PROSTATE TUMOR HETEROGENEITY AND AGGRESSIVENESS REVEALED VIA THE ANDROGEN RECEPTOR EXPRESSION AND ACTIVATION BY TYROSINE PHOSPHORYLATION

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I. LIST OF ABBREVIATIONS

Amino Acid	AA
Androgen Deprivation Therapy	ADT
Androgen Receptor	AR
Androgen Response Elements	ARE
Antibodies	Abs
Benign Prostatic Hyperplasia	BPH
Biochemical recurrence	BCR
Bone metastasis	BM
3,3'-diaminobenzidine tetrahydrochloride peroxidase	DAB
Dihydrotestosterone	DHT
Digital Rectal Examination	DRE
DNA binding domain	DBD
Formalin fixed paraffin embedded	FFPE
Gleason Score	GS
Gonadotropin-releasing hormone	GnRH
Growth Factor	GF
Growth Promoting Factor	GPF
Heat shock proteins	HSP
High risk	HR
Immunohistochemistry	IHC
Insulin-like growth factor 1	IGF-1
Interleukin-6	IL-6
Intermediate risk	IR
Janus activated kinase	JAK
Ligand binding domain	LBD
Lower urinary tract symptoms	LUTS
Low risk	LR
Lymph nodes	LN
Neoadjuvant Hormonal Therapy	NHT

N-terminal domain	NTD
Percentage	%
Phosphate Buffer Saline	PBS
Phosphorylation/phosphorylated	p
Prostate Cancer	PCa
Prostatic Intraepithelial Neoplasia	PIN
Prostate Specific Antigen	PSA
Protein kinase	PK
Radical Prostatectomy	RP
Seminal Vesicles	SV
Signal Transducer and Activator of Transcription 3	STAT3
Src Homology2	SH2
Tissue Microarray	TMA
Trans Urethral Resection of the Prostate	TURP
Tumor/Nodes/Metastasis scoring system	TNM
Tyrosine Kinase	TK
Tyrosine residue	Y

II. Abstract:

Purpose and Objective: Prostate cancer (PCa) figures among leading causes of cancer deaths in North America. To date, androgen-deprivation therapy (ADT) is the gold standard treatment for patients experiencing biochemical recurrence (BCR) after radical prostatectomy (RP) or radiation therapy. However, ADT invariably fails and is followed by castration resistance (CRPC) and further progression of the disease. The host lab study on signaling through the Fer tyrosine kinase in PCa has led to the identification of the androgen receptor (AR) as a Fer substrate, activated by phosphorylation on the tyrosine (Y) 223 residue, a step followed by AR interaction with Fer and the accumulation of these complexes in the nucleus of PCa cell lines established from metastases. Moreover, the analysis of these proteins by immunohistochemistry (IHC) revealed that Fer and AR are at high levels in the tumor cell nucleus of advanced CRPC patients. The current **hypothesis** is that activated pY223AR reflects tumor cell aggressiveness and may serve as a potential biomarker of clinical relevance in PCa. The **aim** was to quantify activated –Y223 phosphorylated AR, along with AR proteins using antibodies (Abs) that recognize epitopes located in the C- vs. N- terminal domains of the protein, in hope to distinguish full length AR (C-epitope) and all forms (N-epitope), including small variants lacking the C-terminal ligand binding domain (LBD).

Methods: IHC was performed using AR C- and N- terminal Abs and specifically raised pY223AR Abs on sections from human prostates covering the whole spectrum of prostatic proliferative diseases and metastases from PCa. The cohort included, 40 healthy men (donors; < 40 y/o), 5 benign prostatic hyperplasia (BPH), 326 RP hormone-naïve cases or untreated a for prostatic indication, 25 neo-adjuvant hormone therapy (NHT) cases, 29 advanced CRPC cases on palliative ADT and 54 cases comprising 14 paired primary tumors and corresponding lymph node (LN) metastases, 19 tumor extensions into seminal vesicles, and 21 bone metastases. Titration was performed to establish optimal staining conditions. AR and pY223AR levels (0-3+) and cytoplasmic vs. nuclear distribution were quantified and expressed in percentages (%) relatively to the number of total cells counted and by H scores. The updated clinical database of the RP cohort was used to determine the predictive value of studied markers. Results were statistically analyzed to determine significant differences between subgroups.

Results: Cell by cell quantification of AR using C- and N- terminal Abs showed that the number of negative cells increased with progression, up to 16% in ADT/CRPC cases. In positive cells, AR was primarily nuclear. In cells of primary tumors stained with C-terminal Abs, the nuclear intensity remained at +1; H scores increased with Gleason score (GS) ($p<0.01$), being most elevated in ADT/CRPC patients, primary tumors and matched LNs, seminal vesicles extension and bone metastases. Quantification with N- terminal Abs revealed similar observations up to GS8, where a shift in higher intensity (+3 and +2 vs. 1+) was observed and persisted in all tissue samples categorized as advanced. Nuclear AR levels did not correlate with BCR.

Quantification of pY223AR showed that prostate epithelial cells of both healthy and BPH cases expressing AR but not Fer were negative for pY223AR. In tumors, the nuclear pY223AR intensity of positive cells and H score increased with GS of primary tumors ($p<0.01$), being most elevated in ADT/CRPC patients. Results were similarly elevated for primary tumors and their matched LNs, seminal vesicles extension and bone metastases. Statistical analyses (KM, COX regression and ROC) showed that nuclear pY223AR H scores significantly correlate with BCR and improve prognostication of standard parameters.

While scoring AR and pY223AR, other cells surrounding tumor foci were analyzed, notably fibroblasts and inflammatory cells. The AR expression pattern in fibroblasts confirmed the literature, with its presence observed from the normal prostate up to cancer of GS7, and loss in GS8 and all subsequent prostate tissues of more aggressive cases or advanced stages. The loss of AR in fibroblasts was predictive of BCR. Of interest, AR was activated (pY223) in prostatic fibroblasts, but only in cancer cases of GS6 and GS7. Inflammatory cells were AR (and pY223) negative in all samples. However, the extent of inflammation had predictive value for BCR and its combination with pY223AR H scores in cancer cells further enhanced prognostic value**.**

Conclusions: Prostate tumor foci and metastases are heterogeneous with regards to cancer cells expressing AR. The AR negative cells constitute up to 16% of total cells in advanced disease. AR negative cells are intermingled among a majority of AR positive cells. AR proteins detected through a N-terminal epitope showed significant relationships with disease progression, which was not observed with C-terminal Abs. AR per se in prostate cells did not correlate with patient outcome. However, AR activation by Y223 phosphorylation in tumor cells had high predictive value, which was enhanced by inflammation. Observations on AR in prostatic fibroblasts in cancer suggest a specific role linked to its activation by Y223-phosphorylation at the transition

from GS7 to GS8. Altogether, these findings proved the proposed hypothesis of the translational applicability of earlier findings in cell lines: pY223AR represents a novel biomarker that has prognostic value in PCa and predicts survival probability.

III. Résumé :

Préambule et Objectif: Le cancer de la prostate (CaP) figure parmi les causes principales de décès par cancer en Amérique du Nord. À ce jour, la thérapie de privation d'androgène (ADT) est le traitement standard pour les patients chez qui une récidive biochimique (BCR) est observée après une prostatectomie radicale (PR) ou la radiothérapie. Cependant, l'ADT échoue invariablement et est suivie par la résistance à la castration (CRPC) et la progression subséquente de la maladie. Les études du laboratoire-hôte sur la signalisation médiée par le tyrosine kinase Fer dans le CaP ont mené à l'identification du récepteur des androgènes (RA) comme étant un substrat de la kinase Fer activant AR par la phosphorylation du résidu tyrosine (Y) 223, une étape suivie de son interaction avec Fer et de l'accumulation de ces complexes au noyau des cellules de lignées de CaP établies à partir de métastases. De plus, l'analyse des protéines dans la prostate par immunohistochimie (IHC) a révélé que Fer et le RA sont exprimés à des niveaux élevés dans le noyau des cellules tumorales prostatiques chez des patients avec un CaP de stade avancé. L'**hypothèse** proposée est que le RA activé -pY223reflète l'agressivité tumorale et pourrait servir de biomarqueur ayant une connotation clinique dans le CaP. Le but était de quantifier le RA activé par sa phosphorylation sur la Y223, de pair avec l'expression du RA à l'aide d'anticorps (Acs) reconnaissant des épitopes localisés dans les domaines C- et N-terminaux de la protéine afin de distinguer la protéine pleine longueur (épitope C-) ainsi que toutes ses formes (épitope N-), incluant les courts variants qui ne possèdent pas le domaine de liaison du ligand localisé en C-terminal.

Méthodes: L'IHC a été réalisée à l'aide d'Acs du RA, C- et N- terminaux et d'un Ac spécifique généré pour détecter le RA activé -pY223 AR, sur des coupes de prostate humaine couvrant le spectre complet des maladies prostatiques prolifératives et de métastases prostatiques. La cohorte comprenait : 40 hommes en santé (donneurs; < 40 ans), 5 cas d'hyperplasie bénigne de la prostate (HBP), 326 PRs de patients n'ayant pas reçu d'hormones ou non traités pour une indication prostatique, 25 cas de thérapie hormonale néo-adjuvante (NHT), 29 cas avancés CRPC sous ADT palliative et 54 cas comprenant 14 paires de tumeurs primaires et leurs ganglions lymphatiques (LNs), 19 cas d'extension tumorale aux vésicules séminales et 21 métastases osseuses. Des courbes de titration ont été réalisées pour définir les conditions

optimales de détection. Les niveaux de RA et RA activé -pY223 (0-3+) et leur distribution cytoplasmique vs nucléaire ont été quantifiés puis exprimées en pourcentages (%) par rapport au nombre total de cellules comptées et par H « scores ». La base de données cliniques mises à jour de la cohorte de PR a été utilisée pour déterminer la valeur prédictive des marqueurs étudiés.

Résultats: La quantification cellule par cellule du RA en utilisant les Acs C- et N- terminaux a démontré que le nombre de cellules négatives augmente avec la progression du CaP, jusqu'à 16% dans les cas sous ADT/CRPC. Dans les cellules RA positives, la protéine était principalement nucléaire. Dans les cellules réactives à l'Ac C-terminal des tumeurs primaires, l'intensité du marquage nucléaire demeurait à l'intensité 1+; les scores H augmentaient avec le stade de Gleason (GS) ($p<0.01$), étant plus élevés chez les patients sous ADT/CRPC, suivis des tumeurs primaires couplées à leurs LNs, les extensions tumorales aux vésicules séminales et les métastases osseuses. La quantification réalisée avec l'Ac N- terminal a révélé des résultats comparables jusqu'au stade de GS8, où l'intensité fut déplacée de 1+ vers +3 et +2, laquelle a persisté à ces niveaux dans tous les échantillons de tissus de catégories dites avancées. Les niveaux du RA nucléaire ne corrèlent pas avec la BCR.

La quantification du RA activé - pY223 a démontré que les cellules épithéliales de cas en santé et d'HBP exprimant le RA mais pas Fer sont négatives quant au RA activé. Dans les tumeurs, l'intensité nucléaire des cellules positives quant au du RA activé et le score H augmentent avec le stade de GS des tumeurs primaires ($p<0.01$), étant plus élevés chez les patients sous ADT/CRPC. Des résultats similaires ont été obtenus pour les tumeurs primaires couplées à leurs LNs, les extensions tumorales aux vésicules séminales et les métastases osseuses. Les analyses statistiques (KM, régression de COX et ROC) ont démontré que le RA nucléaire activé - pY223 et les scores H corrèlent significativement avec la BCR et augmente la valeur pronostique des paramètres standard.

Au cours de ces analyses, nous avons aussi étudié les autres cellules situées tout près et entre les foyers cancéreux, notamment les fibroblastes prostatiques et les cellules inflammatoires. Le patron d'expression du RA dans les fibroblastes est en accord avec la littérature, soit sa présence dans la prostate normale et dans le cancer jusqu'au stade de GS 7, puis perdue au GS8 ainsi que dans les fibroblastes retrouvés dans tous les autres tissus prostatiques ayant des caractéristiques agressives ou représentant des stades avancés du CaP. La perte du RA dans les fibroblastes prédit la BCR. Fait intéressant, le RA est aussi activé -pY223 dans les fibroblastes

prostatiques mais seulement dans les cas de cancer de GS6 et GS7. Les cellules inflammatoires étaient négatives quant à l'expression du RA et du RA activé (pY223) dans tous les tissus étudiés. Cependant, l'étendue de l'inflammation prédit la BCR, et son analyse combinée avec les scores H du RA activé -pY223 dans les cellules cancéreuses augmente davantage la valeur prédictive de ces deux marqueurs quant à la probabilité de survie des patients.

Conclusions: Les tumeurs prostatiques et les métastases du CaP sont hétérogènes quant à l'expression du RA. Elles renferment des cellules RA négatives constituant jusqu'à 16% de l'ensemble des cellules tumorales du cancer avancé. Ces dernières sont dispersées parmi une majorité de cellules RA positives. Les protéines -RA détectées via l'épitope en N-terminal ont montré des associations significatives avec la progression de la maladie, non observées avec l'Ac ciblant l'épitope en C-terminal. Le RA lui même ne corrèle pas avec l'évolution clinique des patients. Cependant, son activation -phosphorylation du résidu Y223dans les cellules tumorales a une haute valeur prédictive, accrue lorsque couplée à l'inflammation. Nos observations sur le RA dans les fibroblastes prostatiques suggèrent un rôle étroitement lié à l'activation du motif Y223 phosphorylé à la transition du grade de GS7 vers le GS8. L'ensemble de ces observations prouve l'hypothèse proposée de l'application clinique possible dess données antérieures dans les lignées cellulaires : le RA activé -pY223 représente un nouveau biomarqueur qui a une valeur prognostique dans le CaP et prédit la probabilité de survie des patients.

IV. ACKNOWLEDGEMENTS:

At the end of my master, I would like to sincerely thank each and every one who helped to complete my project. It has been a journey of science, work and team work. As I see it, the team I worked with is one of a kind. I want each member of this team to know that I had a great honor and joy working with him/her. Dr. Simone Chevalier, the words are not enough to express my appreciation and gratefulness for everything you've done for me. Thank you for the opportunity, guidance and encouragement. You helped me with every step of the way, and I'm sure that your words of wisdom and encouragement will remain in my mind throughout my life. Thank you for being kindhearted and understanding throughout my master, and thank you for believing in me. Also, thank you for helping me with my presentations, papers and thesis. To Fatima Z Zouanat, I can't be more thankful for everything you did for me during my master. Thank you for teaching me how to do my experiments and helping me with everything in the lab. Thank you for being such a great teacher, supporter and a wonderful friend. You were more than a friend and you will always be. To Dr. Eleonora Scarlata, thank you for teaching me how to cut tissue blocks and prepare slides. Thank you for teaching me how to read and analyze my staining and thank you for verifying my results. Thank you for helping me throughout my master, I truly appreciate everything you've done for me. Thanks to Dr. Lucie Hamel who contributed to my work, by the updating of the clinical database used in my project. To Dr. Fadi Brimo, I really appreciate your words of encouragement and continuous support. Thank you for being a member of my committee that added many positive feedbacks to improve my project. To Dr. O'Flaherty, thank you for being the chair of my committee. Your words of encouragement and support were greatly appreciated. To Dr. Aprikian, I really appreciate your help throughout my master. You are a great mentor and an amazing physician. Thank you for the opportunity to work and conduct my experiments using your cohorts of patients. To everyone in our lab, thank you for a wonderful journey.

V. PREFACE AND CONTRIBUTIONS

This project was done as a continuation of the host lab previous work. After reporting the importance of the Fer tyrosine kinase in PCa, by activation of two key transcription factors through different experiments and studies by the host lab on multiple cell lines, the rationale is to take a discovery from the bench to the bed side. Here we are continuing these studies on human tissues from PCa patients to investigate if Fer activation of one of its substrates the androgen receptor (AR) or pY223AR has clinical relevance and to also assess AR expression in parallel.

The author (Turki Altaylouni) contributions to this thesis can be summarized as follows:

1. The Introduction (Chapter 1) was completely written by author.

2. The first paper "CELL BY CELL QUANTIFICATION OF ANDROGEN RECEPTOR (AR) IN THE PROSTATE AND PROSTATE CANCER REVEALS TUMOR HETEROGENEITY AND DIFFERENTIAL NUCLEAR EXPRESSION OF AR PROTEINS", (Chapter 2):

The experiments consisted of the analysis of AR expression on tissue sections from different patients stained by immunohistochemistry (IHC). The author performed the followings:

- Sectioning of formalin-fixe paraffin-embedded (FFPE) blocks from individual cases already gathered for the department of Pathology and obtaining sections from already available tissue microarrays (TMAs).
- Staining of sections using C-Terminal AR antibodies (Abs), carried out on the complete sets of tissues or cohorts. Prostate sections from young males, metastases (bone and LN) and seminal vesicles (SV) were also stained using N-terminal AR Abs.

- Scanning of all slides stained for AR C-terminal expression with the Aperio Image Scanner System.
- Quantification and statistical analysis of AR in C-terminal.
- Quantification of AR N-terminal expression of normal prostate from healthy males, SV and metastases (bone and LN).
- Statistical analysis of AR N-terminal expression of the complete sets or cohorts.
- The writing of the paper as a first author with help from the supervisor.

3. The second paper "FER-ACTIVATED ANDROGEN RECEPTOR (pY223AR): A NOVEL PREDICTIVE BIOMARKER OF PROSTATE CANCER PROGRESSION" (Chapter 3):

The experiments consisted in the analysis of activated AR expression by IHC on a whole cohort of PCa patients. The author performed the followings:

- Sectioning of all the FFPE blocks from individual patients and TMAs already prepared.
- Staining the complete sets of sections using pY223AR Abs.
- Scanning of the complete series of slides using the Aperio system.
- Quantification and statistical analysis of pY223AR expression.

Writing the paper as a first author with help from the supervisor.

4. Discussion and Conclusion (Chapter 4): was completely written by author.

Contributions of other lab members, previous MSc students and the author's supervisor:

- All TMAs used in this study were prepared by Dr. Eleonora Scarlata and Dr. Fadi Brimo.
- The histopathological database of the radical prostatectomy (RP) cohort was built by Dr Brimo and Dr Scarlata. Dr Lucie Hamel built and updated the clinical database, which was next anonymized.

- Generation and characterization of polyclonal pY223AR antibodies (Abs) in rabbits was achieved by Dr Hamel.
- Purification of pY223AR Abs was done by Ms Fatima Zahra Zouanat.
- IHC using N-Terminal AR Abs (except the ones mentioned above as done by the author) and cell by cell quantification were done by a previous MSc student, Tarik Benidir, and verified by Dr. Scarlata and Dr. Brimo.
- Scanning of sections stained for AR N-terminal expression using the Aperio system was done by Dr. Scarlata.
- Verification of quantification of AR C-terminal and pY223AR was done by Dr. Scarlata and Ms Zouanat and confirmed by Dr Brimo.
- Help with statistical analysis: Ms Zouanat, Dr. Shaghayegh Rouzbeh, Yogesh Bramhecha and Dr. Jacques Lapointe.
- In the first paper, Dr. Scarlata helped creating a Table and M. Benidir created two images.
- Supervisor Dr. Chevalier, generated the hypothesis and helped with the project, the paper and thesis plan and revision, follow-up and guidance.

This project is part of a Prostate Cancer Biomarkers Discovery approved by the Ethics committee of the MUHC-RI involving Dr. Lapointe, Dr. Chevalier and Dr. Wassim Kassouf, along with Dr. Aprikian and Dr. Brimo.

1. Chapter #1

INTRODUCTION

1.1 THE PROSTATE

1.1.1 Embryology

The embryos of mammalians have the ability to evolve into a male or female phenotype. In genetically normal individuals, this development is determined at origination and conception and at the embryonic stage, this is reflected by the cooperation of four important parts: The Wolffian and Müllerian ducts, the urogenital sinus and the fetal gonads.[1][2]

In males, sexual differentiation is determined by the influence of androgens, notably testosterone, which is produced by Leydig cells of the fetal testis. The first part of sexual differentiation consists of the regression of the Müllerian ducts and stabilization of the Wolffian ducts. The 2nd part consists of the formation of the convoluted epididymal duct and the vas deferens. Androgens also act to masculinize the urogenital sinus and the external genitalia. This process involves the formation of the prostate, in addition to the prostatic utricle, the closure of the labial-scrotal lobes and the formation of the penis.[2][3] The prostate starts to appear as epithelial buds growing from the walls of the urogenital sinus at the location of the Müllerian tubercle. The buds form branching cords that evolve to a network of tubules and alveoli.[4]

1.1.2 Anatomy

The normal prostate gland in the adult has a volume of approximately 20 g. It is around 4 cm in width, with length of 3 cm, and a depth of 2 cm. The position of the prostate is posterior to the pubic symphysis, anterior to the rectum, above the perineal membrane and below the urinary

bladder (Figure 1). The prostate continues with the bladder at the base, and then creates the external urethral sphincter at the apex. The prostate is surrounded with a capsule that consists of elastin, collagen, and smooth muscles.(5)

Fascia surrounding the prostate consists of three layers on the anterior, lateral, and posterior aspects. They allow the dissecting nerve fibers' containing layers without dissecting prostate tissues.(5) In anterior fascia, the deep dorsal vein of the penis is present. This layer is a direct continuity of the capsule. The lateral layer and the levator fascia are joined. The posterior layer is covered by the rectovesical fascia. This rectovesical fascia surrounds the prostate and seminal vesicles. It also extends above and ends as a fibrous plate below the urethra at the level of the external urethral sphincter.(5)

The prostate is supported by the puboprostatic ligaments anteriorly and by the external urethral sphincter and perineal membrane inferiorly. The seminal vesicles are located above the prostate and under the base of the bladder. Each vesicle joins its corresponding ductus deferens to form the ejaculatory duct before entering the prostate.(5)

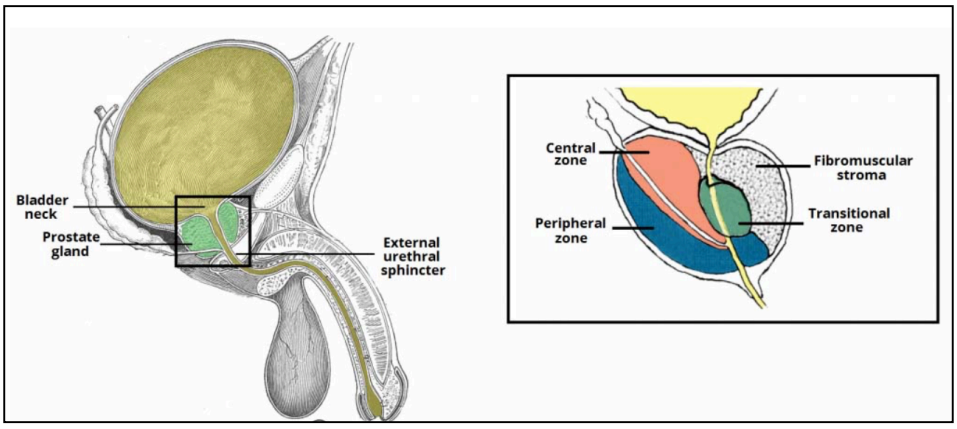

Figure 1 The location and anatomy of the prostate (left) and its zones (right).(6)

Adapted from:http://teachmeanatomy.info/pelvis/the-male-reproductive-system/prostate-gland/.

The prostate is divided into 3 zones (Fig. 1): (1) transition. (2) central. (3) peripheral. It is composed of approximately 70% glandular tissue and 30% fibromuscular stroma.[5]

Transition Zone: This zone represents only 5% of the prostate volume. It appears as pear shaped lobes surrounding the proximal urethra. The epithelium in this zone is composed of transitional cells similar to the bladder. This zone is where benign prostatic hyperplasia (BPH) commonly occurs.[5][7]

Central Zone: This zone is surrounding the ejaculatory ducts. It represents 25% of the prostate volume. It surrounds the transition zone from the angle of urethra to the bladder base. Glands in this zone are complex showing tall columnar, pseudostratified and papillary infoldings. Only 5% of adenocarcinomas are found in this zone.[5][7]

Peripheral Zone: This zone represents 70% of the prostate volume. It covers both posterior and lateral aspects of the gland. Also, it is the area that is palpated during digital rectal examination (DRE). 70% of adenocarcinomas are found in the zone.[5][7]

1.1.3 Histology

Histologically, the prostate is composed of glands or acini comprising three interrelated epithelial cell subtypes forming the epithelium and surrounded by fibromuscular stroma. The epithelial cells are: (1) the secretory or luminal cells which are located along the glandular lumen. They normally produce several proteins liberated in acini and found in the semen. Best known proteins expressed by luminal cells are prostate specific antigen (PSA), prostatic acid phosphatase (PAP) and AR. They are columnar and contain prominent nuclei at their base;[7] (2) the basal cells form a layer which separates the secretory cells from the basement membrane surrounding acini. They are considered as stem or reserve cells and can undergo differentiation

into luminal cells and myoepithelial metaplasia. Their presence helps to differentiate benign conditions from adenocarcinoma. There are conflicting data on AR expression in basal cells.[8] Among their marker is P63 and high molecular weight cytokeratins;[7] and (3) the neuroendocrine (NE) cells are irregularly distributed within acini, dispersed in secretory and basal layers. They cannot be distinguished without specific stains or markers like chromogranin A and serotonin among others.[7]

The fibromuscular stroma has its own cell constituents: (1) The fibroblasts which contribute to the synthesis of the extracellular matrix. They are positive for AR and other stains; [7] and (2) smooth muscle cells produce fibers ensuring contractility and elasticity.

The stroma also hosts other cellular components, notably (1) the endothelial cells forming the inside layer of blood vessels. They are important for the extravasation of leukocytes during inflammation and interact with motile cancer cells during invasion to form metastases. Very minimal AR staining is present in these cells;[7][9] and (2) the inflammatory cells, whose presence may indicate prostatitis, BPH or cancer conditions. Inflammatory cells are negative for AR.[7][10] Additional elements close to the prostate are the urothelium which lines proximal of prostatic ducts,[7][10] ejaculatory ducts and seminal vesicles lined by two layers of pseudostratified epithelium. They have large, hyperchromatic nuclei and could contain intranuclear inclusions.[7]

1.1.4 Function

The prostate has several functions that are important for male sexual responses. These functions include, the production of fluid for semen, the closing of the urethra up to the bladder during ejaculation and the hormone metabolism of testosterone.[11][12]

During seminal emission, mature sperms housed in the epididymis at the level of testicles are transported via the vas deferens into the urethra through the ejaculatory ducts, which lie within the prostate gland. One part of the semen is produced by prostate secretory cells and to some extent, neuroendocrine cells. Other parts are from the seminal vesicles and the secretions from the bulbourethral gland below the prostate. All of these fluids are mixed together in the urethra. The prostatic secretions are important for the proper functioning of the sperm and therefore also for male fertility.[11-14] The protein content is lower than 1% and includes PAP and proteolytic enzymes, like PSA. In addition, the hormone -like substance spermine ensures sperms motility.

During ejaculation, the prostate and the bladder sphincter close the urethra to prevent the semen from entering the bladder. In contrast, during urination the central zone of the prostate closes the prostate's ducts preventing the urine from entering [12-14]

Androgens are important for prostate function.[11] The main hormone is testosterone, which is produced mainly by Leydig cells in testicles. In the prostate, testosterone is transformed by the enzyme 5 alpha-reductase into its biologically active metabolite, DHT (dihydrotestosterone).[11] It is DHT that predominantly regulates the prostate secretory cell function due to its higher binding affinity to the AR.[11]

1.2 COMMON PROSTATIC DISEASES

1.2.1 Benign Prostatic Hyperplasia

BPH is a non-cancerous condition and does not increase the risk of PCa. BPH is a diagnosis made histologically. It is characterized by the proliferation of the cellular elements epithelial and

stromal of the prostate, preprogrammed cell death impairment (apoptosis), or both. These histological changes usually lead to the enlargement of the prostate. BPH arises in the transition and central zones. It may restrict urine flow from the bladder.[15][16]

BPH is considered a part of the aging process in males and is hormonally dependent on the production of testosterone and DHT. Statistically, it is estimated that 50% of males demonstrate histopathologic BPH by age 60 years and the percentage increases to 90% by age 85 years.[15] Risk factors that may increase the likelihood of developing BPH includes aging, family history, abdominal obesity, low physical activity, diabetes and hypertension.[17]

Clinical manifestations of BPH

Males with BPH may not have any symptoms. Signs of BPH start when the enlarged prostate press on the urethra and bladder. BPH often leads to lower urinary tract symptoms (LUTS), which may require medical treatments.[16] LUTS may include urinary frequency, especially at night, urinary urgency, difficulty in micturition, straining during micturition along with weak or slow urine flow and inability to completely empty the bladder. LUTS may cause frequent urinary tract infections and bladder stones, incontinence, causing urine to leak or dribble and presence of blood in urine.[16][18] When patients are seen in consultation, they are usually asked to answer a questionnaire regarding their urinary symptoms. Based on symptoms, the physician will perform a physical examination and DRE along with urine and blood tests, including PSA,[18] to confirm or rule out BPH. Additional tests may be needed, such as flow rate and residual ultrasound, x-ray or ultrasound, to check the kidneys, bladder and prostate, urodynamic assessment, cystoscopy and prostate biopsy.[18]

Management of BPH

Treatment options for BPH are based on the severity of symptoms and patient preference, which include: watchful waiting for mild symptoms, accompanied by lifestyle changes like to control fluids intake and drink less, especially before sleeping, avoid caffeine and drugs such as diuretics and decongestants. Patients can try to retrain their bladder, by urinating only at specific times of the day. Exercises of the pelvic floor can be helpful.[16][18][19]

Drugs can be used to relieve BPH symptoms. The list includes: (1) Alpha-blockers, which relieve pressure on the urethra by relaxing the muscles; (2) 5 alpha-reductase inhibitors, which prevent the conversion of testosterone into DHT, which helps shrinking large prostates; (3) Combination drug therapy, both an alpha-blocker and a 5-alpha-reductase inhibitor for males with large prostates and moderate to severe symptoms; (4) Phosphodiesterase-5 (PDE5) inhibitors which relax the bladder, urethra and prostate muscles and are also used for erectile dysfunction; (5) Muscle relaxants to decrease bladder contractions, treating incontinence and reducing the urge to urinate.[16][18][19]

Surgery is the treatment of choice for men with severe symptoms, when drug therapy stops working or for patients with urinary obstruction.[19] The most commonly used option is transurethral resection of the prostate (TURP). Briefly by passing a resectoscope carrying an electric current through the urethra, it is possible to reach the prostate and cut and remove tissue around the urethra. However, urinary problems may come back if the prostate starts to grow again. Common side effects include infection, bleeding and retrograde ejaculation.[19]

Other transurethral procedures include: (i) laser prostatectomy (holmium laser enucleation or photo-selective vaporization); (ii) incisions in small prostates with a special tool

to relieve pressure without removing tissue; (iii) electric vaporization using electricity and heat to destroy tissue; (iv) prostatic lifts, by implants to pull the prostate and relieving the obstruction.[18][19]

Prostatectomy is only used in rare cases when other procedures or surgeries can't be done. It may also be performed if there is a complete blockage of urethra or if the prostate is huge. [16][18][19]

1.2.2 Prostate Cancer (PCa)

In Canada, PCa is the most common cancer among men (excluding non-melanoma skin cancers). Also, it is the 3rd leading cause of cancer mortality.[20] Similar statistics apply in most industrialized countries. Therefore, this malignancy is the matter of intense research worldwide to prevent and better diagnose and manage patients.

PCa is a malignant slow growing cancer, however, some grow relatively quickly.[21] It is classified as an adenocarcinoma, or glandular cancer. As mentioned above, PCa is most commonly presented in the peripheral zone. Several concepts were put forward to explain how the cancer begins. The most common is by mutations of normal semen-secreting prostate glandular cells whereas the transformation of stem cells is often invoked.[22] Later in the disease or according to tumor cell aggressive features, cancer cells invade surrounding organs such as seminal vesicles, bladder, ureters and the rectum. In addition, tumor cells gaining access to the bloodstream or lymphatic system spread as metastases, most common sites being lymph nodes (LN) and bones. Metastases to solid organs like lung, liver, brain may also occur.[23]

Initially, PCa causes no symptoms as it is often slowly growing [21] but in later stages it can lead to different symptoms including LUTS, hematuria and pelvic or back pain. Late symptoms may include fatigue, sweating and weight loss.[24]

Risk factors includes aging, family history, race and processed-meat rich diet.[22] About 99% of PCa occur in men of over 50 years of age. First-degree family history increases the risk of PCa by 2 to 3 folds. In addition, PCa is more common in African races.[22]

PCa diagnosis

The only test that confirms a diagnosis of PCa is the histopathological examination of prostate biopsies, obtained through transrectal ultrasound (US). However, prior to a biopsy, less invasive testing can be conducted, including DRE, blood PSA, cystoscopy and prostate imaging.[25]

As mentioned above, PSA is a protein secreted in the semen by normal cells of the prostate and may be liberated in the blood by malignant but also benign cells.[26][27] Levels of PSA tests are usually reported as nanograms of PSA per milliliter (ng/mL) of blood. In the medical practice, physicians consider 4.0 ng/mL and lower as normal PSA level.[26][27]

Screening of PCa using PSA is controversial.[21][22] Studies showed that screening increases cancer detection. However, no decrease in mortality was detected.[28] Current recommendations are against screening, due to the risk of over-diagnosis and overtreatment.[29] Nonetheless, most physicians discuss risks and benefits of PSA screening with their patients.[30]

When asymptomatic men with no history of the disease choose to undergo PSA testing for PCa screening and found to have elevated PSA levels (>4.0 ng/mL), another PSA test is done to confirm high levels. DREs and repeated PSA tests will allow the physician and patient to decide and proceed with further prostate imaging tests and biopsy to confirm PCa.[27] Only about 25% of men with elevated PSA level (>4.0 ng/mL) who undergo a prostate biopsy have a PCa

diagnosis.[31] Despite debates on usefulness of blood PSA tests for diagnosis, PSA levels become meaningful to monitor patients who have a history of PCa. Increased PSA levels after PCa therapy could be the initial sign of a recurrence, commonly called "biochemical recurrence (BCR)".[32]

Prostate imaging modalities include US, which reveal hypoechoic area and magnetic resonance (MRI) structural tissue alterations.[33][34] MRI has several benefits. In low risk disease, it may guide toward active surveillance (AS). In intermediate risk disease, it may guide physicians in determining the clinical stage, and in high risk disease it may be used to also detect bone metastasis. MRI may be useful for surgical planning of robotic prostatectomy.[35]

As pointed out above, prostate biopsy is the test of choice to confirm a PCa diagnosis. Basically, 3 to 6 pieces of prostatic tissues are extracted from each side of the prostate with specialized needles. This outpatient procedure[36] require antibiotics to prevent infection.[37]

The microscopic analysis of biopsies is performed by a pathologist, often specialized in the genito-urinary system, who determines whether cancer cells are present and he evaluates the architecture of glandular patterns, cells, organelles, classified as Gleason Score (GS).[37][38] Figure 2 shows the microscopic appearance of normal prostate glands in 4μm unstained sections.

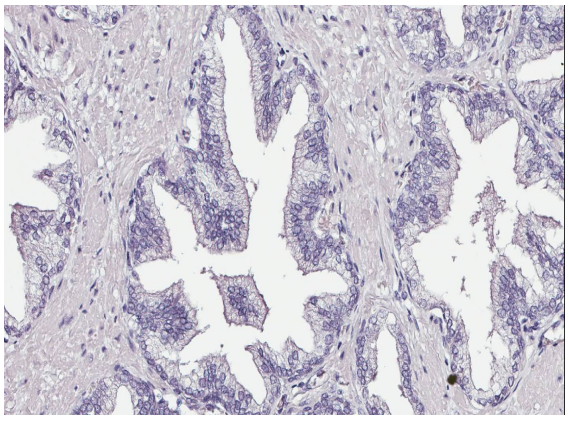

Figure 2: The architecture of the normal prostate gland. Acini and stroma are well defined.

According to the 2014 Consensus on Gleason score grading, there are currently five GS patterns,[37][38] which can be summarized as follows:

- Pattern 1 - Well differentiated carcinoma. Glands looks small, have well-formed shape, and are packed closely. As seen in Figure (3).

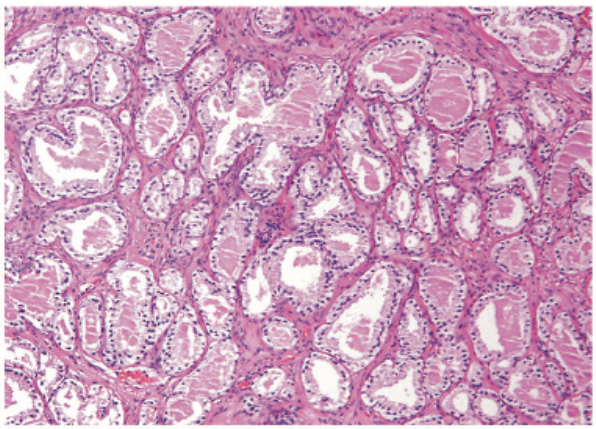

Fig. 3: Gleason Pattern 1.

From: http://pathology.jhu.edu/ProstateCancer/NewGradingSystem.cfm

- Pattern 2 - Moderately differentiated carcinoma. Glands have well-formed shape, but the size is larger and they are less packed, due to an increase in stroma. As seen in Figure (4).

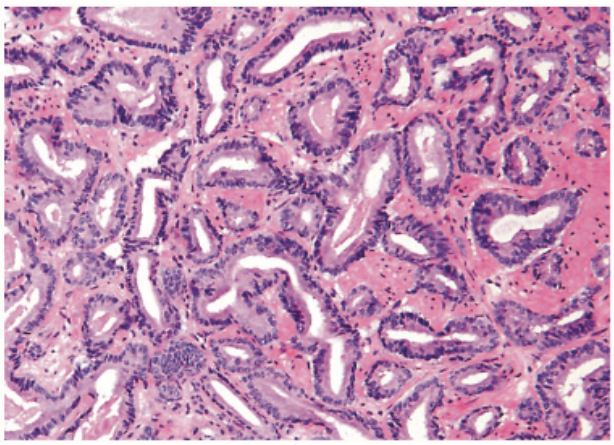

Fig. 4: Gleason Pattern 2.

From: http://pathology.jhu.edu/ProstateCancer/NewGradingSystem.cfm

- Pattern 3 – Also moderately differentiated carcinoma with some darker cells. In addition, several cells started to invade the surrounding tissue and/or show an infiltrative pattern. As seen in Figure (5) on a section stained by IHC for N-terminal AR expression.

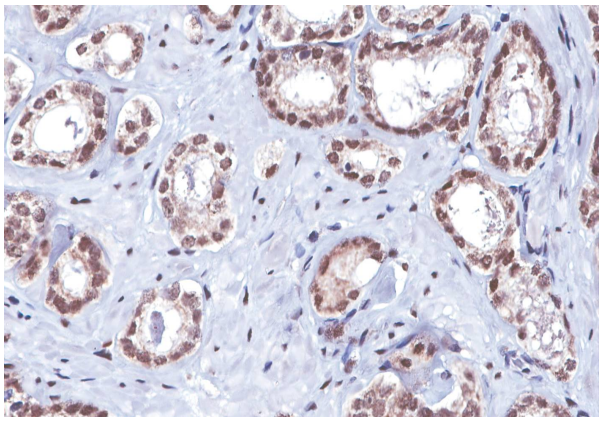

Fig. 5: Gleason Pattern 3.

- Pattern 4 - Poorly differentiated carcinoma. Few cribriform poorly recognizable glands and many cells are invading the surrounding tissue in neoplastic clumps. As seen in Figure (6) on a section stained by IHC for C-terminal AR expression.

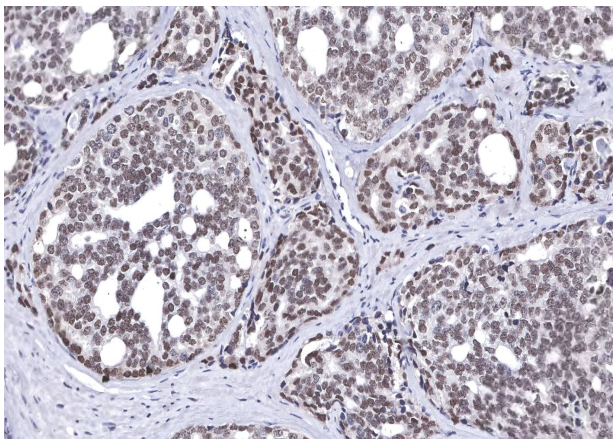

Fig. 6: Gleason Pattern 4.

- Pattern 5 – Anaplastic carcinoma. No glands can be recognized. The majority are just sheets of cells throughout the surrounding tissue. As seen in Figure (7) on a section stained by IHC for pY223AR expression.

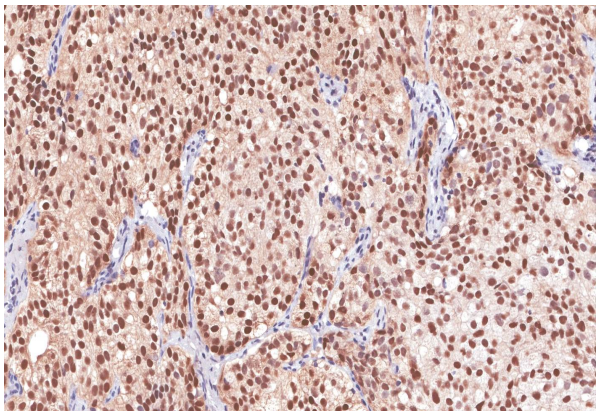

Fig. 7: Gleason Pattern 5.

- Figure 8 Summarizes the patterns based on the ISUP Consensus.[27]

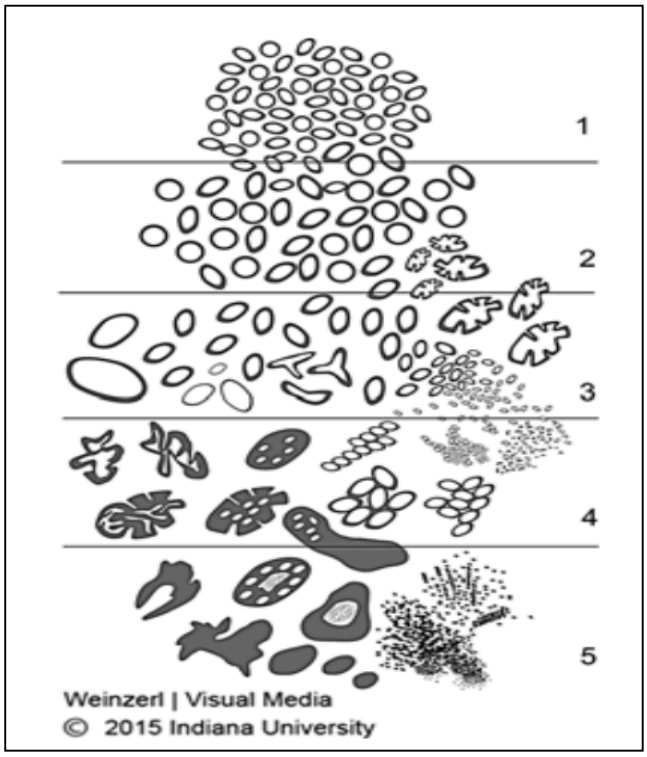

Fig. 8: Histological patterns of the adenocarcinoma of the prostate.[27]

Adapted from:

http://journals.lww.com/ajsp/Abstract/2016/02000/The_2014_International_Society_of_Urological.10.aspx

The assigned GS is the sum of the dominant tumor pattern and the 2nd most frequent pattern in all samples. GS ranges from 2 to 10. Recently, based on the 2014 ISUP Consensus, the widely used reporting of Gleason grades and prognostic grade groups(27) is:

- Gleason score ≤ 6 (Grade group 1) - well differentiated glands.
- Gleason score 3+4=7 which means that 3 is the main pattern (Grade group 2) – contain minimal component of less differentiated glands.
- Gleason score 4+3=7 here 4 is main pattern (Grade group 3) - Mainly poorly differentiated glands.
- Gleason score 4+4=8 or 3+5=8 or 5+3=8 (Grade group 4) – Only poorly differentiated/cribriform glands or minimal components of no gland architecture.
- Gleason scores 9-10 (Grade group 5) – No remaining glandular formation.

Tumor markers

Prostatic tissue samples are stained if necessary for various tumor markers. This may help in determining the origin of malignant cells.(40) However, beside PSA, GS and stage tumor, markers allowing physicians to determine the best management option predicting prognosis are almost unavailable. An improvement in tumor markers for outcome prediction is likely to come in the near future.(41)

Staging

Determining the stage of PCa by evaluating how far the cancer has spread is of high importance. Stage determines the patient prognosis and choosing optimal management options.[42] Clinical staging is done through the TNM (Tumor/Nodes/Metastases) system, which refers to three parameters: the size of the tumor (T), the number of LN affected (N), and the presence of metastases (M). Evaluation of the stage is done through imaging. However, the exact pathological stage is confirmed after surgery by analysis of the prostate.[43]

The TNM staging system can be summarized as follows: (from AJCC 8th edition):[44]

- T1: Cancer cannot be felt or seen.
- T2: Cancer can be felt and is confined to the prostate gland.
- $T3_a$: Cancer extends outside the prostate but not into seminal vesicles.
- $T3_b$: Cancer has spread to the seminal vesicles.
- T4: Cancer has spread beyond the prostate into surrounding tissues other than seminal vesicles.[44]

Pathologic staging (pT):[44]

- pT2: Tumor confined to prostate.
- pT3a: Tumor extends beyond prostate (unilateral or bilateral) or invades the bladder neck microscopically.
- pT3b: Tumor invades one or both seminal vesicles.
- pT4: Fixed tumor or the tumor invades adjacent organs other than seminal vesicles, such as bladder, rectum, muscles, external sphincters or wall of pelvis.[44]

When LNs are affected, N categories include:

- N0: None positive.
- N1: presence of at least one positive regional LN.[44]

If metastasis present, M categories include:

- M0: No metastasis.
- M1: Metastasis is present.
- $M1_a$: Cancer has spread to distant LNs, outside the pelvis.
- $M1_b$: Cancer has spread to bones.
- $M1_c$: spread to other organs such as lungs, liver, or brain.[44]

Risk group stratification has been defined by D'Amico et al at (Harvard)[45] and adopted by the National Comprehensive Cancer Network (NCCN).[46] This stratification takes into account pre-treatment blood PSA level, GS and stage as the three most important predictors. This system allows physicians to categorize patients into groups with the same predicted prognosis in order to compare results of one treatment to another.[45][46]

The D'Amico/NCCN stratification is:[45][46]

- Low-risk (LR): PSA $\leq$ 10; GS $\leq$ 6 ng/mL, and clinical stage T1-T2a
- Intermediate risk (IR): PSA between 10 and 20 ng/mL, GS= 7, or clinical stage T2b
- High-risk (HR): PSA > 20 ng/mL, GS $\geq$ 8, or clinical stage T2c-T3a

Systems stratifying GS 7 into GS 4+3 and GS 3+4 are based on several studies that show different behaviors of disease severity between GS 4+3 vs. GS 3+4.[45-50]

1.3 CLINICAL MANAGEMENT OF PROSTATE CANCER

The treatment of PCa depends on risk group stratification in addition to factors such as age, life expectancy, general health, and a person's views about potential treatments and possible side effects.[47-50]

1.3.1 Active surveillance (AS) and watchful waiting (WW)

If PCa is in the early stage and the treatment may cause more discomfort than the disease, options may at first be AS or WW. In AS, which is the choice for early stage PCa, physicians perform a close monitoring for signs of worsening rather than starting treatment immediately.[51] The American Society of Clinical Oncology (ASCO) recommends AS for patients with LR disease and may also be an option for men with IR disease.[52] The ASCO recommendations testing schedules suggest PSA test every 3 to 6 months, DRE at least once every year and another prostate biopsy within 6 to 12 months, and then a biopsy at least every 2 to 5 years.[52] Treatment will be initiated when signs of cancer progression suggest a more aggressive disease or spreading, or else, blocks the urinary tract or causes pain.[52]

WW may be considered for patients with life-threatening illnesses and life expectancy less than 5 years.[53] No test or biopsy are done during such a management.[53]

1.3.2 Local treatments

Surgery and radiation therapy are recommended to cure a disease confined within the prostate, whereas evidence of spreading beyond the prostate requires systemic treatments.[49][50] Surgery aims to completely remove presumably localized tumors. Diverse procedures may be used depending on stage and patient’s overall health.[50] They include: (i) radical (open, laparospic or

robotic) RP which removes the prostate, SVs and pelvic LNs, according to practice. Sexual function may be preserved if nerve-sparing surgery is feasible.

Main-side effects are sexual dysfunction and urinary incontinence. Drugs or implants can be used to help regain sexual function. Sometimes, surgery can improve urinary incontinence.[54][55] Robotic or laparoscopic prostatectomy are less invasive options with instruments and camera inserted in the abdomen through small incisions. The robotic approach is much more expensive[55][56] but easier than laparoscopic surgery. Both lead to less recovery time and cause less bleeding and pain than the first option, open surgery. However, the sexual and urinary side effects may still occur.[55][56]

1.3.3 Radiation therapy

Radiation therapy is the use of high-energy rays to kill PCa cells. This treatment is performed by radiation oncologists. It usually consists of a certain number of sessions over a specific period of time.[57] Several modalities exist, notably: (i) external-beam radiation which is most common for PCa and focuses a beam of x-rays on the cancer area. Newer techniques of radiation therapy exist, which are more precise and advanced: (1) Conformal radiation therapy (CRT), which decreases the destruction of healthy tissues around the tumor;[57] (ii) Intensity-modulated radiation therapy, which uses computerized tomography (CT) scan to form a 3-dimensional image of the prostate, the information being computerized to obtain the dose of radiation needed to destroy the cancer before external-beam radiation treatment. This method helps to apply high doses of radiation without increasing the risk of damaging surrounding organs;[58] (iii) Proton therapy, which is based on high energy protons rather than x-rays to destroy cancer cells. This expensive option showed similar results to the common methods used;[59] and (iv)

Brachytherapy, a treatment consisting in the insertion of radioactive seeds directly into the prostate, thereby providing radiations in the area of insertion and around. It is possible to perform short time therapy once for 30 minutes using high dose rate, while longer time therapy uses seeds remaining in the prostate and providing low dose radiations for up to 1 year. Brachytherapy may be used in conjunction with other radiation therapies.[60] Side effects include bowel problems urinary symptoms and fatigue. These side effects are not permanent and usually disappear after treatment. However, erectile dysfunction may be permanent if it happens.[61]

1.3.4 Focal therapies

Noninvasive focal modalities are used primarily for men with LR or IR PCa to destroy small tumors, without treating the whole prostate. These are not considered standard of care for newly diagnosed patients. They may use cold (cryotherapy), heat or other means still under study or part of clinical trials. For instance, high-intensity focused ultrasound (HIFU) uses the sound waves energy, directed to the tumor with assistance of MRI scans. They affect urinary and sexual functions in different percentages.[62, 63]

1.3.5 Systemic treatments

Androgen deprivation therapy (ADT)

As the prostate growth is driven by androgens, mainly testosterone and DHT, diminishing levels of hormones has proven to help slow down cancer growth by androgen-deprivation therapy (ADT).[64] ADT can be performed either surgically by bilateral orchiectomy or medically with drugs that alter the hypothalamo-pituitary-testis axis, resulting in the inhibition of testosterone production by testicles. ADT can be administered alone using LHRH agonists as leuprolide, or

LHRH antagonists as Degarelix, or combined with other inhibitory drugs or anti-androgens that affect the AR signaling axis,[65] including newer drugs like Enzalutamide and CYP17 inhibitors, like Abiraterone.[66][67][68]

ADT is used to treat PCa in different situations. It is the gold standard for local recurrence after surgery or radiation and for metastatic disease.[66-69] ADT can be used as a neoadjuvant hormone therapy (NHT) for IR PCa cases, with radiation as definitive therapy and an adjuvant therapy for patients who underwent surgery and whose excised pelvic LNs showed the presence of cancer cells. ADT may be used as palliative or preventive measures of local symptoms for men who are not candidates for surgery or radiation therapy for localized PCa.[66-69]

Side effects reflect the lack of androgens and result in erectile dysfunction, loss of libido, gynecomastia, hot flashes, weight gain, loss of muscle mass, depression, cognitive dysfunction, osteopenia or osteoporosis and metabolic syndrome.[66][67][70] Studies have shown that the benefits of castration far outweigh the risks of side effects. Patients should try to perform certain habits to manage side effects, such as exercising, avoid smoking, healthy life style and diet and following-up their health status with physicians regularly.[69][70]

1.3.6 Castration-Resistant Prostate Cancer (CRPC)

Usually ADT is given for the reminder of the patient's lifetime. However, most hormone-responsive (dependent) PCa eventually becomes castration resistant, as ADT is then not controlling cancer, after 1 to 3 years and continue to grow. Hence these tumors still show

reliance upon hormones for AR activation. However, the recently introduced abiraterone and enzalutamide therapies also fail, allowing further disease progression.[71][72][73]

1.3.7 Chemotherapy

Systemic chemotherapeutic agents get into the bloodstream to reach and kill cancer cells or stop (slow down) growth. Chemotherapy may help patients with advanced disease or CRPC in prolonging survival.[73][74] Standard chemotherapy begins with docetaxel (Taxotere) combined with corticosteroids (prednisone). This combination increases the life expectancy of patients with metastatic CRPC when compared with other chemotherapeutic regimens. An old agent called Mitoxantrone was one of the first chemotherapies approved for metastatic CRPC, but is not commonly used. It is sometimes considered in specific situations.[74][75] Cabazitaxel is another new drug approved for CRPC when refractory to docetaxel-based chemotherapy, thereby improving survival in comparison to mitoxantrone for men whose disease progressed after docetaxel. However, in clinical trials, cabazitaxel was not better than docetaxel. A new combination of bevacizumab (a recombinant Ab that blocks angiogenesis through inhibiting vascular endothelial growth factor A), docetaxel, thalidomide and prednisone appears effective in prolonging survival.[76][77]

Side effects of chemotherapy depend on patients, administered drugs an doses, and the duration of treatment; the list includes tiredness, decrease in appetite, blood disorders, nervous system problems, sexual and reproductive problems.[78]

1.3.8 Immunotherapy

Immunotherapy targets the patient's own immune system to fight cancer. It stimulates the immune system by using cells extracted from the patient or materials manufactured in

laboratories.[79] Sipuleucel-T is the most common immunotherapy (designed to target PAP) used for CRPC. Briefly, blood is collected from the patient before treatment to separate immune cells that are next modified in the laboratory and returned into the patient. The exact mechanism of Sipuleucel-T is unknown. Some studies suggested that it may stimulate T-cell immune response against PAP.[81] Clinical trials have shown increased survival with few or no symptoms[80] and up to 4 months.[81]

1.3.9 Palliative care

When available treatments cannot stop further progression, the focus is to reduce symptoms of terminal metastatic disease in bone and spine and improve quality of life.[82] Pain due to bone metastases can be treated with bisphosphonates. Steroids, surgery and radiation can be used to deal with spinal cord compression. Urinary retention can be treated by TURP. Opioids can improve pain management. Other symptoms may include loss of weight, tiredness and lymphedema in the scrotum or penis.[83]

1.4 THE ANDROGEN RECEPTOR (AR)

1.4.1 Structure of AR

The androgen receptor (AR), also known as NR3C4 (nuclear receptor subfamily 3, group C, member 4), belongs to the super family of nuclear receptor[84] and is best known as the mediator of androgen action by its transcriptional activity at the DNA level.[85] In addition, AR has other functions via mediating androgen action, including the development and maintenance of the male sexual phenotype and, more specifically, prostate cell function.[86][87]

The AR has a molecular mass of 110 kDa and consists of 919 amino acids (AA). Its N- and C- terminal domains refer to the NH2 and COOH groups of the first and last amino acids located at the extremities of the protein, respectively. The three binding domains illustrated in Figure 9 are the N-terminal domain (NTD), DNA binding domain (DBD) and Ligand binding domain (LBD).[86][88-90]

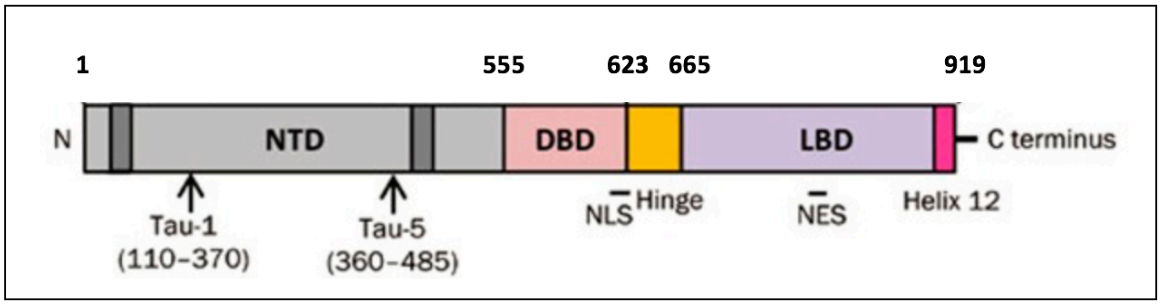

Fig. 9: Structure of AR.[86]

Adapted from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4571323/figure/fig1/

All domains are essential for AR function. The NTD (AA: 1-555) is believed to be innately active, and has the ability to independently activate transcription via androgens in mutants missing the LBD.[86][88-90] Also, the NTD contains the transcriptional activation function (AF)-1. This domain consists of two transactivation units (Tau): Tau-1(AA: 110-370) and Tau-5 (AA: 360-485).[86-90] Tau-1 and Tau-5 contain the nuclear receptor box and the ^{433}WHTLF^{437} motif, respectively, and who both mediate the direct interaction between the NTD and the LBD (N/C interaction). This N/C interaction is important for androgen-dependent gene regulation, stabilization of AR homodimers and slowing the dissociation of ligands.[86-90]

The DBD (AA: 556-623) consists mainly of two zinc fingers. One has a P-box motif which coordinates the contacts of gene-specific nucleotide within the DNA. The other zinc finger has a D-box motif, which is the binding site for DBD/DBD interaction during homodimer

formation.[86-90] The DBD allows AR to bind AR-regulated genes in DNA promoter region of specific genes and leading to activation of NTD and LBD functions and eventually activation of transcription.[86-90]

The hinge region (AA: 625-669) is located between the LBD and the DBD domains. It contains a part of the nuclear localization signal (NLS) for the translocation of AR to the nucleus. AR nuclear translocation happens through the interaction of Filamin-A (FlnA) with the hinge region, the DBD and the LBD. AR nuclear translocation is absent in FlnA negative cell lines, despite continuous androgen exposure.[86][88-90] Additional NLSs are present in NTD, DBD and LBD. The nuclear translocation of AR is regulated by interaction between the NLS present in each domain, suggesting that all domains are essential for AR function.[86][88-90]

The LBD (AA: 669-919) facilitates the binding of testosterone and DHT as AR ligands in the androgen signaling pathway. The LBD contains the transcriptional activation function (AF)-2, which interacts with the steroid receptor coactivator (SRC), the ^{23}FQNLF^{27} motif and the ^{433}WHTLF^{437}motif in the NTD.[86][88-90]

1.4.2 Mechanism of action of AR

The primary function of AR is the direct regulation of genomic transcription. Figure 10 summarizes the canonical androgen/AR signaling pathway.[87]

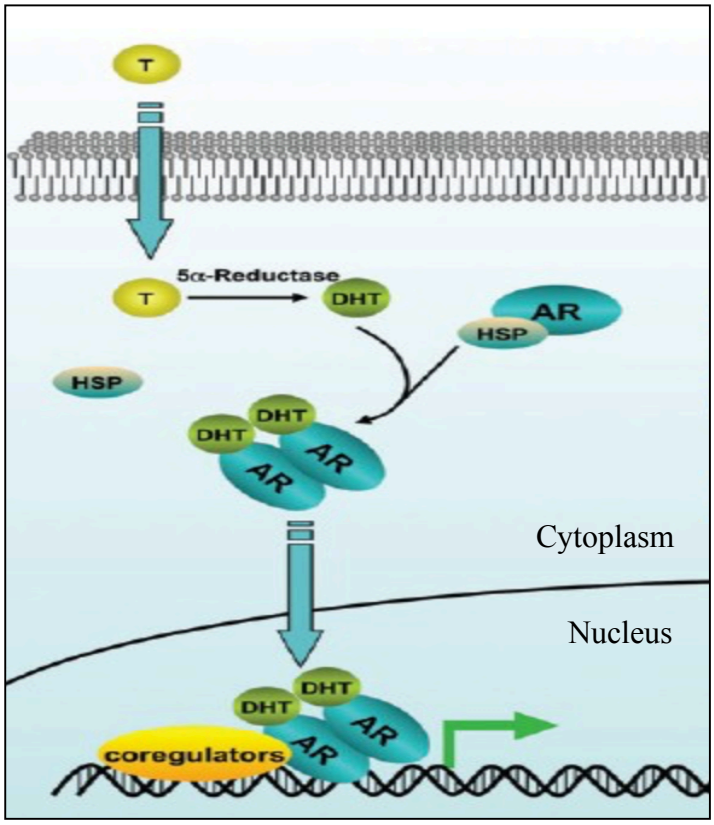

Fig. 10: The canonical androgen/AR signaling pathway.

Adapted from: http://www.carcinogenesis.com/viewimage.asp?img=JCarcinog_2011_10_1_20_83937_f2.jpg

Testosterone diffuses freely in the cytoplasm of its target cells, such as luminal secretory cells of the prostate and binds to AR which interacts with the heat shock protein (HSP)90. This causes conformational alterations leading to the dissociation of HSP90 from AR which translocates into the nucleus and dimerizes. This is followed by binding of the dimer to androgen response elements (AREs), which are specific DNA sequences located in promoter regions of genes regulated by androgens. Additional recruitment of other co-regulatory proteins facilitates the interaction of AR with the transcription machinery in the cell nucleus, causing down or up regulation of the specific transcription of genes involved in cell survival, growth and

differentiation. Transcription results in expanded synthesis of mRNA which is translated by ribosomes specifically producing corresponding proteins.[91][92]

Androgens binding to cytoplasmic AR may cause changes in cellular functions, independently from alterations of gene transcription, for example, ion transport changes.[87][92][93] Beside the canonical pathway, AR participates in cross-talks with other signaling pathways, by interacting with specific cytoplasmic signal transduction proteins regulated by factors other than androgens.[87][92][93] Signal transduction pathways that integrate AR in cytoplasm may lead to alterations in gene transcription by, for instance, phosphorylation of other transcription factors.[87][92][93]

Diverse components of signaling pathway have been associated with androgen-independent progression in PCa patients. This includes interleukin (IL)-6, growth factors, and intracellular kinase signaling. Figure 11 exemplifies signaling pathways interfering with AR in PCa.[87]

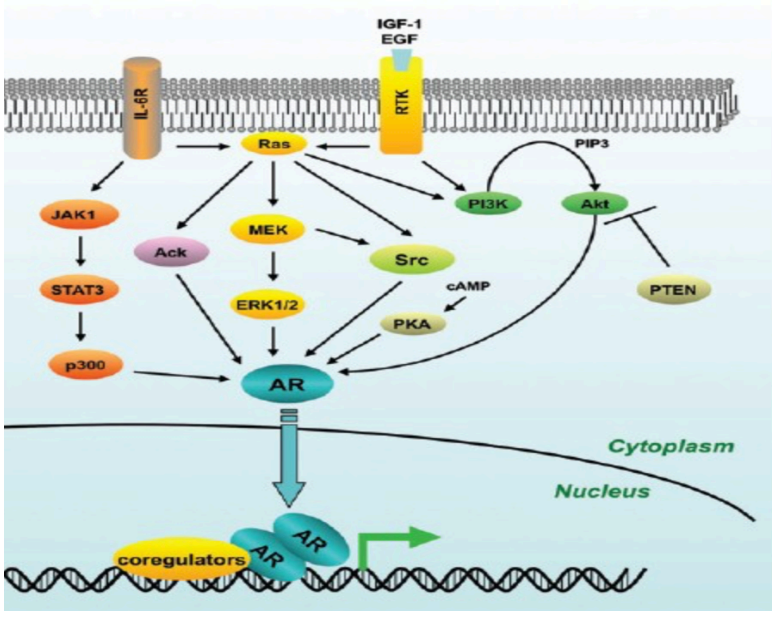

Fig.11: The AR cross-talks with other signaling pathways in PCa.[87]

Adapted from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3162670/

Studies suggests that IL-6 has a major role in maintaining the transcriptional activity of AR in CRPC.[87][94] IL-6 is a cytokine that is secreted by different cells including prostate and immune cells. Once IL-6 binds to its receptor, which consists of two subunits, the ligand binding (gp80) and the signal-transducing (gp130) subunits, the Janus kinases (JAK)-1 activation allows the activation of the signal transducer and activator of transcription (STAT)-3 by phosphorylation (p) of the tyrosine (Y)705 residue and the formation of pY705STAT3 homodimers, then translocated into the nucleus. In addition, based on cell types, the activation of mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) pathways may also occur.[94] IL-6 effects on ligand-independent AR activation and CRPC depends on crosstalks with other signaling pathways.[94][95] IL-6 treatment may inhibit AR and PSA expression in the human LNCaP (androgen-sensitive cell line derived from lymph node metastasis) model in the presence of androgen, through the prevention of p300 recruitment to the PSA promoter. [93][94] On the other hand, the prolonged IL-6 treatment of LNCaP cells contributes to activation of AR and PSA expression.[87][94] This is believed to be due to STAT3 signaling, even with the lack of androgens.[87][94][95] STAT3 directly interacts with the NTD of AR, and importantly the activation of AR transcriptional activity is dependent upon STAT3 activation (pY705) and phosphorylation of the S^{772} residue.[94][95] The IL-6 association with CRPC, allowed it to be explored as a target. [87][94][95]

Beside IL-6, epidermal growth factor (EGF) also leads to the activation of the MAPK pathway in PCa cells.[87][96] The cross-talk between the AR axis and EGF signaling was shown by EGF treatment of the DU145 cell line that does not express AR or PSA. It was found to induce an AR-mediated reporter gene transcription.[96] In addition, EGF can also induce IL-6

upregulation in prostate cancer cells.[87][96] An increased expression of EGF was reported in CRPC and metastatic patients.[96] Moreover, high levels of STAT3 lead to the formation of STAT3/AR complexes in response to EGF and IL-6. Also, STAT3 upregulates the activation of AR transcriptional activity induced by EGF, while androgens increase the levels of STAT3 in an IL-6 dependent fashion.[95]

The insulin-like growth factor (IGF)-1 signaling pathway was shown to have an importance in the modulation of AR signaling and its transcriptional activation. This includes AR phosphorylation, nuclear translocation and cofactor simulation.[97]

The intracellular MAPK pathway is activated by multiple extracellular stimuli. Potential regulators of AR activity via this pathway include the sarcoma-related kinase (Src) and the p42/44 extracellular-signal-regulated kinases (ERK).[87]

AR post-translational modifications

As a protein, AR may be post-translationnally modified in PCa cells and this may play an important role in the AR mechanism of action. Depending on the type and location of amino acid residue affected, post-translation modifications may alter AR transcriptional activity or affect the cellular localization and stability of AR.[96]

Post-translational modifications of AR occur through different processes. Importantly, AR phosphorylation, which occurs as a dynamic process regulated through different kinases,[96] acting on serine, threonine and Y residues. Several Y sites in AR were shown to be activated, including Y^{223} by the Fer TK, Y^{267} and Y^{363} by Ack, and Y^{534} by Src and Etk.[96]

Other post-translational modifications include; (i) acetylation,[97] which promotes AR-mediated transactivation, apoptosis and growth of PCA cells.[97] AR acetylation happens via

androgens and determines its recruitment into the chromatin;[97] (ii) methylation[96], which was found to be closely related to acetylation, as they both occur on the same residues at the NLS of the hinge region. Non-activated AR is found to be methylated; the demethylation of AR occurs with androgen stimulation, allowing the acetylation of AR;[96] (iii) ubiquitination,[96] which occurs on lysine residues in the LBD. The AR ubiquitination status affects its transcriptional activity, stability and localization;[96] and (iv) SUMOylation,[96] which links the small ubiquitin-like modifier (SUMO) to lysine residues in AR. This process affects the transcription of AR, DNA replication and repair.[96]

1.4.3 AR as a therapeutic target

The androgens/AR signaling pathway has become a key therapeutic target in PCa since its discovery by Huggins and Hodges in the early 1940s[64] who essentially showed that surgical castration reduced blood PAP levels and was beneficial to shrink metastases in patients with advanced disease. This discovery has led to the use of different drugs as pharmacological anti-androgens in ADT and AR inhibitors in advanced PCa.

As mentioned, AR antagonists like LHRH antagonists block androgen production through the hypothalamo-pituitary-testis axis, the aim being to decrease disease progression by targeting androgen-sensitive or responsive tumor cells.[100] Currently used anti-androgens primarily target the N-terminal LBD of AR. Such anti-androgens are still under development.[101]AR ligands can either be steroidal or nonsteroidal. Reagents that activate AR transcriptional activation are for non-PCa uses.[102]

1.4.4 Altered forms of AR

Splice variants of AR missing the C-terminal LBD of the protein, but retaining the ability

to engage transcriptional machinery and promote the regulation of known and potentially new sets of target genes have recently been identified. Not only are AR variants constitutively active, but studies showed that they predict a general resistance to anti-androgens. Figure (12) depicts the structure of splice variants in relation to the normal full-length AR.[98]

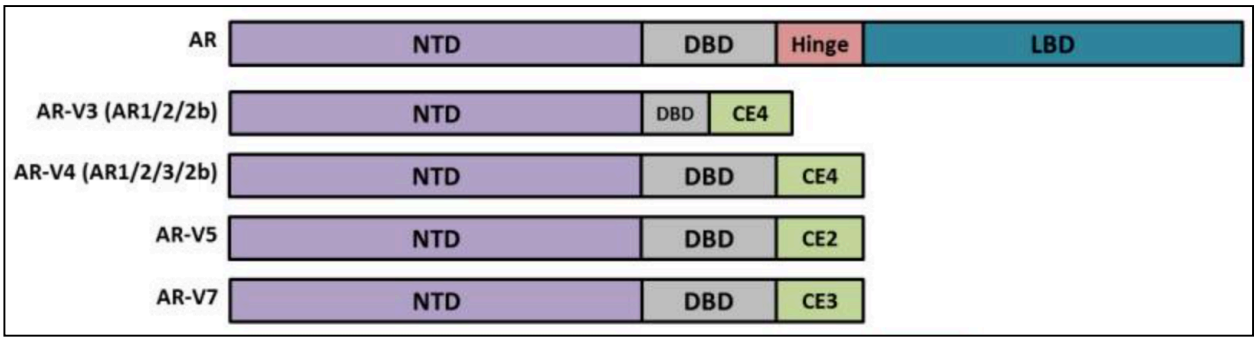

Fig.12: The structure of full length AR and common splice variants.[98]

Adapted from: http://www.mdpi.com/1422-0067/14/7/14833/html

These modified forms especially the subtype AR-V7 caught a lot of attention in the past years, due to its strong relation to progression. AR variants are more readily detected in CRPC compared to hormone-naïve cancers, and may emerge due to the selective pressure of AR targeted therapy.[103][104][105]

1.5 THE FER TYROSINE KINASE AND ITS SUBSTRATES

1.5.1 Structure of the Fer TK

The human Fer gene was discovered in 1989.[106] The Fer TK protein has a molecular weight of 94 kDa and consists of 822 amino acids.[106] Its structure contains a catalytic domain similar to other oncogenic TKs. However, as it lacks a transmembrane domain, Fer is known as a non-receptor TK.[106] The human *fer* cDNA in humans had a sequence homologous to the Fes/Fps

TKs and so it was added to the family composed only of Fes and Fps.[106][107] Fer is distinguished from other cytoplasmic members of the Src, Abl, Btk, JAK and other subfamilies by the presence of an amino-terminal Fer/CIP4 homology (FCH) domain.[108][109][110] The structure of Fer is shown in Figure 13.[112] The FCH domain is followed in the C-terminal side by a domain for oligomerization, a central Src Homology (SH)2 domain for interaction with tyrosine-phosphorylated partners, and a carboxy-terminal kinase domain responsible for its catalytic activity [111] containing an ATP binding subdomain, a NLS and an autophosphorylation site at the Y residue 714 (Y^{714}).[111][112]

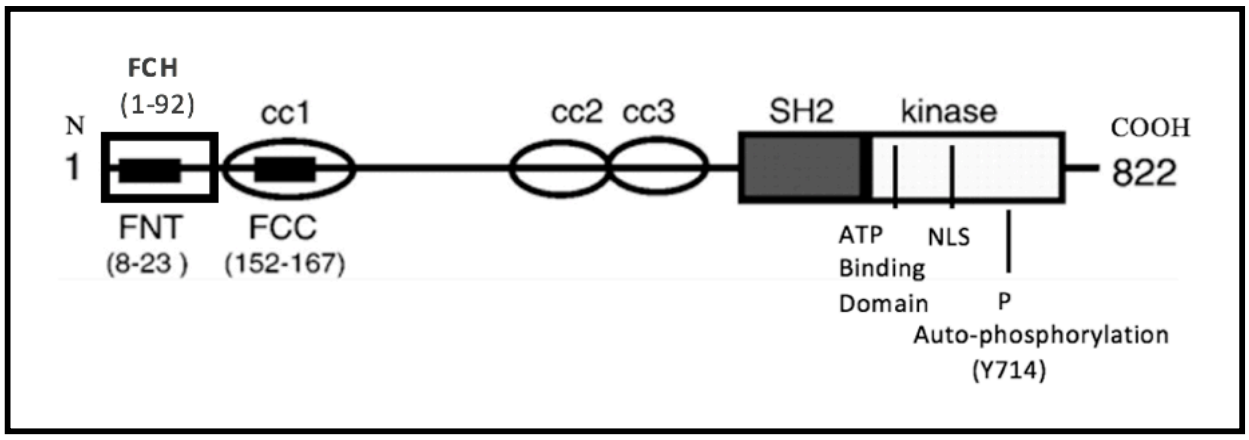

Figure 13: Structure of Fer.[112]

Adapted from; https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2175119/

1.5.2 Fer in PCa

The host lab reported that the Fer protein was non-detectable in extracts from normal human prostates, with low levels in benign extracts and elevated up to 20 fold higher in PCa tissues.[113] Fer was present in all metastatic human PCa cell lines, with increased nuclear to cytoplasmic ratio based on cell line aggressiveness.[113] Fer expression was also studied in our host lab in the

canine species as dogs spontaneously develop BPH, PIN and PCa with aging.[113] In canines, Fer expression was upregulated in dividing *vs.* resting epithelial cells of the prostate.[113] *In vivo*, the Fer protein was not detected in extracts from normal dog prostates, but high levels were detected when basal cell metaplasia was induced through castration and subsequent estrogen administration.[113] In contrast, Fer expression was not detectable when androgenic supplementation renewed the differentiated epithelium.[113]

1.5.3 Substrates and Binding Partners of Fer

The host lab has demonstrated the importance of Fer in the regulation of cell survival and growth using human PCa cell lines.[113] Accordingly, attempts were done to identify Fer interacting proteins. The search on known proteins forming complexes with Fer in fibroblasts was not successful in human PCa cell lines.[113]

Fer and IL-6

Further studies were carried out to find signaling pathways involving Fer in PCa. The literature reports on co-transfected *fer* and *stat3* cDNAs in COS cell line had showed Fer activity on STAT3.[114] Moreover, activated STAT3 and IL-6 were correlating with advanced PCa in patients.[115] The host lab showed the IL-6 induction of Fer tyrosine phosphorylation/activation in cell lines, through the IL-6R β-subunit, gp130, forming complexes with Fer.[110][116] In addition, IL-6 regulates the Fer intracellular distribution from the cytoplasm to the nucleus of PCa cell lines.[110][116]

Fer and STAT3

Within this mechanism, Fer was shown to phosphorylate/activate STAT3 at Y^{705}, form complexes via its SH2 domain with activated pSTAT3, translocate with pSTAT3 into the

nucleus and control transcription of genes involved in growth mediated by IL-6.[110] Interestingly, even under IL-6 stimulation, STAT3 was not phosphorylated/activated and did not translocate to the nucleus when Fer was knocked down with siRNA.[110] Additional studies (unpublished) suggest that Fer acts in concert with JAK1 and JAK2 and may hold a critical decision of activating STAT3 in AR negative (PC3) tumor cells.

Fer and AR

An earlier study reported that exposure of AR positive LNCaP cells to IL-6 allows the AR transcriptional activation of the PSA gene and growth in a process requiring pSTAT3 activation.[117] Since Fer activates STAT3 under IL-6 in PC3 cells, the host lab investigated and confirmed that Fer is also in cause in LNCaP and that under IL-6, Fer phosphorylates/activates AR at Y^{223}, forms additional complexes (via its SH2 domain) with AR, migrates to the nucleus and controls transcription of genes like PSA and growth.[116] This suggests that in PCa, Fer is able to work in both AR positive and AR negative cells, activating key transcription factors (TFs) linked to PCa progression, as substrates and binding partners in the absence of androgens.[116] This mechanism may lead to tumor cell adaptation (AR positive) during ADT and selection (AR negative) in CRPC and metastatic progression of the disease.

1.6 IMMUNOHISTOCHEMISTRY (IHC)

IHC is a technique allowing the detection of proteins or antigens using specific antibodies in cells within tissue sections and in vitro.[118] It has multiple applications in the medical field, including in cancer research discovery of new biomarkers, assessing the presence of a new protein and its levels with specific Abs. [118]

Advantages of IHC methods: [118]

- Fast and readily available. at relatively affordable costs;
- Usable on fixed tissues that represent no risk for human health and shows good preservation of cell morphology.

Disadvantages include the difficulty in optimization and reliable quantification of a protein, its success is controlled by the quality of tissues and specificity of antibody, and the scoring together with the interpretation of results need well trained and dedicated personnel.[118]

2. Hypothesis and Objectives

Hypothesis:

Based on literature data on AR expression and its variants in PCa tissues and cell lines, together with the host lab findings on IL-6 mediated Fer activation in AR negative and positive PCa cell lines, and leading to the Y-phosphorylation of key TFs (STAT3 and AR) playing determinant roles in PCa progression, it is proposed that: Cellular heterogeneity of prostate tumors and metastases regarding AR expression and activation by Y223 phosphorylation, is closely linked to progression, and particularly when tumor cells evolve under ADT and CRPC and reach the metastatic stage of the disease. Moreover, prostate tumor cell heterogeneity is likely to reflect prostate epithelial cell subtypes present in normal and hyperplastic glands. Accordingly, the full length AR and its several variants would be differentially expressed in the nucleus of prostate cells and in tumor cells as a function of PCa progression. Moreover, since Y223 is located in the N-terminal portion of the AR amino acid sequence, it is likely that levels of activated AR would be observed in all AR protein forms and increase with stages of disease. Accordingly, activated AR may be a potentially new biomarker of prognostic value.

Objectives:

- To assess and quantify AR expression in cells of the normal prostate and in prostate tumor tissues from different cohorts of patients, using antibodies recognizing epitopes in the C- and N- Terminal ends of the protein to detect the full length AR vs. all forms including variants, respectively;
- To verify if AR levels in tumor cells correlate with patient outcome;
- To assess and quantify the expression of pY223AR in the normal prostate and PCa tissues of the same cohorts of patients;

- To investigate the correlation between pY223AR expression and PCa patients' outcome and severity of disease.

Chapter #2

"Cell by Cell Quantification of Nuclear Androgen Receptor Reveals Cellular Heterogeneity of Prostate Tumors and Differential Expression with Progression"

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Abstract

Introduction and Objectives: Prostate cancer (PCa) is a leading cause of cancer deaths in North America. There is no cure for advanced disease; patients failing surgery or radiations invariably fail androgen-deprivation therapy (ADT), become castrate-resistant (CRPC) and progress to the metastatic stage. Studies point to anomalies in the androgen receptor (AR) protein in advanced PCa, with expression of full length and shorter variants. We aimed to quantify AR by its N- vs. C-terminal ends from early to end-stage of disease.

Methods: Tissue sections from a series of 479 cases were used to cover a wide range of conditions, with prostates from healthy young males, benign prostatic hyperplasia (BPH), and primary tumors from radical prostatectomies after neo-adjuvant hormone therapy and hormone-naive, and from advanced patients on ADT. Pairs of primary tumors and lymph nodes, along with unmatched bone metastases and seminal vesicles were included. Immunohistochemistry was performed using AR C- and N- terminal antibodies (Abs). Cells were counted and staining intensity (0-3+) determined to express scoring data in percentages (%) and H scores. Statistical analysis included ANOVA and survival analyses.

Results: C- and N- terminal AR Abs revealed a primarily nuclear localization. Cell by cell quantification showed that almost all luminal cells (99%) were positive in both normal and BPH cases, with the majority (87-91%) displaying a 1+ intensity. In the basal layer, the number of AR negative cells (0 intensity) varied between 49 to 58%, implying that half were AR positive. AR H scores differ between luminal and basal cells ($p<0.01$). The two AR Abs revealed that in cancer, negative tumor cells constituted 4-5% of total cells in early stages and increased to reach up to 14-16% in ADT/CRPC cases. In stained cells of primary tumors, the nuclear AR intensity remained highest at +1 using C-terminal Abs, whereas with N-terminal Abs the intensity shifted

from low (1+) in Gleason score (GS) 6 and 7 cases to high (+3) and intermediate (+2) from GS 8 and above. In both instances, AR intensity and H scores increased with GS ($p<0.01$) but did not predict biochemical recurrence (BCR). Of interest, AR detection via N- but not C- terminal Abs yielded highest intensity and H scores and distinguished ADT/CRPC patients and metastases from lower grade cancers ($p<0.01$). AR was equally detected with the two Abs in prostatic fibroblasts, expressed in normal, hyperplastic and cancerous prostates up to GS7 and, as expected, disappearing at GS8 and higher and more advanced stages; this AR loss had predictive value for BCR. Inflammatory cells surrounding tumors did not express AR. However, the extent of inflammation correlated with patient outcome.

Conclusion: Altogether, these findings point to PCa progression being associated with a higher number of AR negative cells in tumors and metastases. This occurs concomitantly with changes in AR proteins expressed, and present at particularly high nuclear levels when detected by their N-terminal ends in ADT cases and metastases and therefore supporting expression of variants in advanced PCa. AR HR scores in tumors did not predict BCR whereas AR loss in fibroblasts near tumor foci and the extent inflammation predicted patient outcome.

Introduction

Prostate cancer (PCa) figures among leading causes of cancer deaths in industrialized countries. To date, no curative therapy exists for patients experiencing biochemical recurrence (BCR) after radical prostatectomy (RP) or radiation therapy (Rx). At this point, androgen-deprivation therapy (ADT) is the gold standard treatment to control the disease but ADT invariably fails and is followed by castration resistance (CRPC) and further progression to the metastatic and lethal stage of disease.[1-4]

The androgen/androgen receptor (AR) signaling axis remains a major target in the PCa field, despite ADT failure and beyond CRPC.[5-10] Tumor progression does occur despite depletion of circulating androgens but local androgen synthesis, high levels of activated AR and expression of shorter forms of AR or variants lacking the C terminal end of full length AR may concur to misregulation of AR-driven transcriptional activation of genes [10] and resistance to treatments.[5][7]

Currently, molecular signatures have gained great attention including in PCa. While the upregulation of AR transcripts is being reported in most studies, results on the AR protein in prostate tissues and in some instances, metastases, have led to inconclusive and conflicting findings. For instance, the AR expression during the transition from androgen-dependent or response to CRPC is unclear. While some reports showed a correlation with disease progression and poor outcome [9][11-13], others have either showed no prognostic significance of AR[14] or an inverse correlation with Gleason Score (GS).[15] Most reports showed that AR is exclusively nuclear in prostate cells, benign and malignant with negligible cytoplasmic staining.[11][14-17] Cytoplasmic AR received greater attention in recent years. Variable cytoplasmic staining was

found in prostatic intraepithelial neoplasia (PIN) [14] and a higher cytoplasmic to nuclear AR ratio in PCa was shown to correlate with androgen-independent disease.[18]

Another puzzling issue at present is the impact of AR negative cells in this disease. A few reports have attempted to evaluate the percentage of AR negative tumor cells with respect to different stages of disease progression. Studies have either relied on semi-quantitative analysis (i.e. above or below 50%) or have focused on a single disease stage (localized or advanced only). [9][14-16][19-20] Combining the results reveals a variety of conflicting conclusions. Certain authors reported that the percentage of AR negative tumor cells increases with dedifferentiation [15] and causes a 2.5 times greater risk of death.[20] Others showed either no correlation between the percentage of AR negative cells and GS [9][18] or even the opposite, i.e. a decrease of AR negative tumor cells with disease progression.[14][16]

Along this line, attempts to address AR expression in the normal prostate in terms of the percentage or number of AR positive and negative epithelial cell subtypes also led to controversies. For instance, AR staining in the normal epithelium has been noted negligible for cells of the basal layer but substantial in the luminal glandular cells,[9] whereas substantial nuclear basal staining was also reported in normal and hyperplastic cases.[16] Among reasons to explain such discrepancies, it is worth emphasizing cohort or sample size, variations in types of tissues analyzed, qualitative or semi-quantitative assessments, epitopes recognized by AR antibodies (Abs), etc. Nevertheless, the question of AR expression in the prostate and primary prostate tumors and metastases remains of great interest in view of AR variants and AR negative cells that may be selected during progression whereas AR positive cells adapt and use non androgenic factors to grow and invade.

The aim of the present investigation was to perform a cell by cell quantitative analysis of prostate tissues and metastases to determine the number of cells expressing or not the AR and in which compartment and whether the recognition of AR by a C-terminal epitope present in the full length protein vs. a N-terminal epitope present in all known forms, including variants, will matter during progression. We report for the very first time a comprehensive analysis of AR which shows that PCa progression is associated with a higher number of AR negative tumor cells intermingled within a majority of AR-expressing tumor and metastatic cells, and observed concomitantly with changes in nuclear AR expression detected at particularly high levels by the N-terminal AR epitope and thereby supporting accrued expression of variants. Our study also confirms AR data in prostatic fibroblasts along with no expression in inflammatory cells nearby tumor foci.

Material and Methods

Clinical cohort

Study protocols (BMD-10-1160) were approved by the Ethics Board of the MUHC-RI. The study included 479 cases, consisting of radical prostatectomy (RP) cases, hormone-naïve (n=326) and neo-adjuvant hormone therapy (NHT) for 3 months prior to RP (n=25), cases of transurethral resection of the prostate (TURP) for symptomatic BPH with urinary obstruction (n=5) and PCa under palliative ADT (n=29), tumor extension in seminal vesicles (n=19), metastases to lymph nodes (LN) (n=14) and bone (n=25) and representative cases of normal prostates retrieved at autopsy from men aged 45 or less (n=40), whose death was unrelated to a prostate disease. Clinical outcome data were available for the RP cohort. Staging was performed in accordance with the 7th edition of the American Joint Committee on Cancer (2010).[21] BCR was defined by two successive rising blood PSA at 2 ng/mL or more over post-treatment nadir level (cf. Phoenix definition).[22]

Tissues and immunohistochemistry

Formaldehyde-fixed paraffin-embedded (FFPE) blocks were retrieved from the Pathology departments at the MUHC (FB) and Sacré-Coeur Hospital (LRB) (bank of normal prostates). Representative prostate tissues from RP cases, extension to seminal vesicles, metastases to bones and LNs, the latter being paired with the primary tumors, had be assembled in tissue microarrays (TMs), consisting of 1mm cores, 3 tumor cores per case and for the RP cohort, benign cores punched from FFPE blocks with no cancer.

IHC was performed with AR Abs recognizing epitopes located in the C-terminal vs. N-terminal ends of the AR protein sequence. The C-terminal consisted in rabbit polyclonal IgGs

raised against a short synthetic peptide mapping within the last 50 amino acids (C-terminus) of the human AR full-length AR protein (Santa Cruz Biotechnology; AR(C-19), sc-815, at 250 μg/ml). The N-terminal Ab was a mouse monoclonal directed against an epitope located in the N-terminal end (amino acid 299-315), present in all known forms of AR, including variants (NeoMarkers, Thermo Fisher Scientific, Inc., Fremont, California; clone 441, at 200 μg/ml).

Sections (4μm thick) were deparaffinized in xylene, and rehydrated using an ethanol gradient followed by rinsing with distilled H20. Slides were incubated in 0.2 M citrate buffer (pH 6.0) for 10 min at 95°C for antigen retrieval, blocked with 0.3% hydrogen peroxide (Sigma Aldrich, Germany) for 20 min, followed by washes with PBS. Slides were then immersed for 30 min in protein blocking reagent (Chemicon Millipore) and incubated overnight at 4°C with AR Abs, at concentrations of 1/250 for both C- and N-terminal, unless otherwise mentioned. Following washes in BPS, sections were incubated with biotinylated secondary Abs, and the streptavidin and horseradish peroxidase (HRP) enhancer (Chemicon International, Millipore) for 60 min. AR reactivity was revealed following the addition of the DAB substrate (kit from Nichirei Biosciences, Tsukiji, Chuo-ku, Tokyo.) Sections were subsequently counterstained with Harris Hematoxylin.

Quantitative scoring of AR in prostate cells

All stained sections were scanned using an Aperio image scanner system. Quantification of the cell reactivity in tissues and the counting of cells was assessed at 40x magnification, both by eyes and through the Imagescope software. Staining was scored by two independent individuals (T.A/T.B). The quality of staining and scoring was reviewed and confirmed by a PCa-trained research associate (E.S), who randomly scored 25% of stained tissues, also reviewed by a genito-

urinary pathologist (FB). The scoring was done blinded to patient data. Cytoplasmic and nuclear staining was assessed in the normal and benign epithelium, luminal and basal layers, and malignant cells in tumor foci along with stromal fibroblasts, endothelial cells, and inflammatory cells. A minimum of 500 cells were counted in each section, including negative (-) benign and tumor cells. Positive cells were analyzed according to the staining intensity on a scale of 1-3+ (1=weak, 2=moderate, 3=strong) to calculate H scores = Σ (Pi(i)) x100, where i is the staining intensity, and Pi the percentage of cells and each intensity yielding values between 0-300.[23] Exemple: 5% AR(0) + 15% AR (1+) + 60% AR 2(+) + 20% AR (3+)= 195.

Statistical analyses

AR H Scores were analyzed in normal, benign and tumor cells among disease stages (i.e. from localized to advanced) and in metastases. Kruskal-Wallis ANOVA plus comparison of mean ranks was used to analyze H Scores and % of cells with negative (intensity of 0) staining and one-way ANOVA plus Bonferroni post hoc test to analyze weak (1+), moderate (2+) and strong (3+) nuclear staining. Statistical analyses (Kaplan Meier, Cox univariate and multivariate, and ROC curves) were performed to determine the clinical significance of the expression of AR proteins with progression and outcome using cases of the RP cohort. Differences were considered significant at a $p<0.01$

Results

IHC optimization

Protein expression relying on IHC staining of cells in tissues often leads to conflicting data, partly explained by differences in methodology and reagent specificity. The optimization of experimental conditions was thus carried out on consecutive sections of PCa tissues from three cases. Figure 1 illustrates typical titration data of stained tumor cells from an advanced case with each Ab (Fig 1A: C-terminal; Fig.1B: N-terminal) in the cytoplasm vs. the nucleus. As expected, increased staining was observed with increasing concentrations of Abs until reaching a plateau at 1/250 for both Abs. Similar results were obtained for PCa tissue samples of the three cases. At this optimal dilution and no or minimal background staining in tissues and at higher concentrations, 10-12% of tumor cells intermingled among AR positive cells, remained AR negative (Fig. 1). The C- and N-terminal Abs stained preferentially the nucleus of 88-90% of tumor cells, with high intensity (3+) in 60-73% of cells, moderate (2+) in 9-18% and low (1+) in 8-10% of them. Cytoplasmic staining was low or weak (1+) in 10-20% of tumor cells, when revealed with the N-epitope Ab, whereas the proportion of cells displaying cytoplasmic AR was in the order of 40-45% with the C-terminal Ab, with intensities varying from 1+ to 2+ and 3+. Overall the vast majority of tumor cells (90%) had high levels of nuclear AR while 55-80% of them had low cytoplasmic AR. The ability to score varying AR intensities in the two compartments of tumor cells with the two AR Abs provided a window for subsequent analysis.

The specificity of two AR Abs was also ascertained by Western blotting after SDS-PAGE, using the recombinant human AR protein (110 kDa) as well as extracts from representative cell lines, including 22RV1 cells expressing AR full length at 110 kDa and several variants in the 65-75 kDa range (data not shown).

AR expression in normal and benign prostate tissues

The quantification of epithelial cells expressing the C-terminal epitope -full length AR vs. N-terminal epitope -all forms of AR was achieved using normal prostates of men of less than 45 years old and benign prostate BPH -TURP cases. Essentially, AR was primarily nuclear (Fig. 2). Results in Table 1 show that 99 to 99.4% of luminal cells were stained with the two AR Abs, in both normal and BPH cases, with the majority (87-91%) displaying a 1+ intensity. Moderate 2+ staining was seen in less than 10% of luminal cells, irrespectively of Abs used.

Cells in the basal layer yielded similar AR results in normal and BPH cases with the two Abs. As depicted in Table 1, the number of AR negative cells (0 intensity) varied between 49 to 58%, implying that 42 to 52% were AR positive. Their staining intensity was low (1+) in 30% to 51% of cells. Accordingly, AR H scores were different ($p<0.01$) between luminal and basal cells, being 114 vs 42 for the C-epitope ($p<0.01$) and 116 vs 52 using N-epitope ($p<0.01$).

The AR expression in benign areas of prostate tissues obtained from PCa patients (RP cases) was investigated in TMA cores, and quantified according to the proximity to cancer cells, close within the same core (≤ 1mm) and far (> 1mm) in another core. Again, AR was mainly nuclear (Fig 2A: C-terminal; Fig. 2B: N-terminal). The number of cells expressing AR remained in the same order of magnitude, 99-99.3% positive luminal and 47-57% for basal cells (Table 1). The main observation was the change in the distribution of cells expressing AR over the 1+ to 3+ intensity range, when comparing benign areas close to tumor foci vs far, in another block of the same case and glandular cells in "true" BPH cases. For instance, AR intensity was at 2+ in the benign area close to tumor foci and 1+ when far from cancer. Luminal H scores showed an increasing trend ($p<0.01$) as glands get close to cancer. For instance, the AR Abs for the C- and N- epitopes yielded H scores of 175 and 194, respectively, when close to tumors compared to

153 and 154 ($p<0.01$), respectively, when far from cancer. H scores for the C- and N- Abs were also lower, 107 and 113 ($p<0.01$), respectively, in luminal cells of BPH cases. Taken together these findings on similar H scores for the two AR Abs support that normal and benign (BPH) cells of the prostatic epithelium express the full length AR. Over 99% of secretory or luminal cells are AR positive and 50% of basal cells, the other half being AR negative.

AR expression in tumor cells of localized and advanced PCa

The counting of cells expressing AR using C- and N- terminal Abs in the cytoplasm and nuclei at intensities from 0 to 3+ provided data on the percentage of AR negative cells at various stages of disease. Figure 3 shows the results on unstained tumor cells with the two Abs. AR negative luminal cells of normal and BPH prostates and benign cells far from cancer and close are at less than 1% of all glandular cells, with the exception of the C-terminal Abs at 2% for benign cells close to cancer. In tumor foci, AR negative cells increased with GS in a similar manner with both Abs, up to 10% for the C-epitope and up to 8% for the N-epitope. The average number of AR negative tumor cells increased significantly ($p<0.01$) with progression, being at highest levels - 14% (N-terminal) and 16% (C-terminal) of all cancer cells, in primary tumors of CRPC patients with advanced disease and under palliative ADT (Fig 3A: C-terminal; Fig.3B: N-terminal).

The results on AR expression in tumor cells of the different categories of cases analyzed are summarized in Figure 4, which illustrates their distribution based of staining intensities. Panel A shows the tumor cell staining in the cytoplasm (left panel) and nucleus (middle panel) using the C-terminal Ab. The cytoplasmic staining remained at 20% and unchanged with progression (left panel), whereas nuclear AR behave differently (middle panel). Basically, the number of tumor cells expressing nuclear AR at 1+ intensity was relatively constant, in the order

of 40-50% with increasing GS, similarly to benign cells far and close to tumor foci and lower than the 80% of luminal cells at 1+ intensity in normal and BPH prostates (Table1). A trend towards a decrease in the number of tumor cells expressing AR at 1+ in hormone-treated cases, NHT and ADT, was observed. Accordingly, tumor cells with moderate levels (2+) of AR was 30 % in benign luminal cells and tumor cells of GS6 and GS7 and 40% in higher GS and hormone treated NHT and ADT cases. These variations also translated in more tumor cells expressing high levels of nuclear AR (3+) from 15-20% in to 25-30%. When reported as AR H scores, there was an increase with Gleason score of primary tumors ($p<0.01$), being most elevated in ADT/CRPC patients (Table 1).

Similarly, the AR detected with the N-terminal Ab was primarily nuclear and varied with progression (Fig. 4B), whereas cytoplasmic staining remained low and constant with progression (not shown). Basically, the nuclear intensity and H scores increased with GS ($p<0.01$), and were most elevated in ADT/CRPC patients. The most striking observation was the intensity shift from low AR levels in the nucleus (1+) to elevated (+3) and moderate (+2) levels of AR proteins recognized through the N-epitope and in more tumor cells. This was observed in half (50%) of tumor cells in patients who received NHT prior to RP and 70% in CRPC cases on palliative ADT compared to 20%- 25% for tumor cells in these two categories with the C-epitope -full length AR ($p<0.01$), implying changes in nuclear AR proteins expressed in primary tumors, according to the hormonal status -NHT prior to RP compared to hormone-naive, and in resected tissues of CRPC cases on palliative ADT. However, when clinical relevance was assessed using the updated clinical database of hormone-naive RP cases, Kaplan Meier plots (Figs 5 A and B) revealed no predictive value for BCR of nuclear AR H scores assessed with the two AR Abs for the C- vs. N-epitopes.

AR expression in metastases

Given the above observations in NHT and CRPC/ADT cases, AR expression was interrogated in primary tumors and matched LN metastases, in tumor cells extending to seminal vesicles and bone metastases. Similar to the other IHC reactions, a subset of tumor cells dispersed among AR positive cells were not stained with the two AR Abs. They represented 8 % of total cells in both primary tumors and LN metastases, 7-8% for tumor cells in seminal vesicles and 6-8% in bone metastases. In positive cells, AR was primarily nuclear. Its distribution, according to staining intensities in the nucleus of tumor/metastatic cells is shown is Figure 6. The AR Ab staining the full length protein (C-epitope) showed more cancer cells at low intensity with 1+ > 2+ > 3+ (Fig. 6A). The opposite situation was observed for the Ab detecting all forms of AR (N-epitope), i.e. more cells expressed elevated nuclear levels, with 3+ > 2+ > 1+ (Fig 6B). The difference was statistically significant ($p<0.01$) and observed for primary tumors and matched LN metastases, tumor extension to seminal vesicles and bone metastases. Primary tumors and their LN metastases behaved similarly. Collectively, these findings speak for an enhanced expression of all forms of AR proteins vs. full length AR observed in the cell nucleus of primary tumors and their corresponding LNs collected at time of RP and seminal vesicles showing signs of invasion as well as in bone metastases generally present in advanced disease.

AR expression in cells found in the stroma

AR expression in cells of the stroma, fibroblasts, inflammatory and endothelial, was quantified in parallel to epithelial and cancer cells. Results in fibroblasts are shown in Figure 7 (left panel) for AR detected through the C-epitope. The number of fibroblasts not expressing AR was similar in normal and BPH prostates (49%), and the remaining AR expressing fibroblasts

displayed varying staining intensities, 24% at 1+, 18% at 2+ and less than 9% at 3+. The AR expression in fibroblasts of prostates from patients with PCa was different, with the number of stained fibroblasts and their staining intensity decreasing from far to close to tumor foci according to GS6 and GS 7, until complete disappearance at GS 4+4 and above. The loss of fibroblasts was predictive of BCR (Fig. 7, right panel). The two Abs yielded similar results. No staining of fibroblasts was observed with both AR Abs in primary tumors of the matched LN series.

Inflammation was studied in AR-stained sections of the RP cohort. Inflammatory cells were negative with both AR Abs (Fig. 8, left panel). Nonetheless, inflammation was categorized as mild, moderate and high grades, according to the literature,[24] based on the number of infiltrating lymphocytes in close proximity to tumor foci (Fig. 8). Only 47 cases of the 326 cases with localized PCa showed some inflammation in the TMA cancer cores: 15 were mild (inflammatory cells < 100), 22 moderate (inflammatory cells = 100-500) and 10 high (inflammatory cells > 500). Kaplan-Meier survival analysis revealed that in this cohort, inflammation adjacent to cancer was predictive of BCR (Fig. 8, right panel).

Endothelial cells in blood vessels were occasionally stained but the vast majority, 95% were AR negative. No difference was observed between disease stages and progression. Also, no significant correlation with BCR - patient outcome, was observed.

Discussion

The primary goal of the investigation on AR through two Abs raised against peptides located in the C- and N- terminal ends of the protein is to verify if diverse forms of AR could be distinguished in prostate tumor cells with progression. The strategy used of cell by cell quantification on a wide variety of tissues reflecting distinct clinical stages of the disease has revealed the status of such key regulatory proteins, acting as transcription factors in the nucleus of tumor and metastatic cells evolving in different contexts. New information was obtained on AR expression in diverse cells present in the human prostate from normal to hyperplastic and malignant states. The intention was not to develop a method for direct translation in the clinical - pathological setting but to better understand the prostate physiopathology and how tumor cells may evolve in different contexts.

A first point to emphasize is the importance of titration of Abs on consecutive tissue sections to ensure minimal background and optimal staining of cells expressing AR. This optimization on TURP primary tumors from advanced CRPC patients on palliative ADT and a RP localized PCa case of GS7, confirmed the nuclear AR localization in epithelial, fibroblastic and endothelial cells, and a nuclear predominance in tumor cells. Although cytoplasmic staining was observed, and more so with the C-terminal Ab, no consistent changes were observed while increased nuclear intensity was statistically significant with progression, rather than a cytoplasmic escalation as shown in a recent study.[25]

Among interesting features was the AR status in non-malignant epithelial cells. Almost all luminal cells (> 99%) of the prostate express AR, from young adults to BPH cases and PCa patients. The novelty resides in changing number of cells within this luminal population displaying higher AR levels in cancer patients in comparison to a low intensity found in 90% of

luminal cells in normal and BPH prostates. This process was accentuated in luminal cells when glands were closer to tumor foci compared to far from tumors. These findings confirm the fully differentiated state of luminal cells expressing AR, which in the epithelium is required for transcriptional activity of genes triggered by androgens such as kallikrein3 (PSA).[26] Androgens and AR are also critical prerequisites for luminal cells survival that otherwise rapidly die upon castration, surgical or pharmacological like ADT.[26]

The cell by cell quantification approach we used also clearly revealed that 42-52% of basal cells in the prostate of young adults and patients with BPH or PCa express low levels of AR. Moreover, this basal subpopulation of the prostate was altered when cancer had developed, with a redistribution in categories expressing moderate and high AR levels. This was again accentuated in basal cells of glands close to tumor foci. Thus AR positive basal and luminal cells are similarly influenced in cancer as opposed to BPH. Therefore "normal-looking" luminal and basal cells expressing AR in the prostate of cancer patients may have undergone molecular changes associated with transformation but not yet phenotypically detectable by light microscopy. As results are similar with both Abs, we can propose that full length protein is mainly the one involved. Hence AR negative basal cells, which represent around 60% of cells of the basal layer in normal and BPH prostate remained in the same proportion in cancer, far or close to tumor foci. Therefore, cells of the basal layer exist as two subpopulations relative to AR expression. The AR negative ones likely comprise a low number of "true" stem cells (still difficult to identify) [26], a few neuroendocrine cells, as we and other pioneers have identified through markers like chromogranin A, VEGF-A, serotonin,[27] and quiescent basal/reserve cells believed to regenerate the epithelium whenever needed.[27] A step towards this function may be to express AR but remain non differentiated in the basal layer. Indeed, we demonstrated that

around 40% of basal cells are AR positive. This commitment may be necessary for the rapid androgenic response leading to the renewal of the secretory epithelium after castration. [11][26][28] This cycle may be transposed to AR positive cancer cells, which predominate at all times in tumors and metastases. As elegantly demonstrated by intermittent ADT, most differentiated luminal-like AR positive cells would die under ADT, leaving behind the equivalent of AR-positive transformed basal cells that can regenerate the differentiated population at each ADT cycle, until they adapt and use other means to survive, grow and differentiate, as monitored by PSA. Cell response to non-androgenic growth promoting factors and expression of constitutively active AR variants may be the cause.

Our findings on AR quantification in cancer cells of primary tumors and metastases would support this concept and deserve consideration. The bulk of cancer predominantly consists of AR positive cells. Our approach revealed relatively similar behavior for the two AR Abs in the hormone-naive RP cohort, where the majority of cells (around 50%) express AR at low levels, with 22-30% displayed moderate 2+ and 10-20% elevated 3+ levels. The resulting H Scores did not predict BCR using the database of the RP cohort, although the cohort characteristics regarding outcome has met expectations in an earlier study,[28] correlating with PSA at diagnosis, prostate GS and TNM but with not age and confirming the clinical significance of other biomarkers.[28]

It is conceivable that quantification of small variants would show significance, as reported, AR-V7 is associated with unfavorable BCR-free survival if expressed in primary tumors of a high-risk patient cohort with lymph node metastases at time of surgery.[29] The availability of specific Abs that recognize the diverse known AR variants will eventually allow studies to establish their clinical significance.

Of particular interest was the differential AR expression patterns detected by the two Abs in hormonally-treated (ADT) NHT prior to RP and advanced CRPC patients. The AR detected via the N- epitope of the protein showed that the majority of cells (up to 60-70%) expressed elevated AR levels in their nucleus, while 25-30% of cells express the AR splice seen by the C-epitope Ab. Similar results were observed in primary tumors that had paired LN metastases, tumor cells invading seminal vesicles, and bone metastases. This suggests that not only full length AR is detected in these cancer cells but also smaller forms not recognized through their C-epitope. These findings support the expression of constitutively activated small AR variants along with full length AR.[30-32] The patient's response to abiraterone and enzalutamide in CRPC patients would imply the functional full length AR, that may also form dimers with variants.[30][33-34] Adaptation may promote progression through crosstalks between signaling pathways and the AR axis [35-36] and require post-translational modifications of AR, including tyrosine phosphorylation, as we reported in cell lines.[27] From our data, it appears that conditions favoring expression of diverse AR proteins detected through their N-terminal epitope imply an aggressive disease at time of RP, as revealed by paired primary tumors and LNs metastasis, invasion of seminal vesicles and hormonal therapy -NHT, prior to RP. These two conditions are also met in advanced CPRC and likely in cases with clinically proven metastases requiring surgical resection. AR increased expression was observed in advanced disease and high H score value using N- terminal Ab in bone metastases. This suggests overexpression of additional forms of AR vs full length in tumor cells surviving and growing under no or low androgenic stimulation, as reported for slice variants.[37-39]

PCa progression also imply the development of androgen-independent state as evidenced by increasing number of AR negative cancer cells. We confirmed their presence, by titration

experiments performed with the two Abs and showed a similar plateau of unstained cells, despite increasing Abs concentrations. However, AR negative tumor cells represent a small percentage of all cancer cells present in primary tumors and metastases. In low GS6 localized cancers, they constitute around 4% of all cells. This percentage increased to 6 and up to 10% with increasing GS in primary tumors and in metastases and reached 14-16% (average; and seen with the two AR Abs) in advanced CRPC/ADT patients. These changes were significant. Their identity (stem, neuroendocrine) remains to be established. The AR negative cells are not affected by therapies targeting the canonical androgen/AR pathway and may use diverse factors to grow, and emerge with progression. The fact that they remain as minority of cells dispersed among AR-positive tumor cells may not matter if they secrete powerful peptides, like interleukins, neurosecretory products, like gastrin-releasing peptide and VEGF-A, acting in paracrine and endocrine fashion on nearby or distant AR positive tumor cells.

Our findings on AR increased expression or overexpression in tumor cells of advanced disease cases, growing in an environment devoid of circulating androgens or else surviving and growing under low androgenic stimulation speak for constitutive activation of splice AR variants. In addition, bone metastases had a high H score value at 222 using N- terminal Ab, suggesting an additional role in metastatic disease which has also been previously reported.[37-39] As we showed, quantifying AR using C-terminal Abs counting only long form of AR reveals the absence of the shift to high intensity in contrast to the N-terminal Abs. AR expression remains low intensity throughout different disease stages. The presence of the shift with N-terminal is believed to be due to the high expression of AR splice variants with disease progression which are missing the C-terminal. Notably, some splice variants of AR were shown to be more potent in their capacity to transactivate genes such as PSA [40] and also distinct gene profiles.[41]

What is interesting is that the % of AR negative tumor cells increases from 0.3% to 14% and 15% using N- and C- terminal over the whole spectrum of disease progression ($p<0.005$). Our observations support that two concomitant changes take place under ADT, an increasingly higher number of AR negative tumor cells while having the greatest proportion of tumor cells strongly expressing AR. These findings support the notion of AR positive tumor cells adapting, while AR negative tumor cells are being selected, under the influence of non-androgenic growth-promoting factors. Based on these observations, future therapies should aim to prevent the adaptation and selection of these cells.

This comprehensive study on AR expression in prostate cells allowed us to confirm the reported reduction of AR expression in prostatic fibroblasts [(42)] from normal to benign to PCa with no detection in GS≥8. This interesting finding is suggestive of a loss of function as normally, androgens act via the AR in prostatic fibroblasts and regulate the production of growth factors fulfilling multiple functions, including normal homeostasis of the prostate epithelium.[(43)] Loss of these functions thus occurs in parallel with disease progression when AR-regulated stromal growth factors are no more expressed. As such, further dedifferentiation and glandular breakdown happens.[(19)] Our results are in line with this study as well as with a more recent report on localized and recurrent PCa.[(19)(43)] Also, statistical analysis showed that the total loss AR expression in prostatic fibroblasts worsens patients' outcomes, in agreement with the literature. [(19)(43)]

Among cells often present in the stroma are inflammatory cells, which did not express AR. Nevertheless, their quantification revealed interesting results eluded to in other reports,[(44)] and showing that high levels of inflammatory cells worsen outcome of patients. This could alter chemokines and cytokines liberated in the microenvironment and affect tumor cells. This area of

research is being explored and may help understand the interplay taking place between the diverse cell subtypes present in the prostate and metastases.

Endothelial cells were found to express AR in a patchy manner in the tissues analyzed, a finding which supports a previous study describing the AR presence and functionality in vascular endothelial cells of the prostate.[45] Although hypothetical, the authors postulate that AR expression may represent a potential player in endothelial cell proliferation for angiogenesis.[45]

Collectively, our observations support heterogeneity of epithelial and fibroblastic cell populations expressing nuclear AR or not in the prostate, prostate tumors and metastases. Molecular changes of AR expression in cell subtypes of the prostate epithelium close to tumor foci are not phenotypically detectable clinically. Hence they may reflect modifications caused by diverse factors and other cells. Fibroblasts are themselves heterogeneous in their AR expression and together with inflammatory cells, they predict BCR. As such changes in AR expression in epithelial and cancer cells with progression do not predict BCR, but appear to particularly contribute to further progression. This is supported by our quantitative data in advanced CRPC/ADT cases, with more cells being AR negative while the AR positive ones express new forms of the proteins, such as constitutively activated variants along with the full length AR. Splice variants could have significant predictive value if studied separately using specific antibodies. In conclusion, clinical heterogeneity reflects tumor cell heterogeneity and AR functional heterogeneity, which is best detected in advanced disease.

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Table 1.

Nuclear AR expression in Prostate and Pca																
			C- Terminal Ab							N- Terminal Ab						
			Percentage %				Hscore (0-300)			Percentage %				Hscore (0-300)		
Case	TISSUE	#	0	1+	2+	3+	BASAL	LUMINAL	CA	0	1+	2+	3+	BASAL	LUMINAL	CA
	Young adults	40	(L) 1	87	9	3	57±13.5	114.0±12.5		(L)0.52	88	9	3	57±11.7	116±9.3	
			(B) 54	33	8	5				(B)56.0	35	5	4			
BPH	PURE	5	(L) 0.7	92	7	0	42±9.6	107±5.3		(L) 0.60	91	6	4	52±7.79	113±2.17	
			(B) 58	42	0	0				(B) 48	52	0	0			
	BENIGN	67	(L) 1	57	29	13	75±50	154±47.53		(L) 0.71	57	31	11	69.5±41.99	154±65	
	FAR		(B) 50	28	20	2				(B) 53	26	18	2			
	BENIGN	55	(L) 2	45	29	24	95±32	175.±35		(L) 0.54	29	47	24	91±54.40	194±65	
	CLOSE		(B) 46	19	29	6				(B) 43.9	22	26	8			
LOCALIZED																
Pca	Gleason 6	68	4	53	28	15			154±43	3	52	26	18			160±63
	Gleason 3+4	150	6	50	26	18			156±29	4	55	28	13			150±49
	Gleason 4+3	78	8	54	22	16			146±38	5	49	30	16			156±52
	Gleason 4+4	10	10	44	29	17			153±52	7	48	26	19			156.5±62
	Gleason >8	20	10	40	30	20			160±46	6	42	27	26			172±59
	NHT	25	12	35	27	26			167±55	8	27	23	41			199±58
ADVANCE	ADT	29	16	30	28	27			167±54	14	7	23	55			220±23
	BM	21	10	44	28	18			154±55	6	17	33	44			215±32
	SV	19	8	36	35	21			169±45	7	21	30	42			207±38
	Primary of LN	14	8	38	30	24			170±45	11	26	29	34			186±45
	LN	14	8	38	32	22			168±50	8	18	34	40			206±35

Table 1: H Scores and breakdown of AR immunostaining in all patient cohorts. This table presents each parameter, H Score values, breakdown of H Score (0+, 1+, 2+, 3+).

Legends to the Figures

Figure 1. Titration curves with AR Abs to optimize IHC.

Three cases were used for optimization. Consecutive sections of the tissue from an advanced case are shown, once stained with (A) C-terminal Ab and (B) N-terminal at various concentrations along with the AR localization in the cytoplasm (left panel) and nucleus (right panel). The bars represent the % of AR negative (0) and positive +1/+2/+3 starting from the bottom. Images of AR stained sections with each AR Ab at optimal dilution (1/250) are shown in the right side of Figures at a 40X magnification.

Figure 2. AR expression in epithelial cells of the basal and luminal layers of acini.

Sections stained with (A) C-terminal Ab (top panel) and (B) N-terminal Ab (bottom panel). Similarly, in both panels, benign glands showing weaker staining intensity of nuclear AR in basal than luminal cells. The zoomed images in both panels showing a rare negative luminal cell as well as two negative basal cells. Fibroblastic cells in the stroma are also stained.

Figure 3. Unstained - AR negative cells in benign glands and tumors.

Sections **s**tained with (A) C-terminal Ab (left panel) and (B) N-terminal Ab (right panel).

Each column represents the group of patients indicated at the bottom. The % of AR negative tumor cells increases with disease progression from less than 1% in benign cells to 14-16% in patients under palliative ADT ($p<0.01$).

Figure 4. AR expression in tumor cells of localized and advanced PCa.

Section stained with (A) C-terminal Ab, showing the cytoplasmic expression (left panel) and nuclear expression (middle panel), and with (B) N-terminal Ab, showing the nuclear expression (right panel). Each group of cases (mentioned on the right of each panel) is represented with the distribution of the percentage of cells stained with the nuclear intensity represented in 4 columns, from (0) or no stained cells and each intensity defined by +1, +2 and +3 in the abscissa.

Figure 5. Kaplan-Meier curves for AR expression in the radical prostatectomy cohort.

Both curves illustrate the clinical relevance of nuclear AR levels with BCR, using (A) C-terminal (left panel) and (B) N-terminal (right panel) Abs. Cutoffs (showing the lowest p-values possible) and p-values are illustrated on the graphs.

Figure 6. Nuclear AR expression in metastases.

Sections stained with (A) C-terminal Ab and (B) N-terminal Abs. Each group of cases (mentioned on the right of each panel) is represented by 4 columns to show staining intensity, from (0) and each intensity +1, +2, and 3+ indicated in the abscissa.

Figure 7. AR expression in fibroblasts stained with C- terminal Abs.

(A) Distribution of prostatic fibroblasts stained with the C-terminal AR Ab in percentages according to expression (intensity) level for diverse cohorts of PCa patients representing different stages of progression. Each group of cases (indicated on the right) is represented by the nuclear intensity in 4 columns, from (0) or no stained cells to +1, +2 and +3 in the abscissa. (B)

Kaplan Meier curves showing the clinical relevance of fibroblast AR staining with BCR in the radical prostatectomy cohort. The cutoff and p-value are illustrated on the Figure.

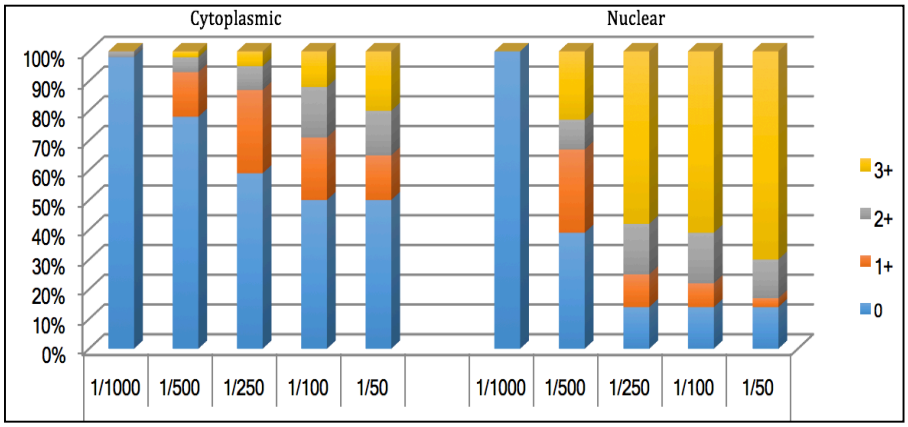
Figure 8. Inflammation adjacent to cancer in the radical prostatectomy cohort of localized PCa.

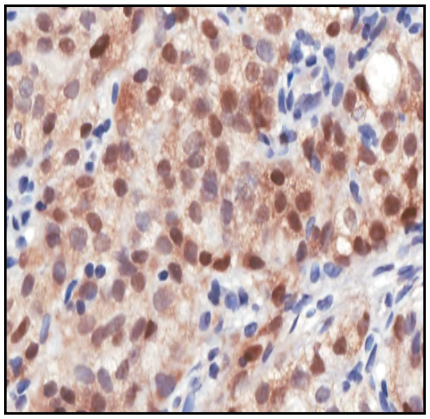
(A) Representative section showing high grade inflammation with infiltrating lymphocytes nearby tumor foci not expressing AR. IHC was performed using the C- terminal AR Ab, as described in Methods. (B) Kaplan Meier curves correlating the extent of inflammation next to tumor foci with outcome (BCR) of patients from the radical prostatectomy cohort. Moderate and high grade inflammation are clinically relevant. Cutoffs and p-value are illustrated on the Figure.

Figures

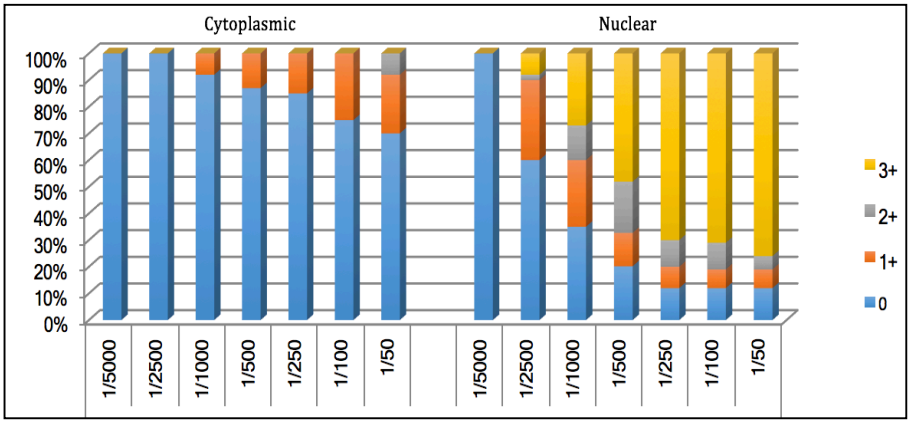
Figure. 1

(A)





(B)



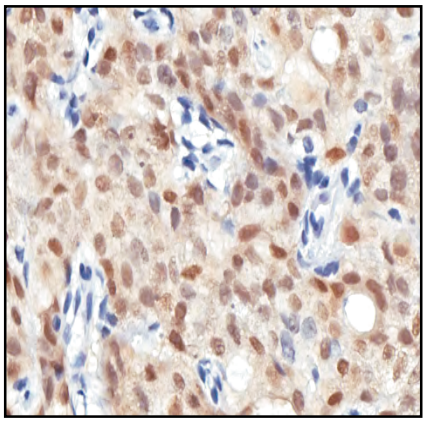
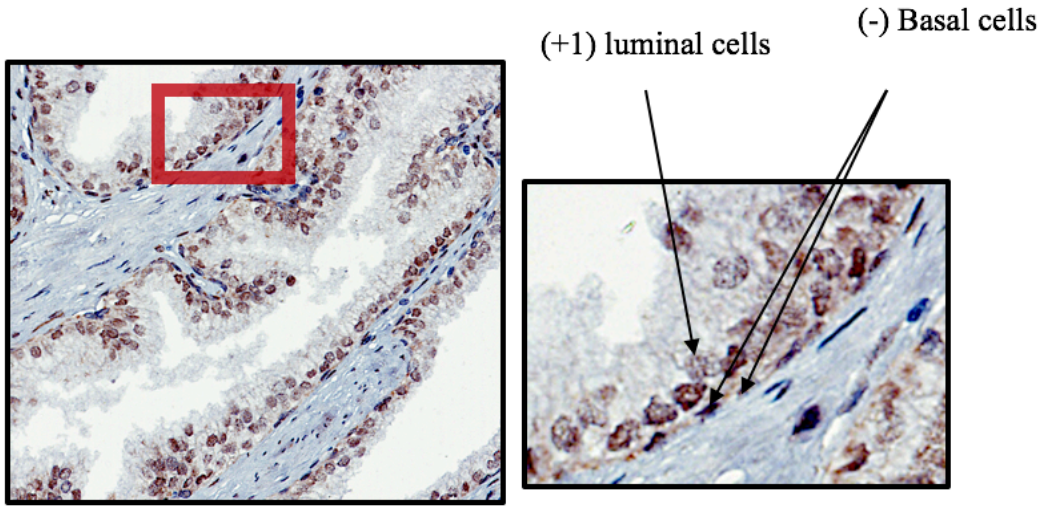


Figure. 2

(A)



(B)

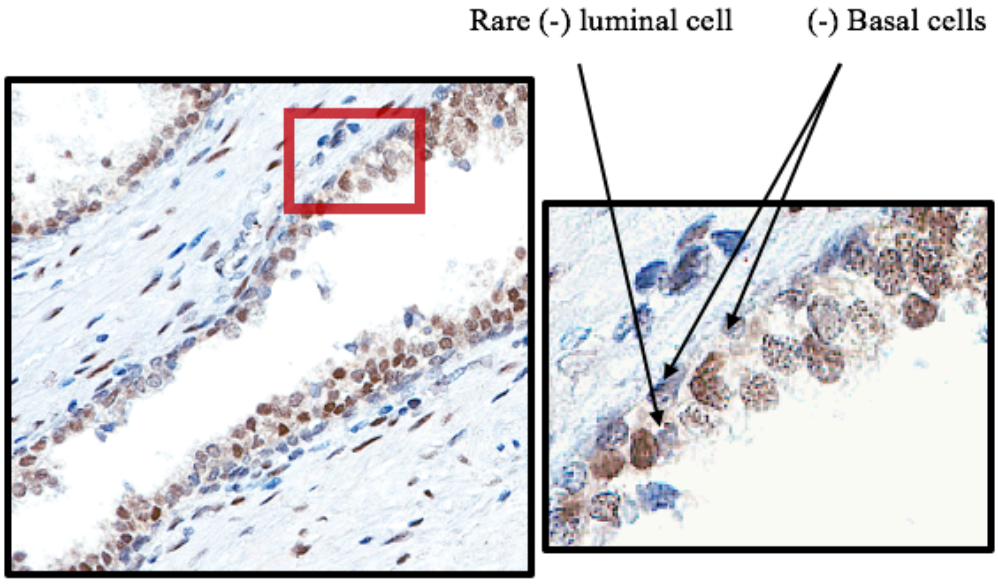
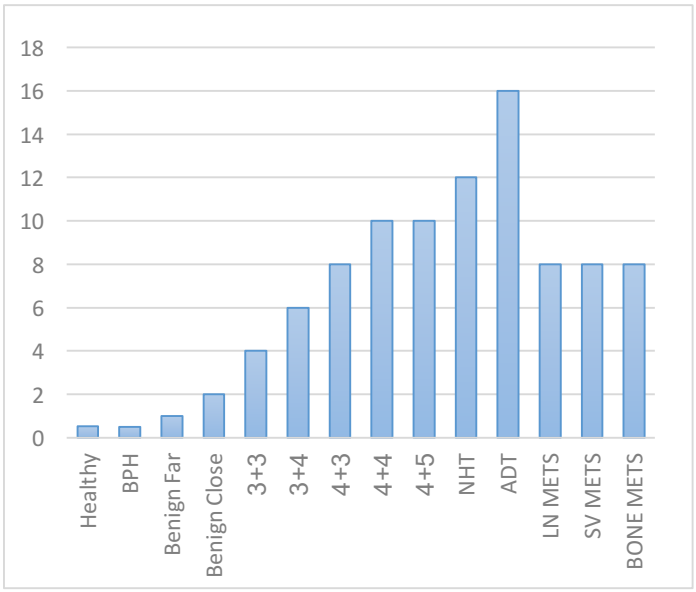


Figure. 3

(A)


18
16
14
12
10
8
6
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0
Healthy
BPH
Benign Far
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3+3
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4+4
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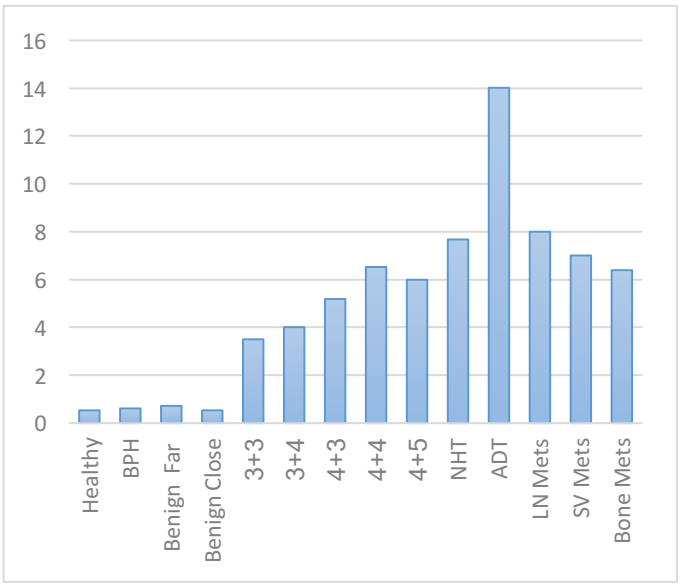
NHT
ADT
LN METS
SV METS
BONE METS

(B)


16
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Healthy
BPH
Benign Far
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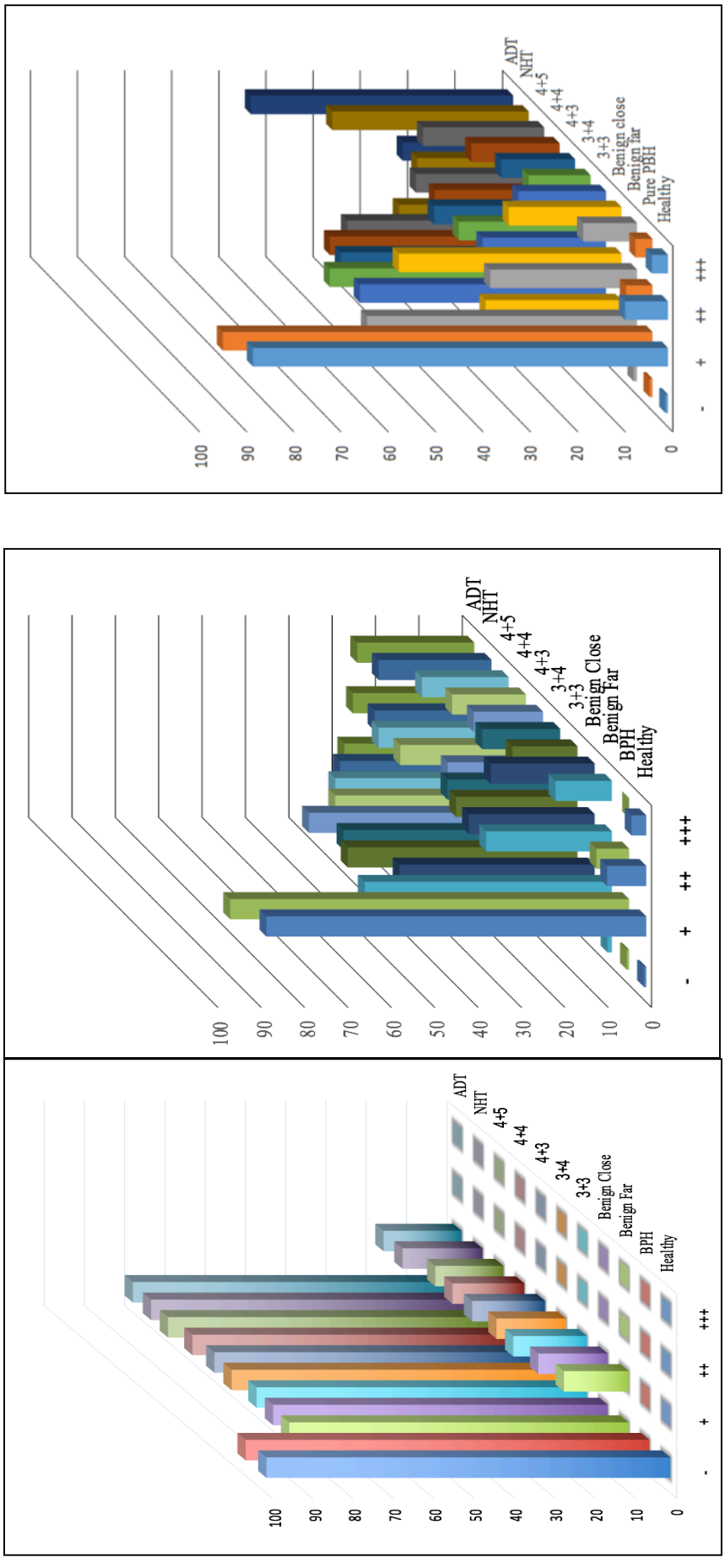
4+4
4+5
NHT
ADT
LN Mets
SV Mets
Bone Mets

Figure. 4


(A)
(B)
ADT
NHT
4+5
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4+3
3+4
3+3
Benign Close
Benign Far
BPH
Healthy
Pure PBH
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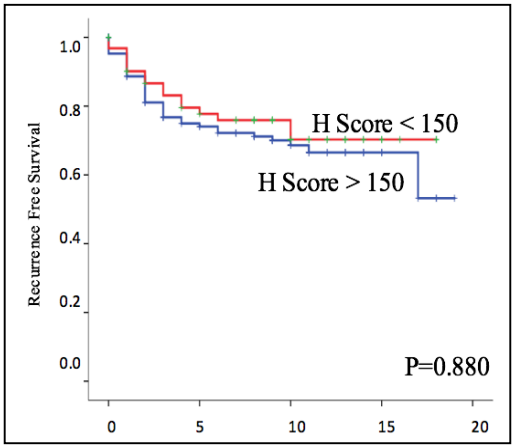
Figure. 5

(A)



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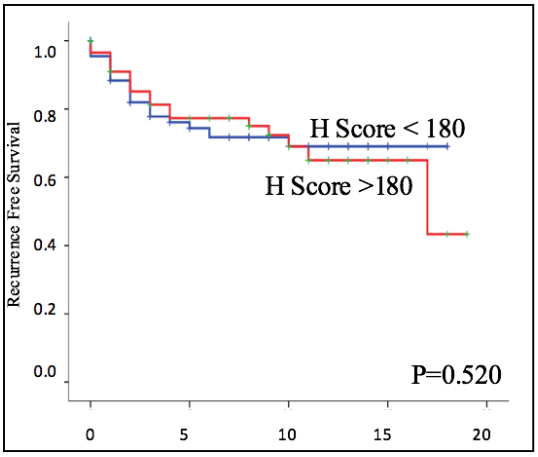
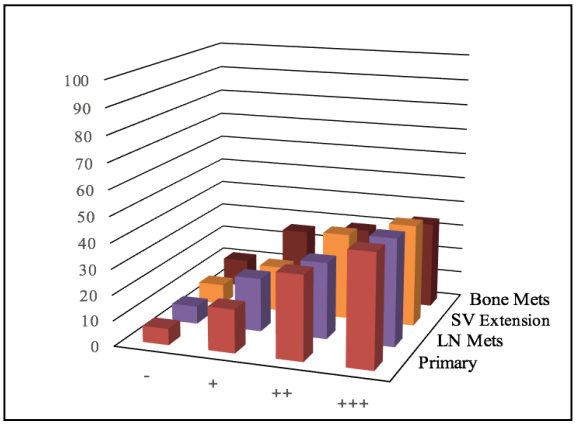


Figure. 6

(A)



(B)

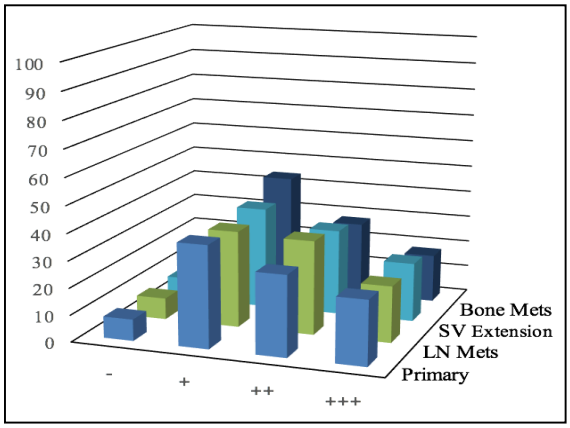
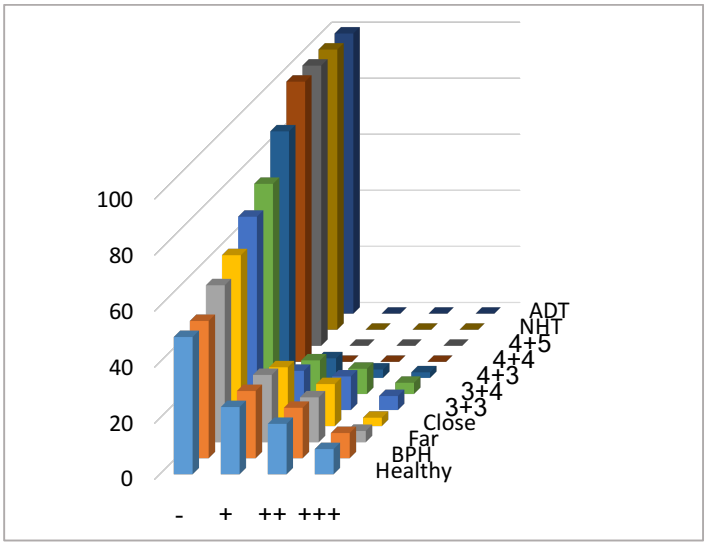


Figure. 7

(A) **(B)**



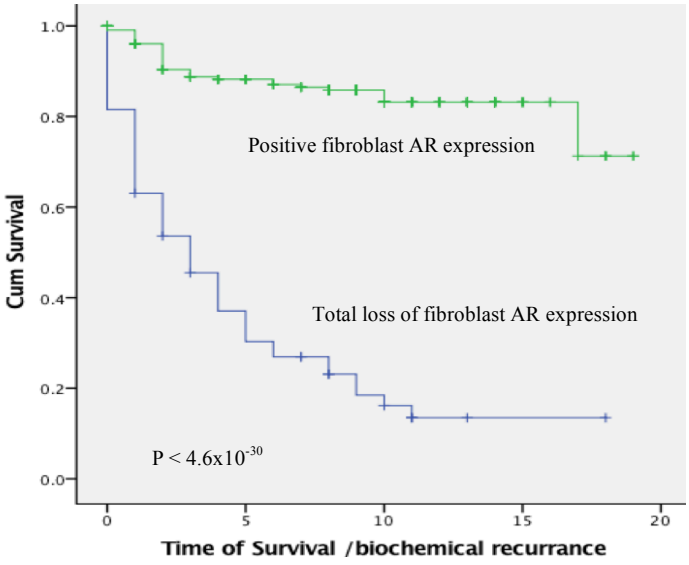
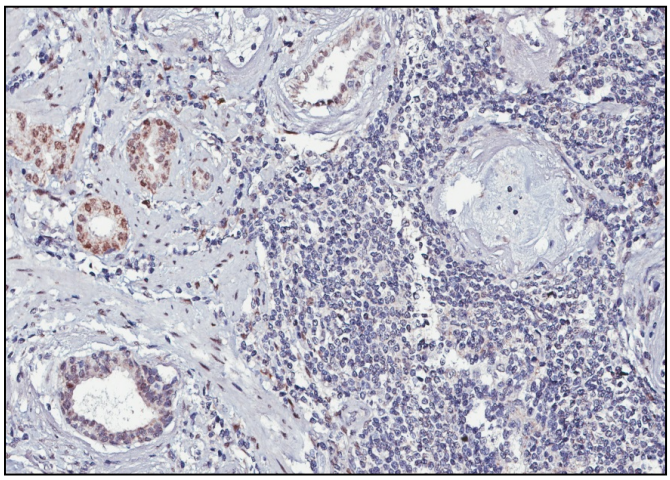
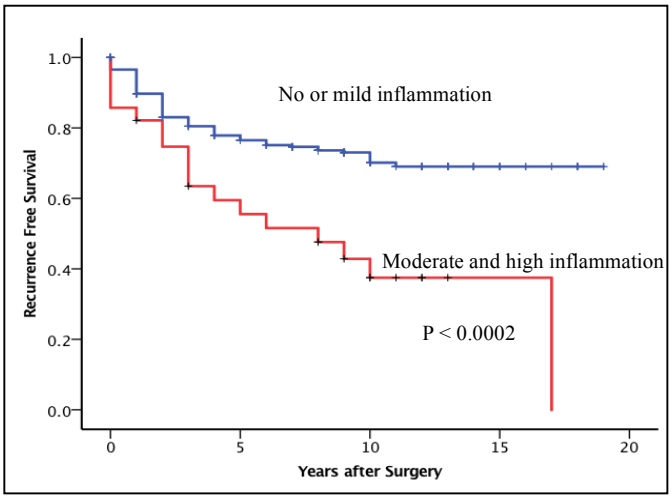


Figure. 8

(A) **(B)**





Chapter #3

ANDROGEN RECEPTOR ACTIVATION BY TYROSINE 223 PHOSPHORYLATION (pY223AR): A NOVEL PREDICTIVE BIOMARKER OF PROSTATE CANCER PROGRESSION

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Abstract

Introduction: Prostate cancer (PCa) figures among leading causes of cancer deaths in North America. To date, androgen-deprivation therapy (ADT) is the gold standard treatment for patients experiencing a biochemical recurrence (BCR) after initial management, but ADT invariably fails and is followed by castration resistance (CRPC) and further disease progression. The host lab reported that the signal transducer and activator of transcription (STAT)3 and the androgen receptor (AR) are substrates of the Fer tyrosine kinase in human PCa cell lines and once activated. i.e. Tyrosine (Y) phosphorylated (p) on Y705 and Y223, respectively, they become Fer partners accumulating into the PCa cell nucleus. We aimed to assess the fate of pY223AR in the prostate and PCa.

Method: Immunohistochemistry using specifically developed polyclonal rabbit antibodies raised against a pY223AR peptide was performed on sections of tissue microarrays and whole blocks to cover the normal prostate of young adults and the whole spectrum of prostatic proliferative diseases. Levels of pY223AR were quantified and expressed in (%) and by H scores. Statistical analyses included Kaplan Meier, COX regressions and ROC curves.

Results: Epithelial cells of both healthy and BPH cases were 100% negative for pY223AR. pY223AR-negative tumor cells constituted up to 15% in ADT/CRPC cases. Activated AR was mainly nuclear. The staining intensity and H scores increased with Gleason score (GS) in primary tumors and with progression to GS$\geq$ 8 ($p<0.01$), being most elevated in ADT/CRPC patients and metastases. Nuclear pY223AR H scores significantly correlate with BCR ($p=10.5x10^{-20}$) and improve prognostication of standard parameters.

Conclusion: Activated AR (pY223) represents a novel PCa biomarker with prognostic value.

Introduction

Prostate cancer (PCa) figures among leading causes of cancer deaths in North America.[1] To date, no curative therapy exists for patients experiencing a biochemical recurrence (BCR) after radical prostatectomy (RP) or radiation therapy (Rx). At this point, androgen-deprivation therapy (ADT) is the gold standard treatment to control the disease but ADT invariably fails and is followed by castration resistance (CRPC) and further progression to the lethal stage of disease.[2][3] Aberrant AR signaling is an attractive emerging concept contributing to PCa progression. For instance, AR phosphorylation is considered as part of mechanisms allowing non-steroidal agonists to alter AR signaling or its co-activators in response to numerous growth factors, including interleukin-6 (IL-6),[4][5] a cytokine clinically linked to CRPC and androgen-independent PCa progression.[6]

Tyrosine kinases (TKs) are known as immediate effectors of AR activation by tyrosine (Y) phosphorylation, as triggered by growth factors, cytokines and neuroproducts.[7] Studies on the Fer TK in prostate cancer has initially shown that Fer expression was critical for the survival and growth of human PCa cells.[8] In addition, the Fer protein was not detected or minimally in extracts from the normal and hyperplastic prostate but found at elevated levels in extracts from PCa tissues.[8] The search of Fer substrates and partners led to identification of the STAT3 (signal transducer and activator of transcription)3 and AR, which are the key transcription factors (TFs) acting downstream of the IL-6 and Androgen-mediated signaling pathways respectively, and determinant for PCa progression.[9-11] Of interest, in PCa cell lines expressing AR such as LNCaP, IL-6 allows the transcriptional activation of AR in a mechanism requiring activated STAT3, i.e. Y phosphorylated on residue 705.[4][10] Our team was first to show through in vitro assays that Fer acts directly on STAT3 and AR by phosphorylating Y705 and 223, respectively.

In PCa cells, this occurs through IL-6 mediated Fer, STAT3, and AR activation, which then become Fer binding partners, via direct interactions of Fer-generated pY-motifs with the Fer SH2 domain.[9][11] Moreover, Fer plays a role in IL-6 mediated growth and AR transcriptional activity on genes, like PSA among others.[12] Finally, Fer, AR and pSTAT3 all co-localize in the nucleus of PCa cells, including in prostate tissues from CRPC patients.[9][11] Altogether, these findings support the aberrant AR signaling in PCa cell nucleus, which through Fer-mediated activation of AR, allows cross-talks between pY223AR and pSTAT3 during CRPC progression.

The identified phosphor Y223 motif regulated by Fer in the AR structure may be of significance by its location in the N-terminal part of the AR protein, and therefore not only present in full length AR protein but its shorter forms like splice variants lacking the C-terminal portion of the protein. AR variants were recently shown to be constitutively activated and are contributing to PCa progression through their own transcriptional activity on distinct sets of genes.[13][14]

Based on the above observations, polyclonal antibodies (Abs) were raised in rabbits using a synthetic pY223AR peptide as antigen. Immunoglobulins were purified and the specificity of the pY223AR Abs was ascertained, both biochemically and immunohistochemically. In the present investigation, we report on the cell by cell quantification of activated pY223AR expression in PCa tissues of different cohort of patients. We show that nuclear levels of activated AR (pY223AR) increase with disease progression and represents a novel biomarker of advanced disease predicting survival probability.

Material and Methods

Clinical cohort and tissues

The study protocol was approved by the Ethics Board of the McGill University Health Centre (MUHC) Research Institute (RI) (BMD-10-115-1170). Formaldehyde-fixed paraffin-embedded (FFPE) blocks from a total of 479 cases were retrieved from Pathology departments of the MUHC and Sacré-Coeur Hospital. The cohort included 40 normal prostates from adults below 45 years-old, 326 radical prostatectomies (RP) covering Gleason scores (GS) 6 to 10 from treatment-naïve cases and 25 cases that had undergone neoadjuvant hormonal therapy (NHT) prior to RP, assembled in Tissue Micro Arrays (TMAs) along with 5 Trans Urethral Resection of the Prostate (TURP) from benign prostatic hyperplasia (BPH) cases and 29 advanced cases that had received hormone therapy (ADT) following biochemical recurrence (BCR). The other 54 cases of the series included in TMAs consisted of 14 primary tumors with matched lymph node (LN) metastases, 19 tumor extension to seminal vesicles and bone metastases from 21 different cases. In addition to the study cohort, three separate tissues were used for optimization, one was GS7 RP cases and the others are TURP from advanced CRPC cases.

Clinical outcome data (updated yearly) were available for the RP cohort. In our Centre, BCR is defined by two successive rising blood PSA at 2 ng/mL or more over post treatment nadir (cf. Phoenix definition).[15]

Immunohistochemistry (IHC)

IHC was performed using specifically developed polyclonal rabbit Abs produced using a pY223AR peptide cross-linked to Keyhole Limpet Hemocyanin. Immunoglobulins were purified from rabbit serum by affinity chromatography and specificity was validated by biochemical

methods. Briefly, 4μm thick sections were deparaffinized in xylene, and rehydrated using a decreasing ethanol gradient followed by rinsing with distilled water. Slides were incubated for 10 min in 95°C with 0.2 M citrate buffer (pH 6.0) as the antigen retrieval step, following by the blocking of endogenous peroxidase with 0.3% hydrogen peroxide (Sigma Aldrich, Germany) for 20 min, then immersed for an additional 30 min in protein blocking reagent to minimize non-specific binding of primary Abs (Chemicon Millipore). The slides were incubated overnight at 4°C with the pY223AR Abs at a 1/100 dilution unless otherwise specified, followed by incubation with biotinylated secondary antibody and streptavidin horseradish peroxidase (HRP) enhancer (Chemicon International, Millipore) for 60 min and then visualized by the DAB substrate kit (Nichirei Biosciences, Tsukiji, Chuo-ku, Tokyo.) Sections were subsequently counterstained with Harris Hematoxylin.

In some instances, AR Abs were used to ensure AR expression when pY223AR yielded no staining. AR Abs were a rabbit polyclonal IgG (Santa Cruz Biotechnology; AR(C-19), sc-815, at 250 μg/ml) detecting a C-terminal epitope of the human AR full-length AR protein and a mouse monoclonal anti-human AR Ab (NeoMarkers, Thermo Fisher Scientific, Inc., Fremont, California; clone 441, at 200 μg/ml) directed against an epitope (AA 299-315) located in the N-terminal end and present in all known forms of AR, including variants.

Titration of pY223AR Abs was performed on consecutive prostate sections from the three cases mentioned above at increasing dilutions of Abs to determine the optimal dilution for best staining with minimal background. Staining was analyzed in three representative areas of these three cases PCa tissues.

Quantitative scoring of pY223AR staining

All stained sections were scanned using an Aperio image scanner microscopy system. Quantification of staining as well as the counting of cells was assessed at 40x magnification by eyes and by the Imagescope software. The staining was scored by T.A who was blinded to cases. A second scoring was performed by an independent individual (E.S.) to randomly score 25% of cores or sections. No difference between random observations was observed. The scoring and data were verified by F. B. Cytoplasmic and nuclear stainings were assessed in normal (luminal, basal) and tumor/metastatic cells along with stromal fibroblasts, endothelial cells, and inflammatory cells in the same tissue samples. A minimum of 500 cells were counted in each section. Negative cells (-) were counted. Positive (+) tumor cells were analyzed according to the intensity on a scale of 1-3 (1=weak, 2=moderate, 3=strong) to calculate H scores = Σ (Pi(i)) x100, where i is the staining intensity, and Pi the percentage of cells and each intensity yielding values between 0-300.[16] Example: 5% pY223AR (0) + 15% pY223AR (1+) + 60% pY223AR 2(+) + 20% pY223AR (3+)= 195.

Statistical Analysis

H scores of pY223AR were analyzed in normal, benign and tumor cells among disease stages (i.e. from localized to advanced disease). Kruskal-Wallis ANOVA plus comparison of mean ranks was used to analyze H scores and negative % nuclear staining; one-way ANOVA plus Bonferroni post hoc test was used to analyze percentages (%) of cells displaying staining intensity qualified of weak (1+), moderate (2+) and strong (3+). Survival statistical analyses were done to determine the significance of pY223AR expression on outcome.

Results

IHC optimization

To enable an optimal detection of pY223AR and find out the best concentration for ideal quantification, consecutive prostate tissues sections from three patients (1 GS7 and 2 advanced under ADT) were used for IHC in titration experiments at dilutions ranging from 1/1000 to 1/50. Figure 1 shows representative titration curves obtained with pY223AR Abs in the cytoplasm and the nucleus of tumor cells. Essentially, AR was activated and detected with pY223AR Abs in the nucleus of up to 90% of cells, with a plateau of 10% unstained cells reached at the 1/100 dilution and no further increased in positively stained cells at a 1/50 dilution. The 1/100 dilution was chosen for the rest of the study. At this dilution, it was possible to score activated pY223AR at all three intensities (1+ to 3+) in stained positive cells. Also, there was minimal cytoplasmic faint staining, with 80-88% of cells unstained or negative cytoplasm with clean background (Figure 1). Titration curves showed similar results with other tissues used in the optimization experiments.

The specificity of the pY223AR Abs was ascertained using the human recombinant AR protein phosphorylated in vitro through the Fer TK catalytic domain, as reported,[20] followed by Western blotting using Abs to detect pY, AR and pY223AR. Extracts from LNCaP (positive controls) and PC3 (negative control) exposed to IL-6 were included and confirmed the quality of pY223 AR Abs detecting one 110 KDa band in the kinase assay and in LNCaP exposed to IL-6 (data not shown).

AR is not activated in epithelial cells of normal and benign prostates

We previously reported that the Fer protein is not detected in normal and hyperplastic prostate.[8][11] Since Fer was identified as the TK activating AR in PCa cell lines,[10] and that almost all luminal cells express AR and ~ 40% of basal cells (unpublished observations), we reasoned that AR activation would not be detected in the normal and hyperplastic prostates. This issue was addressed by IHC with pY223AR and AR Abs, using a TMA generated with 40 prostate blocks from adults below 45 years old and 5 BPH TURP cases. Results in Figure 2A shows a totally negative core of normal prostate tissue stained with pY223AR (all cores from normal prostates were negative). The parallel staining with AR Abs confirmed its expression with the C- (Fig. 2B) and N- (Fig. 2C) terminal Abs. This is also illustrated in Table 1 as well the absence of activated AR in BPH tissues. Therefore, AR activation is not detectable in the epithelium of the normal and hyperplastic prostate.

Activation of pY223AR in benign cells adjacent to tumor foci

We previously observed that the epithelial cells of hyperplastic prostates from BPH patients and corresponding cells in glands of prostates from cancer patients differ in expression of several proteins (unpublished data). As AR is expressed in luminal cells and also in basal, although in less cells, we assessed AR activation in prostate tissues from 67 RP cases of localized PCa, assembled in a TMA along with cores of benign glands far (distance > 1mm, another core) or close (distance of 1mm or less, within the same core) to tumor foci of each patient. AR activation was primarily nuclear and, detected in luminal and basal cells, far and close to tumor foci. However, quantification of activated AR showed a preferential distribution in luminal vs. basal cells, with 74% of luminal cells vs. 24% of basal cells being positive, but both at a low 1+

intensity. This resulted in a significant difference between luminal vs. basal H scores, at 122 vs. 40 ($p<0.01$). In benign glands close to cancer, the higher pY223AR activation translated in increases in H scores to 151 in luminal vs 56 in basal cells ($p<0.01$). Adjacent cancer cells showed an H score of 163. There was a significant difference in AR activation in cancer cells vs luminal benign cells close to them ($p<0.01$) (Table 1). These findings suggest that molecular changes have occurred in benign cells of the prostate of PCa patients.

AR activation in localized and advanced PCa

The above results have indicated that AR is not activated in epithelial cells of normal and hyperplastic prostates, whereas nuclei of epithelial cells in acini far from tumor foci of the same patients were pY223AR positive, implying activation. In addition, AR was activated at higher extent when closer to the cancer foci and further enhanced in cancer cells, suggesting a gradient of AR activation, raising the possibility of further AR activation in more aggressive cancers. This was investigated in primary tumors of RP cases, naïve to treatments. Figure 3 and Table 1 summarize our observations.

The pY223AR staining in positive tumor cells was analyzed in the cytoplasm (Fig. 3A) and the nucleus (Fig. 3B). A majority of cells (80% and more) had no cytoplasmic pY223AR and a low % (below 20%) displayed at low intensity. There were no significant changes ($p>0.05$) with stages. Activated AR was primarily nuclear (Fig. 3B). Staining intensities showed low levels, mainly +1 in low grade cancer, and interestingly, shifted to higher intensity +2 and +3 in high grades cancer (GS8 and above), NHT cases and ADT. The increase in staining intensities and H scores with GS were significant ($p<0.01$). Highest nuclear levels of activated AR in tumor foci were ADT/CRPC patients (H score=211) (Fig. 3B and Table 1). The analysis of negative or

unstained cells revealed a significant increase with progression ($p>0.05$), representing up to 16% of tumor cells in advanced cases (Fig. 3C).

While scoring tumor cells, AR activation was noticed in the stroma, notably in prostatic fibroblasts. Their expression of pY223AR in fibroblasts surrounding tumor foci was quantified and data are summarized in Figure 3D. They were found to be totally negative in prostates from adults below 45 years and in BPH patients. They became positive and expressed low level of activated AR, at a +1 intensity, in the presence of cancer foci of GS6 and GS7 cases and disappear at higher GS and in more advanced disease. Their positivity was maximal when surrounding tumor foci of GS 3+3 > 3+4 > 4+3, and when they were located in benign areas, close > far to tumor foci for GS 6-7 cases. As a whole, a gradient of AR activation is seen in low grade cancers, with no staining in GS8 and over. These findings are in line with reports establishing that AR is expressed in fibroblasts of the normal and BPH prostates and lost in high grade PCa.[17][18] Hence the fact that AR activation is only detected in cancer of low grade suggests a role for activated AR at the transition from GS3+4 and GS4+3.

AR activation in tumor extension to seminal vesicles and metastases

Based on these findings, we wondered if such AR activation translates into metastatic cells. Small subsets of primary tumors and matched LN metastases collected at RP were stained with pY223AR Abs, along with seminal vesicles showing signs of invasion or extension from primary tumors and bone metastases. Results of AR activation, quantified by counting the number of stained cells and intensity are shown in Figure 4 for the nucleus (panel A) and cytoplasm (panel B). AR activation was mainly nuclear in tumor or metastatic cells from all tissues. In continuity with observations made above in advanced CRPC patients on ADT, NHT cases and GS8-9 RP

treatment naïve cases (Fig. 3), cancer cells in these sets of tissues also displayed high levels of pY223AR, starting with staining elevated intensity at +3 followed by moderate +2 and low 1+, and with significant differences between subgroups ($p<0.01$). Results of nuclear AR activation were similar in primary tumors vs. their LN metastases, cancer cells extending to seminal vesicles and distant bone metastases (Fig. 4B and Table 1). Cancer cells displayed some low AR activation in the cytoplasm, which was not significantly different between groups of tissues (Fig. 4A). of note, LN metastases showed cells with moderate and high pY223AR staining in their cytoplasm, which was not observed in matched primary tumors, tumor extension to seminal vesicles and bone metastases. Representative images are shown in Figure 4C.

Clinical significance of nuclear AR activation in PCa

Survival analyses were next performed to interrogate the clinical relevance of AR activation in the nucleus of PCa cells. For this purpose, we used the updated clinical database of the treatment-naive RP cohort. Kaplan-Meier analysis shown in Figure 5 demonstrates that nuclear pY223AR H scores correlate with BCR (log rank, $p<1.5x10^{-20}$) at H scores ≥160. The combination of pY223AR H scores with blood PSA at diagnosis (Fig. 6), GS of prostate at surgery (Fig. 7) and clinical stage (Fig. 8) showed that patients with pY223 H scores ≥ 160 had worse outcome (BCR).

Importantly, in univariate analysis (Table 2), pY223AR H scores (<160 vs ≥160) significantly predicted BCR ($p<3x10^{-11}$), independently of pre-operative PSA, GS and tumor stage and based on the hazard ratio of 5.46, activated AR performed better than these parameters. Age was not predictive. This also holds in multivariate analysis, by the highest hazard ratio 3.21, compared to other characteristics. Moreover, analysis of receiver operating characteristic (ROC)

curves shown in Figure 9 supports that pY223AR H score improve the prognostication value of other standard prognostic markers.

Discussion

This investigation reports for the very first time on a new biomarker in PCa, which is the AR activation by phosphorylation of Y223 in patient clinical samples. It represents a translational aspect of a study that went from the TK Fer phosphorylating the AR under IL-6 exposure of human LNCaP cell line, to the identification of the Y223 site, shown to affect AR transcriptional activity on specific genes, including PSA.[10] To move further and in view of testing clinical significance, we generated specific Abs recognizing the Fer-activated pY223 motif of AR through kinase assays *in vitro* (unpublished observations). In this study, an IHC method was developed to ascertain whether this activation of AR on Y223 has clinical relevance. The observations we made on a wide variety of prostate tissues covering the normal prostates of adults below 45 years old and a whole spectrum of conditions and stages that are characteristic of this disease, including treatments.

We first demonstrated that activated AR (pY223 motif) is predominantly detected in the nucleus of prostate cancer cells and associated metastases along with prostatic fibroblasts but at a specific time-point in the disease trajectory. Normal and benign prostate tissues are totally negative for pY223AR. This is likely due to lack of expression of Fer in normal and hyperplastic prostate and alternative activation pathways.[8][11] Our initial studies had showed Fer overexpression in PCa, cell lines and tissue extracts, along with a determinant role in PC3 cell survival and growth.[11] Fer is particularly critical in the IL-6 pathway, regulating both STAT3 and AR transcriptional activity in LNCaP cells and allowing crosstalks between these pathways, and thereby allowing growth and PSA expression to occur in an androgen-independent manner.[10][11] Being a substrate of the Fer kinase,[10] we had predicted that AR Y-phosphorylation and nuclear accumulation would not be observed in normal and hyperplastic

prostate but only in cancer cells, where indeed AR was activated. This included in epithelial of prostate from cancer cases. Interestingly pY223AR levels increased with disease progression. This was demonstrated by quantification of pY223AR, showing a significant increase in tumor cells displaying high staining intensity from GS6 to GS9 and highest levels in advanced CRPC cases on palliative ADT and in prostatic metastases to LNs and bones. Importantly, there was a shift of intensity from low to moderate and elevated levels in higher grade cancer and cases under ADT and in metastasis. Calculated H scores also showed a significant increase with progression. Survival statistical analyses showed a highly significant correlation of pY223AR and BCR. These observations suggest that in line with findings in cell lines,[10] this post-translational modification of AR is important in patients' tumor and metastatic cells for AR transcriptional activity during progression. Indeed the pY223 motif of AR is located in the transcriptional activation function (AF)-1, which largely contributes to AR transcriptional activity.[19] This activated motif would be particularly relevant within the context of recent observations on AR variants conserving the (AF)-1 domain but lacking the ligand (androgen) binding domain at the C-terminal end of the full length protein.[20][21] The fact that they are transcriptionally active may be related to activation of the pY223 motif in the ATF-1 domain, promoting aberrant AR signaling and possibly explaining the relatively short-term and also no response to abiraterone and enzalutamide in advanced disease.[14][20][21]

Our findings on activation of AR in RP patients who had received NHT prior to surgery is intriguing. The number of cells having nuclear AR over-activated - high level, at intensity of 3+, is the second most important of the whole series of primary tumors analyzed. Given that ADT over 3 months is sufficient to induce the death of luminal cells in benign glands and to reduce circulating PSA to zero or nadir as a consequence of cancer cell apoptosis (luminal-like),

such a treatment may simultaneously induce molecular changes, such as TK expression and activation leading to AR activation, in surviving cancer cells that express AR. This is unlikely affecting the fate of patients, whose RP followed soon after ADT. Nonetheless, these results support an upregulation likely due to ADT. In advanced disease, this refers to selection pressure, which often explains the emergence of cancer cells with new features post-ADT. It is noteworthy that in the dog prostate, Fer is up-regulated by ADT or surgical castration and levels are further enhanced upon estrogenic supplementation after castration, causing basal cell metaplasia, whereas Fer levels become non detectable when androgens renewed the secretory epithelium.[8] Such hormonal manipulations had significant consequences on enzymatic activities of TKs and phosphotyrosine phosphatases (PTPs),[22][23] controlling levels of Y-phosphorylated proteins specifically in cells of the basal layer.[24] This raises the interesting possibility that some of these mechanisms may apply in cancer cells. Altogether, the interplay between activated AR, TKs and PTPs deserve further investigation if in the end, the activation AR full length and its variants contribute to progression and are also regulating metastatic cells.

Among interesting observations, it is worth emphasizing that the fibroblast AR activation is present only in cancer cases. Indeed, pY223AR in stromal fibroblasts was absent in normal prostate and BPH tissues. The presence of pY223AR in low grade tumors of RP cases but its disappearance in high grade disease and absence in advanced and metastatic disease is intriguing. Since the AR protein expression in prostatic fibroblasts is loss in high grade tumors and advanced disease,[17][18] the inactivation of AR observed in fibroblasts (not detected with pY223AR Abs) of cases of GS8 and above was expected. Collectively, these results support a role of AR activation at a precise time point in PCa in the disease. Additional studies are required to determine whether activated AR in fibroblasts is implied in tumor development or progression

or else, when tumor cells move a step further in their transition from low to a high risk cancer.

The access to reliable clinical data gave us the opportunity to relate the histopathological findings on activated AR in primary tumors to the clinical outcome of patients. Statistical analyses showed a significant correlation between nuclear pY223AR H scores and BCR. The cutoff of H score 160 yielded highest significance in comparison to other cutoffs tested. In addition, the H score of pY223AR correlated with patient outcome when combined with standard parameters in Kaplan Meier analyses. Indeed, regardless the pre-operative PSA, GS and stage, patients falling in the pY223AR $\geqq$ 160 category always has worst outcomes. Univariate and multivariate analyses showed very significant P values and hazard ratios in further support of pY223AR H scores significantly predicting BCR, independently from other standard parameters. Finally, ROC curve showed that pY223AR H scores add to the prognostication value of standard parameters.

In conclusion, the quantification of nuclear activation of AR through its pY223 motif in different cohorts of patients clearly established activated AR as a PCa specific new nuclear phosphoprotein, not detected in epithelial cells of normal and hyperplastic prostates but present in tumor cells and at increasing intensity with gradation of GS. The highest levels observed in patients receiving ADT prior to surgery or in advanced stage as well as in metastases support an involvement in tumor cells that survive ADT and are more aggressive in late disease stages. Clinical relevance and significance was demonstrated for patient outcome by predicting BCR, independently of standard parameters and adding to prognostication value by higher hazard ratio. Altogether, these findings support that this post-translational modification of AR in its (AF)-1 domain may contribute to the transcriptional activity of full length AR protein and of its variants

during progression. Above all, pY223AR represents a novel prostate cancer biomarker that has prognostic value as it predicts survival probability.

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Tables

Table. 1

Nuclear pY223 AR expression in prostate and Pca										
Patient	Tissue	N	Percentage %				Nuclear pY223 AR Hscore (0-300)			Hscore Significance
			0	1+	2+	3+	Basal	Luminal	Cancer	
	Young adults	40	**(L) 100**	0	0	0	0	0		no Sig (basal to luminal) (p>0.05)
			(B) 100	0	0	0				
BPH	Pure	5	**(L)100**	0	0	0	0	0		
			(B)100	0	0	0				
	Benign	67	**(L) 26**	39	21	14	41± 44	122± 54		Sig (basal to luminal), to autopsy and pure BPH. (p<0.001)
	Far		**(B) 69**	18	7	5				
	Benign	55	**(L) 10**	48	22	19	56± 52	152± 61		Sig (basal to luminal), to autopsy, pure BPH and benign far from cancer. (p<0.001)
	close		**(B) 53**	14	9	8				
Localized										
Pca	Gleason 6	68	**8**	51	25	16			150±53	Sig to luminal and basal of autopsy, pure BPH and benign far from cancer. (p<0.001)
	Gleason 3+4	150	**9**	48	23	20			154±46	Sig to luminal and basal of autopsy, pure BPH and benign far from cancer. (p<0.001)
	Gleason 4+3	78	**9**	48	23	20			154±62	Sig to luminal and basal of autopsy, pure BPH and benign far from cancer. (p<0.001)
	Gleason 4+4	10	**11**	**28**	**29**	**31**			**172±58**	Sig to luminal and basal (p<0.001). Sig to Gleason 3+3,3+4 and 4+3 (p<0.01)
	Gleason >8	20	**12**	26	26	36			185±53	Sig to luminal and basal (p<0.001). Sig to Gleason 3+3,3+4,4+3 (p<0.01). No sig to 4+4, (p>0.05)
	NHT	25	**13**	18	26	43			200±28	Sig to luminal and basal (p<0.001). Sig to all Gleason (p<0.01).
Advanced	ADT	29	**16**	12	17	54			211±35	Sig to luminal and basal (p<0.001). Sig to all Gleason (p<0.01).
	BM	21	7	25	31	36			196±54	Sig to luminal and basal (p<0.001). Sig to all Gleason (p<0.01).
	SV	19	8	24	29	39			199±42	
	Primary of LN	14	13	27	24	35			180±55	
	LN	14	6	23	31	41			207±48	

Table 1: H Scores and breakdown of pY223 AR immunostaining in all patient cohorts. This table presents each parameter, H Score values, significance (if present), breakdown of H Score (0+, 1+, 2+, 3+).

Table. 2

	Univariate		Multivariate	
Variable	Hazard ratio	P-value	Hazard ratio	P-value
Age	1.02	0.16	1.002	.917
Preoperative PSA, <10 vs. >10 ng/ml	2.47	0.0001	1.445	0.138
GS, ≦3+4 vs. >3+4	4.55	$1x10^{-11}$	2.18	0.004
Stage, ≦T2 vs. ≧T3	3.85	$1x10^{-9}$	1.92	0.01
pY223AR H score, <160 vs. ≧160	7.46	$1.5x10^{-17}$	5.21	$3x10^{-11}$

Table 2: Univariate and multivariate analysis pY223AR H score significantly predict BCR, independent of other standard parameter shown in the table.

Legends to the Figures

Figure. 1 Titration curve with pY223AR Abs.

Three cases were used for optimization. The representative example shown is a section from a primary tumor of the GS7 patient stained with pY223AR Abs at various concentrations, along with the localization of the staining in the cytoplasm (left panel) and nucleus (right panel). The bars represent percentages of cells stained with pY223AR Abs as negative (0) and positive +1/+2/+3, starting from the bottom.

Figure 2. The Y223 motif of AR is not activated in epithelial cells of normal and benign prostates.

Sections were stained with (A) pY223AR Abs; (B) C-terminal AR Ab; and (C) N-terminal AR Ab. In (A), normal glands are totally negative for pY223AR whereas AR expression was confirmed in the same normal prostate tissues.

Figure 3. AR activation in localized and advanced PCa.

Quantification of pY223AR was performed by counting cells as described in Methods. Results are illustrated to show percentages of cells expressing activated AR at a defined intensity represented by columns, from (0) no stained cells, to a low intensity at +1, moderate at +2 and elevated at +3 (indicated in the front of Figs) and throughout the different disease stages analyzed (indicated on the right). AR activation is shown in the **(A)** Cytoplasm and **(B)** Nucleus of tumor cells. **(C)** The percentages of tumor cells displaying no AR activation (0 or negative) in localized cancer is shown as a function of GS in the RP cohort and in the NHT and ADT/ CRPC cases; **(D)** Status of activated AR -pY223AR in prostatic fibroblasts and illustrated similarly to

Figs 3A and B, by Distribution of cells (%) based on intensities from 0 to 3+ in the same cohorts of patients.

Figure 4. AR activation in seminal vesicles extension and metastases.

Quantification of pY223 AR in cancer cells of primary tumor tissues and their matched LN metastasis, tumor extension to seminal vesicles and bone metastasis, in the (A) Cytoplasm and **(B)** Nucleus, as in Figs 3 and similarly illustrated; **(C)** Representative images of pY223 AR expression in sections of: (1) a primary tumor tissue and (2) its LN metastasis; (3) tumor extension to seminal vesicles extension; and (4) bone metastasis.

Figure 5. Kaplan-Meier analysis of pY223AR H scores with BCR.

The clinical relevance of nuclear pY223AR H scores was correlated with BCR and is shown at s cutoff (<160 or ≧160). The number of patients in each group and the P value are presented on the graph.

Figure 6. Kaplan-Meier analysis of the combination of pY223AR H scores and pre-surgery blood PSA levels with BCR.

Blood PSA levels (> or s< 10ng/ml) were coupled to nuclear pY223AR H scores at the cutoff (<160 or ≧160) to interrogate clinical relevance with BCR as the outcome. The number of patients in each group and the P value are indicated.

Figure 7. Kaplan-Meier analysis of the combination of pY223AR H scores and Gleason Scores with BCR.

GS were coupled to nuclear pY223AR H scores at the cutoff (<160 or ≧160) to interrogate clinical relevance with BCR as the outcome. The number of patients in each group and the P value are indicated.

Figure 8. Kaplan-Meier analysis of the combination of pY223AR H scores and clinical stage with BCR.

Stages T2 and T3 were coupled to nuclear pY223AR H scores at the cutoff (<160 or ≧160) to interrogate clinical relevance with BCR as the outcome. The number of patients in each group and the P value are indicated.

Figure 9. ROC of pY223 AR H scores with other standard parameters.

Sensitivity and specificity of activated AR to improve the prognostication value of other standard prognostic markers.

Figures

Figure. 1

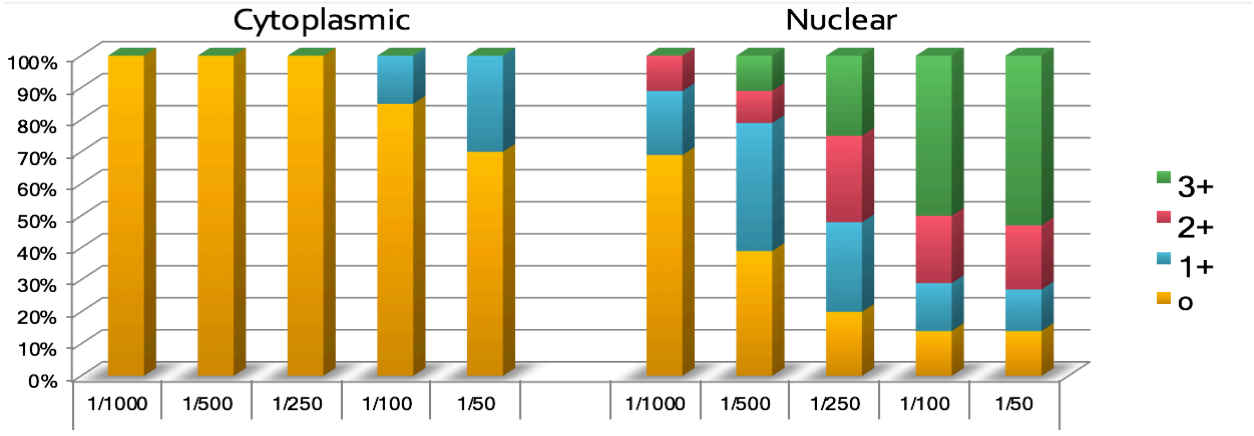

Figure. 2

(A) **(B)** **(C)**

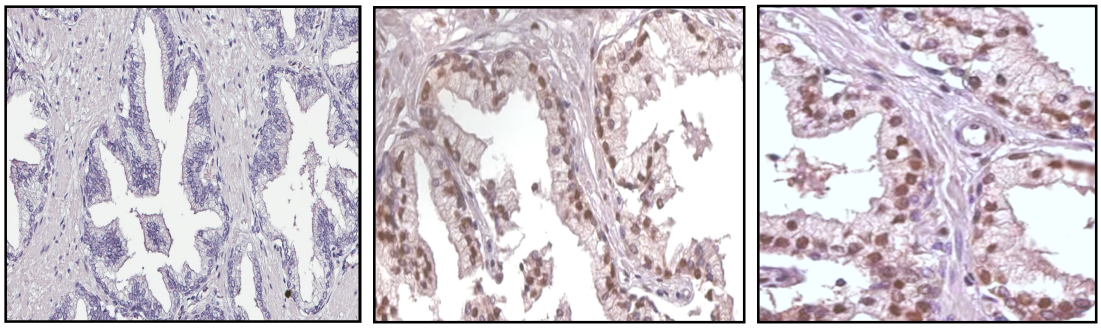

Figure. 3

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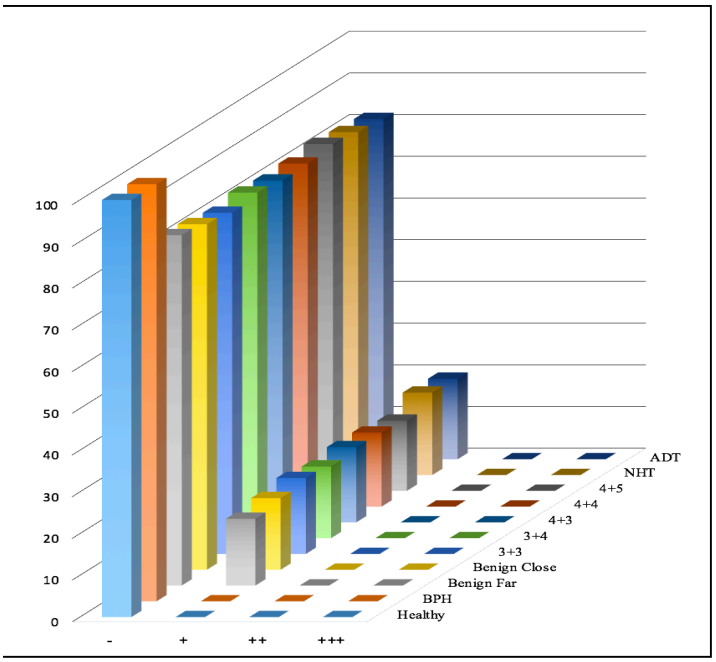

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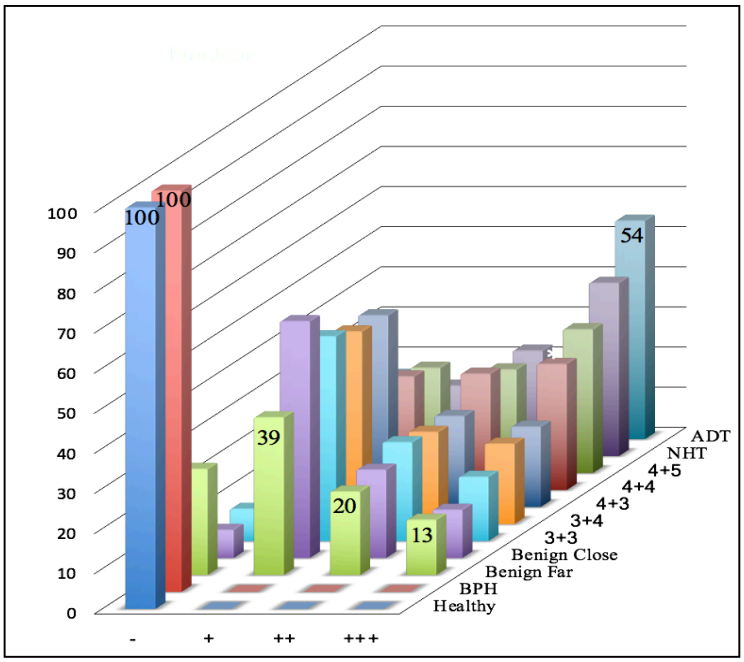

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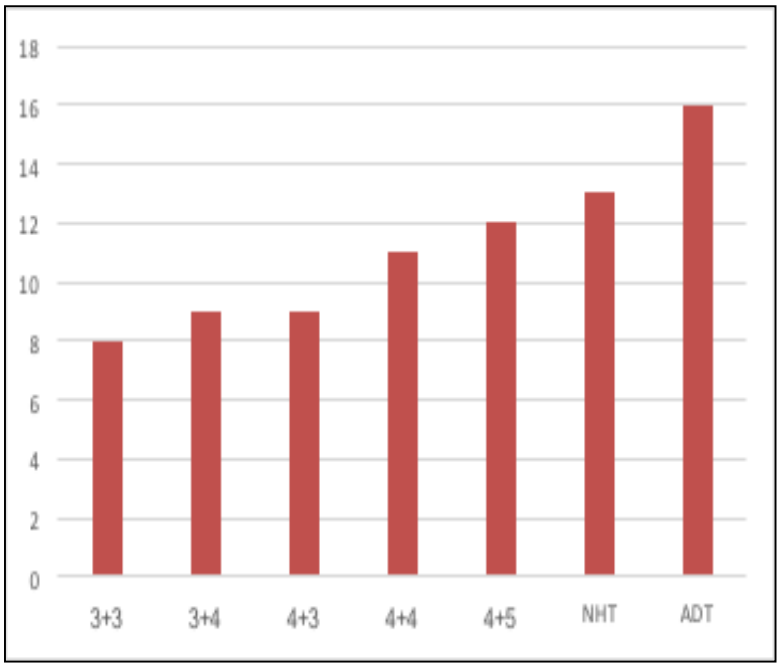

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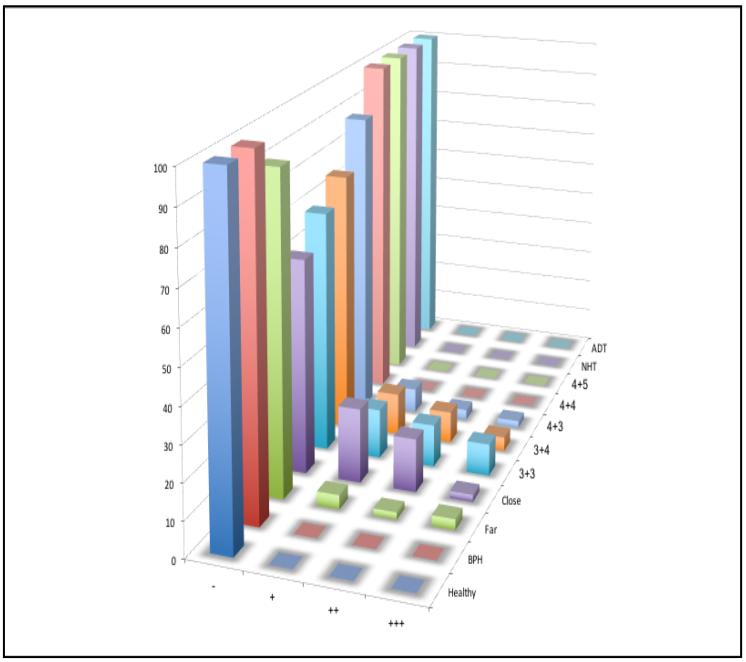
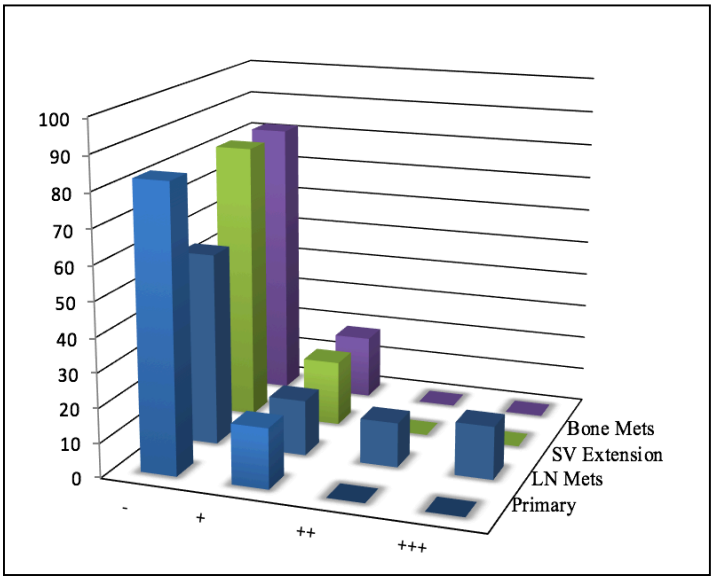


Figure. 4

(A)


100
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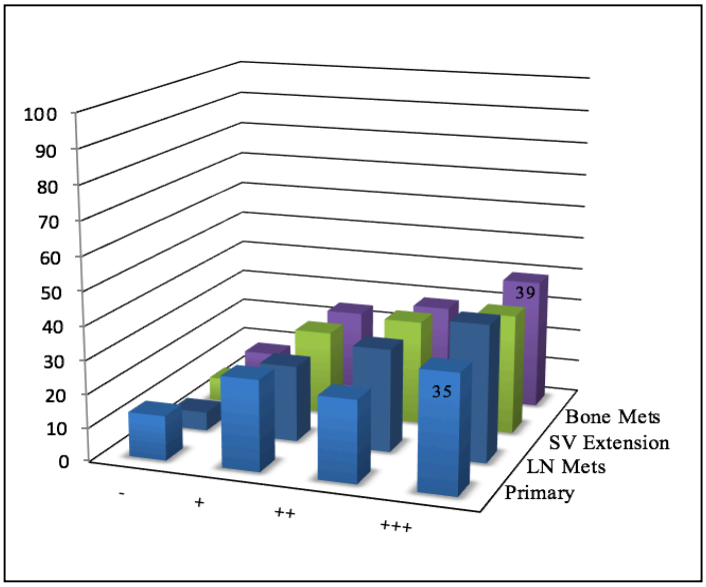
SV Extension
LN Mets
Primary

(B)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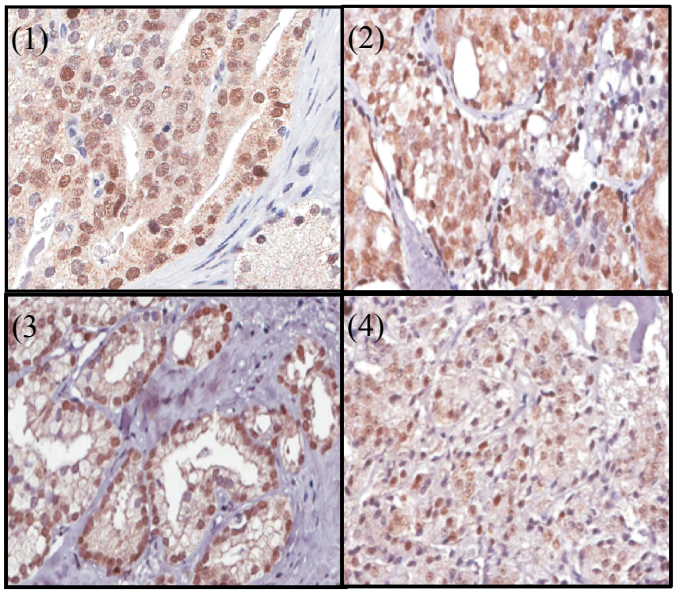
LN Mets
Primary

(C)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(2)
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Figure. 5

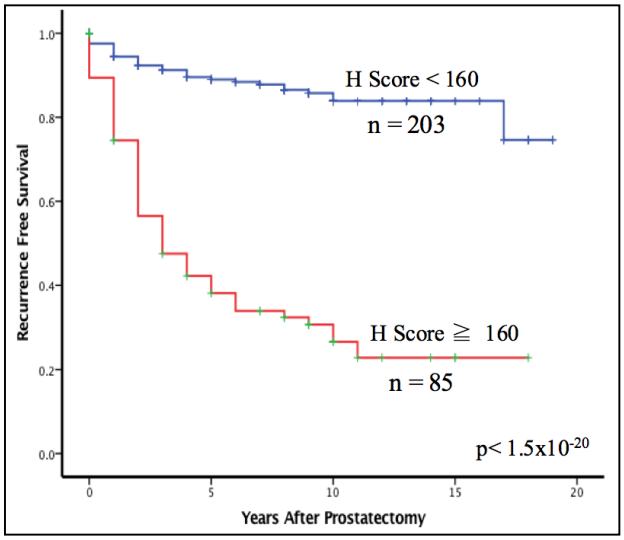


Figure. 6

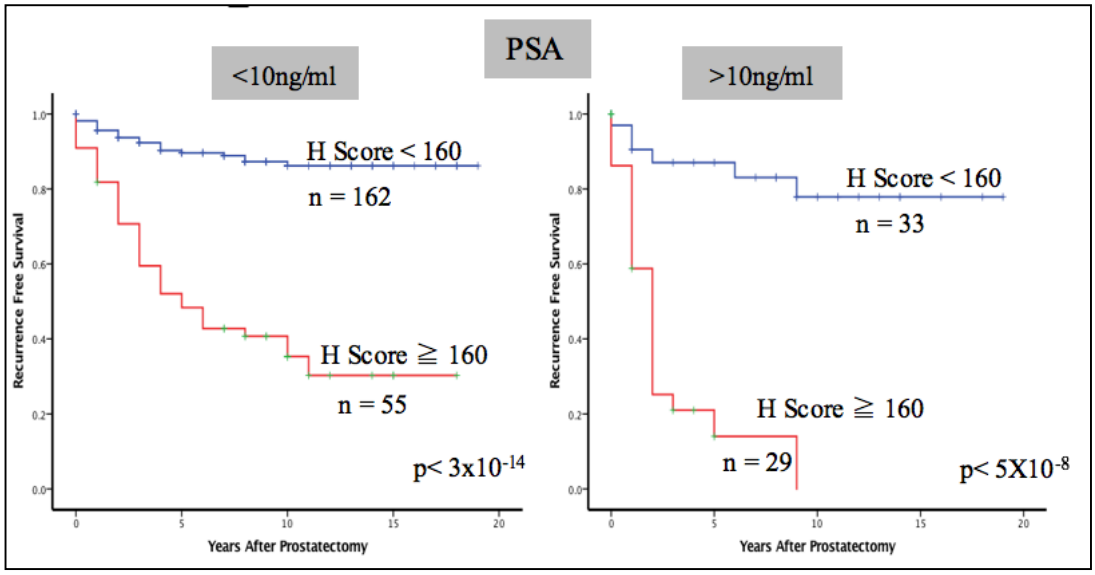


Figure. 7

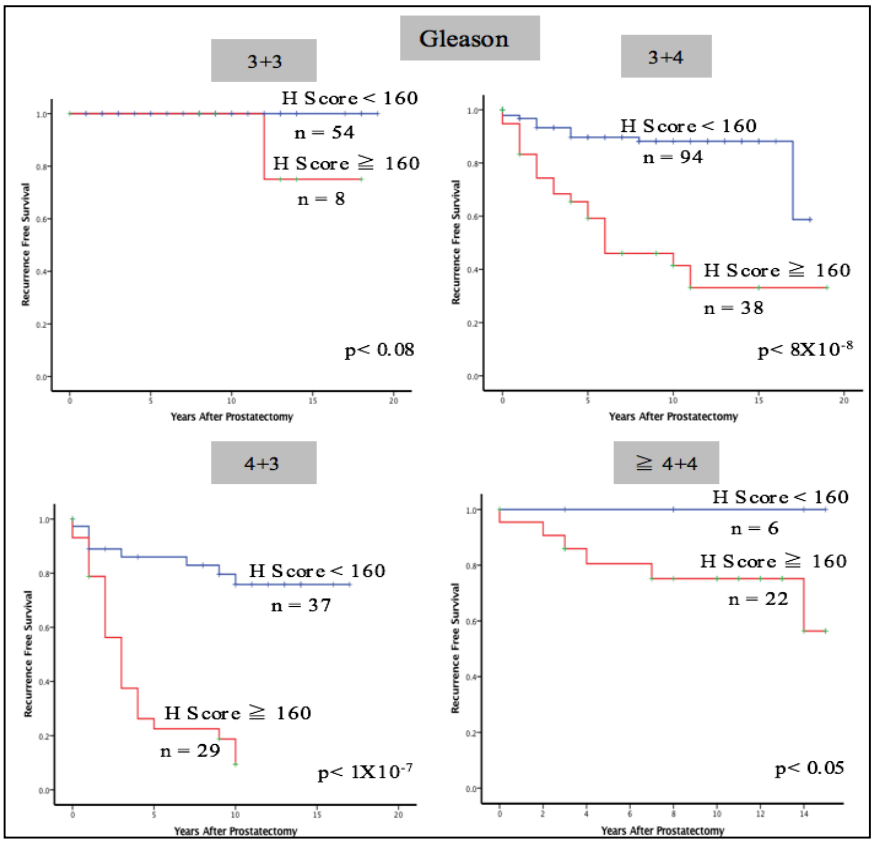


Figure. 8

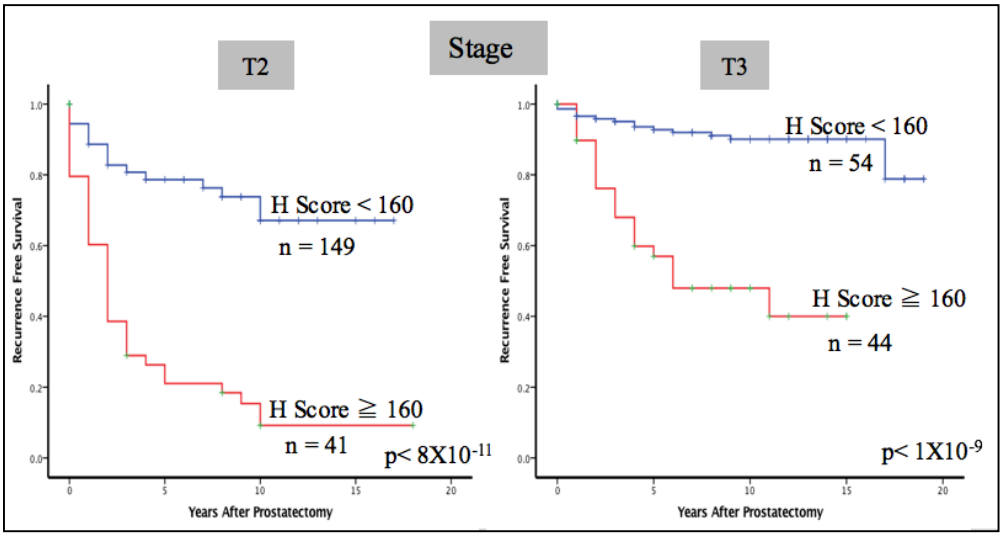
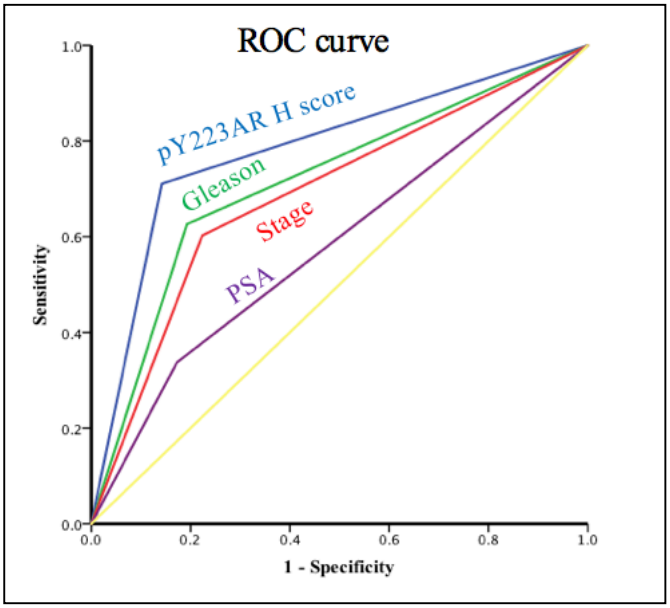


Figure. 9


ROC curve
pY223AR H score
Gleason
Stage
PSA
Sensitivity
1 - Specificity
0.0
0.2
0.4
0.6
0.8
1.0

5. Chapter #4

DISCUSSION AND CONCLUSIONS

5.1 Discussion

The main objective of my thesis was discerning whether previously observed in vitro PCa cell line AR expression and activation had some clinical relevance. Given my medical background, testing the prognostic value of proteins of interest appeared to be a logical strategy to verify their potential as biomarkers. It was challenging but achieved as planned initially. My studies confirmed that the post-translational modification of AR, occurring in vitro through Fer, is an important regulatory mechanism that deserves to be considered for its implication in PCa progression. As proposed, the quantification of pY223AR in tissues from patients with prostate cancer showed that pY223AR correlates significantly with outcome. The quantification of AR was done to strengthen the pY223AR value, by showing that knocking down Fer or inhibiting its activity in cell lines hampers AR activation and has consequences on IL-6 mediated growth and gene expression such as PSA. The fact that Fer is not expressed in normal prostate but present at high nuclear levels in advanced CRPC [110][113][116] is supportive of Fer acting on AR in tumor cells. Without activation, the AR protein as detected by both its N- and C-terminal portions of AR structure, had no apparent role in patient outcome (BCR), using a RP cohort of localized PCa, whereas AR proteins detected from the C-terminal epitope was associated with disease progression. Moreover, this AR study revealed important observations that may shed light on the understanding of this key TF in PCa progression. Our findings on AR, detected through two epitopes located in the N- and C- terminal domains of the protein, support that splice variants are

expressed when tumor cells are of high grade (GS8 and over) and when cancer is aggressive, as witnessed through seminal vesicles invasion, LN and bone metastases. These findings also apply to primary tumors of cases for whom we studied LNs metastases in parallel and patients who received ADT prior to surgery. Therefore, cell characteristics in the higher risk patient category and the patient hormonal status -ADT would contribute to the emergence of AR variants along with full length AR during progression. The role of these AR proteins in progression would therefore be the result of increased expression (AR detected by its N-terminal domain was at highest levels in advanced disease) and activation through Y223 phosphorylation, also observed at highest levels in advanced disease. It is noteworthy that the pY223 motif is present in the N-terminal (AF)-1 domain and therefore in all forms of AR, including splice variants. Our quantitative findings on nuclear AR (all forms and full length alone) in the prostate of adults below 45 years old and BPH patients revealed that AR was found in 99.7% of luminal cell nuclei, with moderate to high expression, while being present in 42.1% of basal cells and at a low expression using both antibodies (C- and N- terminal). In the basal layer, stem cells are suggested to be the origin of PCa once transformed. Precancerous lesions in the prostate appear as proliferation of cells leading to disruption of the basal cell-layer. Cancerous lesions appear as proliferation spreading beyond the basement membrane. Given these facts, the disruption of the basal-cell marker expression could be considered as marker of PCa. What is clearly meaningful is that our study showed that AR is present in subsets of basal cells and missing in other subsets. On the other hand, activated AR is totally negative in normal and hyperplastic prostate cells, known not to express Fer. These observations in human tissues are in line with the host lab discoveries made in cell lines. The absence of pY223AR in prostate tissues from healthy and benign cases is likely due to the absence of Fer.[113] Fer appears as a stress factor, required for

survival and growth signaling mechanisms in PCa cell lines.[113] As AR is a TF and substrate of Fer, its Y223-phosphorylation and translocation to nucleus is not possible likely due to the absence of Fer in prostate tissues from healthy and benign cases. [113]

The situation changed significantly in cancer. The quantification of AR, all forms vs. full length alone, using N- and C- terminal Abs on tissues covering the whole spectrum of the disease has allowed us to better understand how AR may be involved in aberrant signaling pathways due to enhanced expression of AR and Fer, and crosstalks between pathways taking place in PCa cells. As seen in this study, there are differences in AR expression with progression to advanced disease during ADT and metastasis. As the disease progresses, multiple changes affecting AR levels occurred. Our observations suggest that androgen depletion in ADT cases affects AR expression, leading to differences not only in levels but forms present. Increased expression of AR with ADT suggests an overexpression in tumor cells devoid of circulating androgens, or else surviving and growing in such environment. Whether this process is meant for a hypersensitivity to locally-produced androgenic precursors by enzymes of the steroid metabolism chain or other factors as an aberrant signaling mechanism is still to be discovered.

Quantifying AR using C- vs. N- terminal Abs led to strikingly different results. The levels of full length AR (C-terminal Abs) remained at low intensity throughout different disease stages while as quantified, shift in levels to higher expression levels, +2 and +3, indicated that all forms were detected. It is noteworthy that this molecular change leading to the shift in number of cells expressing high levels of all forms of AR vs. primarily full length might not have been perceived without the cell by cell quantification approach we used. The shift is believed to be due to the high expression of "shorter" forms of AR, or in other words, abnormal AR proteins or splice variants appearing with disease progression and not encoded by the known AR transcript.

Importantly, splice variants were reported to be more potent than the full length AR to transactivate genes such as PSA[122] while also transactivating distinct sets of genes.[123] On the other hand, in the presence of cancer, the quantification of pY223AR showed an increase in the number of cells displaying elevated pY223AR at 3+ intensity with progression and highest in ADT cases and in metastases. In addition, a shift of intensity similar to AR using N- terminal Abs was noticed when scoring pY223AR, starting from the high risk patient category of GS8 and over and continued in CRPC cases on ADT and metastases. The quantification of pY223AR clearly showed a strong relationship with BCR with the RP cases and further progression in more aggressive cases. The shift to high intensity is believed to be a combination between elevated levels of AR full length and splice variants, being activated through TKs and maybe Fer, and translating in elevated levels of pY223AR. AR activation may be seen as a gradient, going from most cells displaying no AR activation in low grade disease towards a majority of cells with highest activation levels in ADT and metastases. Ideally, if Abs for all AR splice variants become available, IHC could be used to quantify nuclear AR variants and full length AR and show clinical relevance of variants for BCR. A recent report on AR-V7 showed predictive value and unfavorable outcome in high aggressive cases having LN metastases at time of RP.[123] In clinical practice (pathology lab), the staining of prostate biopsy and also prostate FFPE blocks from the prostate RP specimen with the two C- and N-epitopes along with pY223AR may be helpful for diagnosis and prognosis. One would expect difference in staining intensities between AR levels detected with the two C- and N-epitopes along with pY223AR. Quantifying staining using an imaging software and calculating H scores would reduce the likelihood of variations introduced in reviews made by several non-GU pathologists. Obtaining clues on the severity of disease added to other parameters may improve stratification of patients and in the choice of

treatment options. This would ideally imply an internal parallel calibration with already known sections representing extreme situations using well selected controls, i.e. primary tumor GS in low vs high grade and late disease stages (CRPC on ADT), and also from normal and BPH vs benign areas far and close to tumor foci. Lastly, dedicated personnel with solid expertise would be preferable along with review by a GU-pathologist.

Another important aspect of this study was the quantification of AR negative cells with disease progression. The percentage of AR negative tumor cells increased from 0.3% to 14% and 15% using both N- and C- terminal Abs, over the whole spectrum of disease up to cases reflecting PCa progression ($p<0.005$). The fact that ADT/CRPC cases exhibited the highest number of AR negative cells, while AR expressing tumor cells had increasingly higher levels of this protein in their nucleus, implies that two processes are taking place simultaneously in cell subsets of tumor foci. Based on cell lines data,[94][110][116][117] both cell subsets may grow under the influence of IL-6 and other growth factors, in addition to locally produced androgens (for AR positive cells).[94][110][116][117] The host lab reported on IL-6 mediated Fer activation in AR positive tumor cells leading to AR activation, and growing better, and actually adapting, while AR negative tumor cells are being selected and progressively emerge. Nonetheless, tumors and metastases remain populated by a majority of AR positive cells. The fact that AR negative tumor cells are intermingled among AR positive tumor cells speaks in favor of tumor cellular heterogeneity. This particular location of AR negative cells in tumors and metastases may imply some form of communication between cells subsets. It might be unidirectional, with AR-negative cancer stem and neuroendocrine cells acting on neighboring AR positive tumor cells or else bidirectional, with paracrine influences locally and possibly endocrine at distance. Microvesicles like oncosomes may be involved as well. This would also apply in metastases. The identification

of AR negative cells is a topic of great interest and part of future investigations in the host lab.

Results on AR expression in 40-50% of basal cells and 99% of luminal cells, together with their predictable response to castration or ADT, i.e. apoptosis of fully differentiated luminal cells and survival of AR positive basal cells to repopulate the secretory epithelium, would be a model to consider in cancer. Future therapies should not only deplete androgens, but also target AR positive and AR negative cells that survive, thereby preventing their adaptation and selection, respectively, through the mechanisms aforementioned. The percentage of pY223AR negative cells also increased with progression; their detection started in benign glands adjacent to tumor foci also containing pY223AR negative cancer cells. This most likely reflects the absence of AR itself, as the maximal number of AR negative were in the same order of magnitude. Based on the quantification achieved with AR and pY223AR Abs in adjacent tissue sections and comparisons, it appears that AR negative cells are indeed pY223AR negative. On the other hand, not all pY223AR negative cells are AR negative as activation of AR by TKs (Fer or others) may not occur in all cells. Nevertheless, increases in the number of negative cells for both AR and pY223AR are believed to contribute to tumor heterogeneity and cancer aggressiveness, which are likely determinant for progression and failure of treatments. Indeed, stem cells and neuroendocrine cells would be AR negative cells and current therapies like abiraterone and enzalutamide mainly target AR-positive tumor cells, themselves partly resistant due to the expression of constitutively activated AR variants at the level of transcription. The AR activation process described in this thesis would contribute to AR transcriptional activity. This was shown earlier in the host lab, by site directed mutagenesis replacing Y223 by phenylalanine, and which resulted in altered transcripts of specific genes.[116]

A study recently described how cytoplasmic variations could be a phenotype of androgen-independent cancer.[124] It suggested that while AR in the nucleus acts on the transcription of AR-dependent genes implied in the progression of PCa, cytoplasmic AR could participate in aggressive signaling pathways, promoting extracapsular invasion and towards seminal vesicles.[124] Our findings on cytoplasmic staining using AR N- and C- terminal Abs or pY223AR abs rather showed equal and no consistent changes in levels in high grade cancers and advanced stages, while the increase in nuclear intensity rather than cytoplasmic escalation was statistically significant, in agreement with a recent study.[125]

Cellular elements present in the stroma surrounding tumor foci were considered, since they likely enter into play in PCa development and progression. The AR expression in stromal fibroblasts from the normal prostate to GS grade 7 and its complete loss in advanced PCa along with its activation - pY223AR in benign areas adjacent to cancer solely in GS6 and GS7 cases of the RP cohort and not afterwards are interesting observations suggestive of a loss of function. Fibroblastic AR in the stroma controls and release proteins like placental growth factor, IGF-1 and phosphoprotein-1.[124] These proteins have a role in epithelial growth rescue and homeostasis control.[124] Absence of these growth factors, results in the loss of the capability of fibroblastic AR to control its microenvironment and signaling in nearby epithelial glands, leading to further dedifferentiation and glandular breakdown.[124] Our results using the AR C- terminal Abs and pY223AR Abs are in line with this study,[124] as well as with a more recent report on AR in localized and recurrent PCa.[126] Similar results were obtained with the AR N-terminal Abs (unpublished data; T. Benidir, McGill MSc thesis).

The quantification of inflammatory cells (both AR and pY223AR negative) was carried out while scoring pY223AR staining of prostate tissues from the RP cohort to answer a key

question: Does inflammation contribute to AR activation -pY223AR in cancer cells through the secretion of IL-6 or other cytokines in its microenvironment, resulting in an increase of their Fer activity? Cytokines are produced by both cancer cells and inflammatory cells.[127] Inflammation, as categorized as mild, moderate and high grade by the number of lymphocytes adjacent to tumor foci, had a significant correlation with BCR of the RP cases. As shown, moderate and high grade inflammation alone correlated with BCR, in agreement with another study.[128]

Comparison of statistical survival analyses of nuclear AR (all forms and full length alone) and activated AR were done in continuity to search for significant differences of expression and activation with different stages of disease. AR (all forms and wild type alone) showed no significant correlation of H scores with BCR in the RP cohort. This implies that non-activated AR proteins taken as a whole have a minimal role in tumor cells of primary tumors, despite increases in intensities. Hence results in tumor cells of more aggressive cases and the differential expression noticed with the N- vs C-terminal AR Abs from GS8 and above suggest that splice variants and other complexes, other than AR full length itself, would play a role and predict patient survival once activated on Y223 by TKs like Fer. As previously mentioned, splice variants play important roles in progression, because of their constitutive transcriptional activation, as recently documented for AR-V7. The likelihood that these proteins are fully activated in cancer cells may indeed rely on Y-phosphorylation. So far, beside AR-V7 and AR-V12, most other variants are still under study.[104][105][121] As mentioned, once fully identified and that Abs becomes available, the likelihood is that these AR proteins will significantly correlate with patient outcome when analyzed in different cohorts of patients. The key aspect of our analysis is the clinical relevance of our observations, with very high significant correlations between nuclear pY223AR H scores and BCR. The correlation appears to be very strong and was

recently validated in a CHUM cohort of RP patients (Movember GAP1 initiative). In addition, the H scores of pY223AR correlated with patient outcome when combined with standard parameters. Also, Cox regression and ROC curves showed the significant addition and prognostication value over standard parameters.

4.2 Future Directions

The identification of AR-negative cancer cells in tumors, as neuroendocrine and cancer stem cells is a field that deserves future investigation. The use of immunofluorescence techniques with several probes distinguishing each protein of interest in specific cells and in relation to AR proteins and their activation would add tremendously to the understanding of processes evolving in cells during each patient trajectory through the disease. Fer would be included as well, since it not only activates and binds pY223AR but also translocates in the nucleus with activated proteins as complexes in cell lines. Current study in the host lab have shown that small AR variants are also Y-phosphorylated. This could lead to identification of potential targets for drug design. In situ hybridization with well-designed probes, this would also be an additional mean to investigate the expression of transcripts encoding AR variants revealed in transcriptome profiling. Proteins would be prioritized over transcripts because signaling pathways are ensured by dynamic reactions involving enzymes, and in the present case, Fer, driving its substrates and partners, two TFs in their site of action, the DNA.

4.3 Conclusions

This translational study from a pY-site identified in AR through in vitro assays as a result of Fer, using AR as a substrate and nuclear partner in cell lines exposed to IL-6, has reached its primary

goal. It shows that AR activation in PCa tissues has clinical significance and predicts BCR in patients. The Fer TK may be a factor, given cell lines data, and due to observed Fer expression patterns (absent in normal prostate epithelial cells and overexpressed in advanced PCa). Inflammation at moderate and high levels which also correlates with BCR, accentuates the worsening of disease attributed to activated AR in cancer cells, speaking for liberated cytokines likely contributing to disease progression. Fibroblastic AR, which is activated at specific time points in the disease evolution, may play a role in the transition from GS7 to GS8. Altogether, these findings suggest that this AR post-translational modification on the full length protein and shorter variants is a critical factor to consider in aberrant AR signaling and transcriptional activity during progression. Our hypothesis was confirmed, pY223AR represents a novel biomarker with prognostic value in PCa, predicting survival probability and being most activated in advanced disease. AR activation adds to the value of other standard parameters, and may stands as a more powerful marker for better patient grade stratification and management, especially for the intermediate grade. A promising avenue that is being explored, would be the combination of Fer and pY714Fer, together with pY223AR and pY705STAT3 as a set of markers of progression.

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