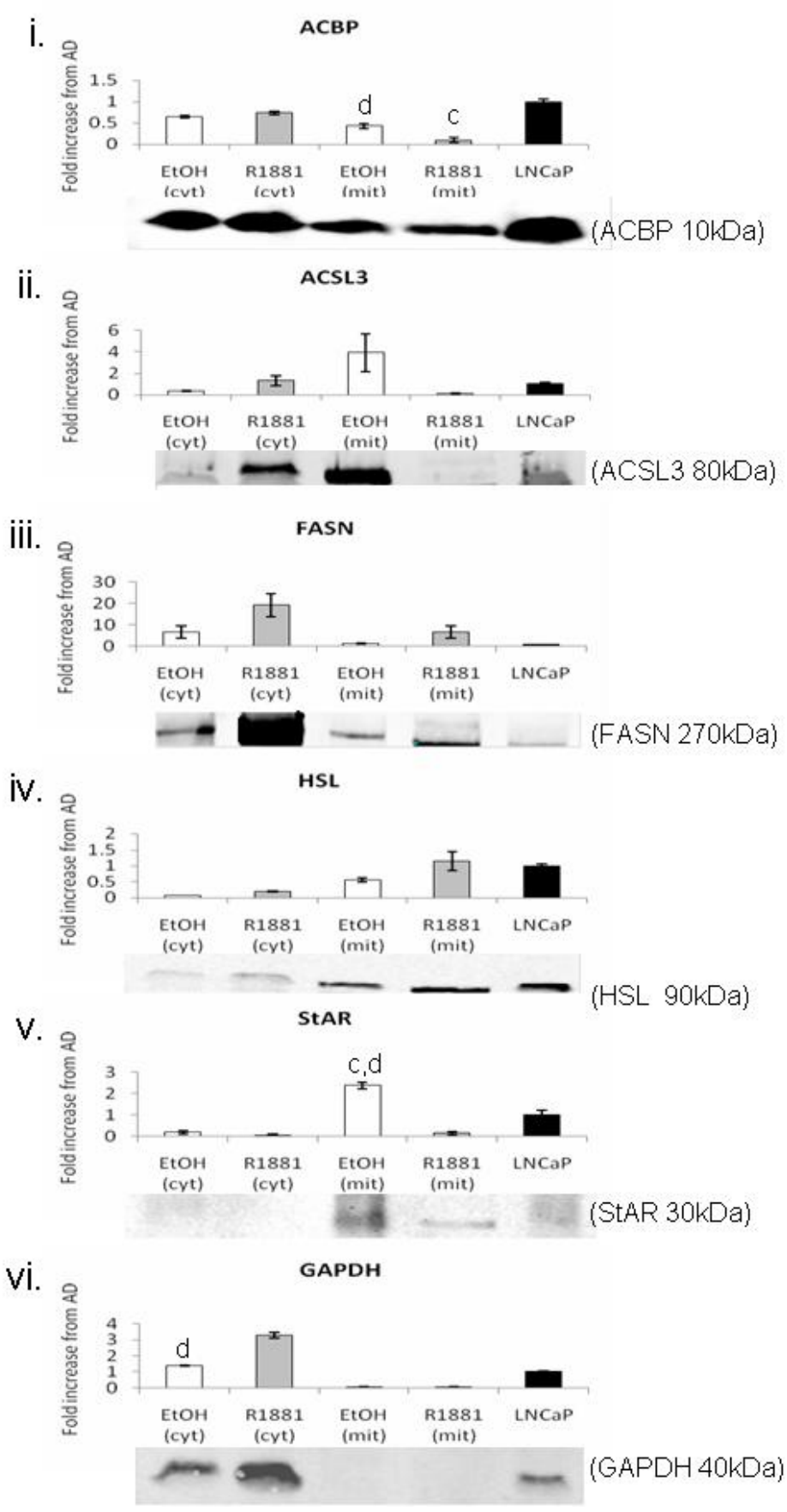


Figure 5.5a: Effect of R1881 on important proteins. Western blot analysis for mean protein levels of ACBP (**i**), ACSL3 (**ii**), FASN (**iii**), HSL (**iv**) and StAR (**v**) and GAPDH (**vi**) in whole cell lysates from LNCaP cells -/+ 1nM R1881. GAPDH was used as a protein loading control.

Mitochondrial and cytosolic extracts from these cells were also analyzed by Western blot for protein expression, alongside untreated whole cell lysates. ACBP, ACSL3, FASN, HSL and

StAR are expressed in the mitochondria of steroid starved LNCaP cells (**Figure 5.5b**); however distinct differences arise in the presence and absence of androgens.

Figure 5.5b: Effect of R1881 on important protein cellular localization. Western blot analysis for mean protein levels of ACBP (**i**), ACSL3 (**ii**), FASN (**iii**), HSL (**iv**) and StAR (**v**) and GAPDH (**vi**) in cytosolic (cyt) and mitochondrial (mit) fractions and whole cell lysates (LNCaP) of steroid starved LNCaP cells +/- 1nM R1881 (n=3 each) as well as representative Western blot depicted below. GAPDH protein localization solely in the cytosolic and cytochrome c protein localization in the mitochondria fractions (data not shown) confirmed efficiency of fractionation experiment. c indicates statistically different from cytosol with same treatment (+/- R1881) ($p<0.05$) and d indicates statistically different from R1881 treatment in the same fraction (cyt or mit) ($p<0.05$).


i.
ACBP
Fold increase from AD
1.5
1
0.5
0
d
c
EtOH (cyt)
R1881 (cyt)
EtOH (mit)
R1881 (mit)
LNCaP
(ACBP 10kDa)
ii.
ACSL3
Fold increase from AD
6
4
2
0
EtOH (cyt)
R1881 (cyt)
EtOH (mit)
R1881 (mit)
LNCaP
(ACSL3 80kDa)
iii.
FASN
Fold increase from AD
30
20
10
0
EtOH (cyt)
R1881 (cyt)
EtOH (mit)
R1881 (mit)
LNCaP
(FASN 270kDa)
iv.
HSL
Fold increase from AD
2
1.5
1
0.5
0
EtOH (cyt)
R1881 (cyt)
EtOH (mit)
R1881 (mit)
LNCaP
(HSL 90kDa)
v.
StAR
Fold increase from AD
3
2
1
0
c,d
EtOH (cyt)
R1881 (cyt)
EtOH (mit)
R1881 (mit)
LNCaP
(StAR 30kDa)
vi.
GAPDH
Fold increase from AD
4
3
2
1
0
d
EtOH (cyt)
R1881 (cyt)
EtOH (mit)
R1881 (mit)
LNCaP
(GAPDH 40kDa)

ACBP expression is predominantly cytosolic (P=0.006); however, in the absence of androgens, localization of ACBP in the mitochondria is significantly increased as compared to in the presence of androgens (P=0.01) (**Figure 5.5b-i**). ACSL3 is localized mainly in the mitochondria in the absence of androgens whereas in the presence of androgens ACSL3 appears to be localized mainly in the cytosol (**Figure 5.5b-ii**). HSL expression appears to be higher in the mitochondria regardless of the presence of androgens (**Figure 5.5b-iv**). FASN expression increases in both cytosol and mitochondrial fractions in the presence of androgen as compared to the vehicle control (EtOH alone) (p=0.01). This result is predicted because unlike the other enzymes being studied, the localization of FASN in the mitochondria is not linked to its overall activity (**Figure 5.5b-iii**). StAR expression remained low in all fractions except for in the absence of androgens when its expression became elevated in the mitochondria fraction (P=0.01) (**Figure 5.5b-v**). To confirm the novel finding that in the absence of androgens StAR becomes active, we also used a fluorescence labeling technique to determine if StAR co-localized with the mitochondria marker, cytochrome c, in steroid starved LNCaP cells. Fluorescence microscopy after cytochrome c, StAR and nuclear DAP1 staining (**Figure 5.5c**) indeed shows co-localization of cytochrome c and StAR in LNCaP cells in the absence of androgens.

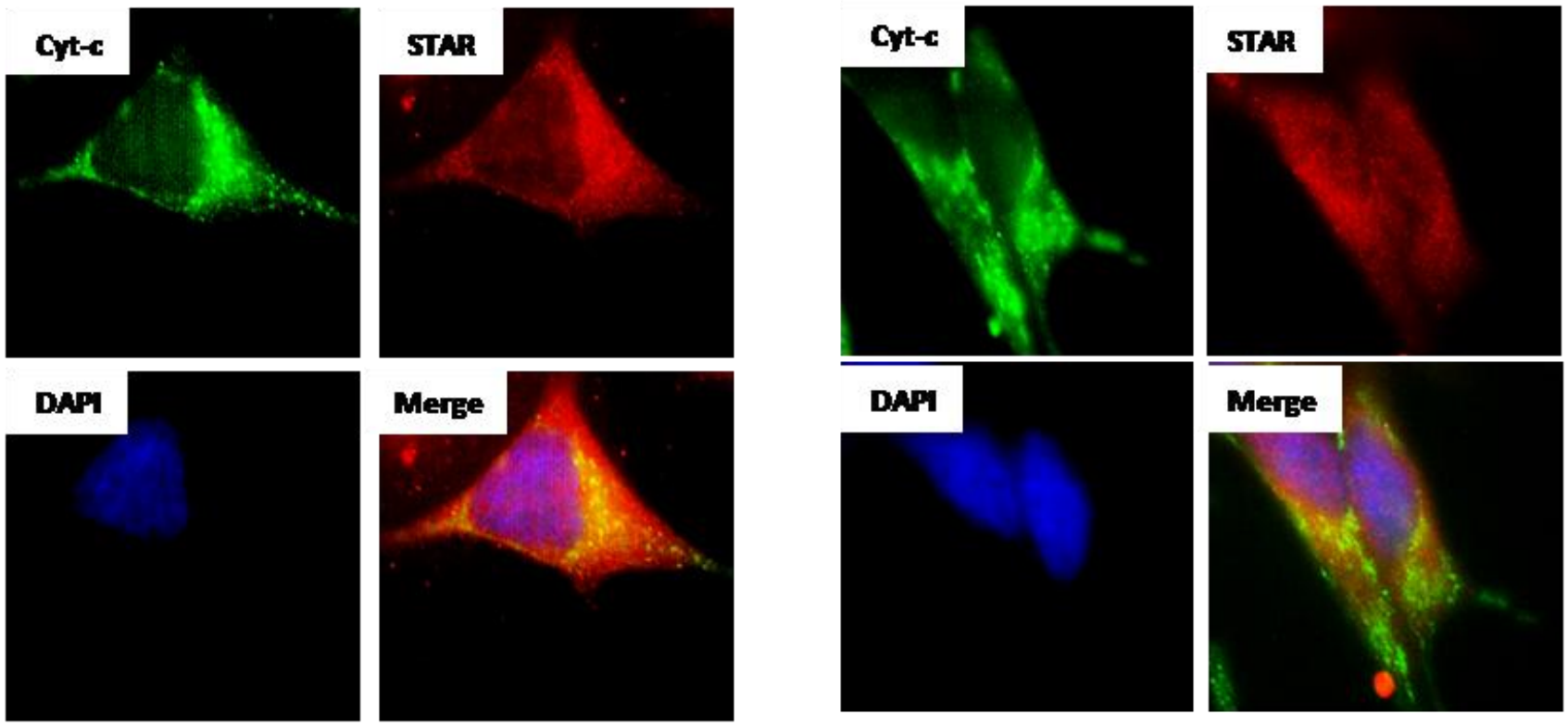


Figure 5.5c: Cellular localization of StAR. Fluorescence microscopy analysis for StAR, cytochrome c (mitochondrial marker) and DAP1 (nuclear marker) cellular localization in steroid LNCaP cells +/- 1nM R1881 (n=2 for each treatment). Cytochrome c localization visualized in green, StAR localization visualized in red, DAP1 localization visualized in blue; co-localization of cytochrome c and StAR visualized in yellow.

These unique and novel findings suggest that the proteins necessary for AA activation of steroid synthesis are present and active in CaP cells in an androgen deprived environment. PBR and ACOT9 subcellular localization were not evaluated in this study as both are known to reside in the mitochondria regardless of activity.

Increased mitochondrial uptake of cholesterol in androgen deprived CaP cells

The functional implication of AA induced steroid synthesis was assessed in the form of mitochondrial cholesterol uptake. Drawing from previous reports, StAR induction by AA leads to the uptake of cholesterol into the mitochondria by an HSL-StAR shuttling interaction [36]. We evaluated the localization of cholesterol, the endpoint of this mechanism, in fractionated LNCaP cells previously treated with radioactively labeled cholesterol +/- 1nM R1881 for 2hrs and 48hrs. In both the presence and absence of androgens, most of the radioactively labeled cholesterol remained in the media (supernatant) of the cells after 2hrs (**Figure 5.6a**). Over time (48hrs) in the absence of androgens significantly more cholesterol was taken into the cell ($P<0.001$) and specifically migrated to the mitochondria ($P<0.001$) than in the presence of androgens (**Figure 5.6b**).

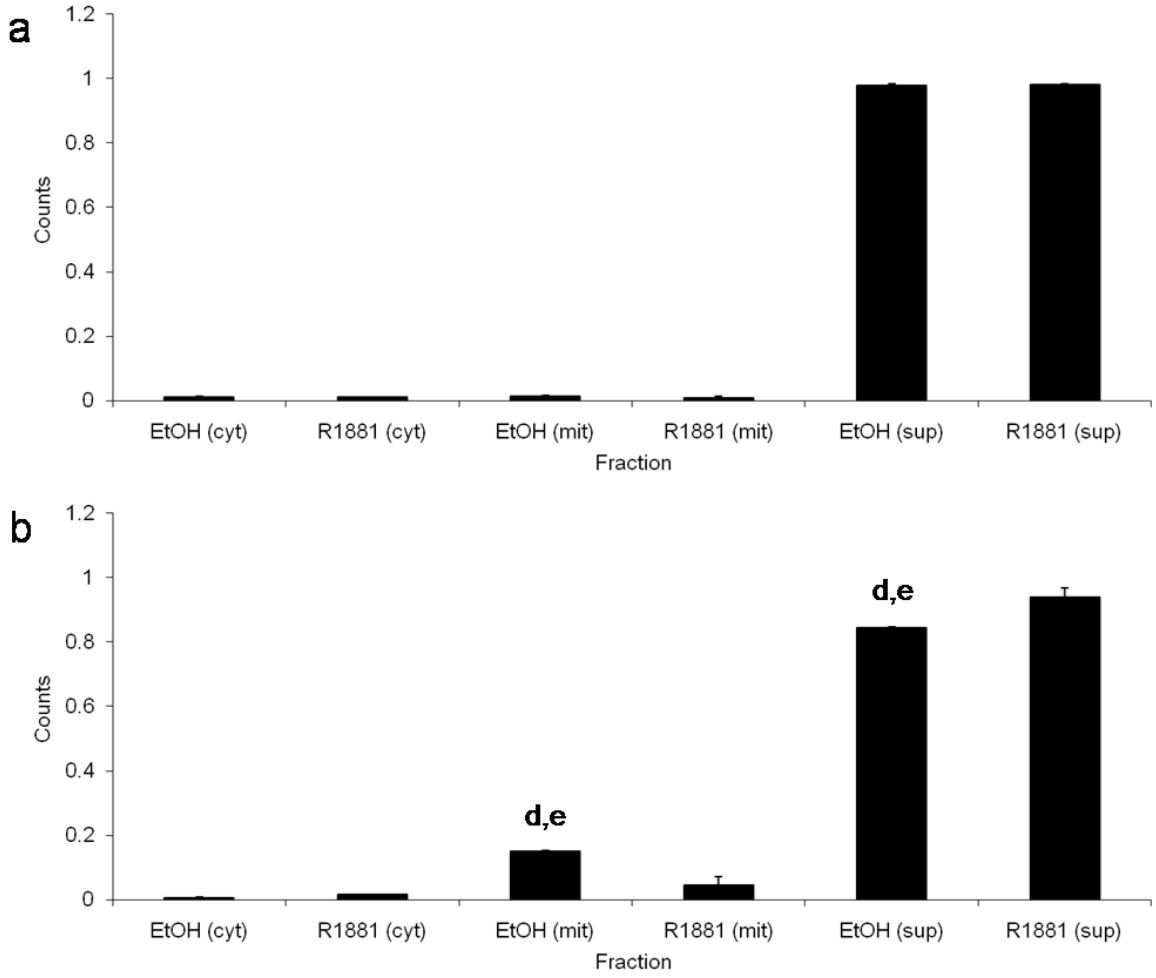


Figure 5.6: Effect of R1881 on cellular cholesterol distribution. ^{14}C-cholesterol content in cellular cytosolic (cyt) and mitochondrial (mit) fractions as well as supernatant media (sup) from steroid starved LNCaP cells +/- 1nM R1881 at 2hr (**a**) and 48hr (**b**) time points (n=3 each) portrayed as fraction of total counts as determined by scintillation count. d indicates statistically significant from R1881 treatment in the same fraction (cyt or mit) ($p<0.05$) and e indicates statistically significant from 2hrs incubation in the same fraction (cyt or mit) ($p<0.05$).

5.4 Discussion

Previously, we and others have shown that CaP cells express and utilize all of the enzymes necessary to *de novo* synthesize androgens from cholesterol in an environment deprived of a circulating source of androgens [3, 7, 8, 12]. In this study, we detail a novel mechanism whereby androgen dependent and AR-mediated production of AA prior to castration can trigger mitochondrial cholesterol uptake for subsequent androgen synthesis in CaP cells in of the absence of circulating androgens after castration (**Figure 5.1**).

We first demonstrate that in the presence of androgens, or prior to castration, CaP tumors produce large amounts of FAs through the induction of FASN. Previously, we have shown that in the presence of androgens CaP cells store these FAs in the form of non-toxic cholesteryl esters (CEs) through a reaction mediated by ACAT2 [27]. Herein, we provide evidence that after castration these CE stores can be utilized by CaP cells to synthesize androgens for AR activation. HSL is an enzyme responsible for cleavage of CEs to form free FAs and free cholesterol. The expression of HSL increases immediately after castration in tumors as compared to pre-castrate levels suggesting that cleavage of these CEs by HSL is an increased event immediately after castration when the supply of exogenous androgens is removed. Furthermore, in CaP cells, it appears that AA is one of the main FAs involved in CE formation by ACAT2 [27] and thus also one of the main FAs involved in dissociation by HSL immediately after castration. Analysis of CaP cells and xenograft tumors by GC-MS demonstrate that AA is produced initially in an androgen-dependent and AR-mediated manner. Furthermore, AA's activating enzyme, ACSL3, significantly increases in expression in CRPC tumors as compared to immediately post-castration. After activation, AA is normally transported by ACSL3 to the outer mitochondrial membrane so that it can bind ACBP in a complex with PBR allowing for it to shuttle into the mitochondria [25, 26, 29]. We have verified that ACSL3 mitochondrial localization is increased in steroid deprived LNCaP cells as compared to in the presence of androgens demonstrating that the trafficking of activated AA into the mitochondria is a likely event in CaP cells. Upon mitochondrial entrance of activated AA, it undergoes a thioesterase reaction to produce free AA which then undergoes metabolism leading to the nuclear transcription of StAR, the rate-limiting enzyme in steroid synthesis [24, 25]. Nuclear StAR transcription in turn leads to increased production of StAR protein at the mitochondrial membrane [34, 35]. In our fractionation experiment, protein expression of StAR increases in the mitochondria in the absence of androgens but does not change in any of the other fractions. Because only newly synthesized StAR can induce steroidogenesis [34, 35], we propose that the observed increase in mitochondrial protein StAR expression is responsible for subsequent steroidogenesis in these androgen deprived LNCaP cells. Furthermore, HSL also appears to accumulate in the mitochondria with StAR. Evidence that the mitochondrial uptake of radioactively labeled cholesterol is increased in the absence as compared to the presence of androgens in LNCaP

cells was also demonstrated further suggesting that in the absence of androgens StAR and HSL are likely working together to shuttle cholesterol into the mitochondria for steroid synthesis to occur [36]. Once cholesterol has entered the mitochondria, as previously demonstrated it readily undergoes reactions with the downstream steroidogenesis enzyme, CYP11A1, to produce pregnenolone, the steroid precursor of androgens [7, 34, 44]. Our circumstantial results from *in vitro, in vivo* and clinical samples support the hypothesis that CaP cells use endogenously synthesized AA to initiate *de novo* androgen synthesis after castration, in an environment deprived of circulating androgens.

This study highlights interplay between two lipid pathways (FA and cholesterol) in progression to CRPC. Expression of genes regulating FA synthesis (FASN) and cholesterol synthesis (HMG-CoA synthase, squalene monooxygenase and farnesyl diphosphate synthase) have been shown to be androgen-regulated and linked to CaP progression [2, 12]. There are many reported cases implicating endogenous FAs and cholesterol in disease progression after castration including cell maintenance and energy stores for rapidly growing cancer cells, production of lipid rafts for various cell signaling events and metabolism to molecules implicated in these cell signaling events [13, 17, 45-48]. We provide data describing a detailed mechanism whereby CaP cells can develop an ability to evade castration-induced apoptosis through the intratumoral production of androgens and subsequent AR activation. This mechanism in combination with many others including increased anti-apoptotic and survival pathway signaling [49-51] and AR activation by alternative growth factor pathways and co-regulators [52-55], ultimately culminate in a castration-resistant phenotype, the fatal form of CaP. By developing a better understanding of how CaP progresses to CRPC through the elucidation of mechanisms such as intratumoral androgen synthesis described here, new and more promising targets including ACAT2, ACSL3, FASN and HSL can be evaluated in hope to provide better treatments for patients and prolong progression to CRPC.

5.5 References

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CHAPTER 6: General discussion and advantages / disadvantages of our approach, conclusions and future directions

6.1 General discussion and advantages / disadvantages of our approach

Since Feldman and Feldman's seminal review in 2001 suggesting that the androgen receptor (AR) plays a significant role in prostate cancer (CaP) progression [1] several publications have built upon this hypothesis: large scale microarray studies have shown that after androgen deprivation therapy (ADT) many androgen-regulated genes including TMPRSS2-ERG gene fusion, fatty acid synthase, PSA and HMG-CoA synthase become re-expressed during progression of the disease [2-6], AR itself has been found over-expressed and / or mutated in human tissue samples collected from patients with late-stage ADT resistant CaP [7-18], knock-down of AR using siRNA, shRNA or inhibitor compounds has been shown to eliminate CaP in various *in vivo* models of progression [19-23] and clinical use of antiandrogens which target AR activation have shown efficacy in patients after ADT has become ineffective [21, 24-28]. Combined, these lines of evidence unequivocally demonstrate that AR is a central mediator of CaP progression.

Successful tumor regression after ADT is characterized by decreased levels of androgens and PSA in the serum; however when the disease progresses some 2-4 years later PSA levels rise once again while serum androgens remain low. This contradiction between low androgen levels and rising levels of an AR-activated gene, PSA, in the serum prompted some to hypothesize that ligand-independent AR activation may be important in disease progression while others opted to investigate how androgens were functioning at the tumor level. By 2004, new developments in sample collection / preservation practices as well as analytical technologies had facilitated sensitive and accurate determination of androgen levels in CaP specimens [7, 29] and results from more recent studies demonstrated that androgen levels measured in the tumor itself were indicative of the true disease state [4, 29-36]. In fact, these studies consistently demonstrated that intratumoral androgen levels remain high after castration and are present in sufficient quantities to activate AR [7], despite the low levels of androgens measured in the serum [31-36]. These studies provided justification for a change in nomenclature in the description of CaP progression after ADT from "androgen-independent" (reflective of a cancer that grows in the absence of androgens) to "castration-resistant" (reflective of a cancer that is simply resistant to castration or ADT) disease [37].

Labrie *et al.*, Mohler *et al.* and others hypothesized that the androgens measured in the tumor may be derived from precursor steroids originating from the adrenal gland that are sequestered and converted by the prostate into downstream androgens [7, 38, 39]. Q-RT-PCR and Western blot studies in various CaP cell lines and human tissues demonstrated all of the necessary enzymes for this process [5, 6, 38, 40]. Furthermore, through experiments involving metabolic radiotracing, researchers were able to demonstrate that upstream steroids could be converted to androgens within the tumor environment [41, 42]. These results supported the potential role of adrenal steroids contributing to disease progression. However, it was also established that mice lack the enzymes necessary for adrenal steroid synthesis [43, 44] and so the observation that progression of the disease to castration-resistance could be mimicked in mouse xenograft models in the absence of adrenal steroid precursors proved to be inconsistent with this adrenal steroid precursor hypothesis [45]. In addition, in humans, efficacy was not observed upon removal of both the testicular and adrenal steroid sources by orchiectomy and adrenalectomy, respectively, as compared to orchiectomy alone, suggesting that the observed intratumoral androgens must have come from an alternative source than the adrenal gland [46, 47].

While these studies were emerging in the scientific literature, several other groups were investigating changes in gene signatures during the progression of the disease using microarray techniques [3, 6, 48]. A recurrent theme arising from these human tissue studies was the upregulation of genes involved in lipid production during progression of the disease to castration-resistance [4, 49-51]. In fact, many of the genes described are linked to the pathways involved in steroid synthesis, including HMG-CoA reductase, farnesyl diphosphate synthase, CYP17A1, fatty acid synthase and acyl-CoA binding protein [4, 49-51].

The research in this thesis describes how upregulation of key enzymes in lipogenesis pathways contribute to the *de novo* synthesis of androgens for AR activation- re-stimulating androgen mediated AR signaling in disease progression to castration-resistance. Herein, I attempt to summarize our results and those from others published during the course of this work in a cohesive discussion that significantly contributes to the knowledge within the field of mechanisms leading to castration resistant prostate cancer (CRPC).

We hypothesized that the observed increase in specific lipogenesis enzymes in human CRPC plays a role in providing substrates for and activation of *de novo* androgen synthesis

within the local environment of the tumor. In turn, the *de novo* synthesized androgens could account for the measured levels of androgens in CaP specimens after castration [31-36].

Support for the hypothesized role of *de novo* androgen synthesis in CaP progression grew from studies published in coordination with this thesis work. One month prior to our publication (**Chapter 2**) Montgomery *et al.* published a study analyzing human tissues from patients with primary CaP and late stage metastatic CaP for testosterone and DHT levels as well as steroidogenesis enzyme expressions [4]. Conclusions drawn from this publication correlated increased levels of tissue testosterone with increased mRNA levels of enzymes responsible for testosterone synthesis in late stage metastatic disease. Prior to this report both observations had been published but never with the hypothesis that the enzymes were being utilized by human CaP cells to make their own testosterone [5, 31-36, 38, 52]. Following this work we reported the discovery that CaP cells are indeed capable of *de novo* androgen synthesis (**Chapter 2**). More specifically, we devised a novel *ex vivo* technique coupled to a sensitive LC-radiometric detection/MS endpoint that allowed us to trace the synthesis of androgens from radioactively labeled precursors in CRPC tumor cells obtained from the LNCaP xenograft model.

There were several advantages to using this particular approach in addressing the first aim of this thesis:

1) by using a mouse based model we were able to discount the potential role of adrenal steroid precursors as being a source for intratumoral androgens (as may be the case in humans) because mice lack the adrenal enzymes needed for precursor steroid formation [43, 44],
2) by using a controlled *in vivo* model of CaP progression the timing and coordination of tissue collection and processing provided optimal conditions for the *ex vivo* metabolism studies and the analysis of low levels of steroids by LC-MS/MS and
3) by tracing the precursor with a radioactive label in our metabolism studies we were able to make direct conclusions regarding the CRPC cell's ability to *de novo* synthesize downstream products.

These advantages allowed for us to uniquely demonstrate that CRPC tumor cells can and do utilize intracellular steroidogenesis enzymes to convert upstream precursor cholesterol (acetate) into androgen.

There were also disadvantages / limitations to using this approach to address the first aim of this thesis:

1) in the LNCaP cell line AR has been reported to be mutated (T877A) [15] in the ligand binding domain pocket and *in vitro* studies have shown that the AR can bind and be promiscuously activated by upstream steroids including progesterone and pregnenolone [12, 15],
2) by using a precursor far upstream of cholesterol (acetate) a significant dilution of signal was inevitable, making it difficult to assess the individual steroids and androgens being produced by this mechanism [53] and
3) androgen catabolism pathways are also known to contribute to tumoral androgens [54] and they were not evaluated in this study, perhaps altering our view of the true tumoral state.

Potential consequences of the first limitation of our model are that the AR mutation found in LNCaP cells may alter the ability of the cells to synthesize their own androgens in a similar manner to that likely found in most human CRPCs. More specifically, because the LNCaP cells do not need DHT for AR activation they may only produce the upstream steroids needed, thus distorting our understanding regarding which steroids are *de novo* synthesized normally in human CRPCs. In some of our follow-up metabolism studies (**Chapter 3**), we indirectly addressed this issue by inhibiting conversion of progesterone to DHT via enzymes CYP17A1 and SRD5A2 in LNCaP cells. In the presence of both CYP17A1 and SRD5A2 inhibitors progesterone induced PSA secretion (measure of AR activation) was decreased, suggesting that precursor steroids were in fact being converted by CYP17A1 and SRD5A2 to downstream DHT prior to AR activation and that precursor steroids were not responsible for direct AR activation themselves. Future studies need to be conducted in a CaP progression model containing a wildtype AR that is capable of binding only androgens (not upstream precursor steroids). Secondly, we compensated for our compromised ability to identify *de novo* converted steroids and androgens due to the observed dilution of signal from precursor [^{14}C-acetate] by using downstream precursor steroid [^{3}H-progesterone] in some of our metabolism studies. While measuring steroid levels in tumors using a sensitive LC-MS assay we discovered that tumor cells produce large amounts of progesterone (**Chapter 2**). Further to this, the progesterone levels were

approximately 10-fold lower in corresponding serum from the mice and when we measured progesterone in the adrenal glands coupled with the kidneys (confirmed by immunohistochemistry) its levels were substantially lower (approximately 200-fold lower than tumor amount). Furthermore, the tumoral expression of progesterone synthesis and metabolism enzymes may be increased during progression of the disease to CRPC as suggested by Q-RT-PCR analysis of tumors obtained from the LNCaP xenograft model. These results suggested that the progesterone in the tumor was in fact produced by the tumor itself and was not circulated from other sources. Using a radioactive tracing approach we confirmed that CRPC tumor cells are capable of converting progesterone to androgens through intermediates in two interlinked steroidogenesis pathways, known as the classical and backdoor pathways, compensating for the issue of dilution of signal. Thirdly, catabolism of DHT via glucuronidation [55, 56] and sulfation [57] has been reported to occur in CaP cells. Not taking these processes into account may limit our ability to accurately measure levels of DHT in our model. Future studies should include the analysis of these catabolites in order to gain a more realistic understanding of intratumoral androgen equilibrium.

Despite methodological limitations, in addressing the first aim of this thesis we have been able to demonstrate that androgens can be *de novo* synthesized in CRPC tumor cells. A month after this data was presented a comprehensive study was published by Dillard *et al.* [58] in which they demonstrate using a similar radioactive labeling approach that *in vitro* LNCaP derived CRPC cells are capable of testosterone synthesis, further confirming the importance of *de novo* androgen synthesis in CaP progression.

Based on the results reported in **Chapter 2**, in all of our follow-up experiments we utilized progesterone as a steroidal precursor to downstream androgens. For example, in **Chapter 3** we utilized this system to address the second aim of the thesis, to investigate the ability of steroidogenesis inhibitors targeting CYP17A1 and SRD5A2 (important rate-limiting enzymes in *de novo* androgen synthesis) to block or alter androgen synthesis. We discovered that ketoconazole (inhibitor of CYP17A1) and finasteride (inhibitor of SRD5A2) do indeed inhibit the metabolism of progesterone in CRPC tumor cells, however not completely. In the presence of both of these inhibitors the cells appear to adapt to use alternative pathways to synthesize DHT in sufficient quantities to activate AR and induce PSA secretion. Furthermore, we also demonstrated that tumors from different stages of CaP

progression are able to synthesize androgens from progesterone with different efficiencies and through different pathways. These studies highlight the evolving ability of CaP cells to adapt to *de novo* synthesize androgens when necessary and the difficulties faced when treating diseases of this form with the current steroidogenesis inhibitor drugs.

The advantages of using this approach to address the second aim of this thesis were that:

1) by investigating the direct effect of steroidogenesis inhibitors on the *ex vivo* CRPC tumors (as compared to systemic effects on the overall mouse) we were able to draw several conclusions about how these compounds affect *de novo* androgen synthesis from progesterone within the tumoral environment and
2) by using the upstream precursor progesterone (as opposed to upstream acetate) in our metabolism studies we were able to show which downstream steroids and androgens were being affected by these steroidogenesis inhibitors.

The disadvantages of using this approach to address the second aim of this thesis were that:

1) the ability of finasteride and ketoconazole to specifically inhibit SRD5A2 and CYP17A1, respectively, in LNCaP cells and CRPC tumors was not evaluated and,
2) currently there are other steroidogenesis drugs in development and their effects on androgen synthesis were not evaluated in this work due to sourcing difficulties.

To address the first disadvantage of the study we conducted Western blot analysis of CYP17A1 expression in the absence and presence of ketoconazole and SRD5A1/2 expression in the absence and presence of finasteride. Results (see **APPENDICES**) confirm that both ketoconazole and finasteride alter the protein expression of their targeted enzymes, validating the use of these inhibitors in altering *de novo* androgen synthesis pathways in our system. Future studies should not only delineate the ability of these inhibitors to alter *de novo* androgen synthesis but also their ability to alter enzyme activity in CaP cells. With respect to the second limitation, experiments are currently underway to evaluate new steroidogenesis inhibitors such as abiraterone acetate and dutasteride using these *ex vivo* CRPC tumor cell radioactive tracing progesterone metabolism assays.

The work described in **Chapters 3** contributes significantly to the field by demonstrating the potential for non-classical mechanisms in which CRPC tumors can adapt

to synthesize androgens in the absence of testicular androgens. Further to this, we demonstrate the ability of CaP cells to evade the inhibition of key enzymes involved in *de novo* androgen synthesis. The ramifications of this discovery apply to the development of therapeutic strategies targeting androgen-AR mediated CaP progression to CRPC.

The third and fourth aim of the thesis was pursued in efforts to understand how CaP cells initiate *de novo* androgen synthesis in the absence of testicular androgens (after castration). Drawing on the previous observation that the expressions of many lipogenesis enzymes are increased during progression of the disease [3, 6, 48] several publications have already demonstrated that lipid products of these upregulated enzymatic reactions are involved in cellular growth, function and signaling events in mediating CRPC onset [59-64]. In our attempts to understand the role of these lipid products in CRPC onset we focused on two specific lipid groups (cholesterol and fatty acids) and their potential role in *de novo* androgen synthesis. We chose to focus on cholesterol and fatty acids because cholesterol is the fundamental precursor of all steroids and androgens and extensive research has implicated fatty acids such as arachidonic acid in the triggering of androgen synthesis in ovary, testicular and adrenal steroidogenic cells [65-68].

Previous publications by Swinnen *et al.* have demonstrated androgen-regulation of several enzymes in both cholesterol and fatty acid synthesis processes in CaP cells [69-72]. In our first study using the *in vitro* LNCaP cell line we probed for the activity of cholesterol synthesis rate-limiting enzyme HMG-CoA reductase as well as cholesterol esterification enzyme acylCoA:cholesterol acyltransferase (ACAT) under the regulation of androgens (**Chapter 4 part 1**). We determined that both HMG-CoA reductase and ACAT are activated by androgens in the endoplasmic reticulum of LNCaP cells and also demonstrated that cholesteryl esters (products of HMG-CoA reductase and ACAT) are produced in substantial quantities in the presence of androgens. The consequences of this observation may be that the CaP cell then has access to large amounts of free cholesterol in times of need, such as androgen starvation at castration. The androgen-regulation of cholesterol synthesis and metabolism demonstrated in these studies suggests an important role for upstream cholesterol synthesis and metabolism in providing precursor for androgen synthesis in CaP.

There were disadvantages to using this approach to address the third aim of this thesis:

1) several of the lipogenesis genes discovered to be altered in human CaP progression to CRPC (steroid regulatory element binding protein (SREBP), farnesyl diphosphate synthase (FDPS), low-density lipoprotein receptor (LDL-r), acyl-CoA binding protein (ACBP), HMG-CoA synthase, HMG-CoA reductase, ATP binding cassette G1 (ABCG1), ACAT, etc.) are not just involved in cholesterol synthesis and metabolism but also in its overall regulation within the cell [3, 6, 48, 58, 73] and in our initial study we only investigated cholesterol synthesis and metabolism processes. We did not include all elements contributing to the regulation of cholesterol and
2) we also attempted to compare the ability of an androgen-dependent CaP cell and an androgen-independent CaP cell to undergo cholesterol synthesis and metabolism processes using two incongruent cell culture models (LNCaP and PC-3, respectively). Our results suggest significant differences in the ability of these cells to maintain cholesterol synthesis and metabolism (LNCaP and PC-3 cells preferentially use different isoforms of ACAT to produce cholesteryl esters); however other factors specific to each of the cell lines that are not related to androgen-sensitivity and progression of the disease could account for this difference.

In efforts to reconcile both of these disadvantages we sought to profile and explore most of the cholesterol regulation processes in the closed LNCaP xenograft model of CaP progression (**Chapter 4 part 2**) as the amount of precursor cholesterol (in the form of cholesteryl esters) in the cell at the time of castration could potentially predict the ability of the cell to utilize free cholesterol to make downstream androgens.

There were advantages to using this closed *in vivo* model approach to address the third aim of the thesis:

1) by investigating all of the cholesterol regulation processes (influx, efflux, synthesis and metabolism) we were able to make conclusions regarding cholesterol substrate availability for *de novo* androgen synthesis within the tumor and
2) the model used more ideally reflects the underlying biology of disease progression which is not possible when focusing only on the two incongruent cell culture models, allowing for a more comprehensive understanding of how cholesterol regulation is altered throughout disease progression in humans. In turn, by understanding how cholesterol is regulated in a CaP cell at different stages of the disease, the potential to

understand how this precursor can contribute to *de novo* androgen synthesis within a castrate environment is significantly greater.

Despite these efforts there remains one disadvantage to this *in vivo* approach to the third aim of this thesis:

1) it is difficult to demonstrate a direct effect of evolving protein expressions and endogenous cholesterol levels on increased *de novo* androgen synthesis specifically without overlooking the cell's need to produce larger amounts of cholesterol and cholesteryl esters for other processes such as membrane formation during tumor growth.

However, in **Chapter 4** we report novel findings that cholesterol regulation processes (influx, efflux, synthesis, metabolism etc.) are affected at the protein level throughout the course of the disease in the LNCaP xenograft model. We verified that key enzymes involved in exogenous cholesterol uptake (SR-B1, LDL-r) and endogenous cholesterol synthesis and metabolism (HMG-CoA reductase, ACAT) expression levels are altered in CaP tumors prior to castration (AD) and at castration-resistance (CRPC) as compared to immediately post-castration (N). We also functionally demonstrated that tumors favor increased cholesteryl ester formation prior to castration, which then decreases or becomes depleted in quantity after castration in the LNCaP xenograft model. *De novo* synthesized cholesterol levels also increased from N to CRPC. Based on the results reported in **Chapter 4** we suggest that the increased *de novo* synthesized cholesterol and large cholesteryl ester stores produced via these regulation mechanisms in the presence of androgens (prior to castration) may be responsible for providing cholesterol precursor for CaP cells to *de novo* synthesize androgens in an environment deprived of exogenous androgens (immediately after castration). In turn, these androgens can trigger the increased transcription of these cholesterol regulation mechanisms at castration-resistance through activation of AR, favoring the production of more cholesterol for further androgen synthesis. To address the limitations of this work, future experiments should involve radiotracing cholesterol distribution in subcellular fractionations of CaP tumor cells to determine the percentage of free cholesterol that can be utilized specifically for *de novo* androgen synthesis as compared to other processes. Furthermore, in this work the mechanism by which the cholesteryl ester stores

could be used in a castrate environment to provide precursor for *de novo* androgen synthesis was not evaluated and subsequently we address this issue in **Chapter 5**.

Fatty acids, another important class of lipids, have previously been implicated in triggering androgen synthesis from cholesterol in ovarian, testicular and adrenal steroidogenesis cells [67, 68, 74, 75] and several reports have documented increased expression of the enzyme responsible for fatty acid synthesis, fatty acid synthase (FASN), during CRPC progression [4, 49-51]. In many steroid-regulated cancer cells, endogenous fatty acid production is increased as compared to non-malignant cells [49] and several studies have demonstrated how these fatty acids contribute to the survival, growth and proliferation of cancerous cells [60, 61, 76]. In this thesis we attempt to understand how increased production of fatty acids may be linked to triggering *de novo* androgen synthesis in a castrate environment. Initially we evaluated the specific fatty acids being produced by CaP cells in an androgen-regulated manner using GC-MS (**Chapter 5**). We demonstrated that myristic acid, arachidonic acid and eicosapentanoic acid are key fatty acids produced by LNCaP cells under androgen regulation. As arachidonic acid has been implicated in triggering androgen synthesis more efficiently than other fatty acids [68] we decided to further pursue its potential role in androgen synthesis in the LNCaP cell model [67, 68, 74, 75]. Addressing this hypothesis we were able to relate the observed changes in cholesterol regulation (**Chapter 4**) and fatty acid synthesis during CaP progression and provide a means for CaP cells to evade castration and aid the tumor in progressing to CRPC through the triggering of *de novo* androgen synthesis in a castrate environment. We uniquely showed evidence to suggest that uptake of cholesterol into the mitochondria (the site of steroid synthesis) is enhanced through an arachidonic acid mediated mechanism in steroid deprived LNCaP cells.

The advantage of our approach in this study to address the fourth aim of the thesis was:

1) using both a mitochondrial fractionation assay and fluorescence labeling technique, we were able to demonstrate localization (and implied activation at the subcellular level) of several key enzymes shown to be up-regulated during CaP progression in human NHT microarrays.

Specifically, we provided evidence that long-chain acyl-coA synthatase-3 (ACSL3) and acyl-CoA binding protein (ACBP) (important in activation and transport of arachidonic

acid) as well as steroidogenic acute regulatory protein (StAR) (rate-limiting steroidogenesis enzyme activated by arachidonic acid) and hormone sensitive lipase (HSL) (responsible for releasing free cholesterol from cholesteryl ester stores for steroidogenesis) are active and contribute to an overall increase in mitochondrial cholesterol accumulation in steroid starved LNCaP cells as compared to cells exposed to androgens. This mechanism, described in **Chapter 5,** demonstrates how increased accumulation of cholesteryl esters and fatty acid (arachidonic acid) prior to castration can contribute to *de novo* androgen synthesis upon removal of the testicular androgen source.

There were also disadvantages of this work addressing the fourth aim of this thesis:

1) mechanistic experiments were solely conducted in the *in vitro* LNCaP cell line,
2) a direct link between increased arachidonic acid induction and induction of StAR was not demonstrated and
3) the proportion of cholesterol being utilized by the cell to specifically produce steroids as compared to other processes that require cholesterol was not determined.

In unreported studies we aimed to partially resolve the first issue by investigating the ability of other *in vitro* CaP lines (PC-3 and DU-145 cells) to synthesize androgens from steroid precursor progesterone (see **APPENDICES**). The data collected demonstrated that progesterone metabolism does occur in other cell lines however to a much lesser extent than that observed in LNCaP cells, which were approximately 3-fold more efficient at making steroids. In future studies investigation of this arachidonic acid mediated steroidogenesis mechanism should be conducted in other *in vitro* androgen-responsive systems such as the VCAP line (that is currently being characterized at The Prostate Centre) as well as at the tumor level (LNCaP xenograft model). To address the second disadvantage of this study, the exact mechanism whereby activated arachidonic acid induces StAR gene and protein transcription in steroidogenic cells is not entirely understood [75] and therefore in future studies inhibition of the arachidonic acid induced StAR mechanisms should be evaluated in LNCaP and other CaP cell lines capable of steroidogenesis. Evaluation of the enzyme kinetics of StAR activation of mitochondrial cholesterol uptake for steroidogenesis should also be considered in order to characterize the cell's dependence on this process amongst others involved in cell growth, survival and proliferation after ADT.

Nonetheless, by combining studies conducted in available clinical samples using data derived from the human NHT mRNA microarray and human NHT tissue microarray at The Prostate Centre at VGH with downstream detailed mechanistic studies in the LNCaP cell line and LNCaP xenograft model, we demonstrated a significant role for increased lipogenesis (in the form of cholesteryl esters and fatty acids) during CaP progression and also highlighted several enzyme targets to inhibit and perhaps develop therapeutics for CaP disease.

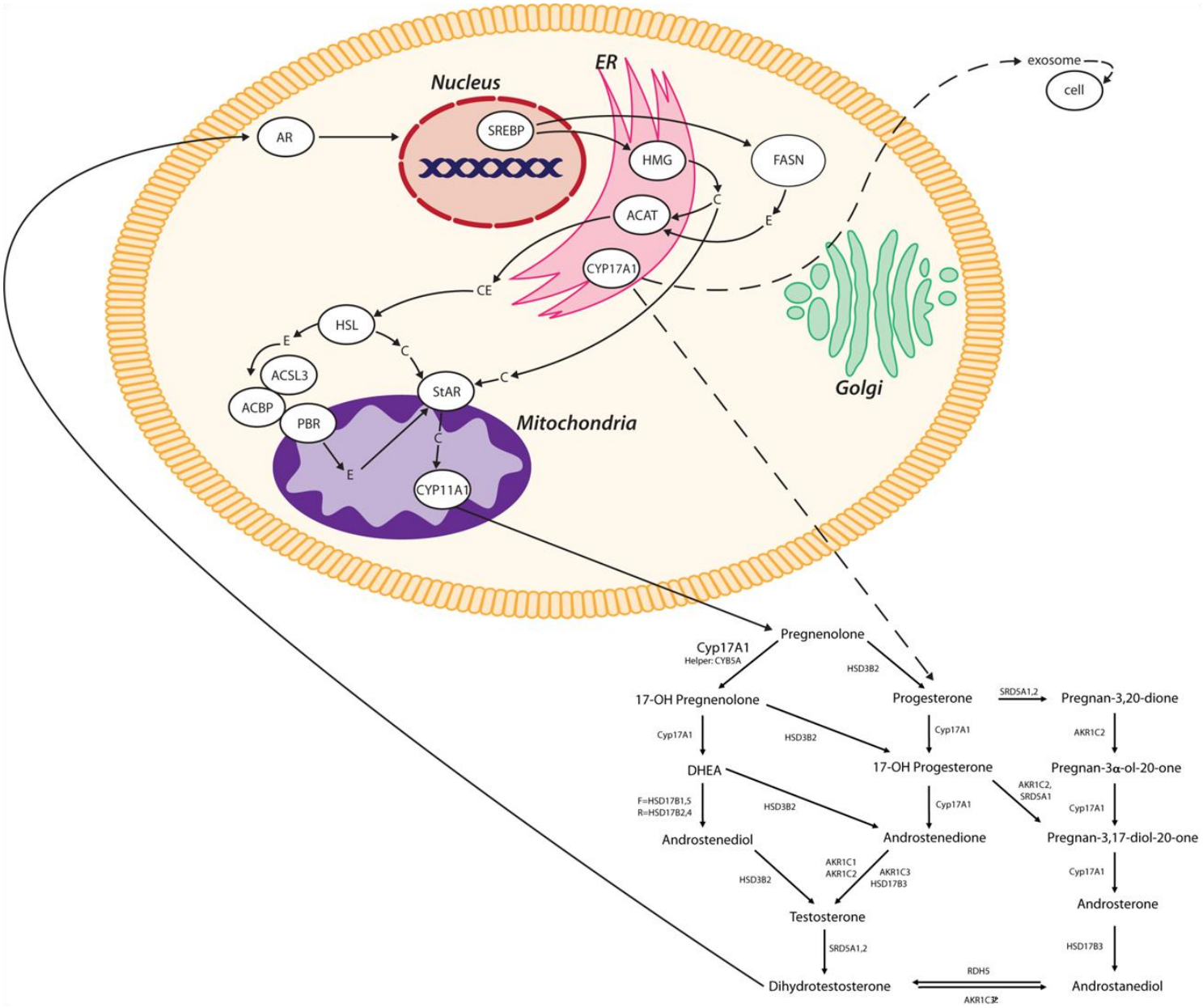
6.2 Conclusions

In addressing the specific aims outlined in **Chapter 1 Section 1.7.2** we can now draw several conclusions regarding androgen-mediated AR activation in CaP progression:

1. **CRPC tumor cells are capable of *de novo* androgen synthesis in sufficient quantities for AR activation,**
2. **Progesterone is a central mediator of *de novo* androgen synthesis via downstream metabolism to DHT,**
3. **Steroidogenesis inhibitors of CYP17A1 and SRD5A2 alter but do not eliminate AR activation as cells find alternative pathways to synthesize androgens,**
4. **Tumor cells originating from various stages of disease progression have different abilities to *de novo* synthesize androgens,**
5. **Androgen mediated cholesterol regulation mechanisms drive CRPC cells to provide substrate for *de novo* androgen synthesis,**
6. **Increased production of specific fatty acids such as arachidonic acid can induce *de novo* androgen synthesis in CRPC cells in the absence of exogenous steroids.**

As summarized in **Figure 6.1** these interlinked processes are likely contributing to adaptive mechanisms facilitating androgen-mediated AR activation in the absence of testicular androgens.

Figure 6.1: Overall schematic diagram of mechanisms studied in this thesis. Depicted are the deciphered pathways leading to androgen-AR mediated CaP progression. SREBP activation contributes to cholesterol (C), fatty acid (E) synthesis via HMG and FASN and cholesteryl ester (CE) formation via ACAT. Upon androgen deprivation the cell releases CYP17A1 exosomes for communication with other cells within the tumor (see **APPENDICES**). Individual cells utilize CEs and *de novo* synthesized C to trigger C uptake into the mitochondria for *de novo* synthesis of androgens (via ACSL3, ACBP, PBR and StAR). DHT in turn activates AR contributing to nuclear localization and the transcription of various survival and proliferation factors including SREBPs. Figure modified from [77].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Pregnan-3,20-dione
Cyp17A1
HSD3B2
AKR1C2
DHEA
17-OH Progesterone
Pregnan-3α-ol-20-one
F=HSD17B1,5
R=HSD17B2,4
HSD3B2
Cyp17A1
AKR1C2,
SRD5A1
Cyp17A1
Androstenediol
Androstenedione
Pregnan-3,17-diol-20-one
HSD3B2
AKR1C1
AKR1C2
AKR1C3
HSD17B3
Cyp17A1
Androsterone
Testosterone
HSD17B3
SRD5A1,2
RDH5
Dihydrotestosterone
Androstanediol
AKR1C3

6.3 Future directions

The work presented in this thesis demonstrates that CaP tumor cells derived from the LNCaP xenograft model are capable of synthesizing their own androgens via several detailed, interlinked mechanisms. The implications of this work are substantial. Reports over the past 10 years have showed that AR can be activated by anti-apoptotic survival proteins and growth factors in the absence of testicular androgens [1, 78-85]. Claims from many of these reports suggest that AR can be activated in a truly androgen ligand independent manner overlooking the potential underlying contribution of intratumoral synthesized androgens. Data presented in this thesis demonstrates that this may not be the case and suggests that androgens may always be present and required for AR activation. These characterized pathways in fact may enhance the ability for AR to bind and be activated by *de novo* synthesized androgens. Studies from human tissues have further suggested that *de novo* androgen synthesis is important in human CaP progression but have not confirmed this by radiotracing techniques [4-6, 52]. In the future it will be pertinent to test the ability of human CaP tumors to make their own androgens, as well as the applicability of this mechanism to all human CaPs (for example does this mechanism happen in all patients or just a select portion of patients) and finally the necessity of this mechanism for CRPC progression in humans (for example do all or just some CaP cells need this mechanism for survival and CaP recurrence).

Our data also demonstrates that CaP cells evade specific therapies targeting testicular and intratumoral androgen synthesis by utilizating many of the multi-faceted pathways, some of which are outlined in **Figure 6.1**. Optimizing current clinical therapies and introducing new drugs targeting the mechanisms outlined in **Figure 6.1** will be crucial to achieving complete blockade of these multi-faceted pathways in clinical CaP. In the 1970's the concept of total androgen blockade (TAB) or maximal androgen blockade (MAB) was introduced by Labrie as a mainstream treatment for CRPC disease [86]. Conceptually the aim of TAB is to develop a therapy that eliminates all androgens and their actions in the body and therefore this therapy appears quite desirable in the treatment of CRPC. At the time, trials evaluating the combined use of orchiectomy (surgical removal of testes) and adrenalectomy (surgical removal of adrenal glands) were initiated [46, 47]. Unfortunately the side effects associated with blocking corticosteroid production in the adrenal glands

proved detrimental to the quality of life of many patients in these trials and against predictions, the CaP often re-appeared [46, 47]. As a result, the concept of TAB received significant negative attention. We now believe that the CaP relapse likely occurred because these forms of therapy were unable to eliminate all of these multi-faceted pathways as well as the interlinked anti-apoptotic survival and growth factor pathways which lead to AR activation in the tumor. Based on new data presented in this thesis and other parallel studies we suggest that TAB, classically targeting testicular and adrenal androgen production, should also include targeting of intratumorally synthesized androgens (**Figure 6.1**). Several other developments in the field have also contributed to the potential re-emergence of TAB: the development of chemical derivatives that mimic orchiectomy (LHRH agonists / antagonists) and adrenalectomy (ketoconazole and aminoglutethimide) allow for less side effects associated with the irreversible surgeries [87-91], new drugs such as abiraterone acetate targeting the androgen synthesis pathways have already proven safe and effective when administered to CRPC patients [92], novel antiandrogens with better AR binding affinities than flutamide, nilutamide and casodex [26] are being developed and evaluated in clinical trials (MDV3100, Medivation, Inc., San Francisco, CA and BMS-641988, Bristol-Myers Squibb, New York, NY) [93] and novel drugs targeting coregulators of AR that act to inhibit AR nuclear translocation are also being developed and evaluated in clinical trials [94-96].

Re-emergence of TAB as a therapy in CRPC disease offers tremendous potential with these as well as other new developments and the field is shifting to explore combinations of these potential treatments. Several clinical trials investigating combinational therapies include classical ADT with steroidogenesis inhibitors, antiandrogens and inhibitors of AR co-regulator proteins and are already in the pipeline (Clinical trial ID NCT00553878, NCT00516815 (dutasteride), NCT00638690, NCT00544440 (abiraterone acetate), NCT00450463 (flutamide), NCT00460031, NCT00039221, NCT00559481 (ketoconazole), NCT00685633 (casodex)) . An example of a successful combination treatment was reported by Taplin *et al.* who recently presented the results from a phase II trial investigating the use of ketoconazole in combination with dutasteride versus ketoconazole alone in 57 CRPC patients and observed a prolonged time to relapse (+5.1 months) in the combination treated group versus ketoconazole alone [97]. Furthermore, Klotz's recent publication outlining the practical use of TAB suggests that benefits are already becoming evident and by combining

antiandrogens with ADT an 8% reduction in mortality of CRPC patients at 5 years as compared to ADT alone can be achieved [27]. The results of clinical trials evaluating the many forms of TAB are greatly anticipated in the field of CRPC disease.

In addition, in this thesis we have highlighted several new (and previously characterized) enzymes for therapeutic targeting as listed in **Table 6.1**.

Target	Potential Inhibitors
HMG-CoA reductase	Statins [98, 99]
FASN	Triclosan, C75, Cerulenin [100, 101]
ACAT	Avasimibe, CI-976, Pactimibe Sulfate [102, 103]
ACSL3	Triacsin C [67, 104]
CYP17A1	Ketoconazole, Abiraterone acetate, VN/124-1 [105, 106]
SRD5A1/2	Finasteride, Dutasteride [107]
AKR1C3	Cinnamic Acid [108, 109]
AR	Flutamide, Nilutamide, Bicalutamide, MDV3100, BMS-641988 [110]

Table 6.1: Potential enzyme targets of inhibition in CaP according to the deciphered mechanisms in this thesis. Drug candidates targeting these enzymes as identified in other studies are also listed.

When we explore the processes in **Figure 6.1** we are able to identify new potential therapeutic targets; for example, inhibition of the synthesis of arachidonic acid and other fatty acids by FASN prior to androgen deprivation therapy (ADT) with compounds such as C75 or cerulenin [100, 101] or inhibition of ACSL3 activation of arachidonic acid immediately after ADT with Triacsin C [67]. These targeted therapies may prevent *de novo* synthesis of androgens from occurring via StAR activation as well as block several survival and proliferation pathways mediated by increased amounts of activated-fatty acids [60, 61, 76]. This in turn may delay the onset of androgen-mediated progression of CaP to late-stage metastatic disease. Inhibition of cholesterol synthesis enzymes (HMG-CoA reductase) [98, 111] as well as the cholesterol storage formation enzyme (ACAT) [102, 103] may offer another novel approach to delaying disease onset by reducing the cholesterol substrate necessary for *de novo* androgen synthesis in a castrate environment. Meta-analysis of several retrospective studies evaluating the use of statins (HMG-CoA reductase inhibitors which block cholesterol synthesis) in CaP patients have not been able to demonstrate a clear

correlation between statin use and prevention of CaP [98, 99, 111]. However, according to our studies, instead of providing patients with statins prior to developing CaP, administering statins to patients already displaying CaP may offer efficacy after ADT when the cells require cholesterol precursor for androgen synthesis. In one study conducted by Platz *et al.* they demonstrated a correlation between statin use and a 50% reduced risk of developing advanced CRPC [112, 113]. Several ACAT inhibitors have also been developed for the treatment of diseases such as atherosclerosis and heart disease [102, 103]. These drugs may be of use in treating patients with CaP when tumor cells are undergoing events triggering the accumulation of cholesteryl ester stores which can be later used as sources of free cholesterol. These examples demonstrate how some of the lipogenesis enzymes identified as being important mediators of *de novo* androgen synthesis in a castrate environment can be targeted by new and / or previously developed drugs in CaP patients prior to and after ADT in efforts of delaying disease progression to CRPC.

Prior to the discovery of intratumoral androgen synthesis as a mechanism contributing to ligand-AR mediated disease progression several groups were already working to develop drugs targeting the androgen axis in efforts to inhibit what was hypothesized to be adrenal steroid conversion to downstream androgens in the tumor. Candidates targeting CYP17A1 (ketoconazole, abiraterone acetate and VN/124-1) [105, 105, 106, 110, 114-116], SRD5A1/2 (finasteride and dutasteride) [107, 117] and AKR1C1-3 (cinnamic acid) [108, 109, 118] have been or are currently being evaluated in pre-clinical studies and clinical trials. The use of CYP17A1 inhibitor ketoconazole in CRPC has been scrutinized for over twenty years while another CYP17A1 inhibitor, abiraterone acetate, has been developed and evaluated in CRPC patients more recently [92, 110]. Both compounds induced significant PSA responses (>50% decline) in most patient studies after relapse from castration inferring that inhibition of CYP17A1 led to decreased amounts of tumoral androgens available for AR activation in the absence of a testicular androgen source. Upon treatment with either ketoconazole or abiraterone acetate levels of downstream steroids derived from CYP17A1 activity decreased in patient serum confirming that both drugs are indeed targeting CYP17A1 [114, 119, 120]. However, abiraterone acetate appears to be a more hopeful candidate than ketoconazole for the treatment of CRPC because: abiraterone acetate is twenty times more potent and more specific to CYP17A1 inhibition than ketoconazole [120], in animal models

abiraterone acetate treatment led to decreased size of organs dependent on androgens for growth whereas ketoconazole displayed no effect on organ weight [120], abiraterone acetate displays only limited toxicity in patients whereas ketoconazole has to be supplemented with corticosteroids to relieve associated side effects [92, 110, 121] and in a phase I clinical trial 52% of patients who developed resistance to ketoconazole treatment displayed further PSA response (>50% decline) on abiraterone acetate treatment despite the fact that both drugs target the same enzyme [122].

Abiraterone acetate is therefore likely more promising for the treatment of CRPC patients than ketoconazole. This example puts forward the notion that basic research such as that described in this thesis can provide mechanistic rationale for clinically observed responses such as those observed with ketoconazole in CaP patients, and this knowledge can be translated into the development of novel, more specific steroidogenesis inhibitors such as abiraterone acetate. As demonstrated in **Chapter 3** CaP tumor cells appear to use alternative modes to synthesize androgens in the presence of steroidogenesis inhibitor ketoconazole (and finasteride). In a study conducted by Attard *et al*. where they demonstrated that CRPC patients who initially responded to abiraterone acetate had reduced serum levels of steroids downstream of CYP17A1 activity than those patients who relapsed (as determined by re-emerging PSA) on this drug exhibited no increase in the levels of these downstream CYP17A1 steroids [92] while in a similar study by Ryan *et al.* patients who had relapsed on ketoconazole did exhibit increased levels of downstream CYP17A1 steroids [122]. This observation suggests that androgen-AR mediated progression of the disease was initially eliminated in these patients through the use of both abiraterone acetate and ketoconazole but that the relapsing CaP occurring after abiraterone acetate (but not ketoconazole) treatment may have occured through yet another alternative androgen synthesis mechanism (bypassing CYP17A1) or that abiraterone acetate resistant CaP might not be dependent on androgens but instead progress as a truly androgen independent disease. This example foreshadows that CaPs will either continue to adapt to bypass new therapies or that CaPs which are initially androgen-dependent may be effectively treated using stronger, more specific inhibitors so that when the disease does progress it does so in a truly androgen independent manner.

This thesis focuses on *de novo* androgen synthesis in CaP, however this is just one of many challenges that face researchers, clinicians and patients in understanding this complex

disease. With the potential addition of new drugs targeting the multi-faceted mechanisms in **Figure 6.1** to the TAB therapies currently being evaluated in clinical trials the potential emergence of completely androgen-independent and AR independent disease is possible. The role of lurking stem cells during CaP progression has been hypothesized and our understanding of how CaP progresses in this manner is only starting to come to light. Stem cells may also be at the root of *de novo* androgen synthesis in these CaP cells. Perhaps in a regressing tumor, stem cells evade apoptosis and begin to differentiate into a heterogeneous tumor population containing AR positive cells. In turn these AR positive cells can increase the production of enzymes needed for androgen synthesis via SREBP induction and begin to utilize and transport these enzymes (via exosomes- see **APPENDICES**) to other surrounding cells for subsequent androgen synthesis within the tumor. These *de novo* synthesized androgens can then trigger further AR activation and the cycle continues resulting in the emergence of a CRPC tumor. This particular theory is quite far-fetched but nonetheless demonstrates that tumor cells go to no end to continue to survive, grow and proliferate in the presence of therapies aimed at eliminated these processes. Developing treatments that completely inhibit progression of the tumor will be a significant challenge to the health care community.

Overtreatment of patients with CaP is already a significant issue, as only 1 out of every 18 men is predicted to benefit from their therapy [123]. With the expanding new drugs and therapeutic regimes currently being evaluated the need for appropriate classification of patients who are likely to respond to specific therapeutic regimes will hopefully help minimize side effects and improve overall survival. This will be another significant challenge for researchers, clinicians and patients to overcome in the future.

While conducting literature searches for this thesis I came across a review from 1985 by Waxman [124] and noticed that conclusions drawn about the advances in CaP research at the time remain the same today in 2009. At first I was disappointed that ~25 years of research had not contributed to a significant survival advantage for patients. Reading through this thesis I hope you have realized that this is not the truth. In fact, this is a very exciting time in the CaP field as new mechanisms and new drugs are being rapidly characterized and developed. Through focused hypotheses, new technologies and a collaborative team effort,

like that exemplified in this thesis, I believe improved quantity and quality of life can be attained in all patients with CaP in the future.

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APPENDICES

Additional manuscript: A novel communication role for CYP17A1 in the progression of castration-resistant prostate cancer[5]

Introduction

CYP17A1 is a member of the cytochrome P450 enzyme family which collectively function in steroid hydroxylation and carbon-carbon bond cleavage reactions [1]. CYP17A1 is responsible for the conversion of C21 steroids such as pregnenolone, progesterone and pregnan-3α -ol-20-one to their 17α-hydroxylated derivatives and further cleavage at the C17-20 bond to produce C19 steroids: dehydroepiandrosterone (DHEA), androstenedione and androsterone (**Figure 1**).

[5] A version of this chapter has been published. Locke, J.A. Fazli, L. Adomat, H.A. Smyl, J. Weins, K. Lubik, A.A. Hales, D.B. Nelson, C.C. Gleave, M.E. and Guns, E.S. (2009) A novel communication role for CYP17A1 in the progression of castration-resistant prostate cancer. The Prostate [Epub ahead of print].

Dr. Emma Tomlinson Guns was the principal investigator on this manuscript. Dr. Ladan Fazli (pathologist) of Dr. Martin Gleave's lab scored all immunohistochemical stainings of prostate cancer tissues and discovered the unique exocrine pattern of CYP17A1 expression. Hans Adomat trained me on the LC-MS analysis of samples from the CYP17A1 activity assays. Jil Smyl and Amy Lubik repeated Western Blot analyses of all human serum samples in order to confirm consistent results from the Western Blot analysis I conducted (no loading standard was available). Kristen Weins collected serum samples from prostate cancer patients for this study. Dr. Dale Hales at the department of Physiology and Biophysics (University of Chicago) provided the CYP17A1 and P450 oxidoreductase antibodies as well as critical comments for the writing of this manuscript. Dr. Colleen Nelson and Dr. Martin Gleave provided critical comments and reviewed the work. I conducted all of the exosome isolations and Western blot analyses. I also produced the first draft and final copy of this manuscript.

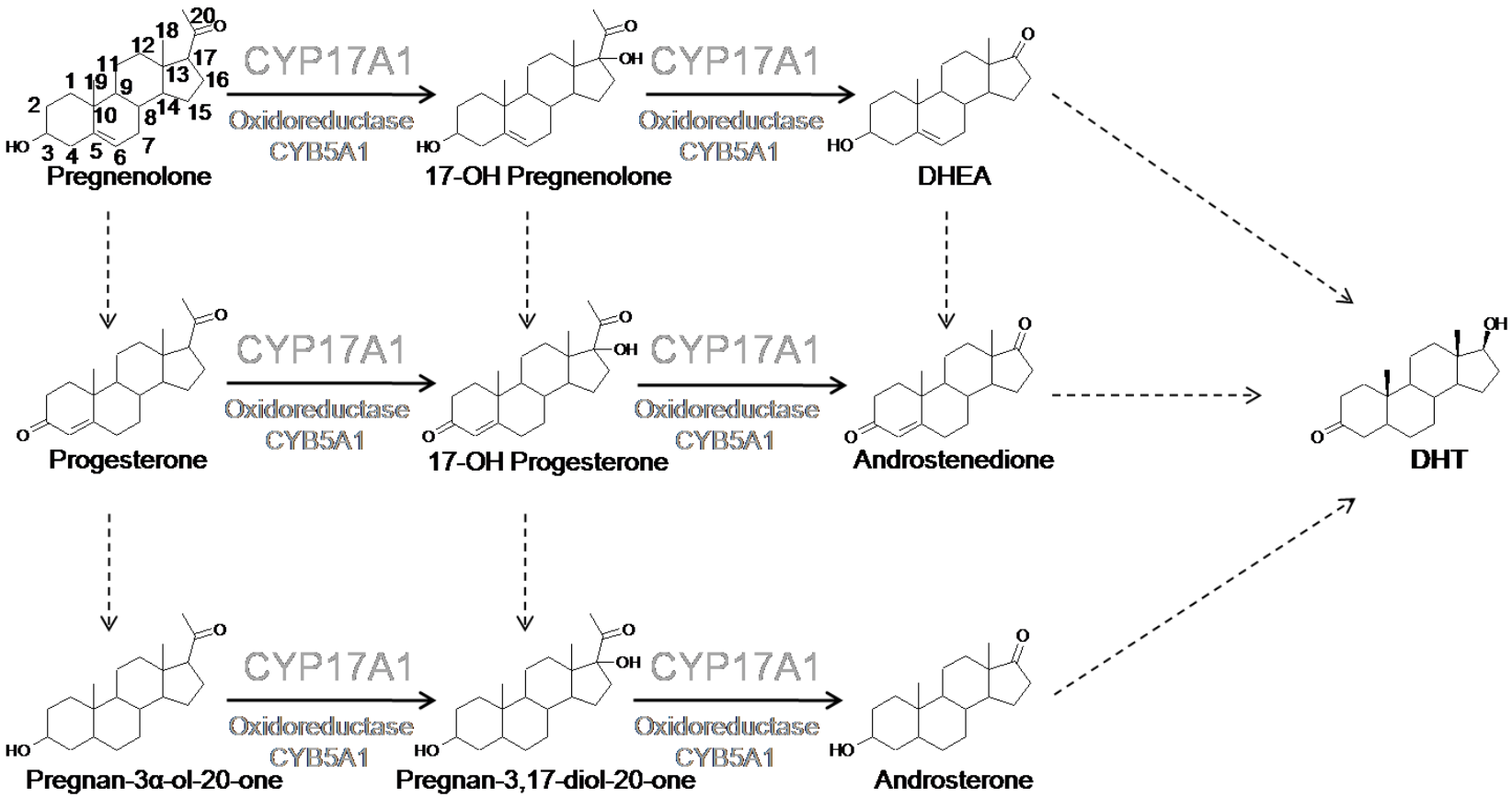

Figure 1: Schematic of CYP17A1 reaction. CYP17A1 initially hydroxylates C-17 on the steroidal entity followed by a lyase reaction at C-17 and C-20 as depicted. P450 oxidoreductase (oxidoreductase) along with molecular oxygen and NADPH are required for both CYP17A1 activities while CYB5A1 is only required for CYP17A1's lyase activity. Pregnenolone, progesterone, 17-OH progesterone and pregnan-3α-ol-20-one are steroid substrates of this reaction ultimately contributing to DHT synthesis. Modified from previously published figure [22].

In humans, the activity of CYP17A1 is regulated by endogenous levels of P450 oxidoreductase (microsomal electron transport chain), NADPH supply and co-localization with helper enzyme, cytochrome b5 (CYB5A1) [2-5]. P450 oxidoreductase and NADPH are required for both steps catalyzed by CYP17A1 while cytochrome b5 is thought to allosterically align C-17 and C-20 during only the C17-20 lyase biotransformation of the substrate [6]. Based on kinetic studies the lyase activity of CYP17A1 has been shown to be rate-limiting in steroid synthesis [3, 7] and is highly dependent on the interactions between the substrate and CYP17A1 protein in its binding conformation [8-11]. Species differences in enzyme structure and different substrate moieties can affect this reaction and alter downstream steroid synthesis pathways dramatically [12, 13]. Using tammar wallaby and mouse testes Auches *et al.* has shown that CYP17A1 more readily converts pregnenolone to DHEA in mammals than progesterone to androstenedione while this trend occurs oppositely in species such as rodents and amphibians [12]. Furthermore, Auchus *et al.* also suggested that CYP17A1 lyase activity is altered in disease [12] based on data that women with functional ovarian hyperandrogenism and polycystic ovarian syndrome produce significantly more 17-OH progesterone and less androstenedione than women with healthy ovaries [14, 15]. This altered activity may result from genetic polymorphisms in the CYP17A1 as many have been documented in the literature regarding patients with polycystic ovarian syndrome and other diseases [16-19]. Nonetheless, because CYP17A1 activity is sensitive to alterations in enzyme and substrate structure and it is the rate-limiting enzyme in steroid biotransformation, its role in steroid-dependent disease development and progression has proven most interesting.

Recently, evidence has suggested a role for CYP17A1 in prostate cancer (CaP) progression as its mRNA and protein expressions have been shown to correlate with stage of disease and relapse of disease after therapy [20, 21]. We and others have confirmed that CYP17A1 contributes to CaP progression at a molecular level [21-23]. Non-localized CaP is commonly treated with androgen deprivation therapy which chemically blocks testicular testosterone production, thereby starving CaP cells of essential androgens. Unfortunately, the cancer often recurs in what is now termed castration resistant prostate cancer (CRPC) and is associated with recurring PSA, tumor growth and metastasis. We and others have previously shown evidence that CaP cells adapt to this androgen deprivation, or castration,

by utilizing steroidogenesis enzymes including CYP17A1 to synthesize their own essential androgens within the local environment of the tumor [21, 22]. Using radio-tracing techniques we further showed that mechanistically progesterone can be *de novo* converted to 17-OH progesterone and androstenedione by CYP17A1 in CRPC tumors [22] and that treatment with ketoconazole (an inhibitor of CYP17A1) impedes this downstream conversion (unpublished data). Clinically, CYP17A1 inhibitors such as ketoconazole [24, 25] and abiraterone [26, 27] have been shown to reduce the circulating levels of downstream androgens: androstenedione, DHEA and testosterone in CaP patients [27, 28]. As both ketoconazole and abiraterone have demonstrated the potential to increase the lifespan of prostate cancer patients in clinical trials [27-30] and elicit enzyme inhibition far upstream that of other therapeutics targeting steroidogenesis, they may offer more effective "maximal" inhibition of androgen synthesis and action in CRPC patients.

In our attempt to profile CYP17A1's role in CRPC disease progression we have discovered that CYP17A1 may not only be a central enzyme in androgen synthesis but that it could also play another role in cellular communication between CRPC cells within the tumor. Upon immunohistochemical analysis of CaP tumor sections we observe that CYP17A1 localisation is orientated in a pattern typical of a secretory protein. Furthermore, detection of the enzyme in human serum as well as further verification that protein levels of CYP17A1 are elevated in CaP patients as compared to healthy controls suggests a functional role of this enzyme in the circulating serum. Although the activity of CYP17A1 was not determined in human serum as compared to positive control human liver and kidney microsomes, further delineation of CYP17A1's expression within human serum exosomes suggests a role in cell to cell communication during progression to CRPC. It is hypothesized herein that secretion of CYP17A1 into serum within exosomes may offer a novel mechanism for the CaP cell to communicate with other cells perhaps triggering *de novo* steroidogenesis in CaP progression to castration-resistance.

Materials and Methods

Materials:

Steroid Standards

Stock solutions of testosterone-16,16,17-d3 (deuterated testosterone) (CDN Isotopes, Pointe-Claire, Quebec, Canada), 4-androstene-3,17-dione (Sigma, Oakville, Ontario,

Canada), 4-pregnen-17-ol-3,20-dione (Steraloids, Inc., Newport, Rhode Island), 5α-androstan-17β-ol-3-one (Sigma, Oakville, Ontario, Canada), dihydrotestosterone (DHT) (Sigma, Oakville, Ontario, Canada), 5β-pregnan-3α-27-diol-20-one (Steraloids, Inc., Newport, Rhode Island), 5β-pregnan-3,20-dione (Steraloids, Inc., Newport, Rhode Island), androsterone (Aldrich, Oakville, Ontario, Canada), pregnenolone (Sigma, Oakville, Ontario, Canada), progesterone (Sigma, Oakville, Ontario, Canada) and testosterone (Sigma, Oakville, Ontario, Canada) were prepared in 100% methanol as mass spectrometry standards.

Human serum samples

Serum was obtained from 36 prostate cancer patients, 24 of whom were undergoing neoadjuvant hormone therapy (NHT > 6 months) and 12 hormone naïve patients. Inclusion criteria required participants to have biopsy confirmed prostate cancer with no evidence of bone metastases. Samples were also obtained from healthy age matched controls (n=10). The men undergoing NHT were being treated for one of three indications: (1) localized prostate cancer managed conservatively; (2) biochemical recurrence following surgery or radiation; (3) management of locally advanced disease. Hormone naïve patients were men with prostate cancer previously treated with definitive therapy (radiation or surgery) or those monitoring localized disease with active surveillance (3 months or more post-diagnosis). Aged matched controls comprise of healthy men without prostate cancer who were asked to participate through a peer nomination process. Participants were asked to nominate a peer (usually a sibling, friend or neighbor) who is similar in age (within 5 years). All serum samples were taken as a fasting morning sample, between the hours of 7:30 and 10:30 am. Sera was then separated by centrifugation at 1500 X g and analyzed immediately or stored at –80ºC prior to analysis. Approval was obtained from The University of British Columbia Clinical Research Ethics Board and patients consented for their serum to be used for the purpose of this research.

Human microsomes

Pooled human liver and kidney microsomes were used for Western Blotting and CYP17A1 activity assays (Celsis *In Vitro* Technologies, Chicago, IL).

Serum and H295 cell exosomes

Exosomes were isolated from fresh human serum and H295 cells as described by Taylor *et al.* [31]. Briefly, serum and cell lysates was centrifuged at 2000 X g for 10 min. and the

supernatant was transferred to a new tube for further centrifugation at 21,000 X g for 20 min. using a swinging bucket Ti 40 rotor (Beckman Coulter, Mississauga, Ontario). The supernatant from this spin was further centrifuged at 200,000 X g for 1 hr. and the remaining exosome containing pellet was reconstituted in PBS. Exosome isolates were stored at -80°C until further analysis. Validation of exosome isolation was done by Western blot analysis for exosome markers heat shock protein-70 (HSP70) [32, 33], glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [34] and Fas ligand (FAS) [31].

Methods:

Immunohistochemistry: Immunohistochemical staining was conducted on tissues from radical prostatectomy patients (from 2000 to 2006) obtained from the Vancouver General Hospital. Benign and cancer sites were identified and marked in donor paraffin blocks using matching H&E reference slides. Cancerous tissues were analyzed by Ventana autostainer model Discover XT ™ (Vantana Medical System, Tuscan, Arizona) using enzyme labeled biotin streptavidin system and solvent resistant DAB Map kit. CYP17A1 rabbit polyclonal antibody was prepared in house by Dr. Hales [35].

Western Blot Analysis of CYP17A1

Protein concentrations were assessed using a Bicinchoninic Acid Kit (Sigma, Oakville, Ontario, Canada) and 15µg of each sample was loaded into each lane. Relative enzyme levels were analyzed using the following antibodies at the indicated dilutions: rabbit polyclonal CYP17A1 (1:1,500) and P450 oxidoreductase (1:1000) were prepared in house by Dr. Hales [35], goat monoclonal CYB5A1 (1:1,000 Abcam, Cambridge, MA), mouse monoclonal HSP70 (1:1000 Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal GAPDH (1:1,000 Abcam, Cambridge, MA) and mouse monoclonal FAS (1:200 Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Protein bands were visualized and quantified using the Li-cor Biosciences Odyssey Infrared Imaging system (Lincoln, Nebraska). Since there are currently no known controls for protein loading with human serum we compensated for this by conducting repeat analyses by two independent researchers who replicated protein determination and Western Blot analysis for CYP17A1 in ten of the same human serum samples. The results from this experiment were reproducible and thus provide confidence in the overall quantitation of CYP17A1 protein expression by

Western blot in each sample. Each condition was conducted in triplicate and Western Blot analysis was conducted three times for each sample.

CYP17A1 activity assay

Steroid standards: progesterone, 17-OH progesterone or pregnan-3α-ol-20-one were added (10µL of 10µ/mL methanolic stock solution) to individual pre-rinsed glass vials and dried down using a Centrivap™ centrifugal evaporation system (35°C for 30 min). Steroids were reconstituted in 500µL of fresh human serum and an NADPH regenerating system added to initiate enzymatic activity (BD Biosciences, San Jose, CA: 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium chloride). Samples were further incubated at 37^0C for 0 and 2hr time periods. Activity reactions were quenched with 50µL of 100ng/mL deuterated testosterone (internal standard) and 465µL of acetonitrile. Samples were vortexed several times and spun down at 1000rpm for 10 min. 500µL of supernatant was removed and dried down using a Centrivap™ centrifugal evaporation system (35°C for 30 min). Samples were reconstituted in 50µL of methanol with vortexing and sonication for 30 minutes, prior to dilution with 50µL of water. Samples were then clarified by centrifugation at 13500 g for 10 min prior to HPLC analysis. 1mg of human liver and kidney microsomes were used as positive CYP activity controls while PBS was used in all blank analyses. For each condition the activity assay was conducted in triplicate.

Steroid Analysis by Liquid Chromatography-Mass Spectrometry (LC-MS)

A Waters 2695 Separations Module coupled to a Waters Quattro Micro was used for LCMS analysis. All MS data was collected in ESI+ mode. Capillary voltage was set at 3kV, source and desolvation temperatures were 120ºC and 350ºC respectively with N_2 desolvation flow at 450 L/hr and Ar collision gas adjusted for $3.5e^{-3}$ mBars for fragmentation when required. The instrument was operated at unit resolution to obtain MS scan and Fragment Ion scan spectra and with reduced high mass resolution settings to enhance MRM sensitivity. Cone voltage (CV) and collision energy (CE) settings were optimized for each standard. Chromatographic separations were carried out using a Waters Exterra 2.1x50mm 3.5µm C18 column equilibrated with 20:80 ACN:H_2O, ramped to 80:20 ACN:H_2O from 0.5-8.0 min, further to 95:5 from 8.0-9.0 min and returned to 80:20 ACN:H_2O from 10.0-10.5 min with a total run time of 15 min. Flow rate was 0.3 mL/min, column temperature 35ºC and 0.05%

formic acid was present throughout the run. MS scan data for metabolite identification was collected using both 22V and 35V for CV.

Results

CYP17A1 is expressed in human CaP tissues and appears to be a secretory protein

Upon immunohistochemical analysis of CYP17A1 expression in CaP biopsy tissues obtained from patients undergoing various treatments we confirm that the enzyme is *in situ* expressed in these samples and also that its expression pattern is typical of a secretory protein. CYP17A1 appears to be localized to the luminal pole of the cells in exocrine secretory mode as depicted in **Figure 2**.

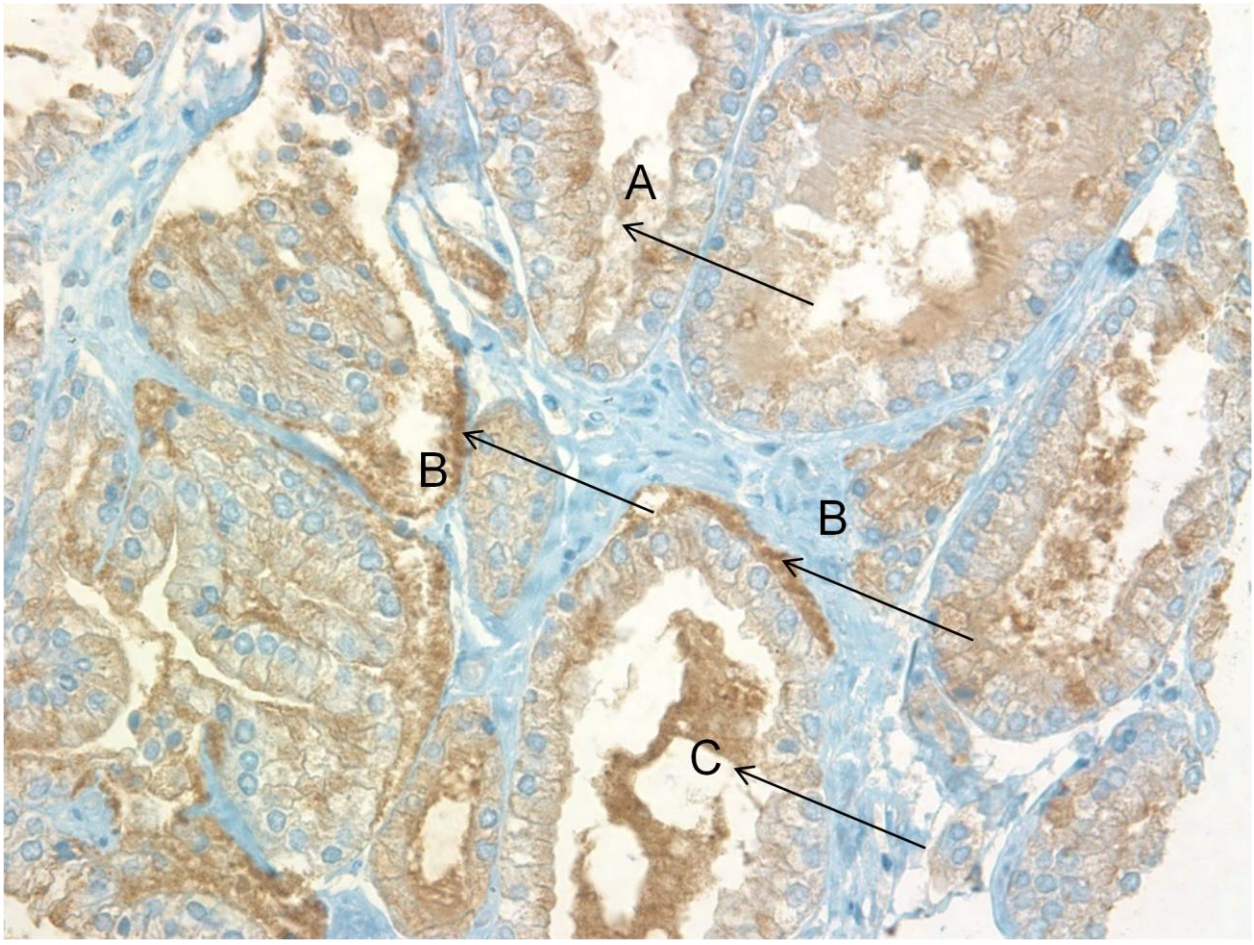

Figure 2: Representative picture of prostate cancer tissue stained for *in situ* CYP17A1 expression. As depicted, CYP17A1 appears to be localized to the luminal pole of human prostate cancer cells in exocrine secretory mode, typical of a secretary protein. The Gleason 3 pattern of prostate cancer shown in the pictures suggests that some acini are still well

differentiated and are able to make glandular lumen. The darker homogenous secretion presents CYP 17A1 in: A) Luminal pole of cancerous acinus; B) in the capillary slit around the cancerous acini and C) inside the lumen.

CYP17A1 protein is present in human sera and increased in samples from CaP patients compared to their age matched controls

The secretory-like expression pattern of CYP17A1 in cancerous prostate tissue prompted us to determine whether CYP17A1 is circulating in human serum. Initially, using Western blotting, we probed for CYP17A1 protein expression in human serum samples and showed that it is not only present in these samples (n=48) (**Figure 3a**) but that its levels were increased in hormone naïve CaP patients as well as those undergoing NHT (>6 months) (n=36) in comparison to healthy controls (n=10) (**Figure 3b**) ($p<0.01$). H295 adrenal cells (n=3) were used as a positive control and HepG2 cells (n=3) were used as a negative control for CYP17A1 expression (**Figure 3a, c, respectively**). We also conducted Western blot analysis of human liver and kidney microsomes to assess for CYP17A1 protein expression prior to activity assays (**Figure 3c**).

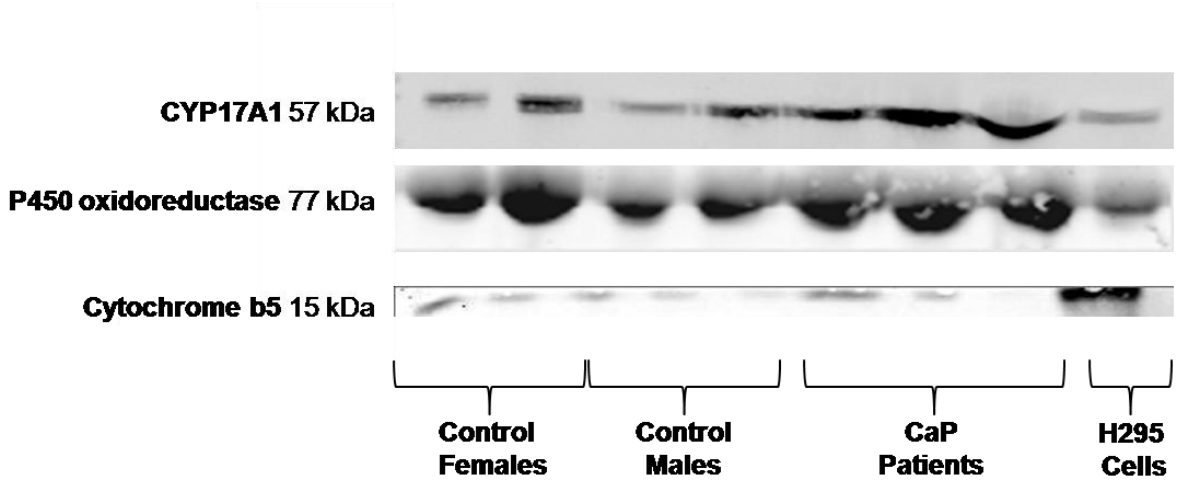

Figure 3a: Representative spectrum of protein expression of CYP17A1, P450 oxidoreductase and CYB5A1 in human serum. CYP17A1 is expressed in human serum (n=48) and appears to be higher in patients with CaP (n=36) as compared to healthy female (n=2) and male (n=10) controls. Enzymes P450 oxidoreductase and CYB5A1 are also expressed in human serum samples however do not appear to be increased in CaP patients'. H295 cells (n=3) were used as a positive control for steroidogenesis enzyme expression. Western Blot analysis was conducted in triplicate for each sample.

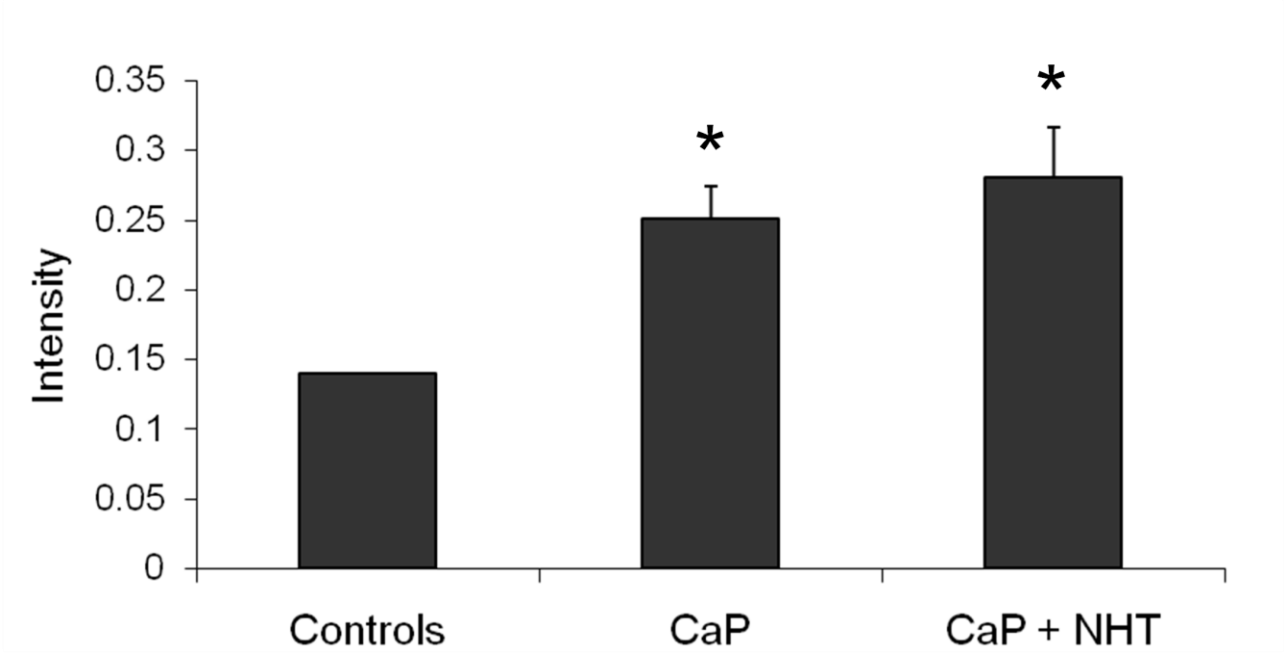

Figure 3b: CYP17A1 expression levels in prostate cancer patients. Mean CYP17A1 protein expression is significantly increased in the serum from patients (n=36) as compared to healthy male controls (n=10) *indicates statistically significant from controls ($p<0.01$). Error bars calculated based on S.E.M. Neoadjuvant hormone therapy (NHT > 6 months) (n=24) does not appear to effect the expression of CYP17A1 in serum as compared to patients with no treatment (n=12). Western Blot analysis was conducted in triplicate for each serum sample.

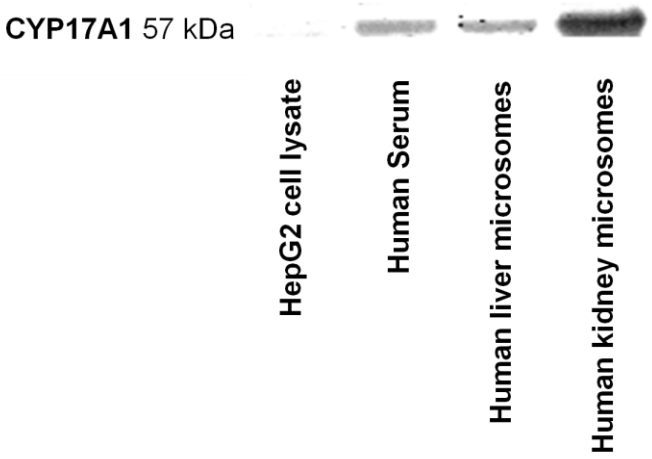

Figure 3c: Spectrum of protein expression of CYP17A1 in human liver and kidney subcellular fractions and HepG2 cells (negative control) for comparison with human serum. HepG2 cells (n=3) were used as a negative control for CYP17A1 expression and blotted alongside human liver (n=1) and kidney microsomes (n=1) for the purpose of

demonstrating a dynamic sample range for CYP17A1 expression as detectable by Western Blot. Western Blot analysis was conducted in triplicate for each sample.

Required P450 oxidoreductase and CYB5A1 enzymes are expressed in human sera

Based on our results suggesting that CYP17A1 is secreted from the CaP tumor we also assessed the expression of circulatory P450 oxidoreductase and cytochrome b5 (CYB5A1), enzymes necessary for CYP17A1 activity. In **Figure 3a** we show evidence of both P450 oxidoreductase and CYB5A1 in human serum, although not with the same observed increasing trend of CYP17A1 seen in CaP patients. The mere presence of these helper enzymes suggests that CYP17A1 may also be active in its circulating form.

CYP17A1 activity could not be demonstrated in human sera

Activity of CYP17A1 in serum was investigated using progesterone, 17-OH progesterone and pregnan-3α-ol-20-one as substrates for both CYP17A1 hydroxylation and lyase biotransformations. We analyzed for their metabolites in serum by LC-MS/MS for retention time match up to standards and MS fragmentation for mass identification. In the cytochrome P450 positive control human liver microsomes (n=3) progesterone (P) is converted to many downstream metabolites over a 2hr incubation period at 37°C, however none of the metabolites appear to be the result of CYP17A1 enzymatic action (**Figure 4a)**. We further probed for activity of CYP17A1 in positive control human kidney microsomes (n=3) and were able to show that progesterone is converted into downstream steroids 17-OH progesterone and androstenedione verifying the validity of the kidney microsome assay to probe for CYP17A1 activity specifically (**Figure 4b**). Upon this validation we conducted further experiments in fresh human serum samples obtained from healthy men while also confirming CYP17A1 detection in their serum. Progesterone does not appear to be metabolized in human serum (n=3) after 2hrs as compared to a 0hr time point and serum free controls (**Figure 4ci-iii**). While we report here only results from progesterone metabolism it should be noted that the metabolism of 17-OH progesterone and pregnan-3α-ol-20-one was also monitored and not observed in human serum samples (data not shown). This suggests that steroid precursors of CYP17A1 are not converted to downstream steroids in human serum.

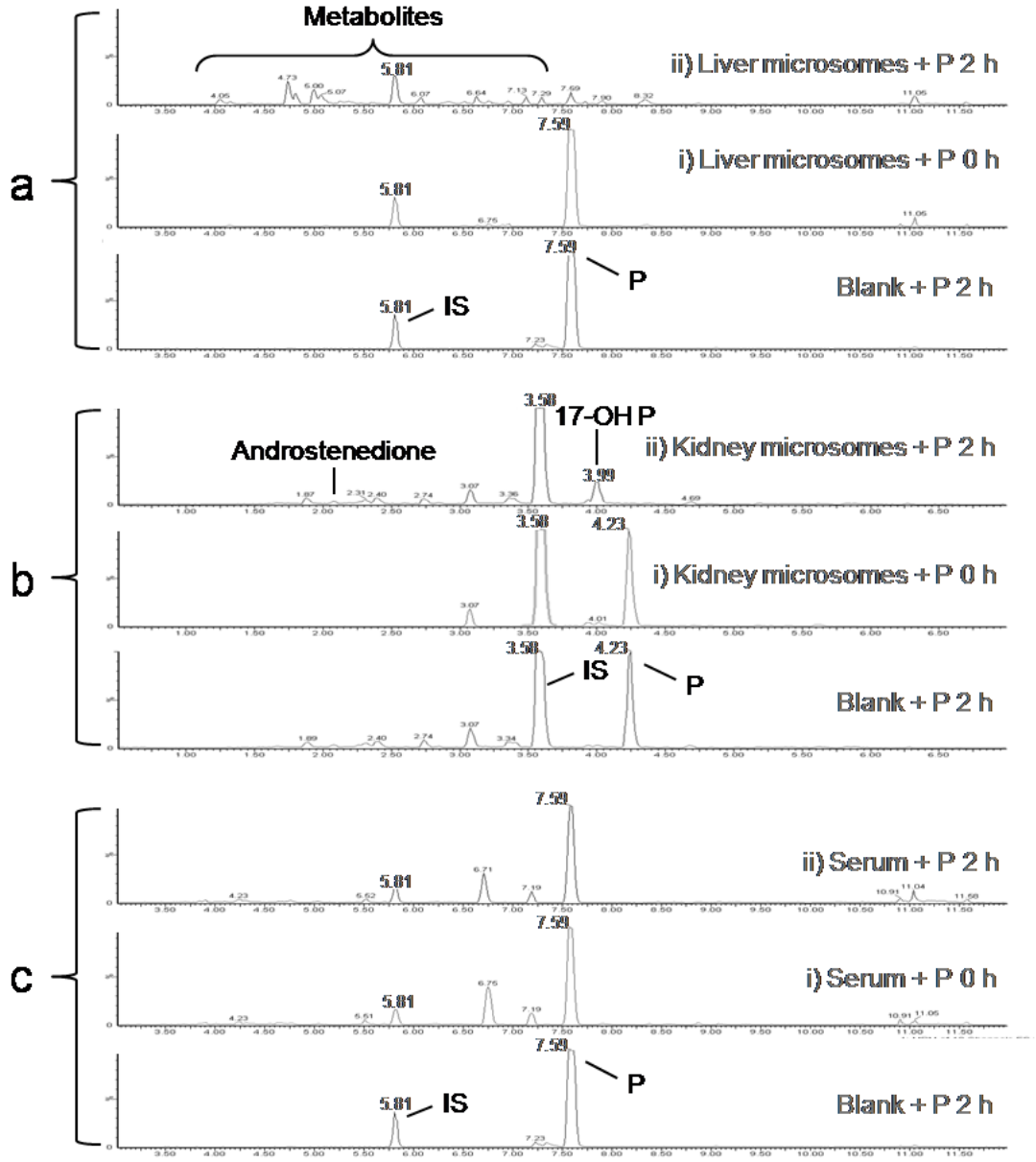

Figure 4: CYP17A1 activity in human serum. Progesterone metabolism in 1mg of positive control human liver microsomes (**a**) and human kidney microsomes (**b**) as well as 500µl of human serum (**c**) after 2hrs at 37°C (**ii**) as compared to 0hrs (**i**). Progesterone appears to be metabolized by human liver microsomes however CYP17A1 products are not present. Progesterone is metabolized by CYP17A1 in human kidney microsomes to 17-OH progesterone (17-OH P) and androstenedione. Progesterone does not appear to be metabolized by CYP17A1 in serum. PBS spiked with progesterone for 2hrs at 37°C was used as a blank control and deuterated testosterone was used as an internal standard in all experiments. Activity assays were conducted three times for each sample and analyzed by LC-MS.

CYP17A1 is expressed in serum exosomes

Exosomes were isolated from human serum as well as H295 adrenal cell media. Validation of exosome isolation was done by Western blot analysis for exosome markers heat shock protein-70 (HSP70) [32, 33], glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [34] and Fas ligand (FAS) [31]. As depicted in **Figure 5** both exosomes from human serum and H295 adrenal cell media express CYP17A1 suggesting that CYP17A1 may be excreted into the media in an exosome mediated transport mechanism. CYP17A1 is also expressed in positive control H295 cell lysates.

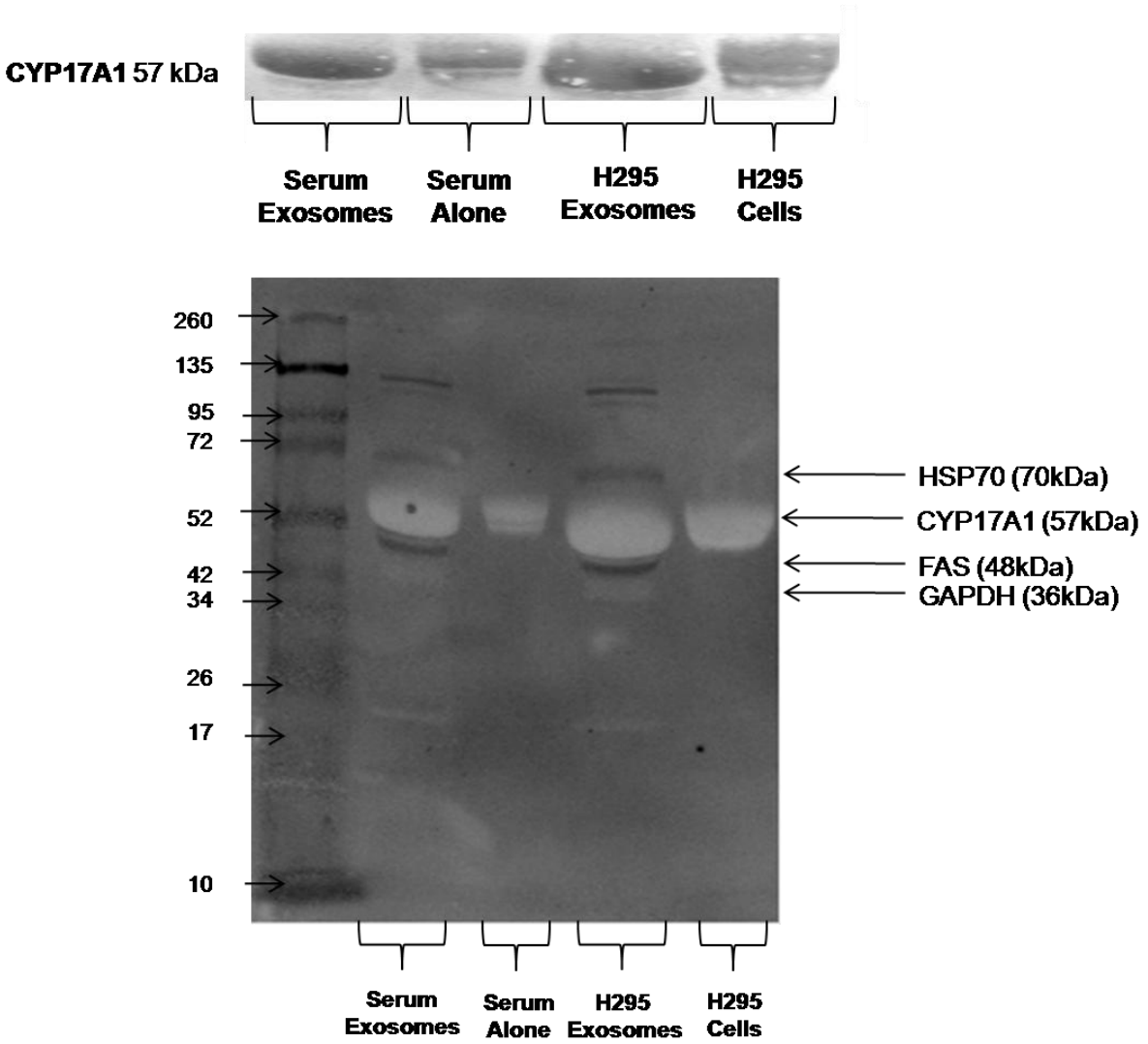

Figure 5: CYP17A1 expression in human serum exosomes. CYP17A1 is expressed in human serum exosomes as confirmed by Western blot analysis. Full human serum, H295 cell media exosomes and H295 cell lysates were also analyzed to confirm CYP17A1 protein

identity. Validation of exosome isolation was confirmed by Western blot analysis of marker ions HSP70, FAS and GAPDH.

Discussion

Many treatments are available for early stage hormone-dependent prostate cancer (CaP); however CaP emerging after androgen deprivation therapy is far more complex and difficult to treat. Castration-resistant prostate cancer (CRPC) emergence has been attributed to a variety of complex interrelated changes at the molecular level involving clonal selection, adaptive upregulation of anti-apoptotic and survival gene networks [36-38], cytoprotective chaperones [39, 40], and alternative mitogenic growth factor pathways [41-44]. Furthermore, most CRPC tumors continue to express the androgen receptor (AR) at levels observed prior to castration [45, 46] and AR is often found amplified [47, 48] and / or hypersensitive [49] suggesting that androgen signaling remains important in CaP cell function and disease progression despite the elimination of the testicular androgen source by chemical castration. This hypothesis was further supported by the documented presence of androgens: testosterone and DHT, in significant quantities to activate AR, in tumor samples obtained from CRPC patients [50]. Labrie *et al.* proposed that in absence of testicular testosterone, adrenal precursors may be utilized by CaP cells to make DHT [51, 52]. We and others have recently shown evidence of *de novo* androgen synthesis from cholesterol within the local environment of the tumor and suggest that this mechanism contributes to CRPC progression by providing ligand for AR activation [21-23]. CYP17A1 is an integral enzyme involved in both adrenal steroid conversion and *de novo* androgen synthesis and is therefore a logical therapeutic target in CRPC disease [5, 53, 54]. Recently in a Phase I clinical trial, Attard *et al.* provided evidence that treatment with abiraterone acetate, a synthetic inhibitor of CYP17A1, caused a decrease in patient serum androstenedione, DHEA and testosterone levels [27, 30] and similarly inhibition of CYP17A1 by antifungal agent, ketoconazole, in CRPC patients was shown to cause a decrease in testosterone synthesis [28, 29]. This accumulating evidence suggests that CYP17A1 plays a fundamental role in CaP progression. However, the exact mechanism of how CYP17A1 becomes one of the rate-limiting enzymes in androgen synthesis during CRPC development remains unknown.

Herein, we verify that CYP17A1 is expressed in CaP tissues *in situ.* Tumor expression of CYP17A1 has been shown to decrease with castration and then following

recurrence to CRPC increase in a pattern similar to that of the CaP biomarker, Prostate Specific Antigen (PSA) [20, 21]. Castration likely causes a reduction in CYP17A1 expression by initially depleting CaP cells of androgens, decreasing AR signaling and inactivating steroidogenesis transcription factors, sterol regulatory element binding proteins (SREBPs) [55]. Re-activation of SREBPs, which in turn are believed to regulate CYP17A1 levels, has been documented by our group and others to occur during progression to CRPC [56, 57]. It is proposed that after androgen deprivation select tumor cells evolve through clonal selection [58] as well as other adaptation mechanisms to induce SREBPs and downstream key steroidogenesis enzymes allowing for androgen synthesis to occur and enable cancer cell survival and progression to CRPC [22, 59, 60]. Using the LNCaP xenograft model we have shown that overall tumor levels of testosterone are increased and associated with SREBP re-expression during progression after castration potentiating a crucial role for CYP17A1 in androgen synthesis within the tumor environment [22].

As previously mentioned, the mechanisms by which *de novo* androgen synthesis is triggered within the local environment of the tumor remain elusive. Various cell signaling pathways involving oncogenes such as AKT and MAPK as well as tumor suppressors such as p53 and PTEN [61] are proposed mediators of many escape mechanisms leading to CRPC [61-64]. Growing evidence implicates many of these networks in tumorigenic processes such as cell cycle dysregulation, angiogenesis and metastasis [41, 65]. Our discovery that CYP17A1 may actually be a secretory protein which is elevated in serum of CaP patients suggests a secondary role for CYP17A1 other than intratumoral androgen synthesis. However, this role does not appear to support steroidogenesis in the serum as we were unable to confirm bioactivity using progesterone, 17-OH progesterone and pregnan-3α-ol-20-one as substrates in our assays. Another potential role for CYP17A1 may lie in cell-to-cell communication within the tumor microenvironment. Yu *et al.* and Lespagnol *et al.* previously implicated p53, a tumor suppressor found to be mutated in the majority of CRPC cells, to regulate the secretion of exosomes [34, 66]. Exosomes are extracellular vesicles, 50-90 nm in size that are secreted by virtually all mammalian cells into circulation and were believed to function in the removal of unwanted material and trafficking of pathogens until the early 1990's [67]. In 1996, van Niel *et al.* discovered a novel role for exosomes in cell-to-cell communication between B and T cells through the expression of HLA/peptide

complexes during immune response [68]. Valadi *et al.* further implicated a role for exosomes in the transfer of functional mRNAs between cells so that host RNA can be translated into proteins in neighboring cells [69]. Cancer cells have previously been shown to release exosomes that contain various proteins that are known to be mediators of escape events during disease progression [68, 70]. Proteins that have known functions in fusion, adhesion and biosynthetic processes have been identified in exosome units isolated from cancer cells [71] and further linked to various signal transduction, migration and adhesion events during tumor development and progression [72]. Interestingly, Lehmann *et al.* recently found that treatment-induced senescence in CaP patients led to increased release of exosomes into the circulating environment [73]. It is therefore reasonable to suggest that the biosynthetically important CYP17A1 enzyme might also be contained within these treatment-induced secreted exosomes. This would provide a potential mechanism for CRPC cell survival whereby CYP17A1 could act as a messenger to surrounding cells to signal them to become treatment resistance by mechanisms such as *de novo* androgen synthesis. Supporting this hypothesis we further confirmed CYP17A1 expression in exosomes isolated from human serum and that its protein expression is elevated in patients' serum as compared to healthy controls. The employment of NHT of greater than 6 months did not statistically increase CYP17A1 protein expression in CaP patients. However, as CRPC does not usually emerge until >9 months NHT [74] and we did not have information regarding patients' duration of NHT after 6 months, it is difficult to make further claims about how NHT affects CYP17A1 protein secretion. As we were unable to show activity within this environment this may be accounted for by Iero *et al.*'s observation that soluble factors and enzymes released in exosomes are likely to act locally or in the immediate vicinity of their releasing site and unlikely to exhibit biological activity further away from the parent cell [70] suggesting that CYP17A1 may act within the local environment of the tumor and not at other distant sites where we obtained our serum samples from. Nonetheless, the presence of essential enzymes P450 oxidoreductase and cytochrome b5 in serum further indicates a functional role for CYP17A1 in neighboring cells through exosome transported units.

In summary, we report exciting and novel findings of CYP17A1, P450 oxidoreductase and cytochrome b5 presence in human serum and suggest that although CYP17A1 does not appear to be active in this environment it may have another important

role. After androgen deprivation therapy levels of CYP17A1 in the tumor decrease during progression to CRPC, however tumor cells often regain the ability to induce CYP17A1 expression leading to androgen synthesis. We show evidence that CYP17A1 may be secreted from the CaP cell as part of an exosome unit and propose that it is used to initiate cell-to-cell communication for global androgen synthesis within the CRPC tumor. This evidence further rationalizes the clinical use of well-characterized and novel inhibitors of CYP17A1 in targeting CRPC progression.

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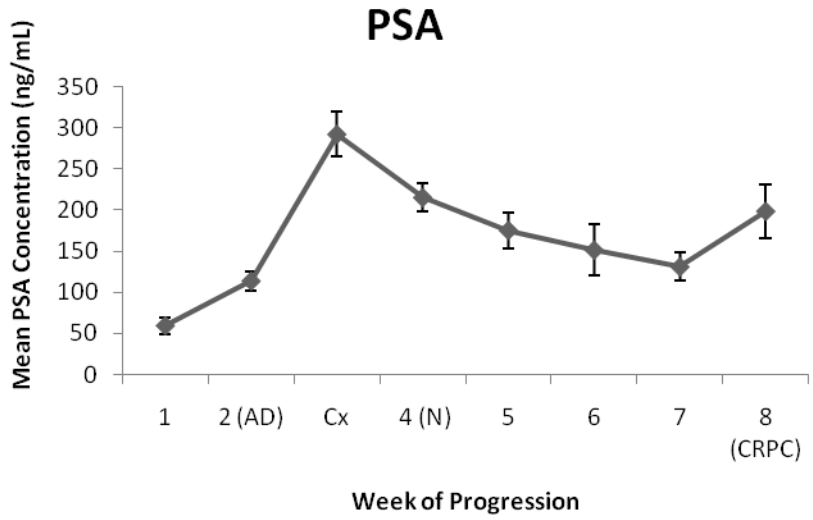
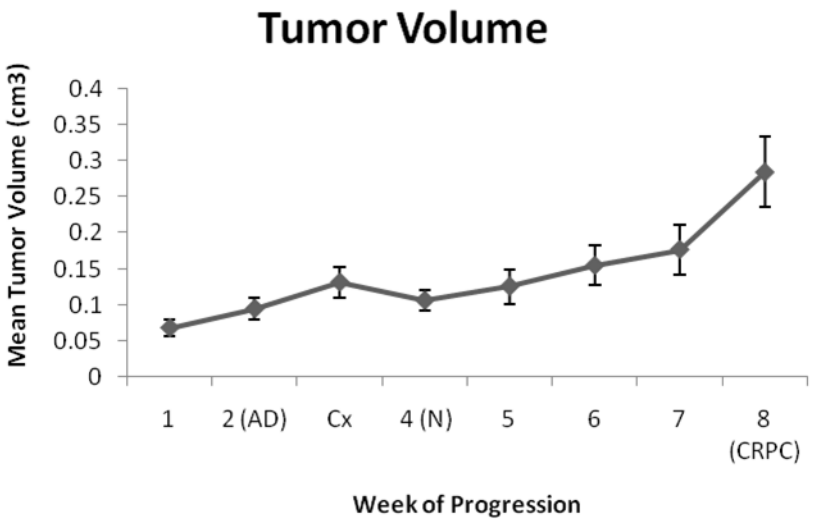
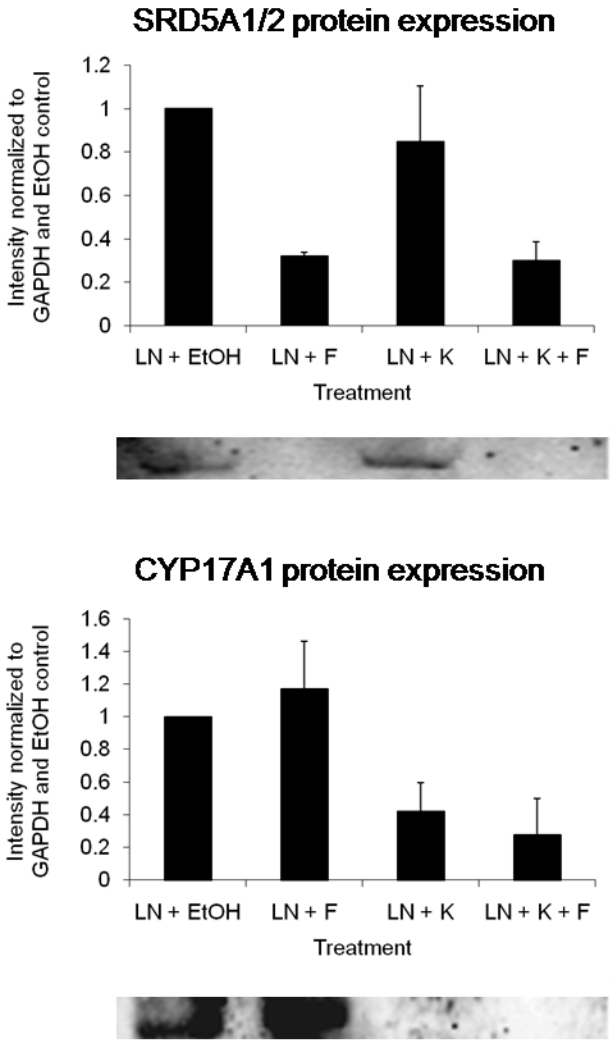
LNCaP xenograft model: PSA and tumor volume profiles





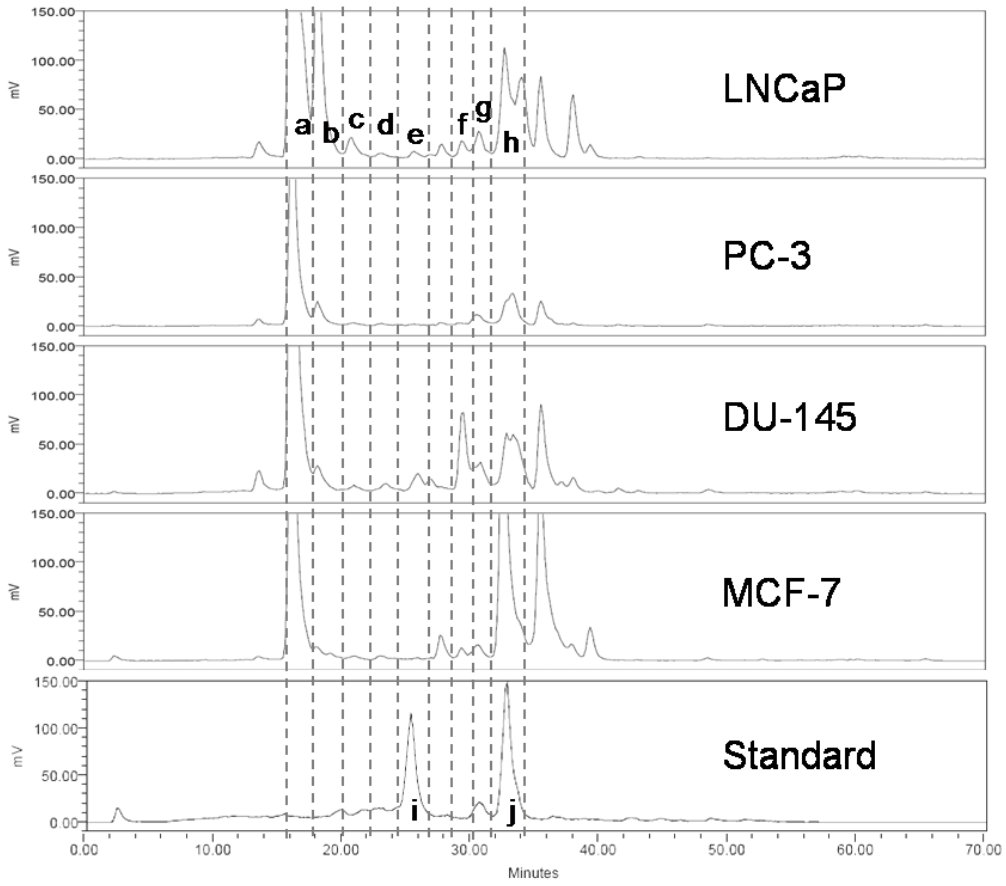
Mean PSA and tumor volume profiles from 14 mice during progression from androgen-dependent (AD), to nadir (N: 8-days post castration (Cx)), to castration resistant (CRPC: 35-days post castration) disease. PSA determined by tail vein sera analyses using the ClinPro kit and tumor volume determined by measurement of length, width and height of tumor by caliper. Tumor volume as depicted as average on each mouse.

Effect of ketoconazole and finasteride on enzyme protein expression in LNCaP cells



Western blot analysis of LNCaP cells in the presence of finasteride (F) and ketoconazole (K) on SRD5A1/2 and CYP17A1 protein expression in LNCaP cells (n=3).

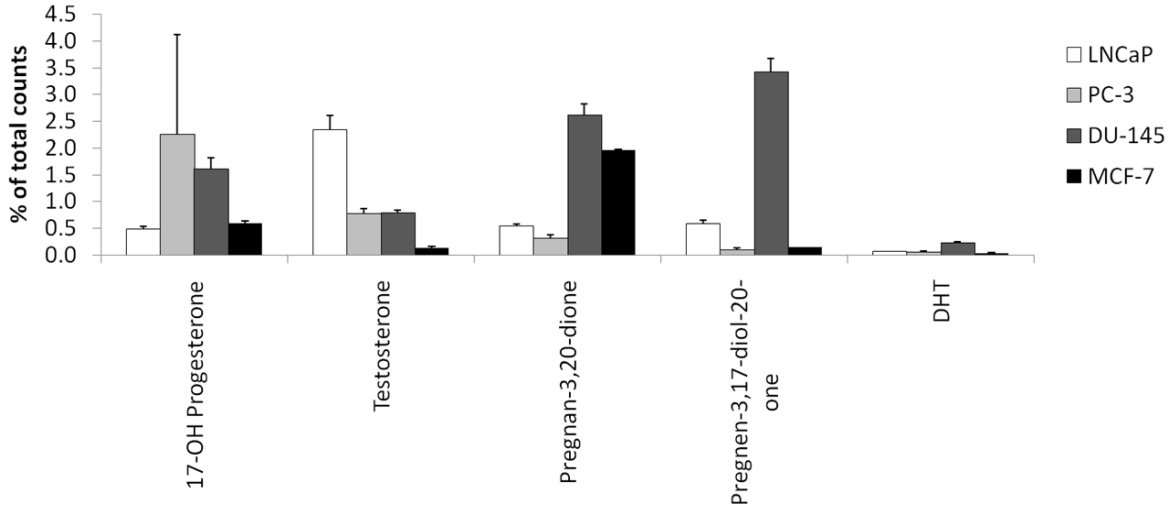
Progesterone metabolism profiles (and metabolites) in LNCaP, PC-3, DU-145 and MCF-7 cells



Table

^{3}H Peak	Steroid Standard	Radiometric RT (min)	SIR/MRM
a	Metabolite 1	15.9	317
b	Metabolite 2	17.9	331
c	Testosterone	20.4	289 > 97
d	17-OH Progesterone	22.7	331 > 97
e	DHT	25.2	287>97
f	Pregnan-3,17-diol-20-one	27.5	331 > 97
g	Androsterone	29.4	273 > 255
h	Progesterone	32.5	315 > 97
i	[^{3}H]-DHT Standard	25.1 +/- 0.1	_
j	[^{3}H]-Progesterone Standard	32.6 +/- 0.1	_

Example chromatographic profile of metabolites from LNCaP, PC-3, DU-145 and MCF-7 cells + [^{3}H]-Progesterone by HPLC-radiometric detection. HPLC-radiometrically identified peaks (a-j) matched up to RT of steroidal standard as determined by LC-MS. SIR/MRM precursor masses and fragment masses were used to identify and quantify steroids listed (table). RT shift of standards [^{3}H-DHT] and [^{3}H-Progesterone] were reported in +/- SEM units (n=7).



Levels of steroidal intermediates measured in LNCaP, PC-3, DU-145 and MCF-7 progesterone metabolism profiles (Mean +/- SEM). Not all standards were available for RT comparison and identification: 17-OH progesterone, testosterone, pregnan-3,20-dione, pregnan-3,17-diol-20-one and DHT were the only steroids positively identified by retention time match-up.

Ethics approval forms

The University of British Columbia

Animal Care Certificate

Application Number: A05-1736

Investigator or Course Director: Colleen C. Nelson

Department: Surgery

Animals Approved: Mice Nude 500

Start Date: **July 1, 2001** Approval Date: **January 6, 2006**

Funding Sources:

Funding Agency:	National Cancer Institute of Canada
Funding Title:	Program on prostate cancer progression - Project 3
Funding Agency:	National Cancer Institute of Canada
Funding Title:	Characterization and Targeting of Androgen Regulated Pathways in Prostate Cancer Progression
Unfunded title:	N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A06-1566

Investigator or Course Director: Emma S. Guns

Department: Urologic Sciences

Animals:

Mice CD-1 60
Mice DDS 120
Mice NU/NU 120

Start Date: **January 4, 2007** **Approval Date:** **October 2, 2007**

Funding Sources:

Funding Agency: Michael Smith Foundation for Health Research
Funding Title: Mechanistic approaches to Androgen-independent prostate cancer

Funding Agency: Heinz
Funding Title: Investigation into the Impact of Lycopene on Lipid Raft Structure and Associated IGF-IR Signaling during Prostate Cancer Progression

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Effects of ginseng and is metabolites on pharmacokinetics in cancer models

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

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102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
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The University of British Columbia
Office of Research Services
Clinical Research Ethics Board – Room 210, 828 West 10th Avenue, Vancouver, BC V5Z 1L8

ETHICS CERTIFICATE OF EXPEDITED APPROVAL: RENEWAL

PRINCIPAL INVESTIGATOR:	DEPARTMENT:	UBC CREB NUMBER:
Susan I. Barr		H05-70455

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

Institution	Site
Vancouver Coastal Health (VCHRI/VCHA)	Vancouver General Hospital

Other locations where the research will be conducted:
N/A

CO-INVESTIGATOR(S):

B. Joyce Davison
Karol Traviss
Kristin Wiens

SPONSORING AGENCIES:

- Centrum Foundation - "Vitamin D Status of Men Undergoing Androgen Deprivation Therapy for the Treatment of Prostate Cancer"

PROJECT TITLE:
Vitamin D Status of Men Undergoing Androgen Deprivation Therapy for the Treatment of Prostate Cancer

EXPIRY DATE OF THIS APPROVAL: August 1, 2009

APPROVAL DATE: August 1, 2008

CERTIFICATION:
In respect of clinical trials:
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The Chair of the UBC Clinical Research Ethics Board has reviewed the documentation for the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal by the UBC Clinical Research Ethics Board.

Approval of the Clinical Research Ethics Board by:

Dr. Gail Bellward

Dr. Gail Bellward, Chair

Certificate of Completion

This is to certify that

Dr Ladan Fazli

has completed the Interagency Advisory Panel on Research Ethics'
Introductory Tutorial for the
Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS)

Issued On: June 5, 2008