Prostate Cancer Circulating Tumor Cells: Automated and Manual Enumeration after Isolation via Size-Based Filtration of Pre-Treatment Patient Samples.

BY

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ABSTRACT

Circulating Tumor Cells (CTCs) have emerged as a potential source of clinical significance for the porpose of clinical use in situations such as diagnosis and prognosis. But with numerous isolating systems currently available, the numbers of captured CTCs vary widely. At this point, CellSearch remains the only FDA-approved system with clinical significance whereby the results could be used to monitor patients with metastatic colon, breast, or prostate cancer. However, its inability to isolate CTCs from non-high risk prostate cancer patients or CTCs that are EpCAM-negative has led to criticism. In this study we have shown that size-based filtration successfully isolates CTCs from patients with localized and metastatic prostate cancer. We have also shown that CTCs can be successfully isolated from low and intermediate risk groups. Additionally, clusters of CTCs were preserved and isolated in all localized risk groups and metastatic patients. Furthermore, we enumerated the isolated CTCs using automated and manual methods in low risk, intermediate risk, high risk, and metastatic prostate cancer. The automated and manual counts were comparable. Moreover, the quantity and size of clusters correlated with the status and stage of prostate cancer.

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LIST OF ABBREVIATIONS

µ	Micro
5AR-1	5-α-Reductase Type 1
5AR-2	5-α-Reductase Type 2
5ARi	5-α-Reductase Inhibitor
5HT	Serotonin
Ab	Antibody
ADP	Adenine Diphosphate
ADT	Androgen Deprivation Therapy
ACTH	Adrenocoricotrophic Hormone
Ag	Antigen
AR	Androgen Receptor
ATP	Adenine Triphosphate
BMP	Bone Morphogenic Proteins
BOO	Bladder Outlet Obstruction
BPH	Benign Prostatic Hyperplasia
CAM	Collagen Adhesion Molecules
cfDNA	Circulating Free DNA
cfRNA	Circulating Free RNA
cfNA	Circulating Free Nucleic Acids
CMV	Cytomegalovirus
CNS	Central Nervous System
CRE	*Cis* Acting Element
CRH	Corticotrophin Releasing Hormone
CRPC	Castrate-Resistant-Prostate-Cancer
CSF	Cerebrospinal Fluid
CTC	Circulating Tumor Cell
CYP17	Cytochrome p17
DHT	Dihydrotestosterone
DNA	Deoxynucleic Acid
DMFO	Diflouromethulornithine
EMT	Epithelial-Mesenchymal Transition
ER	Estrogen Receptor
FGF	Fibroblast Growth Factor
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
GU	Genitourinary
HCG	Human Chorionic Gonadotrophin
HGPIN	High Grade Prostate Intraepithilial Neoplasia
Hh	Hedgehog
HHV	Human Herpes Virus
HMG	High Mobility Group Proteins
HPV	Human Papilloma Virus
HRLPC	Hormone Resistant Localized Prostate Cancer

HSP	Heat Shock Protein
HSV	Herpes Simplex Virus
IDC-P	Intraductal Carcinoma of the Prostate
KLK	Kallikrein
KS	Ketosteroid
LGPIN	Low Grade Prostate Intraepithilial Neoplasia
LH	Luteinizing Hormone
LHRH	Luteinizing Hormone Releasing Hormone
MAPK	Mitogen Activated Protein Kinase
MIF	Mullarian Inhibiting Factor
NAAG	N-Acetyle-Aspartyl-Glutamate
NADPH	Nicotinamide adenine dinucleotide phosphate
OND	Ornithine Decarboxylase
PAP	Prostatic Acid Phosphatase
PCa	Prostate Cancer
PIN	Prostatic Intraepithelial Neoplasia
PRL	Prolactin
PSCA	Prostate Stem Cell Antigen
PSMA	Prostate Specific Membrane Antigen
PSP-94	Prostate Specific Protein – 94
PTH-RP	Parathyroid Hormone Related Peptide
RARE	Retinoic Acid Response Element
RNA	Ribonucleotide Acid
RP	Radical Prostatectomy
RT	Radiation Therapy
SHBP	Sex Hormone Binding Protein
SMT	Seminiferous Tubules
STD	Sexually Transmitted Disease
STI	Sexually Transmitted Infection
TeBG	Testosterone Binding Globulin
TF	Transcription Factor
TGF	Transforming Growth Factor
TRUS	Transrectal Ultrasound
TSH	Thyroid Stimulating Hormone
TURP	Transurethral Resection of the Prostate

INTRODUCTION

1.1 FOCUS OF STUDY

The focus of the study is to evaluate the feasibility of size-based filtration of prostate cancer blood samples as well as enumerating the filtered CTCs and clusters for the purpose of clinical correlations. In this single blinded-study we to utilized pretreatment patient blood samples to assess those labeled as low risk (Protein Serine Antigen (PSA) <10 ng/ml and/or Gleason <6 and/or clinical stageT2a), intermediate risk (PSA 10-20 ng/ml and/or Gleason =7, and/or clinical stage T2b), high-risk (PSA>20 ng/ml and/or Gleason ≥8 and/or clinical stage T2c/T3), and metastatic (osseous, nodal, visceral, regional, and/or distant) prostate cancer. We will isolate circulating tumor cells (CTCs) from a peripheral blood access and assess the morphological circulating tumor cell (CTC) features, as well as CTC enumerations prior to any form of treatments. By numerical CTC counts and morphological features of CTCs, the objective is to find whether size-based filtration is a reliable method to isolate CTCs that can be studied for the classifying the various stages of prostate cancer.

The stratified patients were collected using the D'Amico criteria[1] will each have their pretreatment blood samples analyzed for CTC isolation, CTC automated enumeration, CTC manual enumeration, CTC cluster morphology, and CTC sizes. These findings will allow us to understand the numerical and morphological CTC variation amongst the various cohort groups.

1.2 PROSTATE CANCER

1.2.1 EPIDEMIOLOGY

Since 1984, prostate cancer has been the most common non-cutaneous malignancy in men and constitutes a quarter of all cancers in this category (in North America)[2]. The lifetime risk and death of the disease are 16.52% and 2.37%, respectively (American Cancer Society, 2016)[3]. *Table 1* shows the various incidence and mortality rates amongst the different racial groups. Despite the decline, African Americans continue to have the highest incidence and mortality.

Table 1: The Incidence and Mortality of Prostate Cancer Based on Ethnicity

Ethnic Group	Incidence*	Mortality
Whites	161.4	25.6
African Americans	255.5	62.3
Hispanics	140.8	21.2
Asian-Americans	96.5	11.3
Aboriginals	68.2	21.5

*Values are per 100,000 and collected between 2000-2004
Data collected from the National Cancer Institution (NIH, 2016)4 and American Cancer Society (Cancer, 2016)[3]

Prostate cancer (PCa) has been on the rise, and since the introduction of the PSA in 1987, the incidence peaked dramatically by 1992 (American Cancer Society, 2016)[3]. However, a drastic reduction was evident ("Cull Effect") followed by a steady pre-PSA era-like rise.

Racial differences, as depicted in ***Table 1***, have been attributed to environmental exposures, diet, lifestyle, attitude to health care, and to a much lesser extent, the differences in genomic profiles[5]. Racial differences continue to be a factor in the statistical variations[5]. For example, African Americans are more likely to undergo androgen deprivation therapy (ADT), external beam radiation therapy (EBRT), and watchful waiting, but are less likely to receive radical prostatectomy (RP) than white[6].

According to the American Cancer Society[4], worldwide incidence and mortality of

prostate cancer varies significantly between different nations. Worldwide, it is the fifth most common malignancy and second most common cancer in men[4]. Additionally, the incidence varies according to each counties' socioeconomic status. PCa makes up 11.7% of the overall new cancer cases, with 5.4% in developing and 20% in developed countries[4]. The lowest incidence is seen in Asia, specifically China[3,4,5]. In North America, prostate cancer has been increasing worldwide[3]. Though the PSA could be a significant contributing factor, prostate cancer (PCa) has been increasing prior to PSA screening[2,6]. This could likely be due to increased transurethral resections of the prostate (TURPs)[2,6]. Lastly, for unknown reasons, PCa is highly prevalent in Nigeria and Uganda, and is the most common cancer in the former[7].

As for mortality, the highest has been observed in the Caribbean (32/100,000) and the lowest in oriental Asia and north Africa (<5/100,000). Mortality declined more in countries with more frequent PSA testing than in countries that do not routinely screen with PSA[5]. Interestingly, as people become more 'westernized', the risk of prostate cancer and its mortality increases[8,9]. For example, Chinese and Japanese immigrants to the United States have an increased risk of acquiring PCa compared to the relatives in their home country - Note that Asians still have a lower risk than Whites and African Americans[8,9].

As a disease of elderly, PCa is rarely diagnosed prior to the age of 50 years old (2%). The median age of diagnosis is 68 years, with the majority (63%) of cases diagnosed after 65[10]. Autopsy findings show microscopic evidence of PCa in 30%, 50%, and 75%

of non-cancer labeled patients who are in their 4th decade, 6th decade, and those over 85 years of age, respectively[11]. PSA screening of the most effected patients in the 5th decade has led to a marked increase (50%) in prostate cancer diagnosis from the pre-PSA era to 1992[6,11].

It has been shown[12,13] that the increase of PSA screening is the largest contributor, if not the exclusive contributor, to the substantial clinical stage migration in newly diagnosed patients who now present with more favorable disease, more loco-regional disease, and less metastatic disease. These advantageous findings have improved the overall 5- and 10- year disease-specific survival in all stages to 99% and 91%, respectively[12,13]. The non-palpable T1c presentations account for 60-75% of all newly diagnosed PCa patients[5,6]. There are two published trials[14] that correlate PSA testing with clinical outcomes[15,16]: 1) “Prostate, lung, colorectal, and ovarian cancer screening (PLCO) trial”, and 2) the “European randomized trial for screening of prostate cancer (ERSPC). First, the PLCO trial in the USA showed there is no evidence for prostate cancer mortality reduction after their 76,693 patients were followed for 7 years. However, criticism centered on the short follow up period for a cancer that has a long natural history. The other study in Europe, the ERSPC trial, concluded that after following 162,243 for a median of 9 years, there was significant evidence that routine PSA screening for men 50-69 years of age would reduce the rate of PCa mortality by 20%. However, the financial burden was that to prevent a single PCa-related death, 1410 men would need to be screened with PSA and 48 would need to be treated. Therefore, due to the indolent nature of the disease, morbidity needs to be seriously

considered when evaluating screening and subsequent treatment options. The American Urologic Association (AUA)[17] in 2009 recommended that PSA screening should be considered in all asymptotic men ≥40 years and who have a life expectancy of >10 years.

1.2.2 ETIOLOGY AND RISK FACTOR

1.2.2.1 EPIDEMIOLOGICAL RISK FACTORS

There is consistent evidence that genetic and environmental factors play a role in the risk of PCa. These evidences have been concluded in numerous case-control, cohort, concordance, and metanalysis studies[5]. The risk of PCa is highly associated with the number of effected relatives (especially first-degree), the number of effected members, and the age of diagnosis[18]. There is significant consistence of a higher risk if a brother, rather than a father is diagnosed[5,18]. This supports the theory of X-chromosomal linkage or recessive gene association[19]. *Table 2* shows the various risk of prostate cancers amongst the different family histories. The number of affected relatives and their age of onset (especially if <55 years) play a significant role in the risk factor determination.

Table 2 The Correlation Between the Degree of Family History and Prostate Cancer

Family History	Relative Risk*
None	1
Father Affected	2.17
Brother Affected	3.37
First-Degree Family Member Diagnosed at <65	3.34
>2 First Degree Relatives Affected	5.08
Second-Degree Relative Affected	1.68

*Data Based on Meta-Analysis from[19]

Prostate cancer can be designated as sporadic (85%), familial, or hereditary. In sporadic PCa, there is negative family history[2]. Familial PCa requires >1 effected relative[2]. Meanwhile, hereditary PCa is defined as PCa that effects patients with ≥3 effected men in a nuclear family, or ≥3 successive generations, or ≥2 family members

diagnosed before the age of 55 years[20]. Hereditary PCa affects 43% of all early onset (<55 years) PCa and only 9% of those ≥85 years.

1.2.2.2 GENETIC RISK FACTORS

The importance of *BRCA1* (17q21) and *BRCA2* (13q12) mutations have been extensively studied in breast and ovarian cancer. Likewise, and especially for *BRCA2* mutations, there is interest for their roles in prostate cancer[21]. There is a cumulative risk of 30% by 80 years and an increase of 5-7 folds in the development of prostate cancer in patients with *BRCA1* and *BRCA2* mutations, respectively[21].

PCa susceptible genes include *RNaseL* (Hereditary Prostate Cancer 1 -*HPC1*- region, 1q23-25), *ELAC2* (*HPC2* region, 17p), *MSR1* (8p22-23), *PCAP* (1q42.2-43), and Xq27-28[22-26]. Additionally, SNPs have been associated with PCa with loci located on chromosomes 3, 6-8, 11, 17, 19, and X.

Of the previously listed genetic loci, *HPC1* gene[22] is the best studied. The mutated form is likely autosomal dominant with very high penetrance. Thus, although rare to have, this mutation not only puts a patient at a very high risk of having prostate cancer, but also this form of PCa presents with a higher grade and more advanced disease. *HPC1* encodes for the RNaseL enzyme which is an important enzyme for the innate immune system when combating viruses[27]. Pathogen-Associated Molecular Patterns (PAMP) of viruses (i.e. dsRNA) enter the host cell and activate a 2-5A Synthetase enzyme to produce a '2-5 A' molecule that binds to and activate RNaseL enzyme to eventually cause RNA degradation, interference with cellular protein synthesis, and eventually

apoptosis. This process is facilitated by interferons that act as transcriptional factors to increase the levels of 2-5A Synthetases. The arginine to glutamine substitution in *HPC1* results in SNP R462Q which may cause an increased risk of PCa[27]. (*Table 1*) shows a list of susceptibility genes associated with prostate cancer.

Table 3: Genetic Risk Factors to Prostate Cancer

Functional Loss	Involved Mutated Genes
Inflammatory Mediators	*MIC1, MSR1*
Antioxidant Enzymes	*MSR1, PON1*
DNA Repair	*OGG, CHEK2, BRCA2*
Susceptibility to Infections	*MSR1, RNase1, TLR4*
Apoptosis	*RNaseL*

Table shows the list of genes associated with a functional loss that directly or indirectly increase the risk of mutations and thus malignancy in Prostate Cancer.
Data taken from Campbell-Walsh Urology[2]

1.2.4 THE STEROID RECEPTOR

1.2.4.1 STRUCTURE OF THE ANDROGEN RECEPTOR AND ITS GENE

The Androgen Receptor (AR) gene is located on the X-chromosome (Xq11.2-q12), and thus males have only one copy. Information is used from 8 exons (17% of the total gene) to transcribe and synthesize the AR protein. There is some homology[70] between the genes of AR, Estrogen Receptor (ER), Progesterone Receptor (PR), and glucocorticoid receptor (GR), with the closest being the PR[71]. The AR gene is subdivided into three domains (*Figure 1*) : amino-terminal, DNA-binding, and carboxyl-terminal domain. The regulatory promoter (5') is unique because it contains a GC box, rather than the classic TATA or CCAAT found on genes with affinity to polymerases. As the the initiation site is approached, there is a purine rich *cis*-acting response elements (CREs).

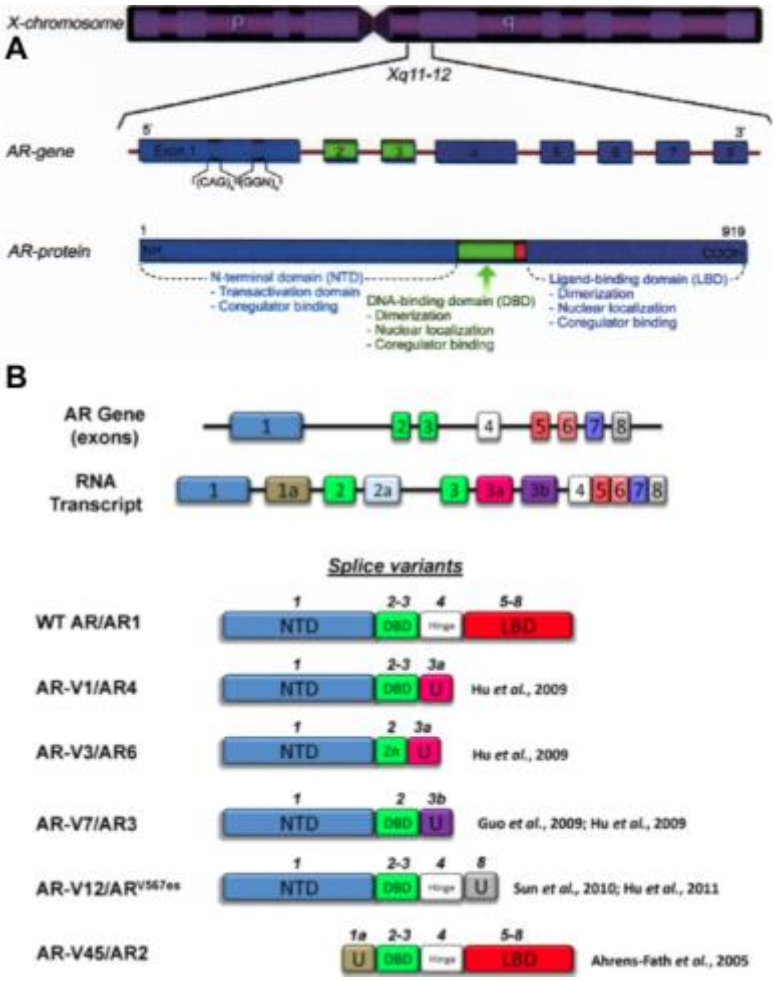

Figure 1: The Androgen Receptor Gene[72,73]

A. The AR is encoded on chromosome X (q11-12). Three unique DNA segments make up the AR gene. 1) The NTD which is a site on the gene that different molecules can bind to for the purpose of regulating the gene's transcription. 2) The DBD is the area of the gene that when translated, gives the AR the ability to bind to other DNA segments. 3) The LBD is the part of the gene that when translated would allow other molecules (i.e. DHT) to bind to the AR. Note that the AR gene consists of only 8 exons (17% of the total gene). Interestingly, other steroid hormones such as ER and PR show high homology rates with the AR. Additionally, AR mutation typically occurs at the LBD and therefore it would not be wise to target it for therapeutic purpose. However, the N-terminus (NTD) can be targeted because no mutations are known to occur there. **B.** Shows the different ways the AR can be spliced to generate similar, but not identical, ARs. This process has been blamed for resistance of prostate cancer to ADT. Currently, the AR-V7 is most studied.

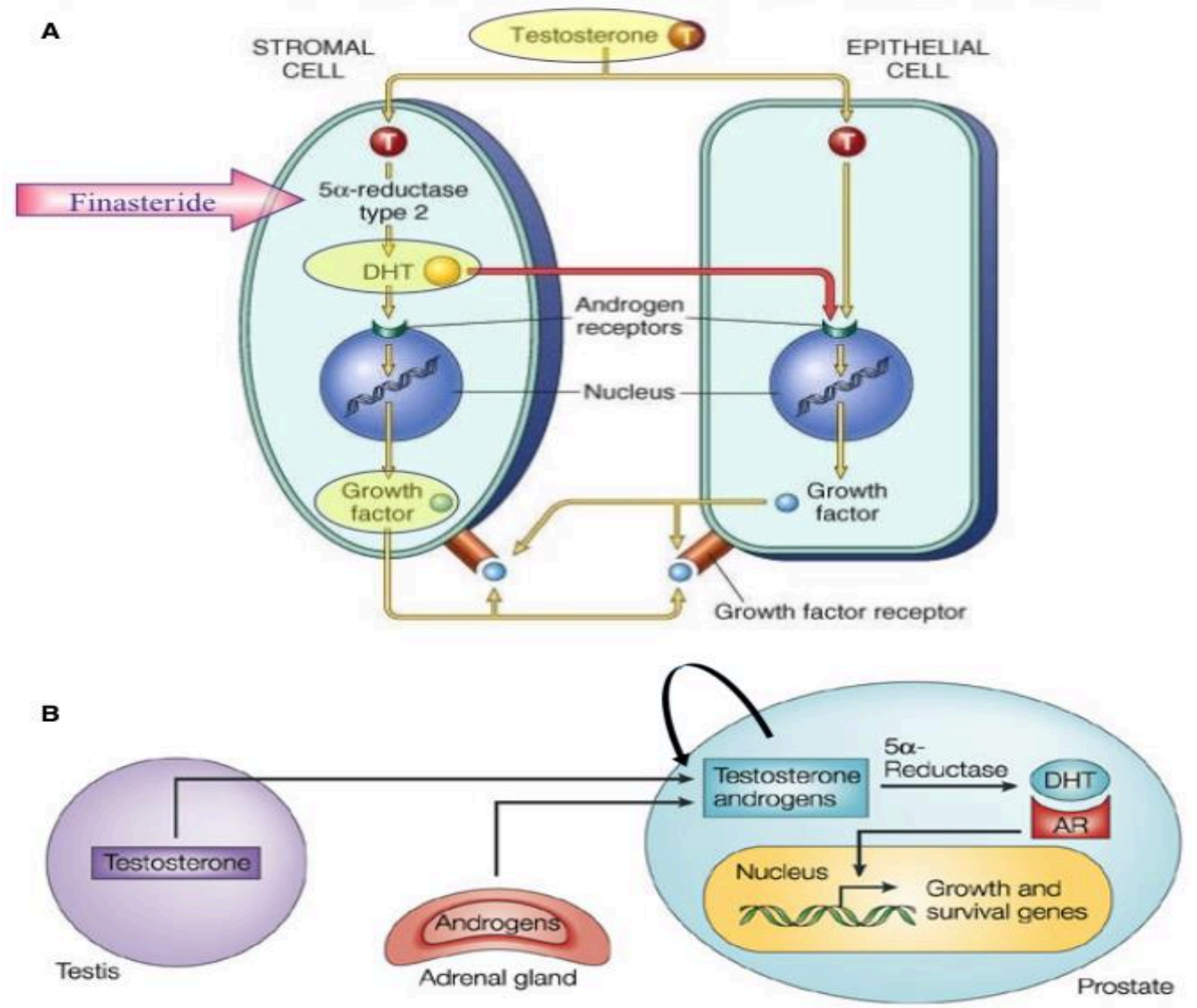

Figure 2: The Role of the Androgen Receptor[74]

A. Shows a schematic overview of the pathway of prostatic growth. On the left is a stromal cell which typically is not involved in prostate cancer, but rather involved in BPH. On the right is a prostatic epithelial (glandular) cell, which is the culprit in most PCa cases. As seen in the diagram, Testosterone (T) enters both cells. In fact, T can behave directly as a ligand to the AR. However, it is much more potent if it is converted first to DHT via the 5-AR enzyme. Once either ligand (T or DHT) binds to the AR, the enzyme-ligand complex behaves as a transcriptional factor (TF) that promotes hypertrophy, hyperplasia, growth, and differentiation. Note: epithelial cells do not contain this enzyme, and thus the source of DHT for the epithelial cells comes from the stroma. Note: the medication Finasteride (Proscar®) is an inhibitor of 5AR. It is used in the treatment of BPH. Proscar® has no role in the treatment of PCa, an epithelial (glandular) adenocarcinoma, because epithelial cells do not have the enzyme. **B.** Shows the different sources of androgens. Androgenic sources include the testis, the adrenal gland, and even the prostatic tumor itself (via autocrine fashion). This is important for targeted therapy.

The AR is activated by a multitude of various factors[57-60,71] that together allow for its expression. (*Figure 2*) shows a simplified overview of the role and function of the AR in prostate cell. These factors are:

1) DNA-Binding Domain:[72,73] The DNA binding domain is an area on the AR that allows it to bind to a DNA segment (known as an androgen/hormone response element) where it will function as a transcription factor (*Figure 1*). This domain is found between exon 1-2 of the AR gene (at the 5' N-terminus of the gene) and consist of two zinc finger motifs which are crucial as they facilitate DNA recognition. Androgen-insensitivity syndrome, characterized by 'resistance' to the AR, is actually in part due to a mutation[57] in the DNA binding domain. This syndrome, also known as testicular feminization, results in a female sex phenotype but an abnormally feminized XY[57] male where the external genitalia (penis, scrotum, testicles) do not form but instead have a vaginal canal with a closed pouched. Also, there are no primary female reproductive organs (i.e. ovaries and uterus), but secondary female sexual character (i.e. breasts) can –and often do develop.

The AR essentially recognizes a palindromic structure of specific CREs. The hormone response element for the AR, like the glucocorticoid and progesterone receptors, has been shown to be class 1[58] and binds to the sequence TGTTCT[59]. Interestingly, the AR is the only[60] currently known receptor that can bind the TGTTCT sequence as well as its inverted version (ACAAGA).

2) Chaperonin[2]: (*Figure 3*) Simply put, these proteins function to fold and unfold the DNA. Examples include the heat shock proteins (HSP). The AR is in equilibrium with the 8s chaperonin complex which functions to prevent the AR from functioning as well as protect it from ubiquitinating proteases. In its free (uncomplexed) form, the AR can be modified (post-translational modification) via phosphorylation or glycosylation, and thus prevent the AR from re-associating with the chaperonin complex. This free form allows

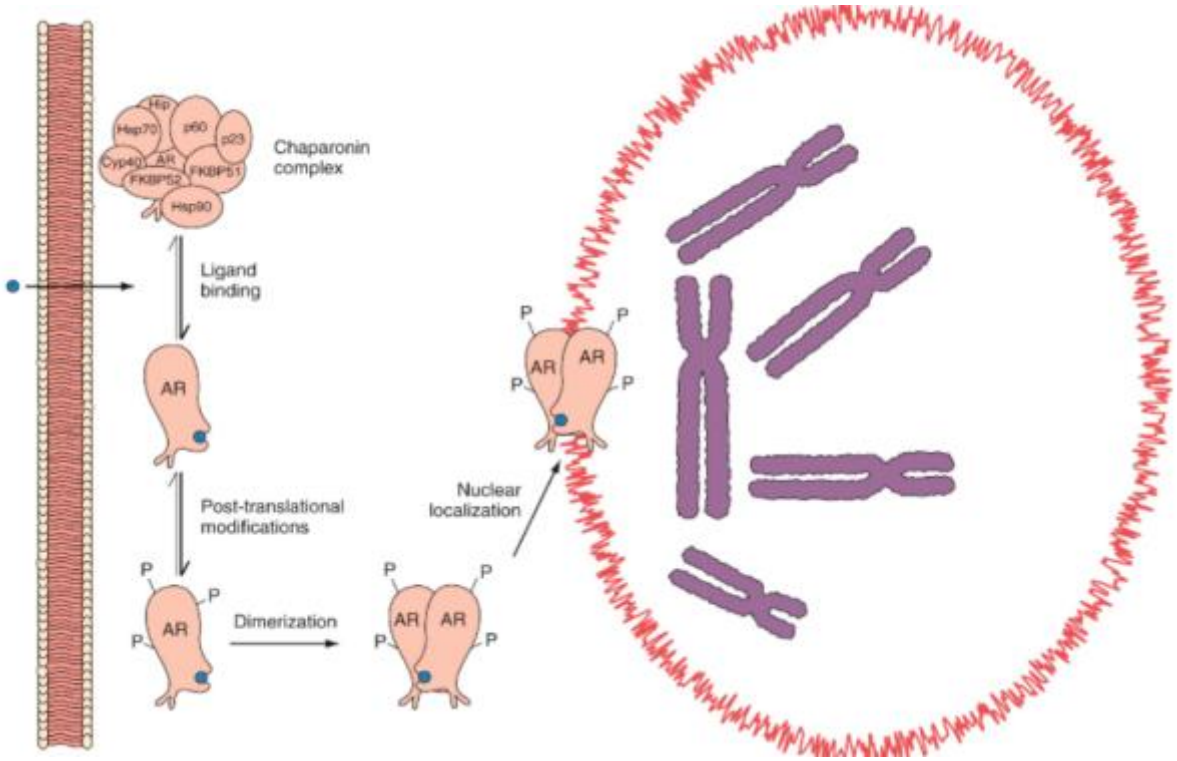

Figure 3: The Activation of the Androgen Receptor[2]
The AR is in constant equilibrium with the 8s Chaperonin Complex (Hsp90, Hsp70, Hip, p23, p60, FKBP51 & 52, and Cyp40). The chaperonin complex conceals the LBD of the AR enzyme. However, when exposed, androgens can bind to the LBD and dissociate from the complex, thus resulting in post translational modification (phosphorylation, glycocylation) that prevent its the re-association with the complex. Subsequently, the AR dimerizes, is phosphorylated, and then translocates to the nucleus to function in a *ligand-dependent* fashion. Sometimes, in the presence of AR mutations, the AR can dissociate from the chaperonin complex and enter the nucleus without the need of a ligand (i.e. DHT). This is referred as *ligand-independent*, and has been implicated as one of the reasons for developing CRPC.

for ligand (DHT)-dependent activation, ligand-independent activation, or degradation by proteases.

3) Ligand-Binding Domain:[72,73] As described earlier, the AR also has a site for ligand (DHT: Dihydrotesteosterone) binding and this is coded by the 3' carboxyl terminus the AR gene. Chaperonins attempt to conceal the carboxyl end (ligand-binding domain) of the AR. An androgen receptor that interacts with the DNA binding domain in the absence of a ligand is behaving in a ligand-independent fashion (*Figure 1*). However, if a ligand was present then this is a ligand-dependent mechanism. Aggressive androgen independent PCa (i.e. CRPC: castrate resistant PCa) behaves in a ligand-independent fashion as one of the ways to maintain the tumor presence. As we shall see later, activation of the AR is characterized by dimerization, modifications, translocation, and gene regulation. AR activation can be done via DHT, testosterone, or a ligand-independent method. Any mutation in the ligand-binding domain of the AR gene will result in either enhanced activation, repression, or ubiquitination. Some examples include:

- Deletion of the ligand-binding domain = constitutively active AR[61]
- Single point mutation (i.e. LNCaP) = allows stimulation (weakly) by other steroids[61].
- Mutations at amino acids 587-794 = inactive AR[61]
- Deletion of entire ligand-binding domain = ligand-independent AR actvation[61].

1.2.5 PHYSIOLOGY OF THE PROSTATE

1.2.5.1 ANDROGENS

Initially discovered in 1936, androgens are steroid-based hormones with many functions including development of primary and secondary sexual organs. Androgens are synthesized naturally by males and females in various body parts including adipocytes, ovaries, testicles, and adrenal gland. For the purpose of this thesis, only male related androgen biology will be discussed. As with all steroid-based hormones, the initial precursor is the lipid cholesterol. Through a serious of reactions, the end products are aldosterone, cortisol, androstenedione, testosterone, estrogen, and DHT. Downstream in the androgen synthesis pathway, testosterone is synthesized and has two options. Although all the previous reactions are reversible, the two options are irreversible. The options are conversion to DHT via 5-alpha reductase (5AR), and estrogen via aromatase. As seen in *Figure 2* the prostate receives growth signals from androgens of the testicles (testosterone) as well as from the adrenal gland (androsteindione). Note that androsteindione is a weak androgen and, on its own, it is insufficient to stimulate any real prostate growth, but does retain the ability to be peripherally aromatized to estrone. Under normal conditions, the adrenal weak androgens do not support any significant prostate growth. As the testes receive the luteinizing hormone (LH) from the hypothalamic-pituitary axis (*Figure 4*), the Leydig cells (*Figure* 5) are stimulated to produce testosterone and some estrogen (in males most of the estrogen is from peripheral aromatization).

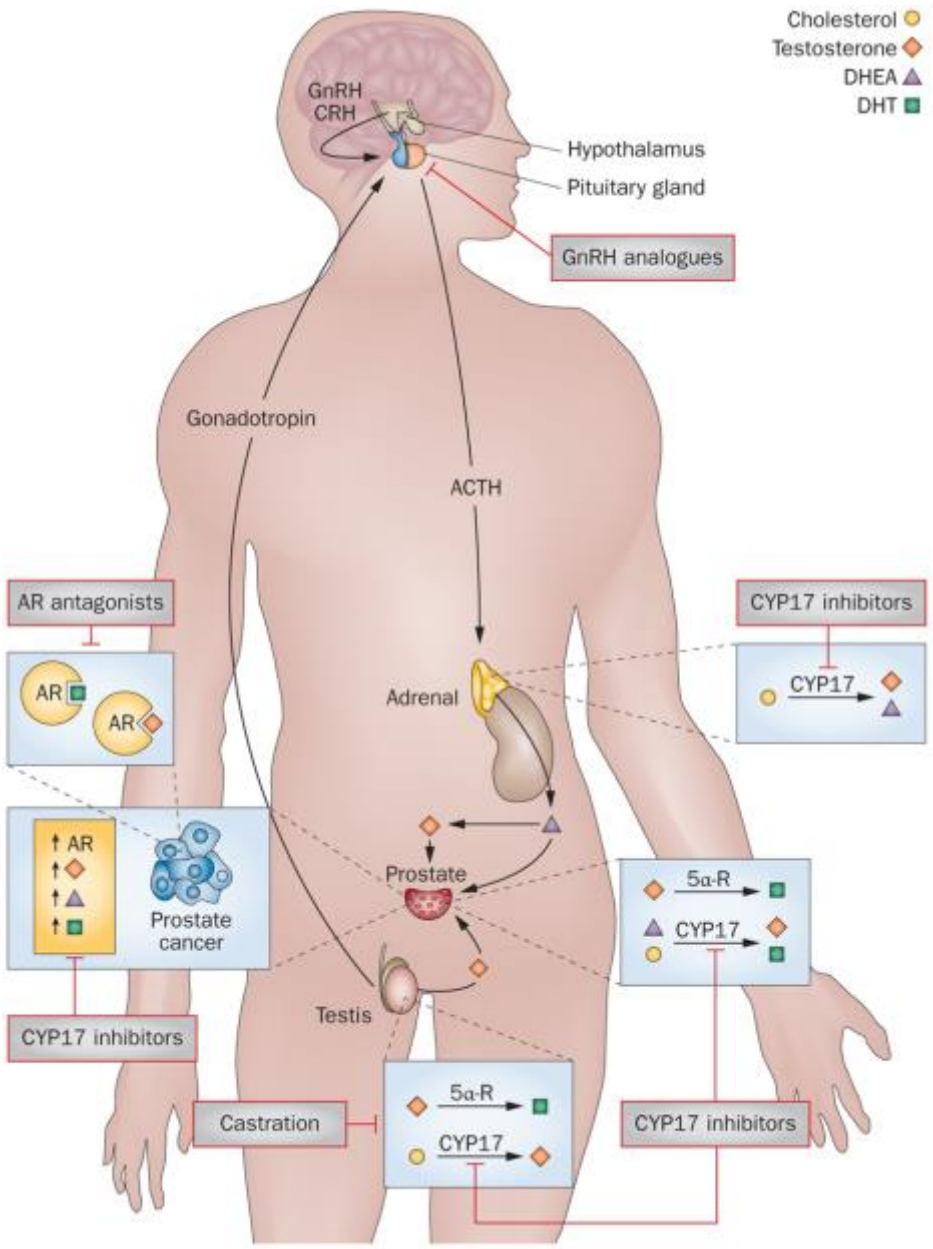

Figure 4: The Hypothalamic Pituitary Axis[75]

The HPA begins at the hypothalumus where GnRH and CRH are secreted vascularly to the adenohypophysis (anterior pituitary gland). Consequently, the pituitary stimulates androgenesis via release of ACTH (Adrenal) and LH (testes). Androgens entre the prostate and are converted to the more potent DHT and eventually result in prostatic growth. Currently, medications are aimed at targeting as many of these androgen pathways in as many organs as possible. This type of treatment is called Androgen Deprivation Therapy (ADT) because it deprives the tumor from the androgens required for growth. This is also called medical castration, as opposed to the older surgical castration method. GnRH Analogues (i.e. Firmagon®) inhibit sthe synthesis of GnRH. AR antagonists (i.e. Biclutamide®) bind to and inhibit the AR. A new class of medications, CYP17 inhibitors (i.e Abiraterone®), inhibits the synthesis of Androgens within the adrenal gland, testes, normal prostate, and malignant prostate. Unfortunately, this new class results in dangers hypotensive hyperkalemia due to the inhibition of other steroid hormone synthesis, namely Aldosterone, and is thus always concurrently administered with prednisone to replace the loss of cortisol and aldosterone.

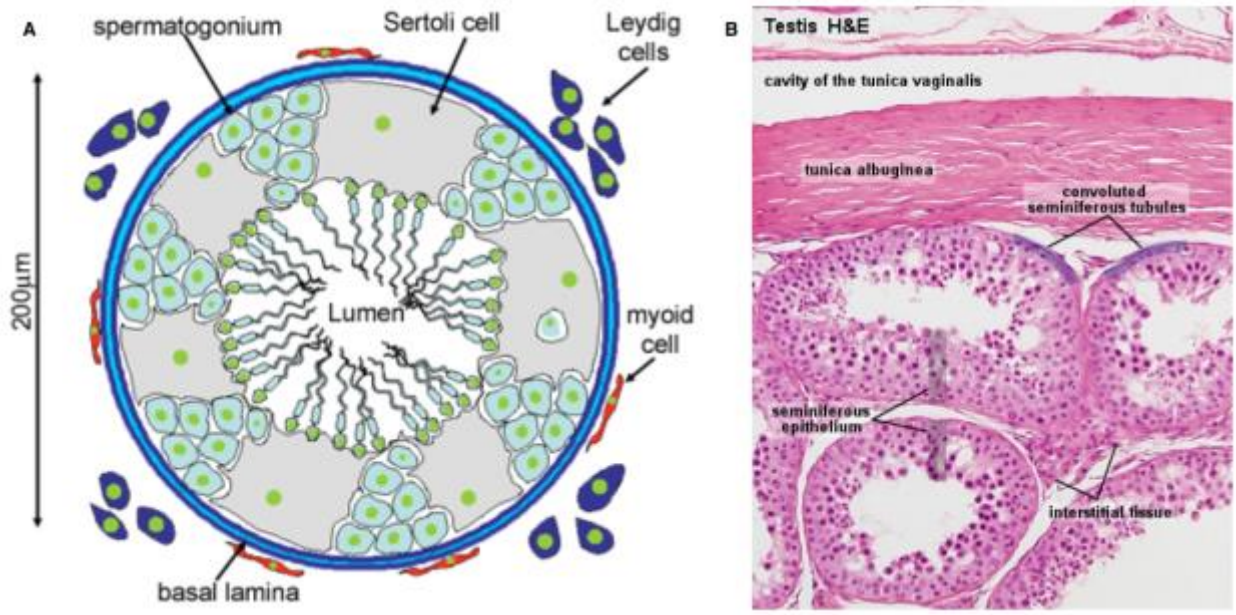

***Figure* 5: Testicular Cross-Section Showing Leydig Cells**[76,77]

A cross sectional image of the testes shown in a cartoon as well in a histological H&E stain slide. **A.** Cross-sectional view of the seminiferous tubules (SMT) showing the spermatozoa maturing in the lumen of the tubules from stem cells (spermatogonium) from within. Also in the SMT are the Sertoli cells which function to: 1) secrete inhibin to inhibit the release of FSH, 2) release of Mullurian inhibitory factor (MIF), a molecule coded by the SRY gene that inhibits mullarionogenesis (development of female GU organs), and 3) formation of the testes blood barrier to prevent autoimmune destruction of the spermatozoa. In the interstitium (around the SMT) there are leydig cells which synthesize testosterone. **B.** An H&E slide of a cross-sectional testes. Within the SMT, the high mitotic activity (blue) is easily visible by the chromatin basophilic stain. A tough membranous layer surrounds and intervenes in the testes is also found: Tunica Albuginea. It is a thick collagen layer that stains strongly for eiosin (pink).

Testosterone:[78,79]

In males, the majority of testosterone (95%) is synthesized by the testes while about 5% is from peripheral conversions of weak androgens such as from androstendione[78]. The spermatic vein contains 40-50 µg/dL of testosterone as opposed to 0.1 µg/dL in peripheral veins (75 times more concentrated)[78]. However, it is unclear[78,79] if the spermatic vein is actually storing the testosterone or contains a high testosterone concentration because it is the structure that releases testosterone into the dilute blood stream. The spermatic vein contains 15% non-testosterone androgens, primarily:

androstendione, dihydroepiandrosterone, and androsternedione[78]. Nonetheless, the human daily Testosterone-blood-production-rate (TBPR) is 6-7 mg/day with an average adult plasma concentration of 611 ng/dL ± 186 to ranges between 300-1000 (or 10.5-34.7 nmol/L in SI units)[78]. With a half-life of less than 10-20 minutes, a complete castration (surgical) will result in castrate-level testosterone within 1-2 hours[78].

In the plasma, testosterone is 98% bound to one of the following proteins: 40% to human serum albumin (HSA)[79], <1% to cortisol binding globulin (CBG, aka transcortin)[79], and 57% to Testosterone-Estrogen binding globulin (TeBG, aka SHBP: sex hormone binding protein)[79]. SHBP has the highest affinity to testosterone while HSA has the least. However, this is overlooked due to the abundance of HSA. Therefore, the bound form is dependent on the affinity of testosterone to the bindings sites as well as the capacity (i.e amount of available binding proteins). Because only free testosterone is bioavailable, protein binding is thus regulated. For example, testosterone decreases SHBP whereas the opposite occurs by estrogen. However, estrogen has 30% affinity to SHBP (vs testosterone). As we shall see later, to reduce bioavailable testosterone, small amounts of exogenous estrogen would increase plasma concentration of SHBP and thus decrease free testosterone levels.

The bound and free forms of testosterone and estrogen are in total reversible equilibrium. The plasma levels of testosterone can vary wildly from person to person and with diurnal variations. For males 25-70 yeas old, testosterone levels do not seem to correlate with age, but they do significantly decrease (<500 ng/dL) in males >70

years old. This makes it somewhat unreliable and difficult to set a standard of expected and accep*table* testosterone levels. As mentioned earlier, testosterone can be irreversibly converted to estrogen or DHT, but can also be metabolized hepatically for renal excretion. Less than 2% of total testosterone is renally excreted, but instead, the hepatic degraded byproduct of testosterone, 17-ketosteroid (glucoronated or sulfated for water solubility), is the form that makes it into the urine. However, measurements of 17-KS are also not a reliable index of the levels of testosterone because many other androgens and non-androgens are hepatically metabolized into byproducts that include 17KS as a functional group.

Dihydrotestosterone (DHT):[79]

Although testosterone is the primary hormone to the human prostate, it is only a pro-hormone because it is DHT that stimulates the prostate (and the seminal vesicles) to grow. Compared to testosterone, DHT concentration in the serum is very low (56 ng/dL ± 20). Although much more potent (3-10x) than testosterone, its tight regulation (binding to proteins) and low availability (low concentration) can mask its potency. Physiologically, DHT functions intracellularly in prostatic epithelial (glandular) cells. In the prostate DHT is 5x more concentrated than testosterone.

The 5-Alpha-Reductase Enzyme (5AR)

There are three recognized isoenzymes for 5AR enzymes, however only two have been studied. This idea of multiple isoenzymes was established in 1985 by Bruchovsky[80] who noticed that the velocity of growth was 10x higher for stroma than epithelia. Type 1 5AR

(located on the extreme tip of chromosome 5p) is primarily found in non-sexual organs (sebaceous glands, scalp, skin – for hair growth) with little amount in the prostatic epithelia or stroma. This type I isozyme is found in normal levels in men with congenital 5AR deficiency. Type 2 5-AR (located on chromosome 2p) is expressed mainly in sexual organs (testes, foreskin, scrotum) and is concentrated in the prostatic fibromuscular stroma[81]; this is the isoform that is mutated in the disease 5AR deficiency. Furthermore, type 2 5AR isoform is expressed in basal and stromal cells[81] of the prostate, but is virtually absent in secretory epithelial cells. Recall that PCa is a disease of epithelial prostatic cells where as BPH is a disease of stromal cells. This points towards the assumption that DHT indirectly stimulates the prostatic epithelia through the basal and stromal cells. Note that short term ADT does not influence the levels of 5AR type 2.

Studies have shown that the more clinically relevant isoform is type 2. There is 49% homology between the DNA of both isozymes. The 5AR inhibitor (5ARi) Finasteride (selective for type 2 5AR)[22] and Dutasteride (non-selective) are equally efficient when treating BPH. This validates the conclusion that type 2 AR is the clinically important prostatic isoform.

When free testosterone passively diffuses into the prostate, the equilibrium (>90%) favors the irreversible conversion to DHT rather than any other diols. This occurs via 5AR type 2 and the cofactor NADPH. Uniquely, due to enzymatic kinetics, the 5AR type 2 enzyme can not be saturated by the testosterone substrate.

1.2.6 PATHOLOGY AND THE GLEASON SCORE

Table 4 shows the 2005 International Society of Urological Pathology Modified System and (*Figure 6*) shows the different pathological features of the Gleason Grading system. In addition, there are two other histopathologies that are often also noted during specimen examination: Prostatic Intra-Epithelial Neoplasia (PIN) and Intraductal carcinoma of the prostate (IDC-P).

Table 4: International Society of Urological Pathology - Modified Gleason System

Pattern 1	Nodules (Glandular units): circumscribed, relatively oval, closely packed but separate
Pattern 2	Same as pattern 1, but loosely arranged, less uniform, some edge infiltration
Pattern 3	Nodules: discrete, small, marked variation in size
Pattern 4	Cribriform and ill defined glands, lamina poorly formed
Pattern 5	No uniformal glandular differential, glands of all sizes, 'comedocarcinoma'

Data Collected From the Society of Urologic Oncology[83]

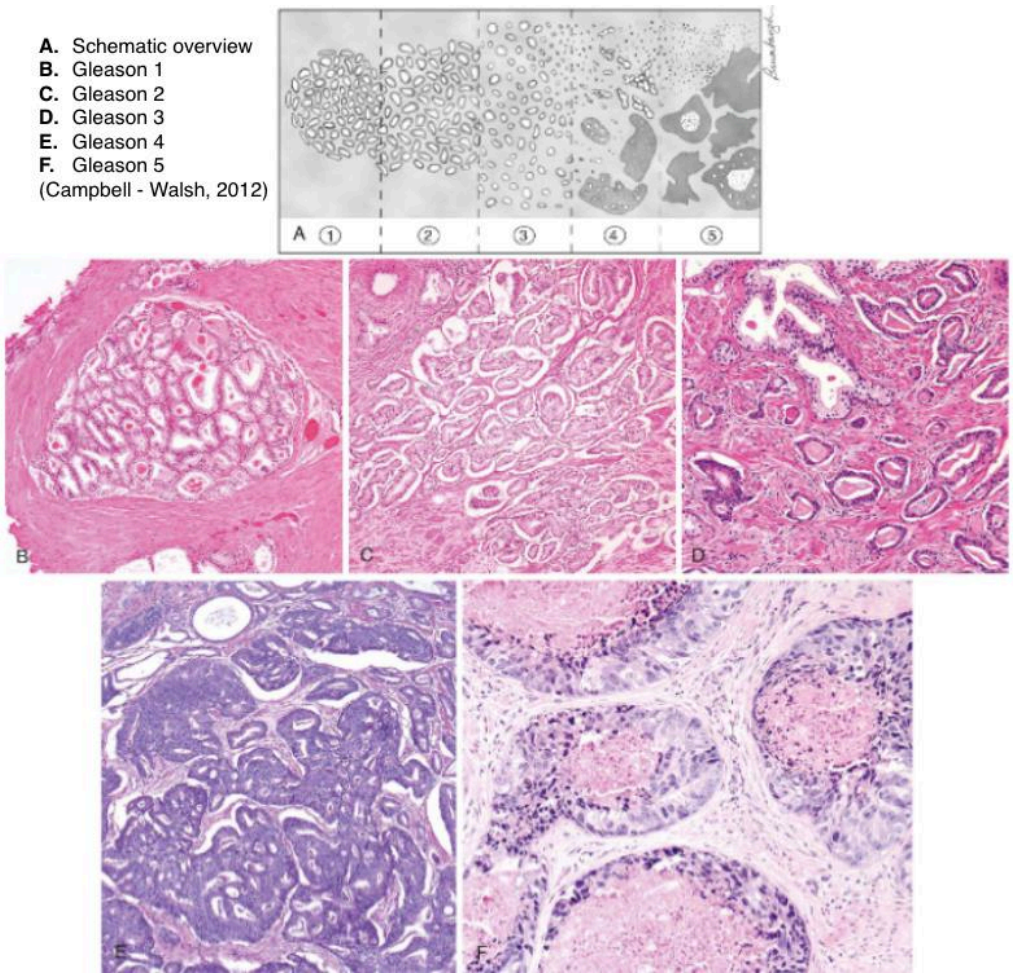

Figure 6: Histopathologic View of Gleason 1-5 [2]

1.2.6.1 PROSTATIC INTRAEPITHELIAL NEOPLASIA

PIN consists of architecturally benign prostatic glands, acini, and ducts. Two types of PIN exist, low grade (LGPIN) and high grade (HGPIN). The difference is the prominence of the nucleoli, and thus interobserver subjectivity is possible during the diagnosis of either LGPIN or HGPIN. Patients with LGPIN have no increased risk for the development of PCa on subsequent biopsies[84]. However, there is controversial and indirect evidence[85] for the association of PCa and HGPIN: 1) PCa samples are more likely to have foci of HGPIN vs. benign samples, 2) The more HGPIN, the more multifocal carcinomas, 3) Similarities in PSA changes between HGPIN and PCa, and 4) Both HGPIN and PCa preferentially involve the prostatic anatomical peripheral zone;

the zone most frequently associated with PCa. The significance of HGPIN detected on TURPs is unclear[86]. Nevertheless, the current standard for HGPIN found on TURP is a no workup for elderly patients. However, the guidelines recommend a clinical workup for young men to rule out aggressive PCa. Due to this ambiguity, most authorities do not classify HGPIN as carcinoma in situ (CIS)[83]. A retrospective study[84] associated HGPIN as a precursor to intermediate or high risk PCa; although the presence of HGPIN is not required.

Due to the histopathological subjectivity in distinguishing LGPIN and HGPIN, there is a huge range (0-25%, mean 7.6%, median 5.2%) in the incidence HGPIN on prostate biopsies[83]. Serum PSA levels and DRE status have no clinical prediction when assessing the risk of PCa in patients with HGPIN. Additionally, on its own, PIN does not increase the PSA.

For those with unifocal HGPIN, it is unnecessary to re-biopsy within the first year in the absence of clinical signs and/or extensive HGPIN (>2 cores). In one study[87], it was shown that a re-biopsy of patients who had HGPIN after 3 years resulted in a 25% chance of PCa. The hypothesis is either previously missed microscopic cancers grew to the level of needle detection or that HGPIN progressed. As such, all repeat biopsies should involve all cores rather than just the previous HGPIN cores.

1.2.6.2 INTRADUCTAL CARCINOMA OF THE PROSTATE

ICD is usually found on radical prostatectomy (RP) pathologies[88] and rarely on benign biopsies. Its presence is highly associated with high-grade disease, poor prognosis, and

advanced disease. Although ICD should not be considered as a pre-invasive finding, it should be treated aggressively even in the absence of documented infiltrating PCa[88].

1.2.7 PROSTATIC SECRETIONS AND THE PSA

1.2.7.1 GENERAL

Along with the seminal gland, periurethral glands of Littre, and Cowper's glands, the prostate is an accessory sex gland that expels ejaculatory secretions which functions to provide a sui*table* and habitual environment for the spermatozoa to function. The ejaculatory volume is 2-6 ml (average 3 ml) and consists of haploid spermatozoa (1% by volume, 100 million/ml) as well as seminal plasma. Most of the seminal plasma volume comes from the seminal vesicles (1.5-2 ml) followed by the prostate (0.5 ml). The unique feature of the expelled ejaculation is its release in a sequential manner[89]. The first fraction of the secretions is low in fructose but high in sperm cells and prostatic secretions (PSA, citric acid). However, it is in the later fractions that fructose (seminal vesicles) is found in significant amounts. The chemistry[90] of the seminal plasma is distinct from vascular plasma in that it has a high concentration of potassium, zinc, and enzymes (i.e. PSA and acid phosphatases).

Recently, expressed prostatic secretions (EPS) have been shown to resemble a unique profile in patients with prostate cancer[90]. EPS from such patients have significantly low concentrations of citrate, but high levels of myoinositol and spermine.

1.2.7.2 NON-PROTEIN SECRETIONS

1) Citrate: This major anion is synthesized in the mitochondria's Kreb cycle. Due to the low activity of aconitase (a catalytic enzyme), the mitochondrial rate of citrate

synthesizes far exceeds its oxidation[91] of citrate. The ejaculatory concentrate of citrate is 500-1000 more than anywhere else in the human body[92]. This anion functions to bind divalent metals such as Ca^{2+}, Mg^{2+}, and Zn^{2+}. Currently, there are studies investigating the use of citrate levels to diagnose prostate cancer[93].

2) Zinc: Of all body organs, zinc is found at the highest concentration in the prostate. Prostate cancer cells lose the ability to retain and accumulate zinc[102]. The decreased levels of zinc result in increase DNA damage[103]. Additionally, zinc is an important protein binder[79] and may effect the fluctuating release of the protein PSA.

1.2.7.3 PROTEIN SECRETIONS

1) Prostate-Specific Antigen (PSA): PSA, also known as Kallikrein 3 –KLK3, is a glycoprotein with serine protease activity[104] and part of a larger serine protease family called Kallikrein (KLK). In fact, there is significant homology[105] between the genes of all KLK proteins (up to 80%[105] with KLK-2 –which has a 20,000x more potent serine protease activity than PSA), thus explaining the close physiological roles and functions amongst KLK proteins. The KLK family of serine peptidases are all located on chromosome 19[106]. PSA is produced almost exclusively by the glandular epithelia[107] of the prostate, which may explain why PSA values are much lower in stromal benign tumors such as BPH. (*Figure 7*) highlights the synthesis of PSA. In addition to the serine protease activity, PSA also has arginine esterase activity through chymotrypsin-like and trypsin-like functions[105]. In the blood, most PSA is enzymatically inactivated by complexing with anti-serine proteases such as α_1-antichymotrpasin (ACT, irreversibly bound) and α_2-macroglobulin (A2M). Only free PSA is enzymatically active.

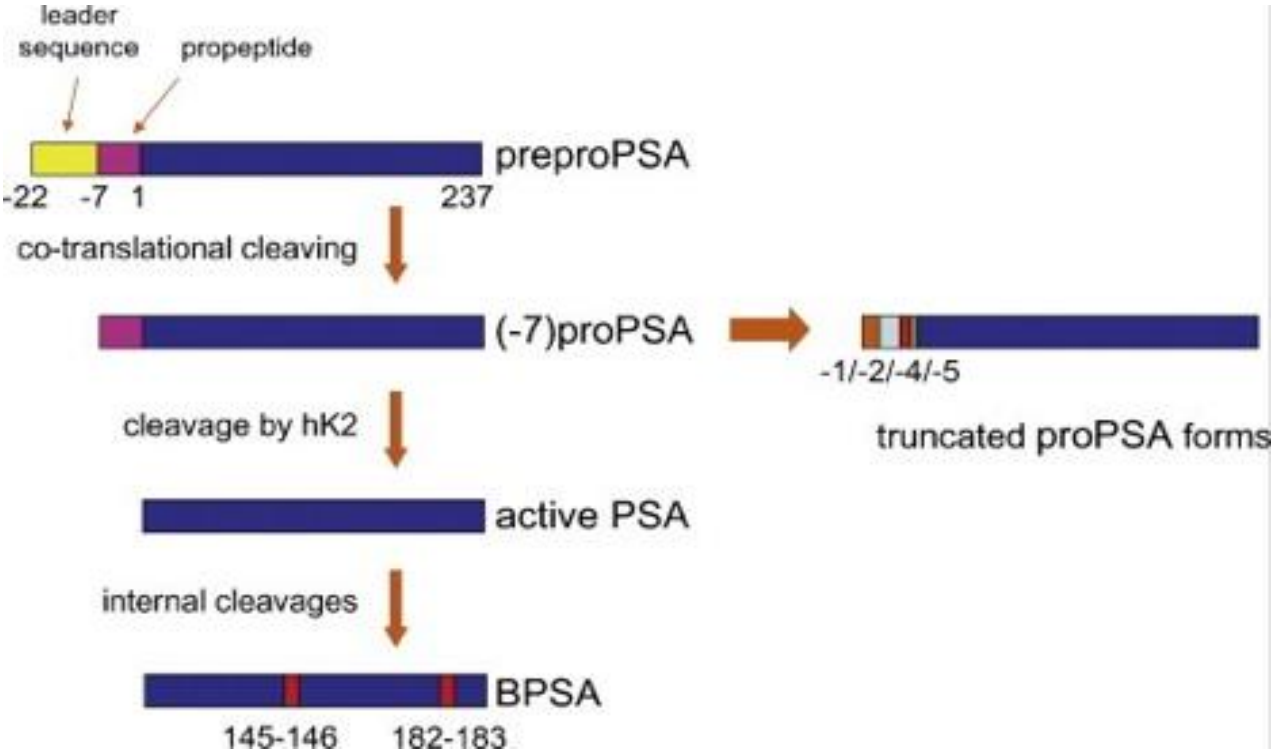

Figure 7: The PSA Gene[108]
The 261 amino acid pre-pro-PSA has 17 of its peptides excised in the endoplasmic reticulum to form the inactive pro-PSA zymogen. The zymogen is then packaged into a vesicle along with KLK2 which cleaves an additional 7 to form the active PSA.

PSA is almost exclusively released by the prostate, with some ectopic expressions in malignant breast tumors, RCC, adrenal carcinoma, normal breast tissue of females, and in breast milk[108]. However, in terms of clinical significance, PSA is largely prostate-specific but not prostate cancer-specific[109]. As one of its limitations, the measured PSA values have overlap between benign (BPH) and malignant cancers (PCa).

When the ejaculate is formed and released into the glandular lumens a seminal vesicle protein called semenogelin I & II behaves as a seminal fluid procoagulant[110]. Semenogelin is a substrate for multiple KLK proteins including KLK 3 (PSA) and KLK 14. PSA simply degrades the free forms of semenogelin I & II, however, it is KLK 14[110] that degrades the semenogelin-zinc complex. See later sections for the clinical use of

PSA as a marker for PCa (Section 1.2.8.1 PSA:).

PSA measurements in prostate cancer patients dates back to 1988[109] and continues to serve in that role; albeit with some controversies. Many other KLK proteins have also been suggested as potential tumor markers in various malignancies. KLK-2's expression increases in metastatic and poorly differentiated prostatic epithelia[111]. KLK-L1 has been found to be hormonally regulated in breast tissue and may be used as a marker in breast cancer. KLK-11 is expressed in amniotic fluid, breast milk, cerebrospinal fluid (CSF), ovaries, the cytosol of breast cancer cells, and up to 60% in patients with prostate cancer. In fact, the KLK-11: PSA ratio significantly reduces the need to biopsy for prostate cancer[112]. KLK-14 may serve as a marker for ovarian cancer[110].

2) Prostate-Specific Membrane Antigen (PSMA): located on chromosome 11p1-12[115], PSMA is a type II integral membrane glycoprotein with three domains[115]: a small intracellular domain, a transmembrane domain, and a large extracellular domain. PSMA has a protease domain that catalyzes N-acetyl-aspartyl-glutamate (NAAG) and hydrolyzes the glutamate tail of the polyglutamate folic acid in the central nervous system (CNS) and intestine, respectively. Although 3 extracellular domain splice variants of PSMA exist, only 1 (PSM'-PSMA) is known to exist in all prostatic tissue (normal, benign, and cancer). The mRNA for the transmembrane domain of PSMA shares 57% homology with that of the transferrin receptor (TfR), a receptor responsible

for the endocytosis of iron-transferin complexes – however, unlike PSMA, TfR has no protease domain[116].

PSMA overexpression has been observed in the neovasculature of many prostate tumors[117]. Specifically, PSMA mRNA over-expression in prostate cancer has been unique. This is because PSMA over-expression occurs in patients who are castrated[117], as opposed to PSA which decreases in such patients. Additionally, the PSMA:PSM' ratio increases[115] in prostate cancer when compared to BPH (0.76 - 1.6) and normal tissue (0.075 - 0.45). This data is supported by other studies that showed an increase of PSMA levels in 47% of patients with prostate cancer vs 5% in those without prostate cancer. Additionally, PSMA overexpression appears to correlate with the histological differentiation, and possibly with stage and grade. The Immuno-SELDI[117] assay used 7E11-C5-Igs against PSMA to distinguish BPH from PCa.

Clinically, PSMA is reliable for low and intermediate risk prostate cancer patients[117]. However, due to decreased expression (via EMT: epithelial mesenchymal transitioning), it loses sensitivity and specificity in high risk and metastatic disease[116-118].

1.2.8 LABORATORY BIOMARKERS FOR PROSTATE CANCER

1.2.8.1 PSA:

In section 1.2.7.3, the PSA molecule was described in details. Here, the PSA will be described in terms of its clinical use. Previously, it was thought that a PSA above 4 is abnormal. However, it is now understood that many factors play a role in determining the accep*table* values for a PSA. When BPH and advanced prostate cancer were ruled

out, a PSA of 8 had a sensitivity and specificity of 94% and 98% for PCa, respectively[122]. But in the presence of bladder outlet obstruction (BOO), a PSA of 10 has a sensitivity of 93% and a specificity of only 65%. Specificity of skeletal metastasis is >70% only if the PSA is ≥60-80 ng/ml[122]. It was also concluded that PSA is far more superior than prostatic acid phosphatase (PAP). Thus, on its own the PSA is most informative unless it is either very low (<4) or very high (>12)[122]. Therefore, it is best utilized in the presence of digital rectal findings, family history, urinary symptoms, age, and the changes of PSA over time. A routine digital rectal examination (DRE) does not result in a falsely elevated PSA. PSA levels have the highest positive predictive value (PPV) than any other test for PCa. When the PSA is >10 ng/ml, a diagnosis of PCa is true in 60% of the cases. However, when at values of 4-6 ng/ml, the PPV is only 20%[122]. The next section explains how PSA sensitivity maybe enhanced by measuring PSA velocity (PSAV) and age-specific PSA levels, while PSA specificity can be enhanced with PSA density (PSAD) as well as free PSA (f-PSA) measurement.

1.2.8.2 PSA DENSITY

PSAD is obtained by dividing the total serum PSA by the prostatic volume (typically obtained during an ultrasound guided biopsy) [122,123]. By normalizing the PSA levels for that of the prostate size, specificity becomes greater. This can be helpful in distinguishing BPH from PCa. A PSAD >0.15 is highly suggestive of PCa[122].

1.2.8.3 PSA VELOCITY

PSAV describes the rate at which the PSA changes overtime. It is a helpful tool to increase sensitivity (i.e. ruling out the presence of PCa)[124]. The idea is that over time, the secreted PSA increases greater in men with PCa than those with either no PCa or

just BPH. The exact value of PSAV is unknown. However, a PSAV >7 ng/ml suggests PCa. PSAV is best detected by measuring the PSA at least three consecutive times in a period no less than 18 months[122,124].

1.2.8.4 AGE-SPECIFIC PSA

This tool helps increase the sensitivity of PSA. As males age, there is more PSA secreted. The maximum accep*table* values in terms of age are <2.5 ng/ml for <50 years, <3.5 ng/ml 50-60 years, and 4.0 ng/ml for >60 years[122,123].

1.2.8.5 COMPLEXED PSA

By acquiring the free vs bound PSA, the specificity of PSA is enhanced, and thus it is more reliable when ruling in PCa[122,125]. As explained earlier, PSA is released by the exocrine prostate gland into the ejaculatory duct and then ultimately to the urethra for expulsion. However, small amounts diffuse into the circulation, most are bound to proteins such as α_1-antichymotrypsin and β_2-microglobulin[125]. PCa-free patients have higher fPSA. The ratio is also higher in BPH patients. The current guidelines (SUO, 2016) recommend a ratio profile for patients who have a PSA of 4-10 ng/ml and a negative biopsy. The accep*table* ratio is approximately 18%. The Immuno-I-Bayer system measures the amount of complexed PSA that is bound to α_1-antichymotrypsin[125]. This assay has shown promising results that increase the specificity with no loss of sensitivity.

1.2.8.6 GENE FUSION

Previously, sarcomas, thyroid cancers, and hematological malignancies were thought to be the only cancers involved in gene fusion from translocation was discovered. However, a breakthrough in PCa genomes[126] suggested otherwise. In 15% of localized

prostate cancer, the 5'-UTR[1] end of *TMPRSS2*[2] (21q22.2) was found to fuse with *ERG* (*ETS*-related gene, 21q22.3)[127]. Other *ERG*'s[3] include *ETV1* (*ETS*[4] variant gene 1, 7q21.2), *ETV4*, and *ETV5*[128].

As *Figure 8* shows, the *TMPRSS2* gene, which is expressed in both benign and malignant prostatic epithelia, is induced by androgens to activate the expression of *ETS*, a gene that controls protein synthesis, cellular adhesion, and cellular invasion. It is not unknown if *TMPRSS2-ETS* gene fusion causes cancer. However, there is a possible link between over-expression of this gene fusion with higher recurrence rate as well as higher pathological stage[129,130].

Currently, there are no known clinical applications in terms of targeting this gene fusion for the purpose of treatment and/or chemoprevention. Because *TMPRSS2-ETS* gene fusion occurs only in PCa, it has been suggested[128] to develop a tool that screens for *ETS* gene fusion-positive individuals.

[1] UTR: Untranslated region
[2] TMPRSS2: Transmembrane Protease Serine 2
[3] ERG: ETS related gene
[4] ETS: E26 transformation specific gene or E-Twenty-Six

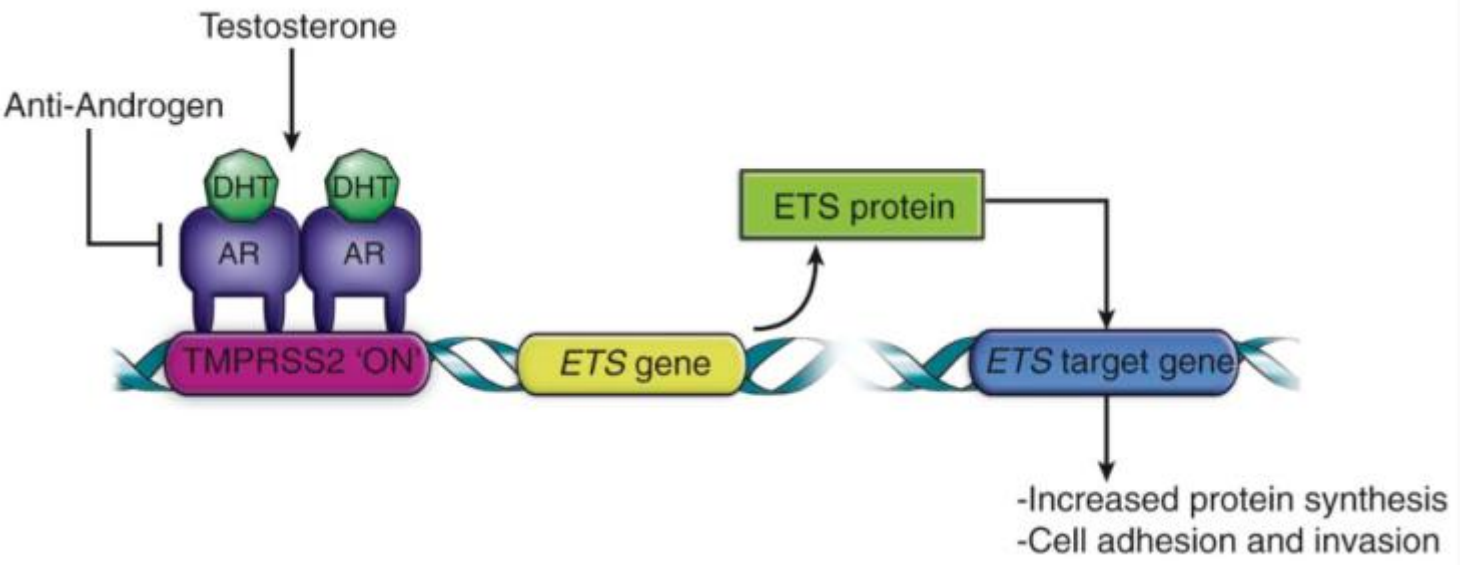

Figure 8: Gene-Fusion as a Risk Factor For Prostate Cancer[2]
During the TMPRSS2-ETS gene fusion, the activation of TMPRSS2 by the androgen receptor-ligand complex leads to enhance and over expression of the ETS gene (i.e. ERG, ETV1). ETS proteins act on ETS target genes that cause increased cellular proliferation and invasion. Although both testosterone and dihydrotestosteron (DHT) bind to the AR, it is usually the more potent DHT that binds. Most of the conversion of testosterone to DHT occurs intracellular at the prostate level. The *figure* does not show this conversion.

1.2.9 DIAGNOSIS

1.2.9.1 THE UCSF CANCER OF THE PROSTATE RISK ASSESSMENT (CAPRA) SCORE

The CAPRA score is based on a study at the university of California San Francisco (UCSF)[131]. It is a nomogram that assess the stratification and risk of the prostate cancer. It relies on age at diagnosis, PSA at diagnosis, Gleason score, clinical stage, and percent of biopsy cores with PCa. It is similar to the D'Amico criteria[1] described, but also accounts for multiple risk factors (age and percent of biopsy cores with PCa).

1.2.10 CLINICAL CHALLENGES IN PROSTATE CANCER

The diagnosis of prostate cancer includes a complete history (especially family history), physical exam (rectal), biomarkers (i.e. PSA, PSMA), and a prostatic biopsy. These findings are all utilized together to diagnose PCa. However, there are two main issues. First, to have any or all these findings, a patient must be suspected of having PCa. This is a disease that rarely shows any signs until when in the late stages. On the other

hand, it would not be financially efficient to screen all patients for PCa. Secondly, PSA levels are measured and rectal exams (DRE) are performed as initial a screening tool. The problem is that both PSA and DREs are not reliable screening tools unless they are abnormal (i.e. PSA= more specific -85%- than sensitive-68%- when using a cut off of PSA >3 ng/ml)[122]. Thus financial budgeting and potential subjective and ambiguous findings can risk physicians missing a prostate cancer diagnosis.

1.2.10.1 MARKERS FOR PROSTATE CANCER

To tackle the previously mentioned issues (section 1.2.10 Clinical Challenges in Prostate Cancer), a simple non-invasive and a relatively cheap liquid biopsy can be performed to aid in the diagnosis of prostate cancer. A liquid biopsy is a simple peripheral blood sample that provides clues about a tumor without actually performing a biopsy on the tumor itself. Liquid biopsies are non-invasive procedures that can give information about tumor markers that may potentially give insight about the behavior of the tumor. Most patients with prostate cancer undergo invasive biopsying to profile the cancer. Those with higher stages are put under ADT. However, the inevitable occurrence of resistance (CRPC) is occurs. Currently, the only method of detecting resistance to therapy is by serial PSA and testosterone levels. However, this method of screening for resistance (using PSA and testosterone) does not give insight about new genomic alterations that may respond to targeted therapy. In this setting, a liquid biopsy can be very important because it is easy to perform, allows for monitoring during treatment, and may also detect genomic alterations that are either accessible to certain therapy or are associated with neoplastic resistance. A liquid biopsy can prove valuable in patients who have a negative biopsy with fluctuating PSA, those in whom solid biopsy

is inaccessible, and patients with tricky anatomy that may preclude proper biopsying. In addition, the rapid access to biomarkers is invaluable in those with metastatic disease

Therefore, it is important to develop a noninvasive biomarker with the ability to monitor tumor behavior in real-time as this can facilitate the development of personalized therapy. There are many markers that are currently being studied: 1) Circulating free DNA or RNA, 2) Exosomes, and 3) Circulating Tumor Cells (CTCs).

1.2.10.1.1 CIRCULATING FREE DNA AND RNA

Cell free DNA (cfDNA) and RNA (cfRNA) can be extracted from liquid biopsies. cfDNA and cfRNA are associated with tumor stage, size, and metastases; thus legitimizing their use as a potential biomarker in patients with cancers[208-210]. There are two main methods for the release of circulating free nucleotides (cfNAs): passive and active release. The release of cfNAs (cfDNA and/or cfRNA) from necrotic or apoptotic cells occurs via passive mechanisms[208]. Immune cells such as macrophages play a role in this passive mechanism. Macrophages release digested nucleotides into the blood stream after they phagocytose necrotic and apoptotic cells[208]. The active mechanism of secreting fragmented nucleic acids remains poorly understood. A weak hypothesis that has yet to be confirmed by other studies suggests that cancer cells (including CTCs) release cfNA to communicate with target cells, and possibly to transform them into malignant cells[208].

There are multiple limitations to using cfDNA and cfRNA as a marker for cancers. First, isolation of cfNA does not give any information about cellular morphology. Second, they

are present at very high levels in the blood of patients with benign disease such as hepatic disorders, diabetes, cardiovascular diseases, BPH, and infections [122-124]. Third, extracted cfDNA and cfRNA do not give insight about their origin[208]. Forth, even if a mutated cfDNA is eventually extracted and somehow confirmed to be from a prostate cancer cell, one cannot assume that it is actually expressed and translated to a protein[208]. Fifth, the amount of extracted cfDNA and cfRNA cannot accurately reflect the the amount of CTCs shed into the vasculature[208]. Sixth, the quantity of short fragmented cfDNA can be too low to develop high throughput analysis, and even if PCR is used to amplify the DNA, wrong nucleotides can be introduced with each amplifying cycle[208]. Seventh, there is a high rate of false-negative and false-positive results[208,211-214]. False-negative results occur because specific isolation of tumor cfDNA cannot happen unless the tumor-specific mutations are previously known. False-positive results occur because tumor-associated mutations can also occur in benign diseases and non-cancerous cells as a result of senescence.

1.2.10.1.2 EXOSOMES

Exosomes are another potential marker that have recently gained interest. It has always been understood that cells communicate hormonally or via direct cell-to-cell contact. However, it was recently discovered that cells can also communicate via extracellular secreted vesicles[132]. These vesicles (also known as extracellular microvessicles -EMVs- or exosomes) are released from a cell and sent into the plasma to communicate with another cell. Inside exosomes, there are metabolites and genetic material including RNA and DNA[132-134]. Exosomes are extremely tiny and measure between 30 nm to a few micrometeres (the majority are around 100 nm)[134]. This recently discovered method

of cell communication has the potential to allow for understanding tumor microenviroment and tumor behavior without the need for invasive tissue biopsy[132,133]. Each exosome presents a sample of material from the parent cell (normal or cancerous cells). Therefore, it can potentially be a source of disease marker[132-134]. Additionally, it is s*table* against many proteolytic and degradation activities[134].

The function of exosomes is not fully understood. However, because they carry genetic material, exosomes have the ability to change tumor and biological behavior[134]. Stem cells have been found to have increased release of exosomes. Most exosomes are heterogeneous in terms of size, markers (i.e. CD133), and content[132]. Most exosomes have a double lipid membrane, but some have a single lipid membrane while others are multilaminar (many vesicles within a single vesicle) [132,133]. Additionally, the content of the vesicles is selective. For example, vesicles do not contain the entire transcriptome, but rather only some genetic material[133]. This is likely, in part, due to the small size of exosomes.

The isolation and enrichment of exosomes can be very challenging. They are heterogenous[132-134] structures found in biofluids (i.e. blood) and have various cell surface markers. One method of isolation involves filtration and the use of peptides (antibodies) against heatshock proteins that are abundantly expressed on exosomes[133-134]. The exosome-peptide pellet is then centrifuged for further isolation[133-134]. But because of their heterogeneity, and the fact that there is no consensus on the expressed markers, isolation remains difficult and challenging. In addition, the isolation

techniques require extreme care. For example, when performing RNA extraction from exosomes (be it exoRNA extraction or direct RNA extraction), one must first isolate the exosomes themselves[132-134]. The initial steps include pre-processing[132,133] and pre-clearing[134]. Pre-processing ensures that no cells are found in the sample. In pre-clearing, one balances the loss of exosomes with 'carrying-over' of other cells (i.e. Platelets – which themselves have RNA)[132-134].

The exosome field in prostate cancer remains in its infancy and still requires more technical research before clinically significant results can be achieved and relied on[135].

1.2.10.1.3 CIRCULATING TUMOR CELLS

This section will give a very brief introduction on CTCs, as they will be heavily discussed in the next section.

Because of extraction and analytical challenges involved in studying cfDNA (cell free DNA), cfRNA (cell free RNA), and exosomes, we have decided that it is more effective and efficient to extract CTCs instead. PCa CTCs can be stained with AR to confirm their prostatic origin. Additionally, CTCs can be more accurately quantifiable. This can be done by counting (enumerating) the CTCs that were identified to be of prostatic origin.

CTC isolation is advantageous because it allows for detailed visual morphological observation (CTC size and cluster). CTCs can also undergo further analysis using fluorescent in situ hybridization (FISH) and immunohistochemistry. This analysis allows for molecular characterization of CTCs. However, there are many disadvantages for

utilizing CTCs in a clinical setting. First, what is a CTC and how does it look like? Second, how can one be sure if a CTC is benign or malignant? Third, which method should be used to isolate CTCs? And fourth, how can CTCs be enumerated. These questions along with benefits and limitations of CTC used will be answered in details in the subsequent section

1.3 CIRCULATING TUMOR CELLS

1.3.1 BACKGROUND

In 1869 Ashworth T.A. showed that circulating tumor cells (CTCs) can be isolated and used to study the parent tumor. Today, the idea is that CTCs can aid in screening, predicting treatment outcomes, and estimating prognosis. Because CTCs could potentially resemble metastasis-initiating tumor cells, they can give insight on the pathophysiological development of seeding not only to regional and distant organs, but also back to the primary parent site where they often return[137]. This phenomenon of 'self-seeding' by colonizing the primary tumor has been blamed for primary tumor progression[208]. It is estimated that metastasis account for over 90% of all cancer-related deaths and that the number of CTCs present in the vasculature directly correlates with disease progression[137]. Therefore, the minimally invasive liquid biopsy can be used as a tool for enumeration to help evaluate the clinical course of cancer patients. The advantage of the low cost minimally invasive biopsy may merit frequent clinical evaluations as well.

1.3.2 PROPERTIES OF CIRCULATING TUMOR CELLS

CTCs differ from other cells in the body. They have specific morphological features. In general, there are two types of properties, physical and biological (immunological). *Figure 9* explores the various techniques utilized today for CTC isolation.

CTCs show properties that are characteristic and specific to them. Biological (immunological) properties take advantage of cellular expression of markers such as epithelial cell adhesion molecule (EpCAM). These markers can be targeted by antibodies. Additionally, certain techniques can isolate only viable CTCs based on the active role of protein secretions. Physical properties also guide many isolating methods. These include electrical polarity, size and deformability (filtration), and centrifugation based on density.

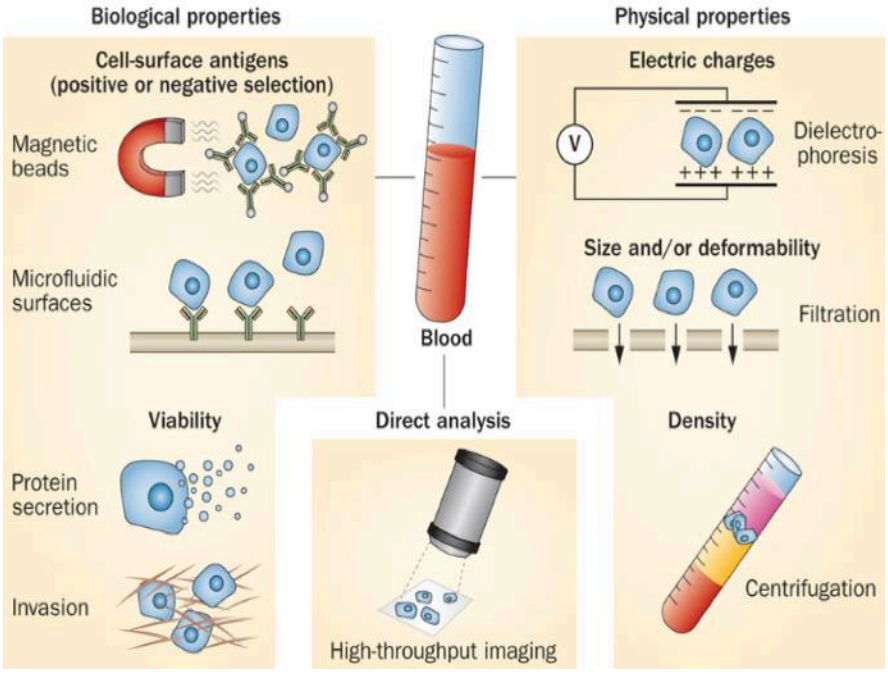

Figure 9: The Different Methods to Isolate Circulating Tumor Cells.[138] [141]. *Figure* taken from article: Circulating Tumor Cells – monitoring treatment response in prostate cancer

1.3.2.1 PHYSICAL PROPERTIES

Physical properties include size, density, electric charge, and deformability. Multiple methodologies attempt to isolate CTCs based on physical properties, including:

1) Density Gradient Centrifugation (*Ficoll, OncoQuick).*[138]
2) Filtration through special filters.[138]
3) Biochip that uses unique differences in size and deformability of CTCs.[138]
4) Micofluidic devices: by combining miltiorific flow fractionation (MOFF) and dielectrophoretics (DEP) cell separation techniques.[138]
5) Dielectrophoretic field-flow fractionation (DEP-FFF) device: allows for the isolation of viable CTCs by different responses to DEP due to differences in size and membrane properties.[138]

1.3.2.2 IMMUNOLOGICAL PROPERTIES

In addition to using physical properties of CTCs, there are a many biological (immunological) properties that maybe employed for CTC isolation. These properties are largely based on the expression of cell surface markers, including EpCAM for positive selection and CD45 for negative selection. Numerous methods have been used and many of them are EpCAM-based. Examples include:

1) Antibody-coated microposts: The disadvantage is the high CTC counts in cancer patients and the frequent detection of positive events in healthy controls[139].
2) EPISPOT (EPithelial ImmunoSPOT) which detects only viable cells after the depletion of CD45+ cells[140].
3) Collagen Adhesion Matrix (CAM) identifies CTCs based on their invasive in vitro properties[138].
4) MagSweeper: Identifies circulating epithelial cells (CepCs) expressing EpCAM[141].

5) CellSearch: Detects only EpCAM positive cells[142].

1.3.3 BASIS OF ISOLATION

1.3.3.1 NEGATIVE SELECTION

Negative selection refers to isolating CTCs by removing all non CTCs from the sample. This can be performed by red blood cell (RBC) lysis (which leaves nucleated cells and platelets) or via immunological depletion of leukocytes (i.e. removing CD45+ cells). The latter can be performed with magnetic beads or the use of a solid support coated with anti-bodies that target CD45, a leukocyte-specific antigen. Obviously - and is usually the case - depletion of both RBCs and leukocytes are used together to negatively isolate for CTCs. Those negatively selected CTCs are then detected by microscopy or flowcytometry. Other detection tools include molecular methods (i.e PCR: Polymerase Chain Reaction) which are usually used after density centrifugation. However, PCR can not be a reliable tool to enumerate CTCs aside from estimating the number of CTCs, because the abundance of leukocytes and their DNA reduces the accuracy of the PCR assay[149]. After RBC lysis, the sample contains mostly leukocytes with some dispersed CTCs. The use of PCR to quantify CTCs is unreliable in this setting[149]. This is because the majority of the sample contains leukocytes, and thus the overwhelming majority of the DNA is from leukocytes rather than CTCs[149]. This obstacle reduces the sensitivity of CTC detection by PCR[149].

The inaccuracy of PCR relates to the fact that blood doesn't only contain CTCs. There are numerous other cancerous and non cancerous products that contain genetic material. The blood contains cfDNA, cfRNA, lymphocytes, apoptotic cells, exosomes,

and CTC fragments. All of these contents will give PCR products. The only way for PCR to work is if there is a specific enough mutation found in CTCs only[149,208]. This mutation has to be found in abundance or it can be missed by the PCR assay.

1.3.3.2 POSITIVE SELECTION

Positive selection[141] refers to isolating only CTCs from a blood sample and discarding the rest of the content. Positive selection of CTCs has been characterized by either physical or biological properties. Physical characterization assesses the differences in deformability/rigidity, size, and dielectrical properties of CTCs. Filtration techniques[143-147] allow erythrocytes and most leukocytes to pass through while retaining the larger sized CTCs. Variations in CTC and leukocyte movements allow for separation and enrichment of CTCs based on dielectrical properties[138,141]. Biological characterization using intracellular and extracellular tumor specific antigens can also be used for positive selection of CTCs. EpCAM expression by CTCs can be directly targeted using antibodies against it via ferrofluids, solid support, or magnetic particles[148]. The issue with this technique is the assumption that all CTCs - and none of the leukocytes- express EpCAM[148,149]. This can be problematic when studying many aggressive tumors that have undergone epithelial mesenchymal transition (EMT)[149]. Refer to section 1.3.4.6 on page 44 for more details on EMT.

1.3.3.3 SIZE-BASED FILTRATION

The first use of filtration for CTC isolation was in 1964[150]. Size-based enrichments of CTCs use membrane filter devices such as ISET (isolation by size of epithelial tumor cells) or MEMs (microelectromechanical system)-based microfilter approachs[143,146]. The use of filters allows for isolation of CTCs based on their physical properties (i.e. size and

deformability). Large cells cannot pass through the filter pores. Likewise, stiff cells find it difficult to squeeze through the filter pores. Both of these features are characteristic of CTCs and therefore, as blood cells flow through the filter pores CTCs are retained. However, recently[151], it was suggested cell stiffness between CTCs and blood cells is not significantly different. Stiffness was determined by the length of time required for CTCs to pass through a microfluidic constriction. A few limitations exist in that study. First, according to the authors, for the experiment to have statistical significance, there had to be a high CTC count in each sample, and this was not the case. Second, only two prostate cancer patients samples were used, the rest were engineered cell lines and mouse cells. Third, most of the CTCs were small and thus had less difficulty passing through the constrictor site. Fourth, the authors admit that just because some cells are not stiff, it doesn't mean that stiff cells do not exist. Additionally, even if CTCs are not stiff, size-based filtration can still isolate CTCs based on size, and thus deformability is not a crucial necessity for size-based filtration.

It was shown[152] that size-based filtration is successful when attempting to isolate prostate cancer cells of all risk groups. Additionally, this antibody-independent system does not rely on immunological properties of CTCs, and more importantly, is not susceptible to miss CTCs that have under gone EMT and lost the ability to express EpCAM[153]. These features allow for objective isolation of CTCs that will allow examination of their morphologies. Furthermore, the uniqueness of this technique is the ability to isolate CTCs across all spectra of prostate cancer risk groups; an ability that the FDA-approved CellSearch lacks. *Table* 5 highlights some of the common

polycarbonate track-etched sized-based filters used today.

Table 5: The Common Available Filtration Devices[154]

Company	Device	Pore Size (µm)	Volume of Blood used (ml)	Filtration mechanism	Application of isolated CTCs
Screen Cell®	ScreenCell® Cyto	7.5	3	Aspiration created by a vacuum tube collector	Cytology, imaging, immunohistochemistry, FISH
	ScreenCell® MB	6.5	6		Molecular Biology
	ScreenCell® CC	6.5	6		Culture
RareCells®	ISET®	8	10	Aspiration created by an electric vacuum pump	Cytology, immunohistochemistry, molecular biology, culture
MetaCell®		8	10-50	Capillary action of the absorbent in contact with the membrane filter	Culture, and then cytology, immunohistochemistry, molecular biology

1.3.3.4 THE CELLSEARCH SYSTEM

The CellSearch system is the only FDA approved[142,149] system for CTC isolation that produce clinically significant results. The system[142,149] enriches CTCs from a 7.5 mL blood sample by incubating the sample with a ferrofluid labeled monoclonal antibody targeting the EpCAM antigen, a membrane protein expressed in a variety of cancers[149]. As with any procedures, there are potential complications. The issues of degradation, coagulation, and aggregation have been resolved with the use of the anticoagulant EDTA and centrifugation prior processing[149]. However, a clear limiting factor is the heterogeneity of EpCAM expression which ultimately decreases the CTC recovery rate from tumor samples; specifically, those with decreasing EpCAM antigen densities (via EMT)[149]. While it is true that the CELLTracks-Autoprep system is fully automated and thus devoid of human bias and errors, the results will always be questionable because CTCs that lack expression of EpCAM will not be detected[142,149]. EMT effects all tumor cells, but more so the more aggressive tumors that are metastatic[159]. When EMT stop EpCAM expression, CellSearch® (and other techniques that rely on EpCAM) can miss CTCs[142,149].

1.3.4.5 LIMITATIONS FOR FILTRATION

Filtration is a reliable, fast, and simple technique with little to no complex instrumentation. Filtration devices (7.5-8.0 µm diameter) were shown to retain 85-100% of CTCs[113]. However, certain filtration limitations do exist and should be anticipated. The filtration kit (ScreenCell®) used in this experiment must process blood samples within 4 hours[113]. Additionally, blood clogging may stop filtration[113]. To avoid the later, EDTA is used as an anti-coagulant. Very rare cells, smaller than 6-8 µm, can be missed by filtration. A study[146] showed that the average detected nuclear CTC size was 4-18 µm and 8-30 µm using CellSearch and filtration devices, respectively. It was shown[113] that that ScreenCell® has a 92.5% recovery. However, they did not use patient samples. To test the recovery rate of ScreenCell® filters in PCa samples, one could do a simple PCR assay on the flow through to determine how many CTCs are lost. However, this method would also detect circulating free DNA. There are also many other limitations in using PCR that were described in details in section 1.3.3.1, but in summary, PCR is not optimum for a multiple reason: The flow-through contains circulating DNA, circulating free RNA, CTC fragments, apoptotic CTCs, and lymphocytes. Hence, PCR quantification cannot accurately resemble the number of CTC in the flow-through. As a result, other techniques based on immunological expressions may be used. However, to increase the sensitivity of the assay, one must not rely on EpCAM only. Markers to be investigated should include: EpCAM+, CD45-, CD15-, CK+, and PSMA+.

Although prostate and breast CTCs are typically smaller than CTCs from other cancers (i.e. cervical and liver)[155], they remain significantly larger than leukocytes[155,156].

1.3.4.6 EPITHELIAL MESENCHYMAL TRANSITION (EMT) AND METASTASIS

For metastasis to occur, tumor cells from the primary parent tumor must acquire the ability to leave the host. Upon doing so, tumor cells must also have the ability to intravasate into the vasculature to become known as CTCs. Once in the vasculature, a CTC must survive the host's protective mechanisms (i.e. immune system). Eventually, CTCs will extravasate to a new site where they are either destroyed by tissue macrophages and the inhospitable new environment, or proliferate to establish a new metastatic site[149,157,158]. The presence of CTCs in the blood plays an important factor in initiating metastatic deposits[157-159]. The majority of CTCs are cleared in the circulation, and when administering CRPC cells to mice[185], but without any evidence of new onset tumor growth[129]. CTCs also have the ability to reseed to their point of origin, a phenomena known as "self-seeding". This method is thought to accelerate parent tumor growth and angiogenesis[162, 163] through the expression of new factors. EMT is alleged to be responsible for metastasis[160,161]. Adherent tumor cells are thought to gain migratory and invasive properties through this process[162]. During EMT, tumor cells lose the ability to express epithelial markers and gain the ability to express mesenchymal and/or stem-cell like markers[163]. In doing so, the phenotype changes from an epithelial to a mesenchymal or stem-cell like phenotype[163]. Through this process (EMT), cells acquire the ability to break away from the primary tumor, extravasate into the vasculature, and also gain the ability to survive in the blood stream[164]. Studies have shown that CTCs in the vasculature acquire mesenchymal markers (when compared to the parent tumor)[164]. The process of EMT can be reversed (Mesenchymal-Epithelial-Transition - MET); probably occurring at the new site of metastasis when CTCs need to settle and proliferate again[162-164].

CellSearch misses the EMT population[142,149], which plays a crucial role in metastasis[163]. On the other hand, EMT cells are not missed by filtration. Specifically, metastasis-initiating microemboli[162] (a.k.a clusters) which generally undergo EMT, are missed by CellSearch but not by filtration[140]. Microemboli, like CTCs, have been shown to have mesenchymal phenotypes[165]. Detecting these clusters is important because they carry a 23- to 50- fold increase in metastatic potential[137]; especially in CRPC patients who express *EMT-related genes*[166].

1.3.4.7 SUPERIORITY OF FILTRATION OVER IMMUNOAFFINITY ASSAYS

In addition to the points mentioned in the preceding section (1.3.4.6: Epithelial Mesenchymal Transition), there are other reasons why filtration is superior to CellSearch®. Frances et al[148], showed that filtration is superior to CellSearch (57 vs 42 CTCs) for the detection of CTCs in metastatic breast, prostate, and lung cancer. Filtration was also found to be more superior for hepatocellular carcinoma[167], pancreatic cancer[168], and non-small cell lung cancer[146,169]. A study[152] compared filtration (ISET®) and Miltenyi® (immunomagnetic separation method) on follow up patient samples. They showed that an increase in CTC numbers correlated with worsening clinical status (via ISET®). However, Miltenyi® (based on immunological phenotypical expressions) showed a decrease in CTC counts in those with worsening clinical status. It is possible CTCs that have undergone EMT to be detected by filtration only. Benign epithelial cells corresponding to the CellSearch® criterion EpCAM+, CD45-, and CK+ can be detected as CTCs and thus produce a false-positive result and a mislabelled CTC[170]. In filtration, false positive CTC detection of benign cells can occur. Examples include normal

epidermal cells (large picnotic nuclei) during blood collection, megakaryocytes (round and pale nucleus), parathyroid or thyroid adenoma cells (large, irregular anisonucleosis nuclei)[169], and benign melanocytic nevi cells[171]. However, the nuclear morphology and the positive stain for androgen receptor (as in this study) easily distinguishes true CTCs from other false-positive detections. This is because the AR-Ab we utilize binds only to AR type 2, which is only found in the prostate. In prostate cancer, EpCAM expression is reduced by 29%[170]. The reduction is more pronounced in higher stage disease (likely due to EMT)[170]. Therefore, CellSearch® is not an ideal system to use for enriching CTCs from PCa samples. As a system that only detects EpCAM-positive cells, at least 29%[170] of CTCs (which are EpCAM-negative) will be missed.

To conclude, size-based filtration has many advantages that include: 1) ease of use, 2) precise counting of CTCs per mL of blood, independently of the volume of treated blood, 3) allows for subsequent cytopathology, and cytological staining, and 4) allows for laser microdissection to isolate specific CTCs of interest for further RNA and DNA sequencing once RT-PCR is performed. There are disadvantages: 1) non-specific, 2) CTCs can go through pores, and 3) CTCs can be damaged. However, these disadvantages are less pronounced than the disadvantages of using CellSearch®, which include: 1) Subjective analysis for CTC identification (i.e. EpCAM+ CTCs only), 2) time consuming, 3) Needs specific makers and anti-bodies, and 4) expensive. Also, some of the limitations of filtration can be minimized. For example, non-specificity can be greatly reduced by doing immunohistochemistry and staining for AR. CTC damage can be minimized by careful manipulation of the filter.

1.3.4.8 ENUMERATION

Enumeration of CTCs can be carried out manually or by automated methods. Each has its own advantages and disadvantages. Manual counts are not ideal because of potential interobserver bias. However, this bias can be limited if a trained clinician scientist performs the counts. An advantage of the manual method is the ability for the researcher to examine each CTC and rule out fragments and artifacts that an automated system may not. On the other hand, automated methodologies are more attractive because of the ability to standardize the process. The downside is the need to validate and remove events the computer counts incorrectly. *Table 6* summarizes the different CTC counts using the various available CTC isolating techniques.

1.3.4.9 THE USE OF CIRCULATING TUMOR CELLS IN CLINICAL DIAGNOSES

The isolation of CTCs can serve as a potential diagnostic tool for numerous types of cancers, thereby, replacing an invasive tissue biopsy with a simple peripheral blood sample. Multiple studies have been done in the past that showed adequate sensitivity and specificity when evaluating the benefits of using CTCs for diagnosing cancers. In pancreatic cancer[172], sensitivity and specificity were 55.5% (95 CI, 40.1-70.9%) and 100% (95% CI, 75-100%), respectively. For bronchopulmonary cancer[173], sensitivity and specificity were 71.9% (95 CI, 60.5-83%) and 52.9% (95% CI, 31.1-77%). Additionally, the use of CTCs shows the potential to diagnose cancers that are not radiographically visible, and to distinguish between benign and malignant tumors. In one study[173], it was shown that a count of >25 CTCs/7.5 ml had an 89% and 100% sensitivity and specificity for malignant rather than a benign lesion. Similar experiments have also been performed to distinguish benign and malignant adrenocortical masses[174,175]. Interestingly, in one study, CTCs were able to predict the development of

cancers. This study[173,176,172] followed 5 COPD patients with annual low-dose CT scans. Within 1-4 years, all patients developed lung nodules that corresponded with histological lung cancer. The patients had detectable CTCs in their blood prior to the radiographic appearance of lung nodules. Therefore, the use of CTCs as a marker in high risk individuals may be used as a screening tool.

Table 6: The Different CTC Isolation techniques In Prostate Cancer & Their Respective Enumerated Results

MECHANISM	CTCs	COMMENTS	AUTHORS
Reset*table* cell trap (RCT)	mCRCP = 257 CTCs/ 7.5 mL		*Qin X, 2015*
Microfluidic/Acoustophoresis Chip	86.5 ± 6.7% recover and 1.1% contamination	DU145 cell line	*Antfolk M, 2015*
Filtration/Automated microscopy	195 CTCs/ 7.5 mL	EpCAM and Anti-PSA	*Nitouroupi TG, 2008*
Nanovalcro Chips (Cytolumina)	Visceral Mets: 0-61 CTCs/mL (vsn 65%, sn 20%) Non-visceral mets: 6-38 CTCs/mL (sn 51%, vsn 27%) No mets: 8-15 CTCs/mL (vl 62%)		*Chen JF, 2015*
CellSearch	Bone mets (mean: 41.12 CTCs/7.5 mL) LN mets: (mean: 2.53 CTCs/7.5 mL) Mean pre and post ADT: 2.7 vs 26.9 CTCs/7.5 mL Gleason <6: mean 0 CTCs/7.5 mL Gleason 7: mean 1.43 CTCs/7.5 mL Gleason >8: mean 38.42 CTCs/ 7.5 mL	EpCAM Positive statistical clinical significance	*Amato RJ, 2015*
Microfluidics NanoveIcro CHIP	Healthy control: 0-2 CTCs/mL Cell lines: 1-99 CTCs/mL	PC3 cell line LNCaP Capture efficiency: 80-95% Positive statistical clinical significance	*Lu Yt 2013*
Microfluidics	20-300 CTCs/7.0 mL	EpCAM PC3 cell line Recovery of 71%	*Wael Harb, 2013*
Ficoll/CellSearch	HRLPC: only 49% of pre-surgical detected Median: 3 CTCs/ 7.5 mL	E-Cadherin and CD133 No clinical correlation	*Pal Sk, 2015*
Microfiltration CellSeive/CellSearch	3.6 CTCs/ 7.5 mL	Cytokeratin positive cells Anonymous PCa patients	*Adams DI, 2015*
Functional & Structural Medical Wire (FSMW) by GIPLUPI/microscopy	221-354 CTCs/ 5L (manual microscopy)	Vein insertion via angiography EpCAM-based Further stain for CK & CD45 Irregular nuclei & clusters counted as a CTC	*Svensson CM, 2015*

EpCAM-based Ficoll centrifugation/NIR dye/Microscopy	Control 1: Killed PC3 cells = 0 CTCs that up-took NIR Control 2: 1-5 CECs/1mL in healthy adults Range findings per 1mL (total cells, NIR cells, percentage) Gleason 6 (low risk, n=2): 8-31, 4-48, 50-89 Gleason 6 (Int. risk, n=9): 1-54, 1-53, 83-100 Gleason 3+4 (int risk, n=15): 0-151, 0-148, 11-100 Gleason 3+4 (high risk, n=5): 8-119, 8-87, 45-100 Gleason 4+3 (int risk, n=1): 4, 4, 100 Gleason 4+3 (high risk, n=5): 8-440, 7-439, 89-100 Gleason 4+5 (high risk, n=1): 57, 46, 81%	Aim: Detect live CTCs that uptake NIR dye Live cells defined as: EpCAM+/CD45-/NIR+/DAPI+ Samples are 2 wks pre-RT Risk based on NCCN Live CTC count increased as tumor progressed Live CTC count decreased with androgen therapy <5 CECs/1mL = normal	*Chen Shao, 2014*
ISET	High Risk samples = 4.1 CTCs/6.5 ml	LNCaP cell line	*Vona G, 2000*
EpCAM based immuno-magnetism (IE/FACS)	CRPC: 1-590 CTCs/7.5 ml	CRPC patient samples	*Magbanua M, 2012*
PSA-ELISPOT assay	Examined PSA in viable secreting cells: Pretreatment: 5/12 samples detected (2-172 CTC/10ml)	LNCaP cell line PC-3 cell line	*Alix Panabieres, 2005*
High-throughput microfluidic mixing device/HB-Chip	CTCs detected in 14 of 15 (93%) patients Median = 63 CTCs/mL Mean = 386 ± 238 CTCs/mL	metastatic prostate cancer	*Stott SL, 2010*
EpCAM based microchip	Detection of CTCs in all prostate cancer patients (7/7) Metastatic Prostate Cancer: 16-292 CTCs/ml	Samples from metastatic and localized prostate cancer	*Nagrath S, 2007*

	Localized Disease: 25-174 CTCs/ ml		
EpCAM based CTC-iCHIP	Recovery rate of: 89.7 ± 4.5%	PC3-9 prostate cancer cell line	*Emre Ozkumur, 2013*
Geometrically enhanced differential immunocapture (GEDI) microfluidic device via anti-PSMA	Median: 54 CTCs/ ml vs. 3 CTCs/ml in healthy donors	Samples from mCRPC patients on taxane chemotherapy CTC defined as nucleated PSMA +/CD45-	*Kirby BJ, 2012*
MetaCell® Filtration device	Localized Prostate Cancer (n=55) CTCs detected in 28 samples (52%) Proliferation capacity in 18/28 samples (64.3%) pT2a: 5 CTCs pT2c: 17 CTCs pT3a= 5 CTCs pT3b= 1 CTC Gleason 5 (2+3) = 1 CTC Gleason 6 (3+3) = 14 CTCs Gleason 7 (3+4) = 8 CTCs Gleason 8= 2-4 CTCs Gleason 9 (4+5) = 2 CTCs	Used actual patients' samples Localized prostate cancer No metastatic samples used CTCs are per 8.0 ml	*Kolostova K, 2014*
CellSearch	N= 231 patients >5 CTCs/7.5 ml = reduced overall survival (11.5 vs 21.7 months, p-value <0.0001)	CRPC patients on Abiraterone+prednisone after Docetaxel chemotherapy	*De Bono et al, 2008*

2. SUMMARY

In summary, prostate cancer is a heterogeneous disease that requires multiple exams and/or procedures to be put together to finalize a diagnosis. These include: biopsy, PSA, and a physical examination. However, diagnosis is not always accurate and is sometimes either missed, disputed, or delayed. Recently, CTCs have emerged as an important tool for analyzing and diagnosing cancers. Currently, the isolation and characterization of CTCs presents a challenge. The only FDA approved system misses many cells. I have thus performed a study to isolate CTCs via size-based filtrations. The CTCs were enumerated and so were the clusters on the filter. This was performed for all risk groups of localized PCa as well as metastatic disease. Filters were stained with an androgen receptor antibody (targeting the N-terminus)[215] to prove that captured CTCs were prostatic in origin. A manual and an automated CTC count was carried out on all filters.

3. MATERIALS AND METHODS

3.1 PATIENTS AND CONTROLS

As per the Health Research Ethics Board of the University of Manitoba (Ethics #: HS14085 (H2011:336) and its affiliated Cancer Care Manitoba center, prostate cancer patients were consented to participate in this research study. We began by collecting peripheral blood samples from all risk groups: low risk (n= 12), intermediate risk (n= 40), high risk (n= 38), and metastasis (n= 7). Risk groups have been assessed as per Dr. Anthony D'Amico's[1] risk classification for localized prostate cancer (Hernandez DJ., 2007). Metastatic patients showed radiological evidence of lymph node (LN) involvement, osseous, and/or visceral metastasis. All blood samples were collected prior to the initiation of any form of prostate cancer treatment (pre-treatment). Note that we did not consider 5-alpha reductase inhibitors (i.e. Dutasteride) or alpha antagonists (i.e. Tamsulosin) as treatment for prostate cancer. Many patients have been taking at least one of these medications for years. Blood Samples were mixed with Ethylenediaminetetraacetic acid (EDTA) and then processed within two hours through a filtration-based CTC isolation mechanism made by ScreenCell®. All samples were obtained from patients with active PCa prior to treatment. All prostate cancer cases were pathologically confirmed via 12-14 core transrectal ultrasound (TRUS) biopsy.

The following sections (3.11-3.15) show the details of each cohort. A simplified version can be found in *Table 7.*

3.1.1 CONTROL SAMPLES

Five (5) samples were taken from healthy young volunteers to account for confounds

and false-positive findings. None of the control samples have any form of any malignancy, including prostate cancer. See *Figure 15*.

3.1.2 LOW RISK SAMPLES

There were 12 pre-treatment patient samples in this group. Five underwent radical prostatectomy (RP) and continue to have undetectable (UD) PSA, while 1 patient, who remains on active surveillance, has progressed to intermediate risk. The rest of the low risk patients continue to have stable non-progressive (chemically and pathologically) disease.

3.1.3 INTERMEDIATE RISK SAMPLES:

In this group, there were 40 pre-treatment patient samples, of which 31 underwent RP, 6 continue to be under surveillance, 1 was treated with androgen deprivation therapy (ADT) only, and 2 underwent cryotherapy (cryo). Of the 31 RP patients, 25 had UD PSA, 7 had biochemical failure (BCF), and 2 continue to be on ADT to achieve UD PSA. Of the 7 post-RP BCF patients, 2 had positive lymph node involvement on pathology. Of the 2 cryo patients, 1 achieved UD PSA while the other had BCF.

3.1.4 HIGH RISK SAMPLES:

There were 38 pretreatment high risk samples: 14 received external beam radiation therapy (EBRT) ± adjuvant/neoadjuvant ADT, 22 RP only, and 2 ADT only. Only 8 of the 22 patients who underwent RP had UD PSA post-operatively. The other 14 BCF patients were put under adjuvant ADT ± salvage RT. Only 1 patient of the RP group developed CRPC as he failed to respond to EBRT.

3.1.5 METASTATIC SAMPLES:

There were 7 pretreatment samples in this cohort, all of which received hormonal and/or

radiation therapy with subsequent palliative chemotherapy. The 4 expired patients had only transient response to ADT. Of the 3 alive patients, only continues to be on ADT.

***Table* 7 Shows an overview of the patient sample list detailed in sections 3.1.1 to 3.1.5**

Patient Cohort	Sample size	Cure	RP [1]	Radiation [2]	ADT[3] ± Chemotherapy [4]	Active surveillance [5]	Stable disease [6]	Progression [7]
Control[8]	5	-	-	-	-	-	-	-
Low Risk[9]	12	4	5	0	0	6	7	1
Intermediate [9]	40	26	31	2	1	6	6	7
High Risk[9]	38	8	22	14	2	0	16	14
Metastasis[10]	7	-	-	-	7	-	-	7

[1]RP: Radical Prostatectomy

[2]Radiation: includes external beam radiation, brachytherapy, and cryotherapy

[3]ADT: Androgen deprivation therapy

[4]Chemotherapy: Docetaxel or Cabazitaxel

[5]Active surveillance refers to no therapeutic interventions, but only routine PSA and rectal exams

[6]S*table* disease: No increase in Gleason grade on re-biopsy, low PSA velocity, and/or no new masses. Refers to status of those on Active surveillance for low risk and intermediate risk patients. But for High risk and metastatic patients refers to status of those who failed to be cured.

[7]Progress: includes biochemical failure, rapid PSA rise, new metastatic lesions, and/or failed initial therapy

[8]Healthy volunteers with no sign of prostate cancer or any other malignancies.

[9]Defined as per Dr. Anthony D'Amico's criteria for the stratification of localized prostate cancer

[10]Defined as positive lymph nodes on CT-scan, lesions on x-ray or CT-scan, osseous involvement on bone scan

3.2 SCREENCELL® FILTRATION CYTO-KIT UNIT

To isolate CTCs, we used a size-based filtration method, the ScreenCell® filtration kits. The procedure was previously explained in details[113]. In brief, samples were initially equilibrated with ScreenCell-Buffer® and incubated at room temperature for 8 minutes. Subsequently, to isolate CTCs we used non-coating filtration units (Cyto-kit) made by ScreenCell® (Sarcelles, France). Only 3 mL of blood was utilized to flow through the

filtration unit into an empty vacuum-behaving tube, after which a thorough wash of the filter was applied with 1.6 ml 1x phosphate-buffered saline (PBS). This qualitative approach relied on the filters' small pores ($1x10^5$ pores/cm^2) of 7.5 ± 3.6µm to allow erythrocytes, leukocytes, and platelets to pass through but not the stiff larger CTCs (15-25µm)[113]. The validation of this technique showed a 91.2%[113] recovery rate[113,178]. ScreenCell® uses a patented buffer composed of Saponin and formaldehyde.

3.3 THE ANDROGEN RECEPTOR ANTIBODY

Our AR-Ab (441): sc-7305 (Santa Cruz Biotechnologies, Dallas, TX, USA)[215] is a mouse monoclonal IgG provided at 200 µm/ml that binds to amino acids 299-314 of the human AR. However, it does not target splice mutations such as AR-V7. The specific step for using the AR-AB involved incubated the filters for 45 minutes in a 37˚ humidified chamber. A 1:50 µl (20ng/µl) of AR Ab conjugated with Alexa Fluor® 488 at 100 µg/2ml was added to the filters. These steps were part of the QFISH/Immunohistochemistry protocol that will be discussed further below.

3.4 ANDROGEN RECEPTOR SPECIFICITY

As described in the previous section (3.3), the CTCs' AR was stained using immunohistochemical techniques with a monoclonal IgG antibody. To show the specificity of the AR-Ab used in this experiment, an isotype control was performed. The AR-Ab used in this experiment is an IgG_1. A control targeting the same IgG subunit (concentration 1:20) was used to determine whether this control stains the AR of CTCs or not. The control Ab is CY5 labeled. A total of 4 pre-treatment samples (2 intermediate and 2 high risk) were used. The samples were each filtered through a ScreenCell Filtration CytoKit Unit as explained in section 3.2. After filtration, each sample stained

for DNA using Q*FISH* (DAPI) and AR (FITC). In addition, the samples underwent immunohistochemical staining for the the CY5 labeled isotype control. The techniques of the staining protocol can be found in section 3.4.

3.5 STAINING WITH DAPI AND ANDROGEN RECEPTOR ANTIBODY

We developed a protocol for staining DNA, telomeres, as well as the androgen receptor. Telomeric staining was concurrently performed to allow for subsequent analysis of CTC telomeres, a portion of the study that is not part of this project or thesis. All filters went through the immuno/FISH telomere hybridization protocol. Initially, we incubated filters at room temperature in 1xPBS for 5 minutes without shaking to allow for rehydration. We then transfer the filters into a freshly made 3.7% formaldehyde/1xPBS solution and then again incubated for 10 minutes at room temperature in a vacuum hood without shaking. After, we applied two 3 minute 1xPBS/50mM $MgCl_2$ washes at room temperature on the shaking platform (Clorkip® Model: 14928) at speed 3. Then, CTCs were permeabilized with 0.05% Triton X-100 (in ddH2O) for 10 min without shaking. Afterwards, three 2 minute washes of 1xPBS/50mM $MgCl_2$ were performed on a shaking platform. Then, CTCs were blocked in 4% BSA/4xSSC for 5 min at room temperature. This was quickly followed by addition of AR antibody (8µl/filter) in 4% BSA/4xSSC, 45min at 37˚, humidified atmosphere (as explained in the previously). From this step onwards, the rest of the experiment was performed in the dark only (this is due to the photosensitivity of the AR-Ab). The filters were again washed three times for 3 minutes each in 1xPBS/50mM $MgCl_2$ on the shaking platform. The filters were then transferred into a freshly made 3.7% formaldehyde/1xPBS solution and incubated for 5 minutes in the vacuum hood without shaking. The filters were then placed in 70%, 90%

and 100% ethanol to dehydrate, each at room temperature for 2 minutes.

CTCs were not stained for cytokeratin (CK). This is because cytokeratin-based CTC counting has been shown to not correlate with the clinical scenario or add any benefit to enumeration[152]. In addition, we have previously shown[179] that prostate cancer CTCs contain very little cytoplasm, and thus CK staining may be missed when working with the 40x non-oil lens. Also, CK staining is not specific for PCa cells the way AR staining is. Lastly, studies have shown that CTCs do not always stain positively for CK[208].

3.6 EXAMINATION OF THE FLOW-THROUGH

After filtration (as described in section 3.2), a total of 6 pre-treatment samples (3 intermediate and 3 high risk) underwent a flow-through (FT) study. To study the FT, 1ml of post-filtration blood was used. The 1ml was diluted with 10 ml of 1xPBS and then centrifuged at 1500 rpm for 15 minutes. The supernatant was removed and another 10 ml of PBS was added and centrifuged again for 15 minutes at 1500 rpm. Three more rounds of supernatant removal and PBS washing/centrifugation occurred. The final product of clear fluid cellular component was centrifuged through a drainage block that dehydrated the solution on a mounting slide. Subsequently, the slide underwent Q*FISH* with DAPI for DNA staining and a immunohistochemical staining with AR-Ab for AR staining. The sample was then microscopically visualized using the 20x, 40x, and 63x oil lens.

3.7 PREPARING FILTERS FOR ENUMERATION

To enumerate CTCs, we utilized an automated and a manual method. After filtration

and subsequent staining, the filter sheath was carefully carved out of the metal brim using a skin punch biopsy (1 cm diameter). The filter sheath, which now had all the collected CTCs was transferred onto a microscope slide (75x26x1 mm). A 5µl drop of Vectashield® Anti-fade Mounting Medium with DAPI (catalogue number H-1200) was applied. A cover slip (20x20mm) was then used to cover the filter. To immobilize the coverslip, a clear nail polish was applied carefully to the surrounding edges of the cover slip. This step is tedious because the minutest amount of nail polish could harm the filter and render it useless for microscopic visualization. Therefore, the nail polish brush was gently brushed on the brim of the bottle prior to application to remove excess nail polish.

3.8 ENUMERATION OF CIRCULATING TUMOR CELLS

3.8.1 AUTOMATED ENUMERATION

The automated method was carried out using the GenASIs® software (version 7.2.1.2) developed by Applied Spectral Imaging (ASI)® which scans up to 9 filters at a time. The filters were all scanned three-dimensionally (3D) using a 40x non-oil lens. After scanning, the program would detect CTCs based on a classifier we developed (unpublished). The classifier included the following parameters: cell diameter= 3-65 pixels, cell brightness= 0.4, inflammatory level =100, cell circularity= 2, signal sensitivity= 1, cell detection scheme= uniform contrast, enabled overlap = 1%, split touching cells = on, and touching borders= off. The scanned frames were reviewed for the correctness of the scanned cells. The automated count only produced CTC counts and was not set up to identify clusters or fragments.

3.8.2 MANUAL ENUMERATION

Additionally, all the filters were entirely manually visualized to count and comment on

CTCs. The filters were three-dimensionally (3D) imaged using the 40x non-oil lens described in the imaging section above. A record was kept regarding morphology, clusters, cluster size, and fragments. A CTC was defined as that which stained positive for DAPI and AR, as well as showing morphologic features such as absence of apoptotic features and the presence of an intact structure. Fragments and clusters were not counted as individual CTCs. However, a record was kept with respect to CTC size as well as the quantity and size of clusters. The entire manual enumeration took between 30-60 minutes per filter. In comparison, automated method per filter lasted for 30-45 minutes to scan and another 3-4 hours to validate by visual inspection.

3.9 CHARACTERIZATION OF CTCS

In addition to comparison of enumerated filters, we also characterized CTCs based on their physical profile. We included information on large CTCs (CTC_{large}), cluster sizes, as well as the amount of clusters found in each filter. The data of the morphological and physical characterizations of CTCs and clusters was drawn from the manual count method as it was more feasible to comment on these physical profiles while counting.

3.9.1 LARGE CTCS

A large CTC (CTC_{large}) was defined as those with a nuclear size larger than 20 µm in the greatest dimension. In addition, the CTC must also meet the criteria (DAPI+ and AR+) mentioned above in the enumeration section of the materials and methods.

3.9.2 CLUSTERS

During manual enumeration of CTCs, we also noted the behavior of clusters. Clusters were defined as 2 or more CTCs with no discernible segregation at the 40x non-oil lens.

The size of the clusters was defined as: $Cluster_{small}$ (2 CTCs), $Cluster_{medium}$ (3-5 cells), and $Cluster_{large}$ (>5 cells and/or >45 μm in diameter).

4. PROJECT

4.1 HYPOTHESIS

1) CTCs are shed into the vasculature from the primary tumor and metastatic foci. The quantity of CTCs in the blood is associated with the stage of prostate cancer.

2) Clusters are also released into the vasculature. The quantity and sizes of clusters are associated with the stage of prostate cancer.

4.2 RATIONAL

In CRPC, the PSA lacks accuracy in assessing disease burden[181]. Therefore, physicians are left to rely on symptoms, imaging, and bone biopsies to monitor progression or response to treatments. Additionally, the PSA is known to fluctuate, and is not always a reliable indicator for the diagnosis of PCa. However, CTC enumeration presents a potentially promising biomarker that can be employed in clinical trials or simply as a tool for following patients in real time[180].

CTCs can also be employed to differentiate localized from metastatic prostate cancer. They can also be used as one of the tools that differentiate the various stages of PCa (see *Table 6*). Currently metastatic disease is confirmed only via radiographic evidence or post surgically when lymph nodes are microscopically examined. The latter is the most reliable method for detecting micrometastasis[181]. However, many metastatic sites are either too small to radiographically visualize, are benign lesions, or can be missed. Additionally, lymph node involvement does not necessarily correlate with haematogenous spread and metastasis[182]. Many studies[172-175] have shown that CTCs can be detected prior to metastasis. Additionally, other studies[172,173,176] have shown that

CTCs can be detected prior to the clinical diagnosis of malignancies. Also, studies[174,175] have shown that CTCs can also profile metastatic vs localized diseases in various cancers.

In this study, we will show that size-based filtration is a reliable tool that can isolate and enrich CTCs. The isolated CTCs will be enumerated to give insight and understanding of the numerical counts found in the various stages of PCa. CTC behaviour (i.e. the ability to form clusters) will also be examined in all prostate cancer stages.

By performing this study, we can validate the reliability of size-based filtration, and show that a higher stage of prostate cancer is associated with a higher CTC count, and more frequent CTC clusters. The efficiency of this techniques lies in the fact that CTC enumeration can be rapidly accessed through a quick, simple, and a non-invasive liquid biopsy.

4.3 AIMS

1) Collect CTCs

2) Enumerate CTCs automatically

3) Enumerate CTCs manually

4) Analyze whether CTC count changes between prostate cancer stages

5) Analyze whether CTC morphology (i.e. clusters) correlate with disease aggressiveness.

5. RESULTS

CTCs are emerging as a clinical marker for understanding cancers. The current FDA approved system, CellSearch®, does not isolate cells from lower risk PCa samples, nor does it account for EpCAM-negative CTCs. Therefore, we have utilized a size-based filtration approach to isolate CTCs from all stages of PCa. The isolated CTCs met morphological criteria as well as stained positive for DAPI and AR-Ab. *Table 8* shows the detailed values for each patient blood sample. *Table 9* highlights the mean, median, and range of each prostate cancer group for the following categories: Automated enumeration, manual enumeration, large CTCs, $clusters_{total}$, $clusters_{small}$, $clusters_{medium}$, $clusters_{large}$, and PSA (refer to section 3.7 for definition of large CTCs and cluster size).

5.1 LOW RISK SAMPLES

The low risk (n=10) enumerated automated (and manual) counts were comparable (from *Table 8* and *Table 9*): mean= 41.4 (43.9), median= 39 (40.5), and range= 24-87 (17-84). The mean (median, range) counts for the measured morphological features were also recorded. Large CTCs counts were 34.8 (28, 8-82). Clusters were also documented in the same manner: $clusters_{total}$= 5.5 (1, 0-40), $clusters_{small}$= 4.9 (1, 0-35), $clusters_{medium}$= 0.6 (0, 0-5), and $clusters_{large}$= 0 (0, 0). See *Figure* 17 for a low risk image.

5.2 INTERMEDIATE RISK

The intermediate risk (n=40) enumerated automated (and manual) counts were comparable (from *Table 8* and *Table 9*): mean= 65.81 (60.2), median= 66 (62.5), and range= 22-91 (15-125). The mean (median, range) counts of measured morphological features were also recorded. Large CTCs counts were 25.9 (19, 1-86). Clusters were also documented in the same manner: $clusters_{total}$= 18.1 (10, 0-77), $clusters_{small}$= 12.33 (7, 0-72), $clusters_{medium}$= 5.38 (0, 0-35), and $clusters_{large}$= 0.15 (0, 0-5). See *Figure* 20 for

an image of an intermediate risk CTC.

5.3 HIGH RISK

The high risk (n=37) enumerated automated (and manual) counts were comparable (from *Table 8* and *Table 9*): mean= 80.03 (69.92), median= 57 (52), and range 21-200 (22-177). The mean (median, range) counts of measured morphological features were also recorded. Large CTCs counts were 17.38 (8, 0-90). Clusters were also documented in the same manner: $clusters_{total}$= 33.81 (30, 2-106), $clusters_{small}$= 5.7 (5, 0-16), $clusters_{medium}$= 15.11 (2, 0-88), and $clusters_{large}$= 11.7 (0, 0-76). See *Figure* 21 and *Figure* 19 for images of a high risk CTC as well as clusters.

5.4 METASTASIS:

The metastatic (n=8) enumerated automated (and manual) counts were comparable (from *Table 8* and *Table 9*): mean= 80.67 (75.63), median= 84 (73.5), and range 15-161 (20-119). The mean (median, range) counts of measured morphological features were also recorded. Large CTCs counts were 18.75 (3.5, 0-65). Clusters were also documented in the same manner: $clusters_{total}$= 75.5 (83, 20-117), $clusters_{small}$= 8.25 (6.5, 0-21), $clusters_{medium}$= 19.63 (9.5, 0-34), and $clusters_{large}$= 47.63 (60, 7-69).

5.5 ISOTYPE CONTROL FOR ANDROGEN RECEPTOR SPECIFICITY

After microscopic examination using AR-Ab and antibody isotype control, the sample showed that the CY5 labelled control antibody (Ab) did not stain CTCs. See Figure 10 for a detailed visual.

5.6 FLOW THROUGH ANALYSIS

The flow-through (FT) was microscopically examined after appropriate staining. Particular attention was paid to the cellular components. In the samples, DAPI+ cells were observed throughout. Androgen receptor staining did not show any clear or

obvious cellular staining, aside from minimal background haze. See Figure 11 and Figure 22.

5.7 MORPHOLOGICAL ANALYSIS

5.7.1 ENUMERATION

Both manual and automated mean counts increased steadily and comparably between the 4 groups (*Figure 13*). Patients with a more aggressive cancer had an average of 17.91% and 13.65% more $CTCs_{mean}$ in the automated and manual enumeration count, respectively. See *Figure* 18.

5.7.2 LARGE CTCS

Large CTCs were less prevalent in more aggressive PCa. The frequencies of the mean large CTC counts were 79.3%, 41.2%, 24.56%, and 22.1% for low risk, intermediate risk, high risk, and metastatic disease (*Figure 14*). This variation shows an average difference of of 19% large CTCs between the four groups. While high risk and metastatic samples showed only a 2.46% difference in the frequency of large CTCs, more apparent variations were observed between low and intermediate risk disease (38.1%). *Figure 14* shows the ratio of large CTCs in all groups of prostate cancer.

5.7.3 CLUSTERS

The overall mean $cluster_{total}$ was higher in a more aggressive disease by an average of 1.5-3 folds. There was a reciprocal relationship between the frequency of the mean small and large sized clusters (*Figure 14*). The sequential pattern from one risk group to the next -and ultimately to metastasis- showed an average decrease of 132.89% and an average increase of 80.53% in the frequency of $cluster_{small}$ and mean $cluster_{large}$, respectively. The statistical correlation for medium sized clusters was not as strong. See

Figure 14 and *Figure 22* for a comparison of clusters in all PCa groups.

5.8 STATISTICAL INTERPRETATION

5.8.1 AUTOMATED VS MANUAL ENUMERATION

There was no statistical difference between automated and manual enumeration [n=95, U-value: 2674, z-score: -1.0479, p-value ≤0.01]. Pearson correlation coefficients were calculated and strong positive relationships were found for the total samples [n=95, R=0.9503, R^2=0.9031], low risk samples [n=10, R=0.9516, R^2=0.9055], intermediate risk samples [n=40, R=0.9109, R^2=0.8297], high risk samples [n=35, R=0.9768, R^2=0.9514], and metastatic samples [n=8, R=0.9466, R^2=0.8961].

Table 8: Raw Data of All Patients Samples Utilized in this study.

The *table* shows the raw data of all the patients' samples used in this study. In total, there were 97 patients. The recorded data include automated CTC count, manual CTC count, number of large CTCs (per manual count), total number of clusters (per manual count), number of large clusters (per manual count), number of medium clusters (per manual count), number of small clusters (per manual count), and PSA.

All values are pretreatment and are per 3ml of blood.

			COUNT			CLUSTERS				
RISK	**PATIENT ID**	**FILTER #**	**Manual #**	**Automated#**	**# Large CTC**	**Total Cluster #**	**Small**	**Medium**	**Large**	**Initial PSA**
Low	MB0276	13AA0651	25	28	22	0	0	0	0	9.05
Low	MB0321	13AA0633	70	87	55	3	2	1	0	3.83
Low	MB0353	13AA0783	51	56	30	8	8	0	0	3.62
Low	MB0468	14AA3332	20	21	8	2	2	0	0	7.22
Low	MB0221	11AA7018	23	39	10	1	1	0	0	2.1
Low	MB0239	12AA3282	37	28	30	40	35	5	0	3.26
Low	MB0244	12AA3171	17	24	17	0	0	0	0	3.14
Low	MB0268	12AA5822	68	NA	68	0	0	0	0	5.58
Low	MB0275	12AA6008	44	45	26	1	1	0	0	9.05
Low	MB0358	14AA1275	84	45	82	0	0	0	0	6
Intermediate	MB0277	12AA5939	58	66	20	30	28	2	0	3.25
Intermediate	MB0390	13AA8142	60	70	8	4	4	0	0	1.23
Intermediate	MB0376	13AA8165	67	71	10	7	6	1	0	19.73
Intermediate	MB0534	15AA0561	55	50	10	8	8	0	0	15
Intermediate	MB0263	12AA3117	50	66	34	9	9	0	0	4.9
Intermediate	MB0339	13AA0173	58	60	30	10	10	0	0	11.15
Intermediate	MB0227	11AA8433	37	49	2	30	28	1	1	7.7
Intermediate	MB0399	13AA8087	66	87	35	9	8	1	0	3.26
Intermediate	MB0462	14AA3256	73	77	39	11	11	0	0	8.9
Intermediate	MB0550	14AA4073	79	84	40	38	30	8	0	5.88
Intermediate	MB0266	12AA5838	80	88	50	33	10	13	0	7.7

Intermediate	MB0527	14AA5188	80	91	48	7	7	0	0	5.4
Intermediate	MB0269	12AA5936	50	57	8	25	20	5	0	2.24
Intermediate	MB0304	12AA8654	31	47	7	6	6	0	0	5.86
Intermediate	MB0256	12AA5800	75	91	58	45	7	33	5	4.07
Intermediate	MB0336	13AA0862	40	54	11	10	10	0	0	8.33
Intermediate	MB0272	12AA5814	78	NA	70	6	6	0	0	14.71
Intermediate	MB0251	13AA8195	60	NA	1	37	35	2	0	4.06
Intermediate	MB0258	12AA3094	37	13	11	5	5	0	0	7.5
Intermediate	MB0323	13AA1157	35	NA	19	4	4	0	0	5.29
Intermediate	MB0257	12AA6190	44	45	13	5	5	0	0	5.7
Intermediate	MB0467	14AA2904	64	60	19	2	2	0	0	3.85
Intermediate	MB0236	12AA3137	76	91	18	7	7	0	0	6.8
Intermediate	MB0377	13AA7693	77	88	33	20	18	2	0	5.02
Intermediate	MB0330	13AA1390	37	58	19	10	10	0	0	5
Intermediate	MB0403	13AA8066	22	22	1	0	0	0	0	5.61
Intermediate	MB0245	12AA3140	15	46	2	0	0	0	0	9
Intermediate	MB0264	12AA6004	71	NA	49	15	15	0	0	9
Intermediate	MB0265	12AA6002	36	40	20	2	2	0	0	3.5
Intermediate	MB0241	12AA3133	39	46	9	60	58	2	0	4.35
Intermediate	MB0248	12AA3487	61	72	12	77	72	5	0	5.09
Intermediate	MB0414	13AA9628	77	NA	40	35	5	30	0	16
Intermediate	MB0234	10AB1102	70	NA	12	10	2	8	0	9.4
Intermediate	MB0416	13AA9657	77	87	33	20	1	19	0	8.9
Intermediate	MB0469	14AA3409	91	NA	50	44	9	35	0	4.93
Intermediate	MB0224	11AA7577	70	72	16	33	17	16	0	9.96
Intermediate	MB0417	13AA9654	67	71	55	1	1	0	0	2.8
Intermediate	MB0465	14AA2993	55	55	28	39	7	32	0	3.8
Intermediate	MB0260	12AA3134	125	132	86	4	4	0	0	4.2
Intermediate	MB0267	13AA5931	65	NA	10	6	6	0	0	11.8

High	MB0171	12AA3344	30	39	14	55	6	4	45	8.74
High	MB0214	11AA8570	44	40	2	40	6	2	32	12.69
High	MB0230	11AA3374	98	107	8	77	0	0	77	4.6
High	MB0232	12AA3176	45	55	26	60	2	42	16	56.69
High	MB0233	12AA3147	90	99	10	40	2	38	0	7.38
High	MB0249	12AA3318	122	146	44	68	0	0	68	10.08
High	MB0253	12AA3161	117	120	47	76	0	0	76	6.15
High	MB0255	12AA6634	44	45	2	6	5	1	0	14.33
High	MB0271	12AA6493	40	56	31	5	5	0	0	52.6
High	MB0274	12AA5850	72	NA	9	48	12	33	3	14.57
High	MB0275	12AA6020	76	44	11	37	4	30	3	16.7
High	MB0297	12AA8680	39	39	1	2	0	2	0	11
High	MB0301	12AA8582	70	79	10	6	5	1	0	15.75
High	MB0319	13AA0408	52	NA	21	3	3	0	0	257.8
High	MB0320	13AA0470	95	100	90	2	2	0	0	12.39
High	MB0337	13AA0954	148	165	0	106	6	88	12	39.8
High	MB0349	13AA2779	34	34	4	66	0	66	0	99.07
High	MB0389	13AA8592	88	NA	4	60	4	54	2	11.24
High	MB0393	13AA8517	30	NA	3	78	16	58	4	29.48
High	MB0394	13AA7666	122	133	12	30	7	22	1	9.77
High	MB0405	13AA7733	33	21	1	4	4	0	0	5.83
High	MB0408	13AA7854	71	77	4	16	15	1	0	8.35
High	MB0410	13AA7848	33	18	2	10	7	3	0	29.65
High	MB0413	13AA8527	44	NA	2	6	6	0	0	26.68
High	MB0418	13AA8127	52	54	5	7	3	4	0	10.79
High	MB0421	13AA7837	40	NA	6	17	10	7	0	8.68
High	MB0426	13AA8630	121	122	34	6	6	0	0	6.93
High	MB0438	13AA8304	140	141	70	34	0	0	34	23
High	MB0444	14AA1032	48	49	6	10	10	0	0	NA

High	MB0445	13AA8476	32	41	5	8	8	0	0	40.02
High	MB0475	14AA2789	22	30	4	9	9	0	0	9.52
High	MB0500	13AA2554	25	NA	5	7	7	0	0	13.06
High	MB0466	14AA3430	177	200	79	87	16	42	29	11.25
High	MB0461	14AA2672	88	91	15	40	12	21	7	11.26
High	MB0454	14AA1295	50	57	17	41	4	17	20	11.63
High	MB0446	13AA8434	40	NA	1	68	6	12	2	23.8
High	MB0452	13AA8412	115	119	38	16	3	11	2	7.9
Metastasis	MB0282	12AA6441	72	91	3	38	4	8	26	47.57
Metastasis	MB0301	12AA8582	60	77	4	94	7	34	53	40.37
Metastasis	MB0352	13AA2478	40	40	2	20	1	4	15	117.46
Metastasis	MB0385	13aa7983	149	172	0	77	6	2	69	1257
Metastasis	MB0355	13AA2467	109	100	54	117	10	33	74	852
Metastasis	MB0364	13AA2806	110	161	65	102	21	11	70	1012
Metastasis	MB0411	13AA8197	119	NA	22	89	17	65	7	173
Metastasis	MB0300	12AA8622	20	25	0	67	0	0	67	6000

		Low Risk (n=10)			Intermediate Risk (n=40)			High Risk (n=37)			Metastasis (n=8)		
Count Type		**Automated**		**Manual**	**Automated**		**Manual**	**Automated**		**Manual**	**Automated**		**Manual**
Enumeration[1]	**Mean**	41.4		43.9	65.81		60.2	80.03		69.92	95.14		84.88
	Median	39		40.5	66		62.5	57		52	91		90.5
	Range	24-87		17-84	22-91		15-125	21-200		22-177	15-161		20-119
Count Type		**Mean**	**Median**	**Range**	**Mean**	**Median**	**Range**	**Mean**	**Median**	**Range**	**Mean**	**Median**	**Range**
Large CTCs (>20µm)[2,3]		34.8	28	8-82	25.9	19	1-86	17.38	8	0-90	18.75	3.5	0-65
Clusters[2,4]	**Total[5]**	5.5	1	0-40	18.1	10	0-77	33.81	30	2-106	75.5	83	20-117
	Small[6]	4.9	1	0-35	12.33	7	0-72	5.7	5	0-16	8.25	6.5	0-21
	Medium[7]	0.6	0	0-5	5.38	0	0-35	15.11	2	0-88	19.63	9.5	0-34
	Large[8]	0	0	0	0.15	0	0-5	11.7	0	0-76	47.63	60	7-69
PSA[9]		5.29	4.705	2.1-9.05	7.02	5.66	1.23-19.73	26.1	12.01	4.6-257.8	1187.43	512.5	117.46-6000

Above are the respective CTC values for all pre-treatment patients with prostate cancer. Values are per 3ml of blood.
[1]Enumeration reflects manual and automated values in all groups.
[2]Reflects mean count per risk group. [3]Large CTC is defined as a CTC with a nuclear size of >20 µm in the greatest dimension.
[4]Clusters were defined as 2 or more CTCs with no discernible segregation at the 40x non-oil lens. The size of the clusters was defined as: [6]small (2 CTCs), [7]medium (3-5 cells), and [8]large (>5 cells and/or >45µm). Note that the [5]total quantity of clusters is presented as a mean and thus does not necessarily resemble the sum of the means of small, medium, and large clusters.
[9]All PSA values are pretreatment**.**

Table 9: Summary of the Data presented in Table 7

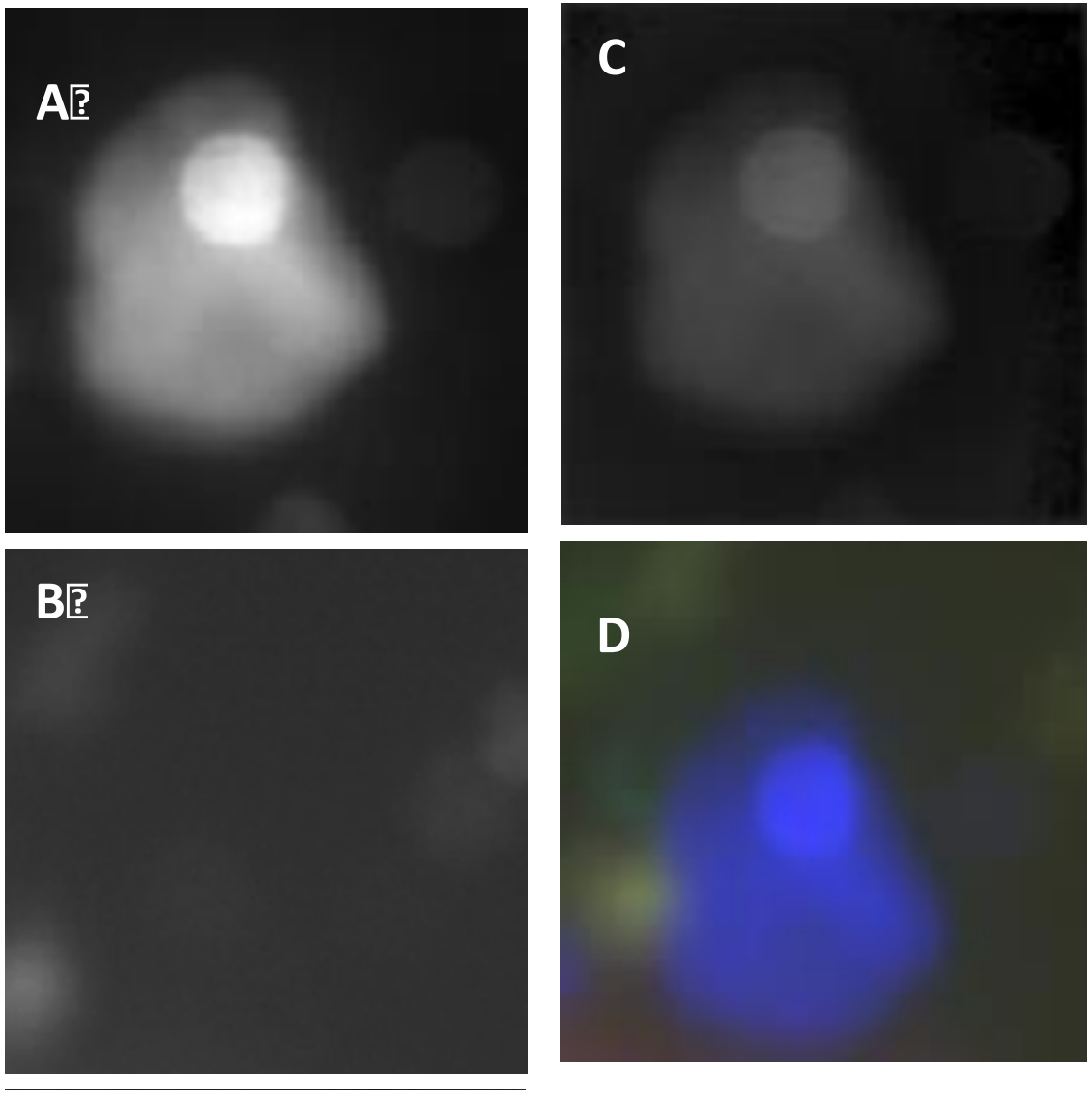

Figure 10: Antibody Isotype Control
This figure shows the sample cellular staining of A: DAPI, B: CY5 labelled Control Antibody, C: FITC labelled Androgen Receptor, and D: The fluorescent combination of DAPI, CY5, and FITC. The absence of CTC staining in "B", shows that all though both the AR-Ab and the Control-Ab target IgG1, only the AR-Ab binds to the CTCs' AR, and thus the AR-Ab we utilized in this experiment is reliable and specific for immunohistochemical staining of CTCs

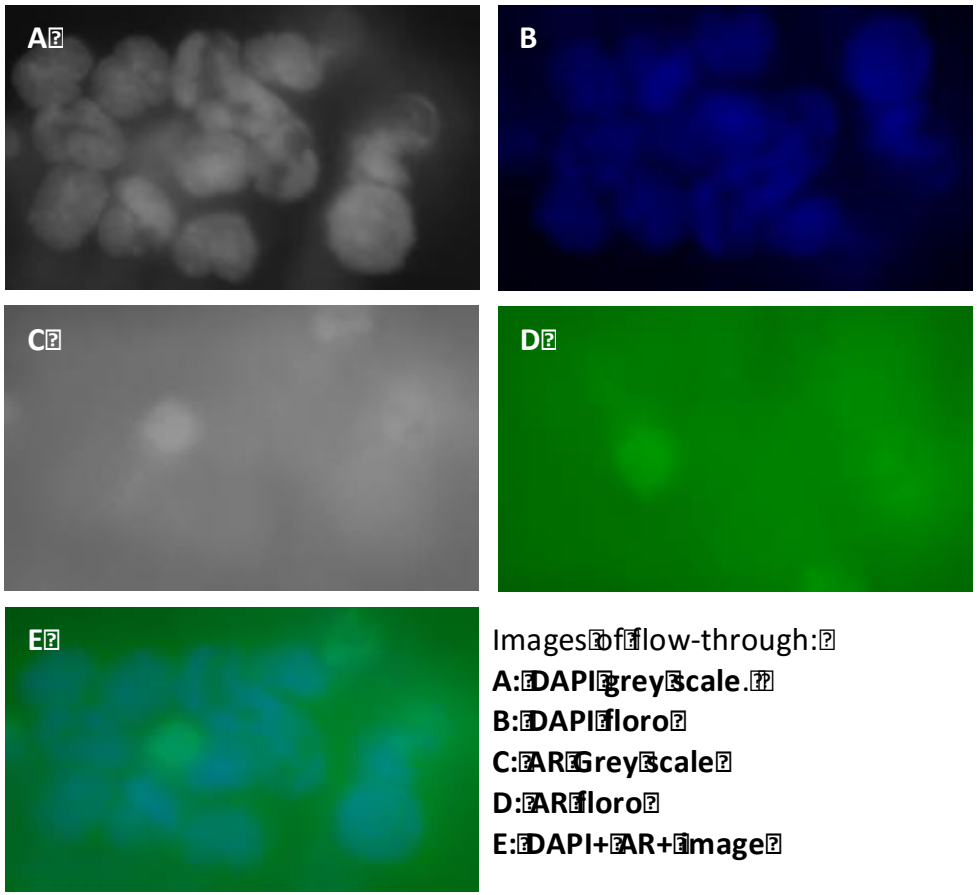

Figure 11: Flow Through Imaging.
The above figure is a microscopic image of a sample's flow-through. Imaging was taken using the A: DAPI gray scale, B: DAPI florescence, C: FITC gray scale, D: FITC florescence, and E: DAPI+/FITC+ florescence. It is readily visible, especially though the gray scale images, that no cells have any obvious staining for the FITC labeled AR-Ab. A small sharp contrast is seen in the center of images C, D, E. However, the absence of a clear DAPI staining renders this rather an artifact instead of a CTC

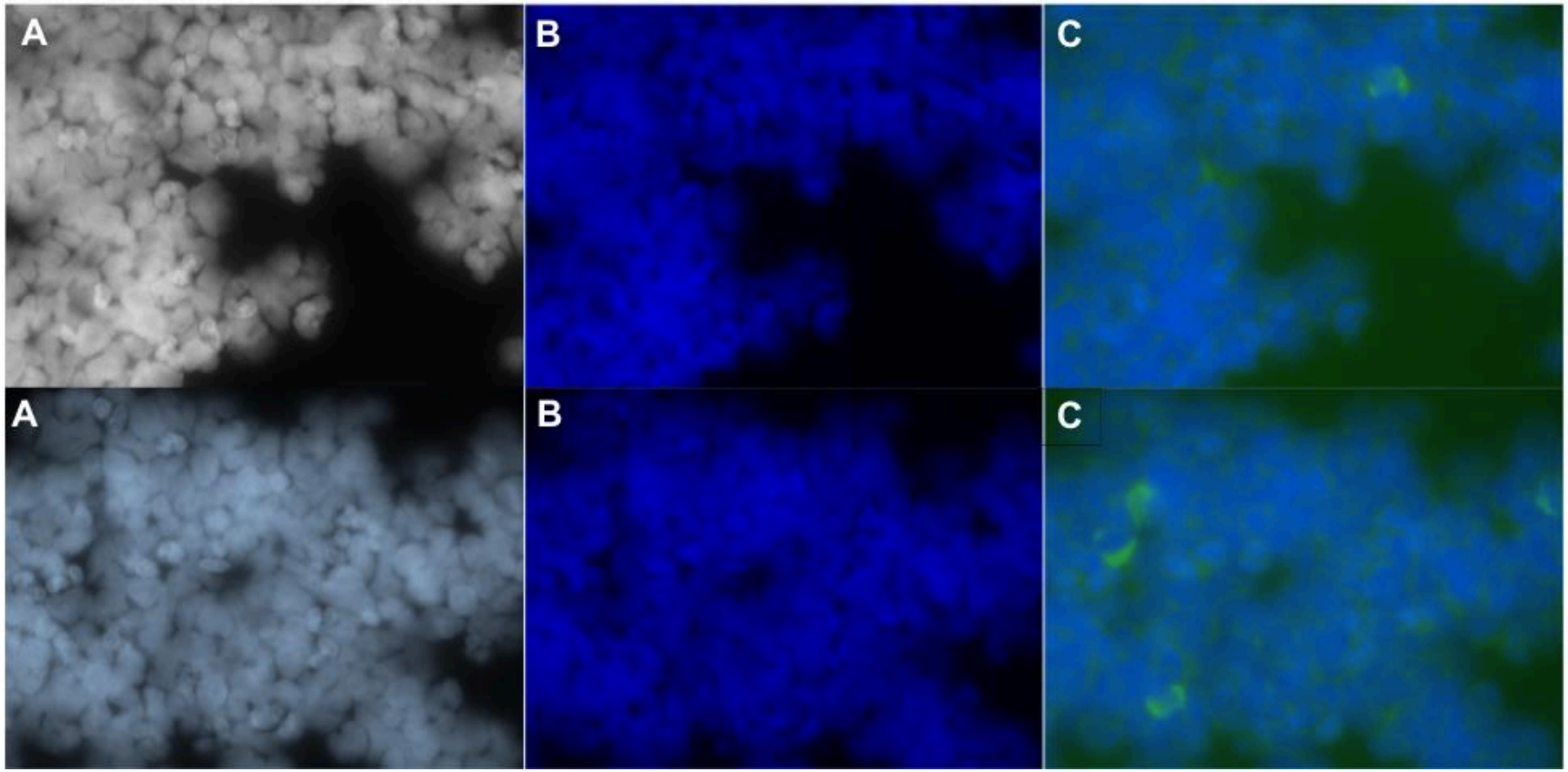

Figure 12: Large Scale Flow-Through Image.
These are large scale images of a flow-through (FT) from a high risk patient. Above, A: DAPI gray scale, B: DAPI florescence, C: DAPI+/FITC+ florescence. The same is found (below), but from a different part of the same slide. On image C, there is clear and visible green (FITC labelled AR). At least 2 and 3 above, and below, respectively. However, careful examination quickly rules out that these are CTCs. The eccentric nuclei of each is a classic image for a plasma cell

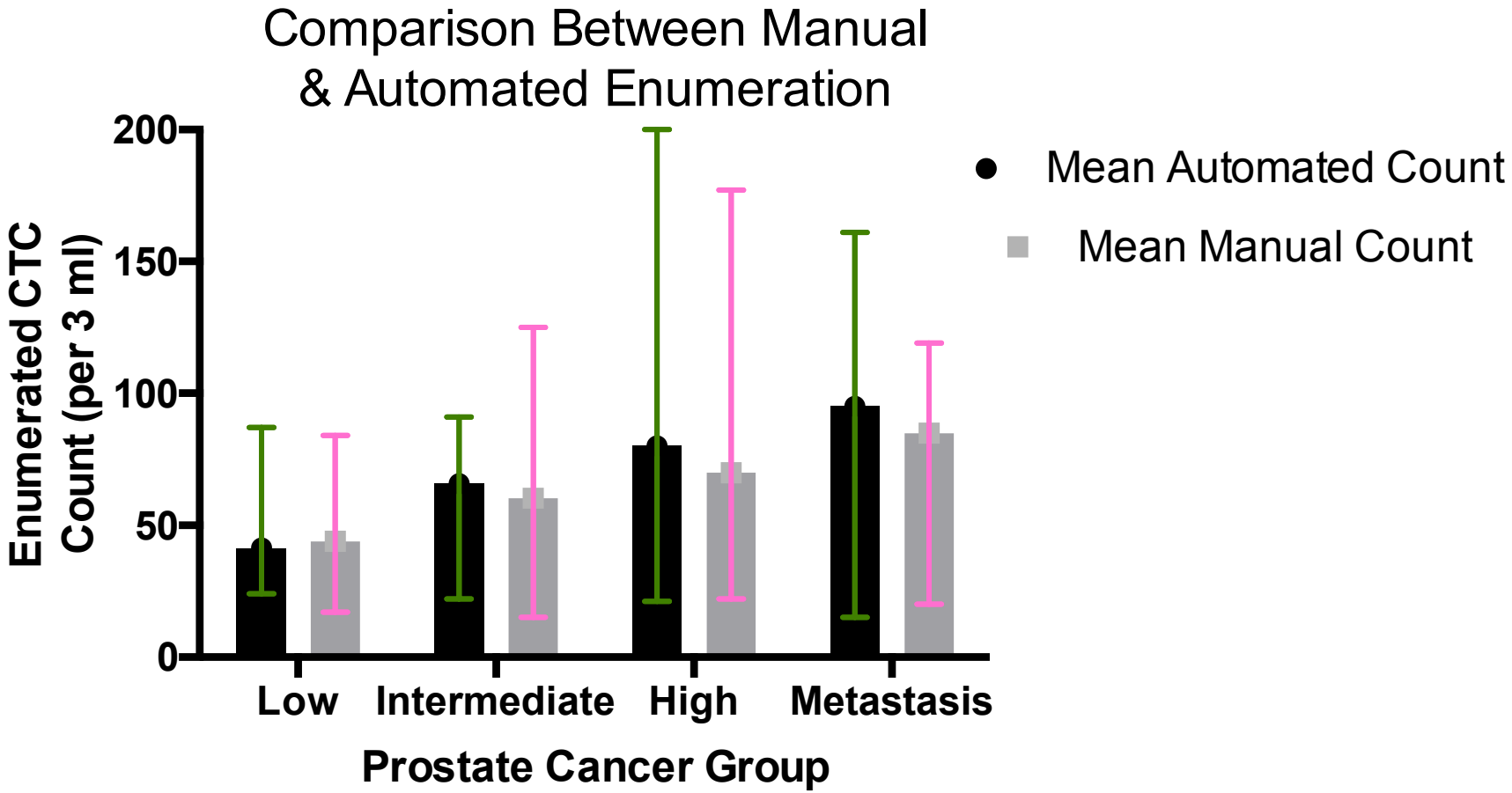

Figure 13: Manual vs Automated CTC Enumeration in all Groups of Prostate Cancer
The above bar graph compares the manual and automated counts in all risk groups of localized prostate cancer as well as metastatic disease. In addition, the vertical linear plot resembles the ranges of enumerated CTCs. The graph is based on data in *Table 9*.

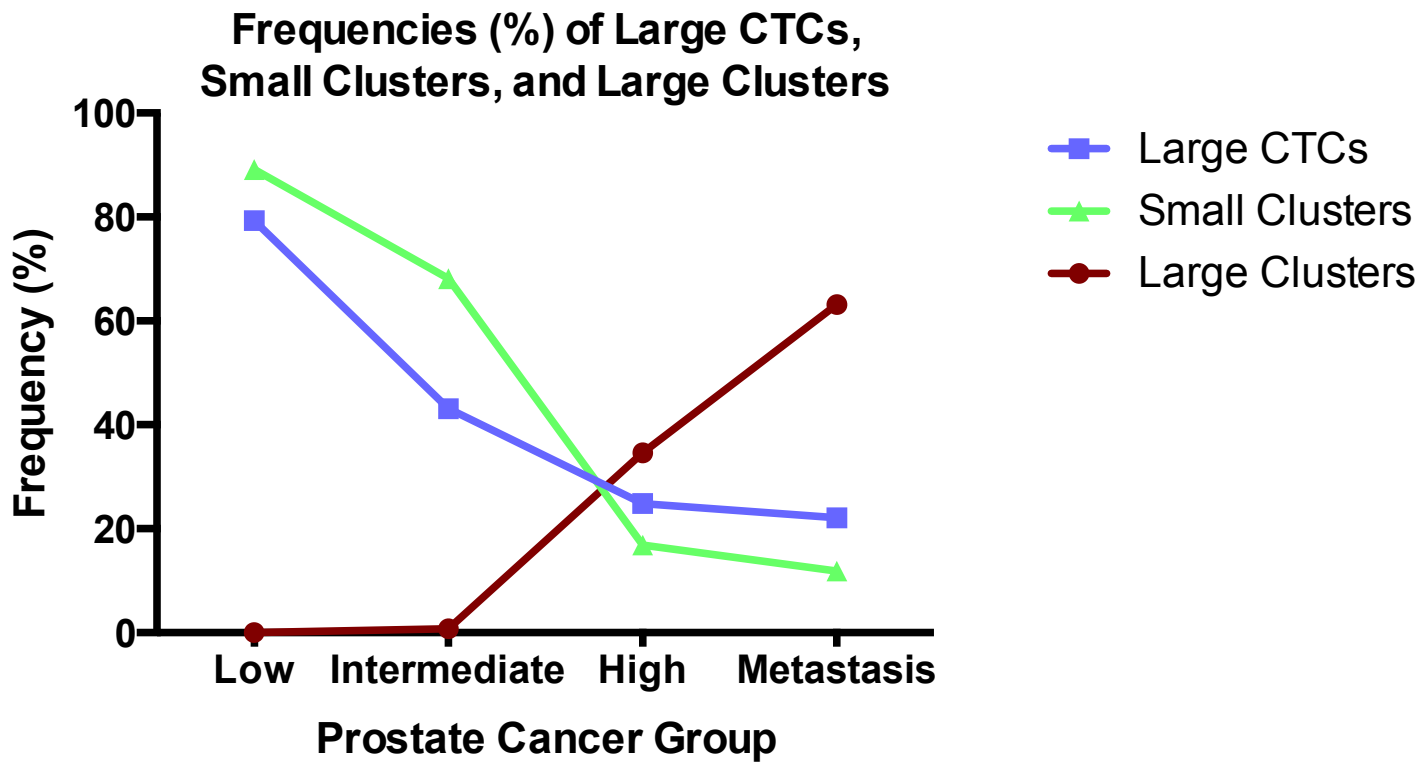

Figure 14: Frequency of Large CTCs, Small Clusters, and Large Clusters

The above linear plots show the frequency of large CTCs, small clusters, and large clusters. This graph is based on data presented in *Table 9*. frequency of large CTCs was determined as the ratio of "Large CTC:Overall CTC count:". Frequency of $cluster_{small}$ was determined as the ratio of "$Cluster_{small}$:$Cluster_{total}$. Similarly, frequency of $cluster_{large}$ was determined as the ratio of "$Cluster_{large}$:$Cluster_{total}$.

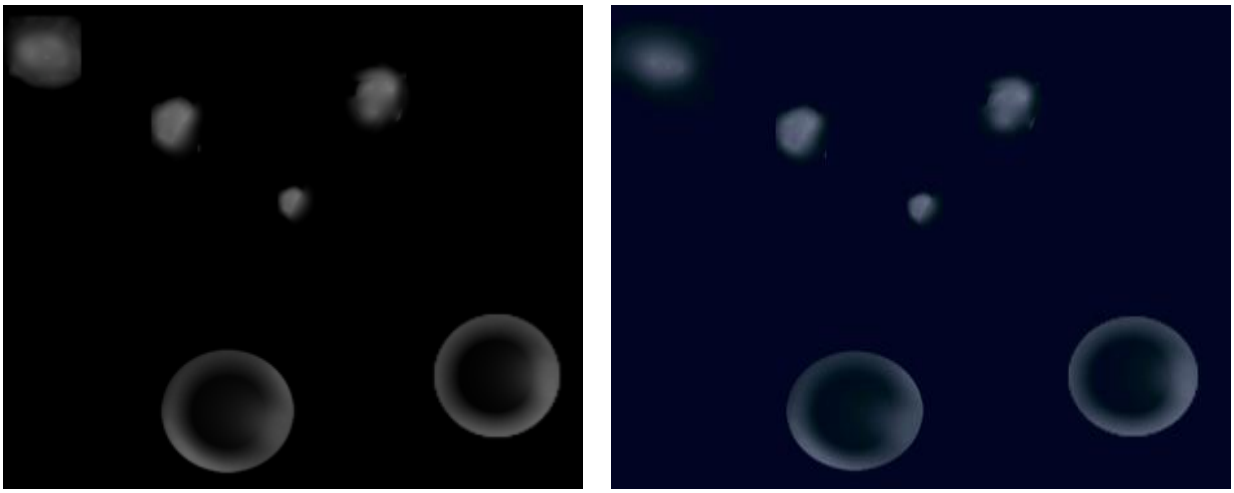

***Figure* 15: Images of the Control Group.**

The above images are of the control group used in this project. On the left is a gray scale image showing two pores with small four unidentified small findings. On the right is a weakly stained DAPI image. The four unidentified objects above the pores are no leukocytes because they did not stain intensely for DAPI and there is no morphological connection. There are no CTCs in the control group.

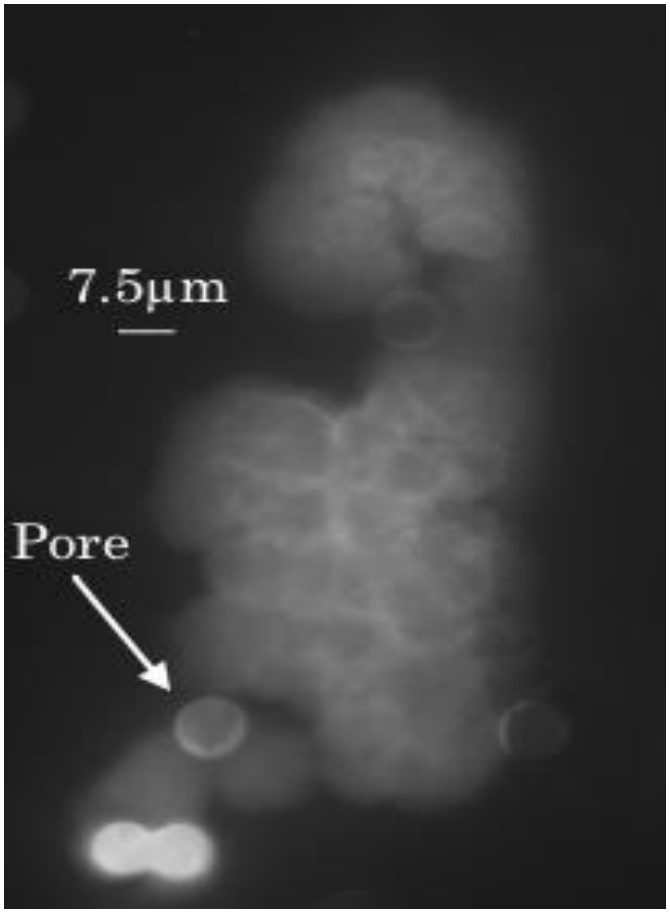

***Figure* 16: A Large Cluster From a Metastatic Sample**
This is an image from a metastatic prostate cancer sample showing a large cluster in gray scale to allow for maximum exposure of nuclear boundaries. In the image, the pore can be easily identified. It measures 7.5 µm. A very large cluster can be seen. The presence of clusters is associated with a metastatic potential[170].

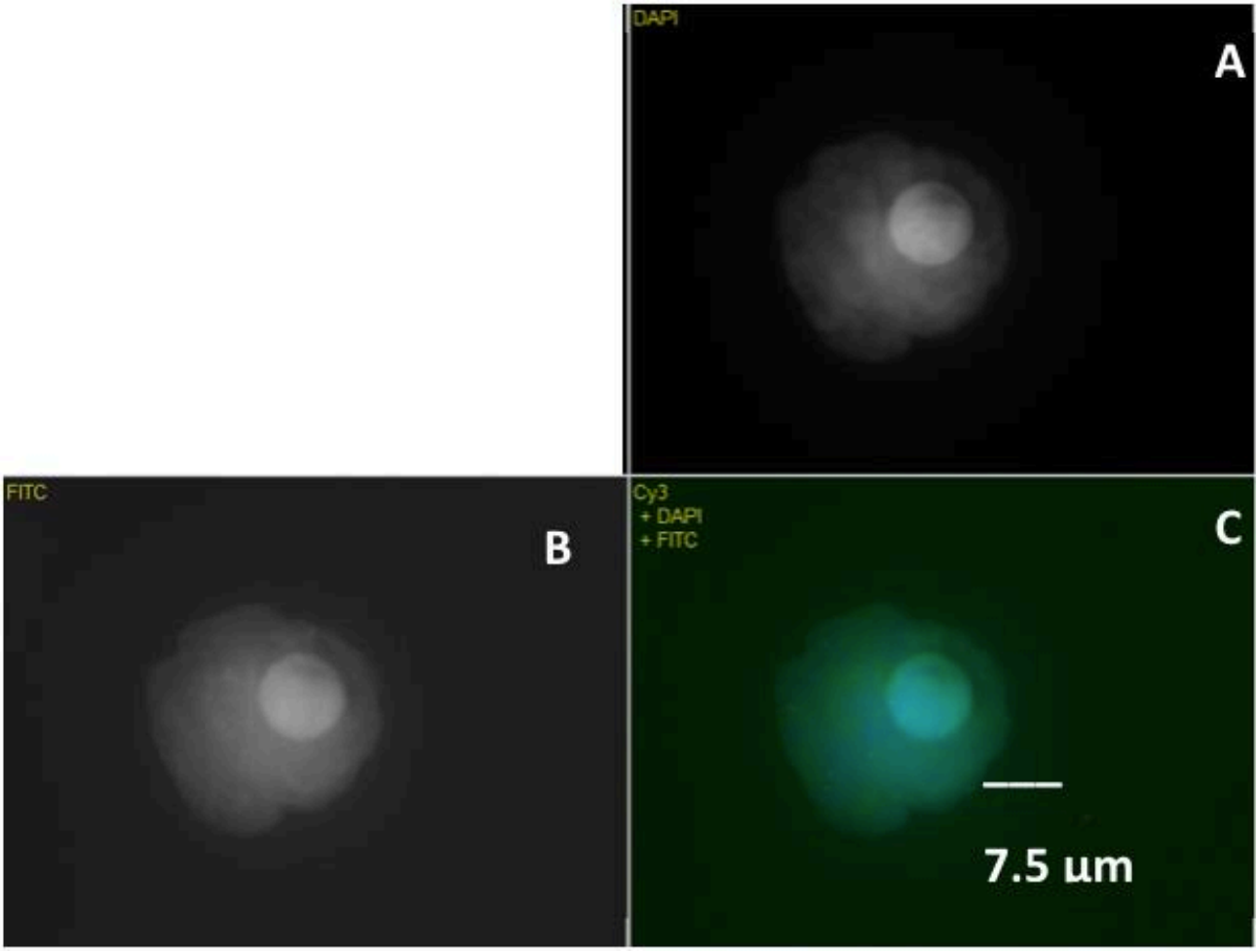

***Figure* 17: Low Risk CTC with Androgen Receptor Staining**
The isolated CTC is from a low risk sample. All images are of the same CTC. **A.** DAPI staining in grayscale clearly demarcates the nuclear border. **B.** FITC showing AR staining in gray scale. **C.** DAPI+FITC with immunofluorescence turned on. There is a clear green tint indicating positive AR staining. The faint blue hue is from the DAPI stain. This CTC has a nuclear size of 30 µm and thus is classified as a 'large' CTC. Low Risk samples showed the highest frequency and ratio (79.3%) of large CTCs

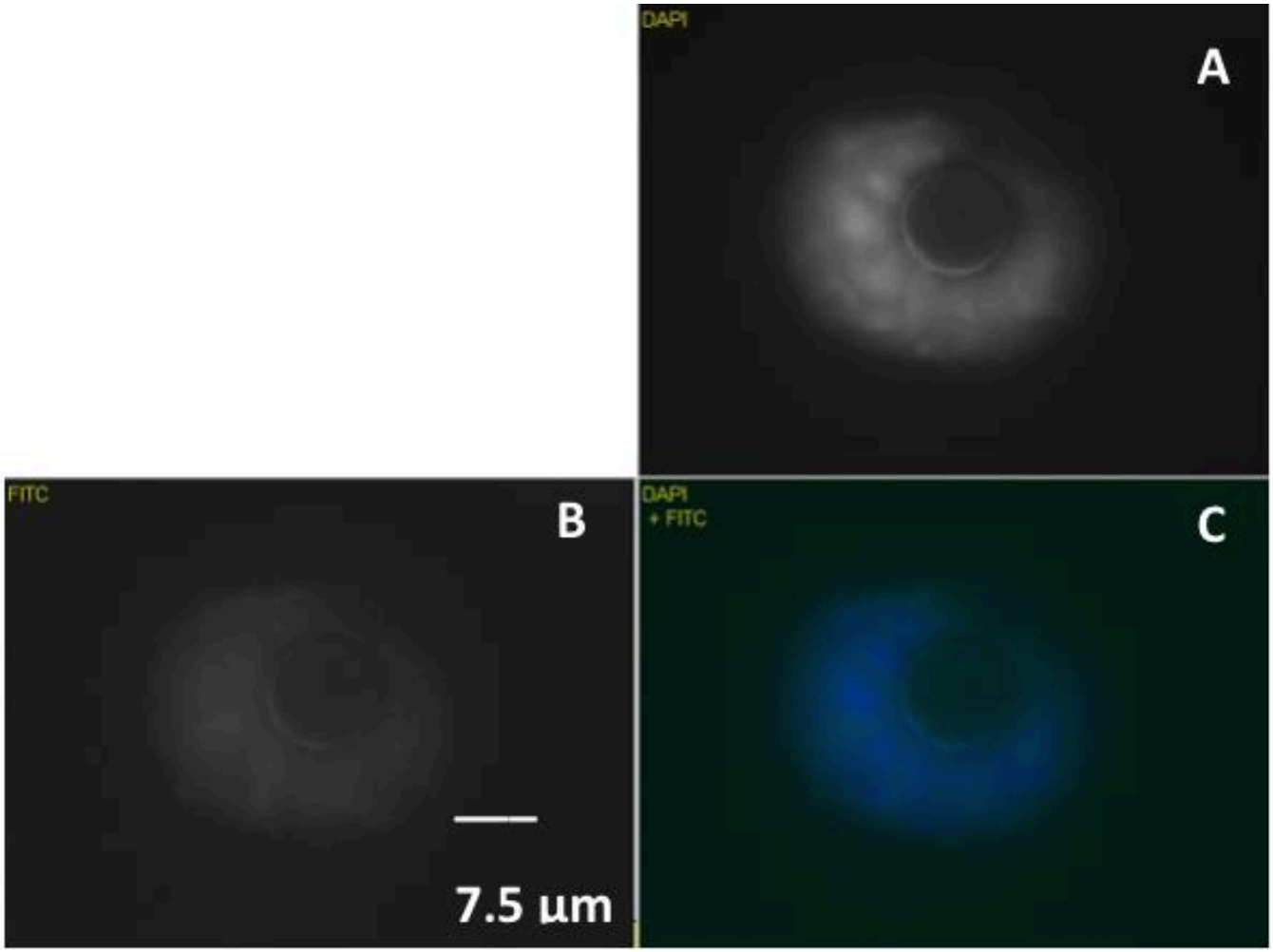

***Figure* 18: CTC from a Metastatic Samples**

This isolated CTC is from a metastatic patient sample. All images are of the same CTC. The grayscale DAPI stain is clearly visible on images **A** (Grayscale) and **C** (florescence on). **B.** Grayscale FITC labeled faintly stains the CTC. Due to tumor aggressiveness and tumor mutation, the AR stain does not intensely stain in some high risk and metastatic samples (as seen in this case). AR mutation (especially splice variants) are one of many ways the AR mutates during higher stage disease. This may explain CRPC. This CTC has a nuclear size of 21 µm and is thus termed a "large" CTC, a rare feature in metastatic samples as only 9.72% of CTCs in metastatic samples were large.

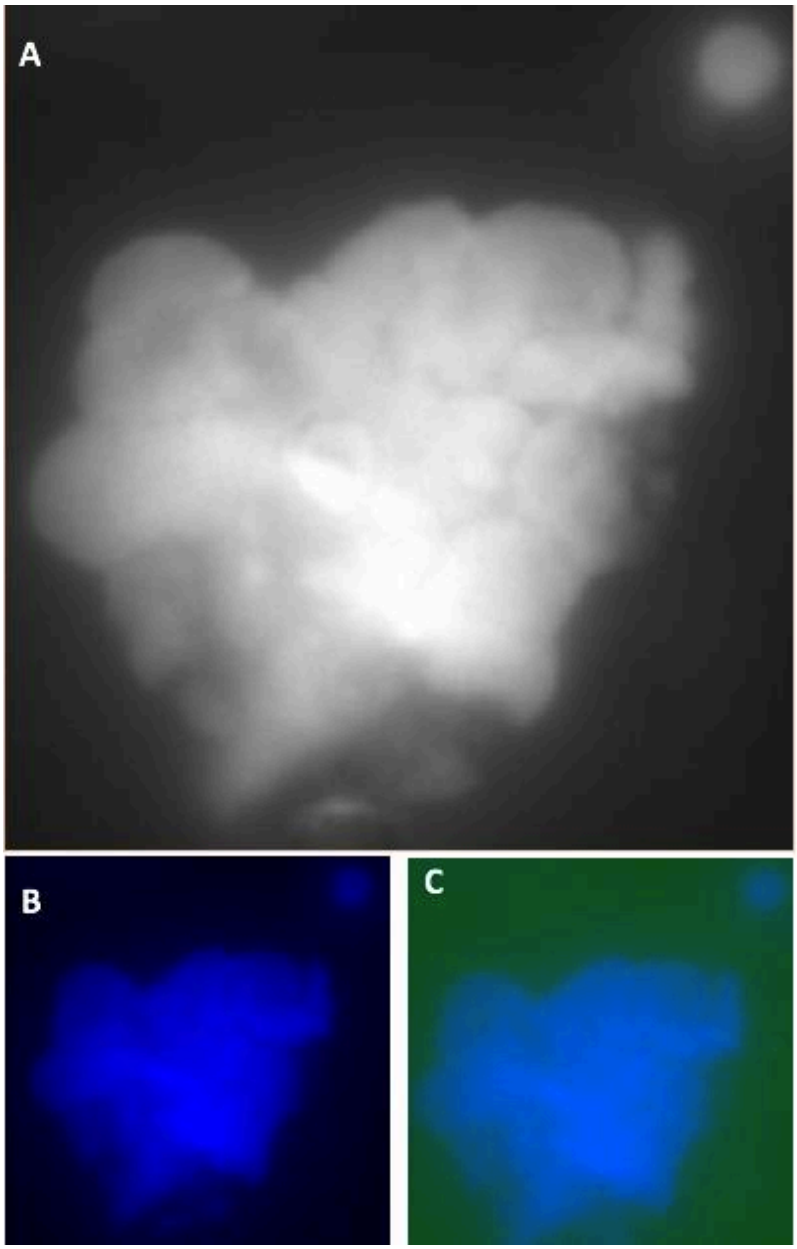

***Figure* 19: Cluster From a High Risk Sample**

This *figure* shows the morphology of clusters. The image was captured from a high risk sample. **A.** Shows a photo with grayscale DAPI turned. The cluster contains numerous CTCs and/or fragments. In this case it is difficult to discern the number of CTCs in the cluster. However, our criteria for $cluster_{large}$ was either >5CTCs or >50μm overall length. This cluster had a length of 72μm and thus was labeled as $cluster_{large}$. **B.** The same image with DAPI fluorescence turned on. **C.** FITC (green) turned on to detect FITC labeled AR-Ab stain. Many aggressive cancers do no stain for AR (or stain lightly) and thus only show as a background haze with no nuclear staining. These findings are consistent with high risk and metastatic samples[170]. In this case, 3D imaging helps distinguish aggressive AR-negative clusters from random artifacts.

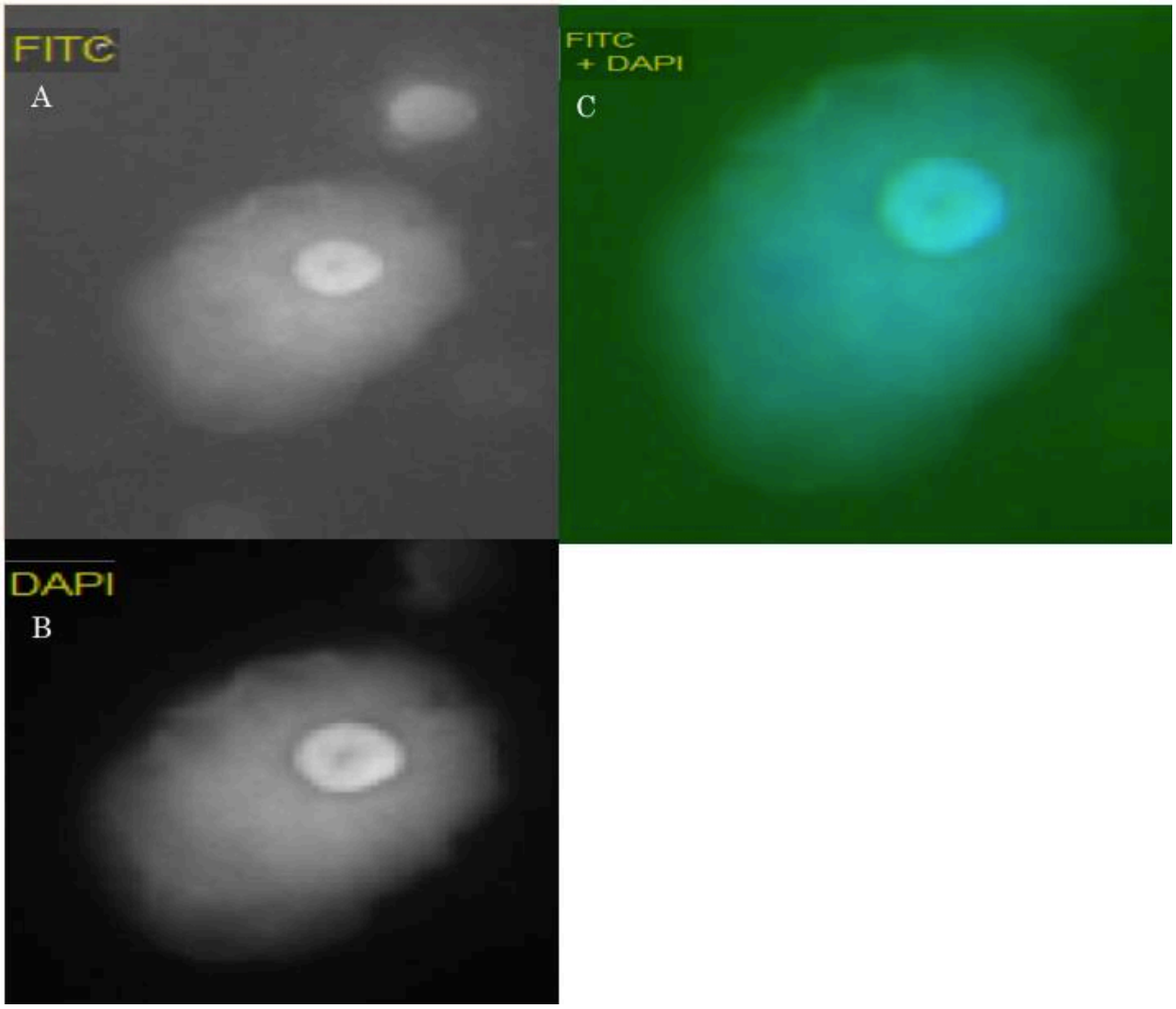

***Figure* 20: Intermediate Risk CTC**

This is a CTC from an intermediate risk sample. **A.** An image showing FITC-labeled AR-Ab staining without fluorescence. **B.** DAPI staining for DNA without fluorescence. **C.** The image shows both DAPI and FITC fluorescence turned on. The FITC labeled AR-Ab emits a green pigment, while DAPI emits a blue pigment. The combination of both results in a mixture of both green and blue pigments. The center of the image is a the filter pore that measures 7.5µm.

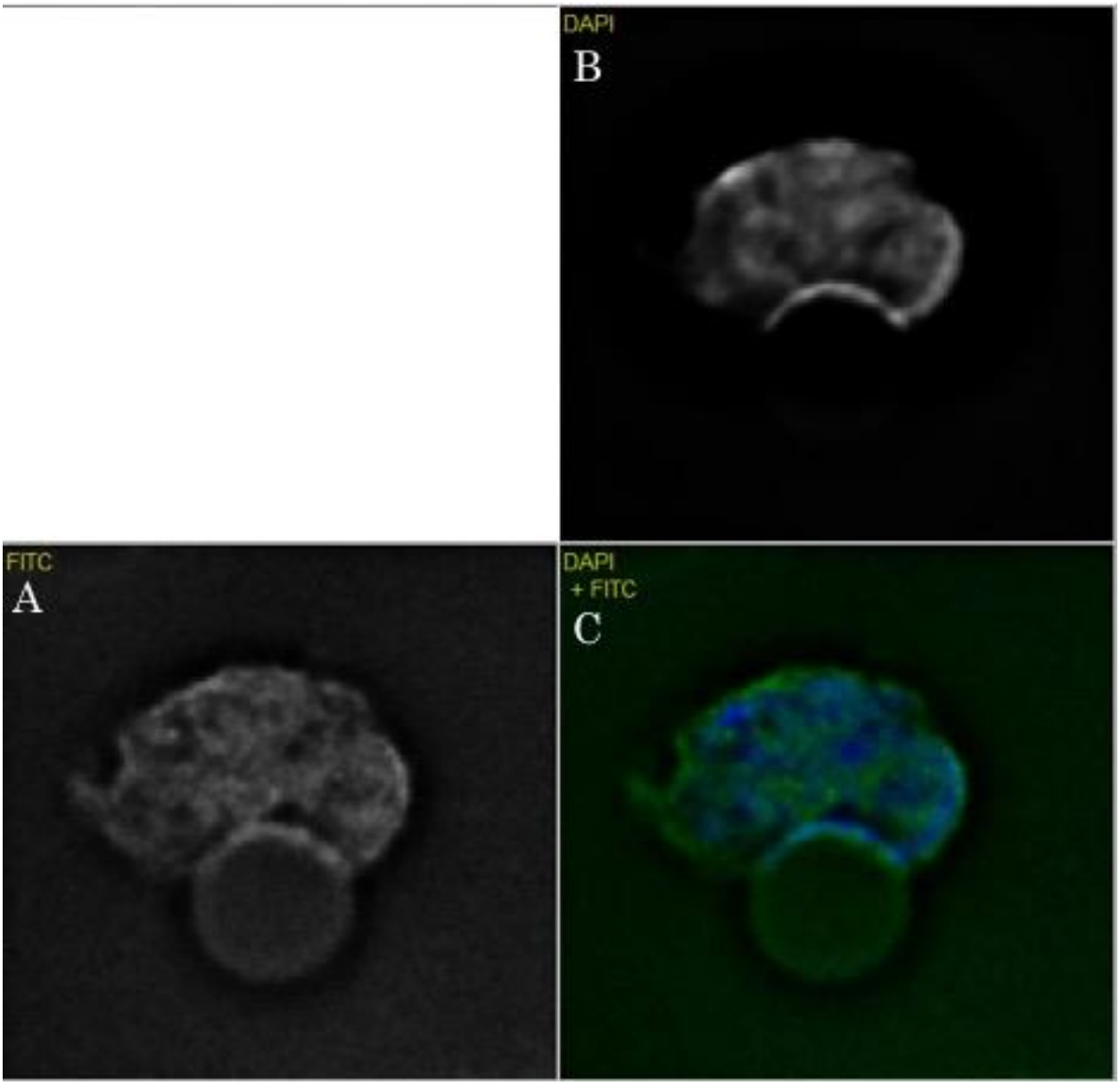

***Figure* 21: High Risk CTC**

A high risk CTC is shown above. **A.** Non-fluorescent FITCH-labeled AR-Ab stain showing a CTC's nucleus at the edge of a 7.5µm pore. **B.** Non-fluorescence DAPI stain showing the same CTC. **C.** An overlying image of both DAPI (blue) and FITC (Green) fluorescence. The AR-Ab stain is dispersed throughout the nucleus, thereby confirming the presence of an Androgen Receptor (nuclear receptor). Within the nucleus is also DNA, shown in the blue DAPI stain.

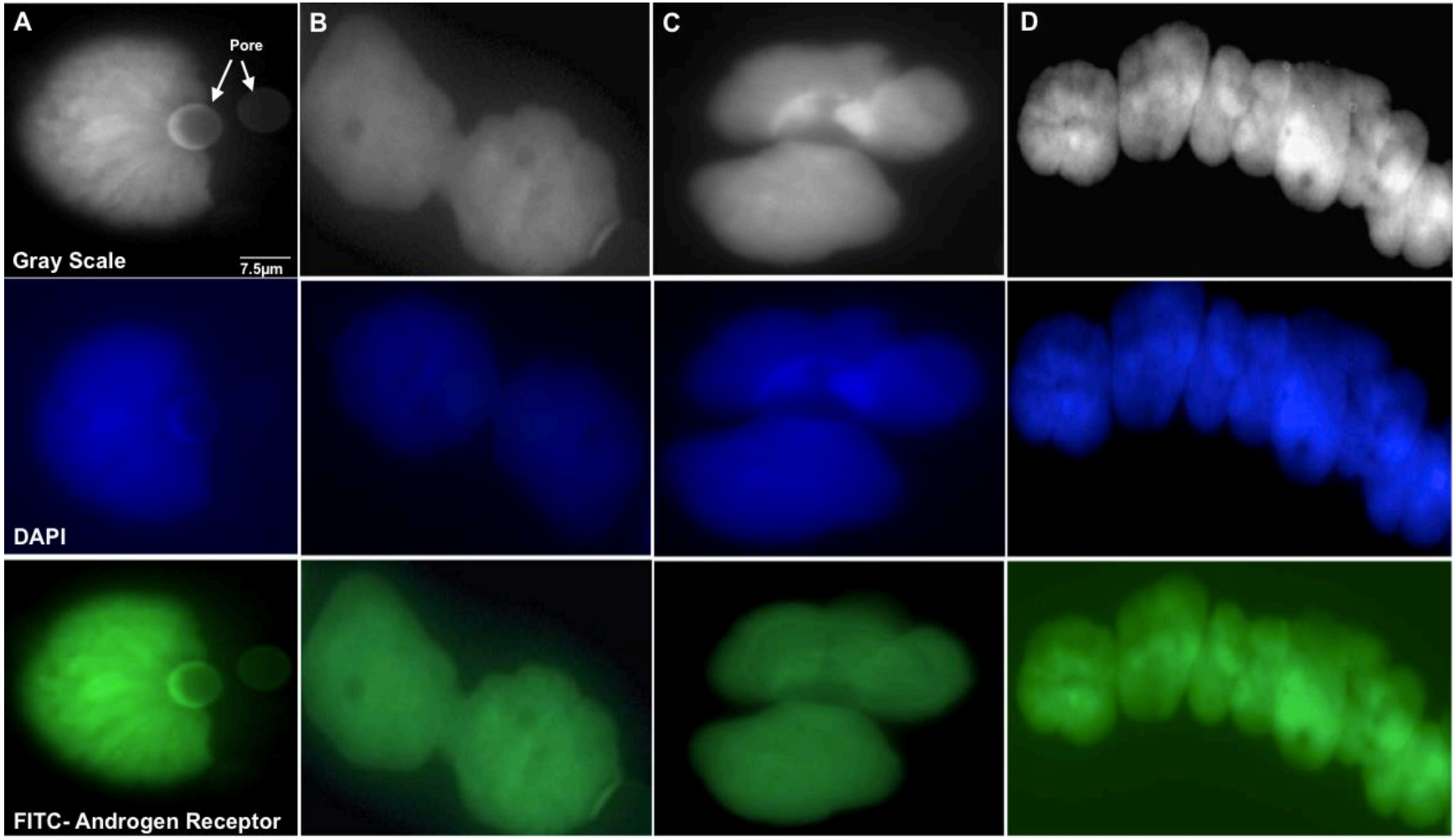

Figure 22: The Different Types of Clusters Seen in Patients with Prostate Cancer.
In this figure there are four images from each of the previously defined clusters. Each image is seen in gray scale, DAPI stain, and with FITC labeled AR stain. Note that the visible structures are the nuclei only **A.** Shows a single CTC with its edge overlying the filter pore. **B.** A $cluster_{small}$ is defined by the presence of 2 CTCs. **C.** A $cluster_{medium}$ is defined by the presence of 3-5 CTCs. The 3 CTCs are partially overlapping each other's edges and are overlying a filter pore. **D.** A $cluster_{large}$ is defined by the presence of >5 CTCs or a cluster that measures ≥50 µm. Overall, large sized clusters were more frequent in high risk and metastatic prostate cancer.

6. DISCUSSION

In this study we isolated CTCs from pretreatment prostate cancer patients of all stages. Isolation was performed by size-based filtration using ScreenCell® filter kits. CTCs were stained with DAPI and AR-Ab. Subsequently, enumeration was carried out manually and via an automated system (GenASIs®). Our data showed that there is no statistical difference between the manual and automated methods (Pearson Correlation R= 0.9503). A strong positive correlation was found between the manual and automated methods for all four groups (R= 0.9109-0.9768 in low risk, intermediate risk, high risk, and metastasis). The quantity of CTCs showed a positive linear slope regression, in that the quantity of CTCs increase in higher stage PCa. The frequency of large clusters was higher if the disease was more aggressive, whereas the opposite is true for large CTCs and small clusters.

As per *Table 6*, there are multiple methods currently used to isolate CTCs. However, large variations in the amounts of detect CTCs exist. Not all samples in the studies listed in *Table 6* had detec*table* CTCs (range: 0-354 CTCs[216]). Currently, the only FDA-approved system, CellSearch®, is only beneficial in metastatic prostate cancer, and this is largely due to its inability to isolate CTCs from lower risk groups. Additionally, CellSearch® cannot detect EpCAM-negative CTCs. A similar method[183] to our study was recently published. They used size-based filtration using the MetaCell® filtration kit. All patients (n=55) had localized prostate cancer. Only 28 (52%) of the samples had detectable CTCs. Additionally, with a mean of 6.5 CTC/8ml (range: 1-17), the CTC count was not as high as had been produced from previous size-based filtration

studies[155]. Our study utilized ScreenCell® size-based filtration and was successful in enriching CTCs in all filters with an overall automated average of 70.8 CTCs/3ml of blood (range: 15-200). Another study[155] showed only 4.1/6.5 ml CTCs in high risk samples. Although a very small sample size was used, their study showed 14 CTCs in Gleason 6, but only 2 CTCs in Gleason 9 (4+5). In our experiment, we had a much higher yield in low risk Gleason 6 disease, with an automated average of 41.1 CTCs/3ml (range: 24-87 CTCs). Other studies using different techniques, as per *Table 6*, have successfully isolated CTCs but unfortunately with very low counts due to loss of cells or very high counts influenced by false-positive events (i.e. artifacts, leukocytes)[148,152,204]. The ScreenCell® filter kit we utilized in this experiment was shown to have an average loss of only 7.5% of CTCs[113]. We have accounted for false-positive results by staining with AR-Ab and also manually validating all automated counts. Therefore, ScreenCell® filtration kit, when used with AR-Ab stain, can successfully and reliably isolate CTCs from all risk groups including low risk PCa. More importantly, it is able to isolate higher counts than was previously isolated using EpCAM based technologies and other filtration kits.

Commonly used techniques for enriching CTCs such as CellSearch® and microfluidics did not reliably yield sufficient CTCs. For example, CellSearch® recovered a mean of 0, 1.43, and 38.42 CTCs/7.5 mL in Gleason <6, Gleason 7, and Gleason >8 PCa[206]. Although CTCs can be successfully isolated when using an EpCAM-based system such as CellSearch®, the findings remain under the influence of subjective analysis for CTC identification due to loss of EpCAM-negative CTCs. Additionally, it is an expensive time-

consuming screening tool that relies on the presence of specific markers. However, our size-based filtration is cheaper ($90 CAD/filter)[113], fast (20 minutes to filter and centrifugation), easy to use, and most importantly does not rely on the presence or absence of specific cell surface markers. Therefore, the results of our study proved that we can isolate a higher CTC count in a more reliable method using filtration techniques. These positive findings were most noticeable in low risk and intermediate risk where we were able to isolate a mean automated count of 41.4 and 43.9 CTCs/3ml (vs 0 and 1.43 CTCs/7.5ml with CellSearch®)[206]. Results from ScreenCell® filtration are not influenced by cell markers that are manipulated during EMT.

In microfluidics, results have been more inconsistent than CellSearch®. Two large studies reported their findings using PC3 cell lines. The first study was published in 2013 and showed that microfluidics isolated 20-300 CTCs/7.0 ml of PC3 cell line[217]. Their recovery rate was 71%. In contrast, another study used the same cell line, and with a recovery rate of 80-95%, they were able to capture 1-99 CTCs/ml[218]. One main reasons why the results from microfluidics should be taken with caution is the recovery rate. The recovery rate of microfluidics has been examined in multiple recent studies and was shown to be 71-93.2%[217-219]. This wide variation in recovery, in part, maybe responsible to the extreme differences in captured CTCs reported within, and amongst the previously mentioned microfluidics studies. Recently, another studied using microfluidics reported a wide range of CTC counts (0-61 CTCs/ml), thereby supporting earlier findings using microfluidic devices[220] .Many microfluidics devices still rely on EpCAM expression, and thus reiterating the similar disadvantages observed in

CellSearch®. The system is also very slow and expensive. Making the system operate faster means increasing the fluid speed which could damage CTCs and fragment them, thereby exacerbating an already weak recovery rate. With ScreenCell® filtration kits, speed and expense are not limiting factors. We were able to capture CTCs in all samples. The ranges (15-220 CTCs/3ml) of our results were also not as drastic as a microfluidic study[217], nor did it include samples with 0 CTCs in other microfluidic studies[218-219]. Based on these observations, size-based filtration is more superior to microfluidics in terms of consistency and reliability.

ScreenCell® size-based filtration had proved to be more reliable than the commonly used CellSearch® and microfluidic devices. We successfully detected CTCs in all PCa stages of our patient samples (n=95); an ability that neither ScreenCell nor microfluidics was able to do. To control, we used blood samples (n=5) from cancer-free healthy volunteers and yielded 0 CTCs in all of five control samples. Our control had better results than microfluidics where it was acceptable to have up to 2 CTCs/ml in this group[218]. Also, the ability for ScreenCell® to consistently detect CTCs in low and intermediate risk samples is untraditional in EpCAM-based systems, but certainly is in accordance with the pathophysiology of solid malignancies whereby it is generally acceptable that tumors continue to shed cells into the vascular until they establish a new metastatic foci or are degraded by the immune system[137,165].

To examine and enumerate the captured CTCs, we enlisted the use of microscopy over EpCAM-detecting flowcytometry. The advantage of microscopy is its ability to allow for

visualization of detailed morphological features of CTCs including size, clusters, cell borders, and cell fragments. Simply, the filter was removed from its metal brim and placed on a microscope slide for microscopic visualization. CellSearch® and microfluidics do not have the simplicity that ScreenCell® has for microscopic visualization. Additionally, neither of the devices allow for any form of commenting on cluster size and shape. Multiple studies have shown that the size of clusters is often reflective of the stage and prognosis of PCa[137,139,154,186-193]. The ability for these two devices to not be able to examine cluster sizes is shortcoming. With ScreenCell® we carefully examined each and every captured clustered and as published[137,139,154,186-193] earlier, we found a strong positive relationship between the size of the cluster and the stage of PCa.

Most low risk prostate cancer patients never progress and in fact die with the disease rather than from the disease[184]. Therefore, it might be unexpected for such patients to have comparable CTC counts to high risk or metastatic diseases. The ability to isolate CTCs in low risk samples was reproduced in other size-based filtration studies[183,205,206], but not in assays the rely on cell surface markers, such as CellSearch®[207] and microfluidics. One study[207] utilized EpCAM-based Ficoll® centrifugation, NIR dye, and microscopy to detect CTCs in prostate cancer. They found 8-31 CTCs in low risk patients. However, draw backs of the study is that healthy control subjects were also found to have CTCs (<5). Also, they did not use the D'Amico criteria, but instead used the National Comprehensive Cancer Network (NCCN) criteria which slightly over estimates staging when compared to the D'Amico criteria. The significance of this is that

a sample that is deemed low risk using NCCN criteria may in fact be an intermediate risk patient as per the widely used D'Amico criteria. Lastly, they still needed to use microscopy, rather than rely on EpCAM only, to allow for detection of CTCs in low risk samples. However, in our study we had multiple advantages. First, we used the D'Amico criteria and thus allow us to compare our results to other studies. Second, we found more CTCs (24-87 vs 8-31) in low risk samples. Third, should there be any EpCAM-negative CTCs, our method (ScreenCell®) would not miss them. Therefore, ScreenCell® is more superior to systems that rely on expressed immunophenotypes.

We found that CTC quantities are not the sole factors that can predict tumor aggressiveness. Other factors that we found include the behavior and morphology of the CTCs. For example, our results show that the frequency of large CTCs is significantly less if the tumor is more aggressive. This finding was recently observed[151] (see later, p.88). Also, it is now known, and we also have shown, that in more aggressive tumors, the ability to form clusters increases in both the numbers of clusters as well as the size of clusters[137]. It is perhaps these clusters, rather than single CTCs, that are responsible for initiating micrometastasis[137, 154]. The ability for ScreenCell® to examine clusters makes it potentially reliable for clinical use to monitor, in real time, patients with PCa.

Although single cancer cells make up the vast majority of CTCs, clusters of CTCs are also observed in liquid biopsies or as microemboli during incidental autopsies[139,186-188]. Mucin secreting adenocarcinomas such as prostate cancer are also associated with

tumor emboli, a feature that correlates with poor prognosis[189-191]. Mouse models show that CTCs are oligoclonical in origin, and stem from a specific focus of the primary tumor that releases a single grouped CTC cluster[192]. Therefore, according to these observations, clusters are not derived from multiple single CTCs that either proliferate or aggregate in the vasculature. On the other hand, other studies show that clusters in mice models form as a result of aggregations between CTCs and cellular fragments[193].

We believe that clustered CTCs have at least two crucial features that facilitate the formation of metastasis. Some of these features have also been hypothesized in the past[171,172]. One feature is evasion of the immune system. Concealed antigens occur in many ways, but one method is due to the physical hindering location of the antigen[194]. Because concealed antigens are not exposed to the host's immune cells, they typically do not illicit an immune response[194,195]. Cluster formation may serve to conceal antigens of CTCs within the core of the cluster. This may occur physically by sheltering CTCs inside the cluster, or biochemically through EMT by terminating the expression of certain antigens that would normally illicit an immune response[157-159]. EMT is known to contribute to CTC's ability to avoid apoptosis induced by immune cells through the increased expression of anti-apoptotic cell surface PD-L1[221]. Increased PD-L1 expression was attributed to EMT, a process that is more aggressive in clusters as opposed to single CTCs[186-188]. Even in invasive tissue biopsies, PD-L1 expression is more likely to be seen in clusters of cells rather than scattered in single cells. In an unpublished presentation at the AACR in 2015 (abstract number 1608), the authors recommended to assessing PD1/PD-L1 expression in CTCs of clusters as well as

leukocytes trapped within the clusters. The significance of clusters is completely missed by the CellSearch® system which counts clusters as a single CTC. During microscopic examination of ScreenCell® filters, we observed clusters surrounded by and directly in contact with a few leukocytes. However, the leukocytes were only in the periphery and not in the core of the cluster[179,198]. Using 3D microscopy and AR-Ab stain, we were able to differentiate CTCs from leukocytes when examining clusters that were in contact with leukocytes. In addition to concealing antigens, clusters are 'stickier' than single CTCs. This is consistent with earlier findings on autopsies that showed CTCs in cluster tightly adherent to the microvasculature in the form of a micro-and-maco emboli[165,192,193]. Clusters can have newly acquired mesenchymal phenotypes[196] and contain platelets, fibroblasts, and extracellular material[139] that allow them to adhere to the vasculature and consequently extravasate[197] to body tissues and organs. Therefore, this ability to adhere (stick) tightly to structures plays a crucial role in amplifying the metastatic potential of clusters. In this study, we were able to show that the frequency of clusters is greater in higher stages of PCa. Additionally, the frequency of larger sized clusters was also more frequent in higher PCa stages. Hence, the results are consistent with previously published papers [124,139,171] that validated the increased malignant potential and metastatic ability of clusters.

There are other studies[199] that studied in the depth the consequences of the increased adherence of CTC clusters. In mice models with breast cancer, clustered CTCs expressed a high level (200 fold) of the cell-cell adhesion marker plakoglobin[199]. These findings further support that clusters have increased adhesion molecules that can

facilitate the establishment of a metastatic foci. However, and more importantly, normal cells that lose contact with their tissue (i.e. single CTCs in the blood) are prone to anoikis, a form of programmed cell death as a result of the loss of adhesion-dependent survival signals. So when clusters continue to express high levels of adhesion molecules like plakoglobin, they may avoid anoikis due to adherence-independent survival and also prolong their half-life. Moreover, clusters are virtually always coated with platelets, and sometimes with fibroblasts and other endothelial cells; all are factors that support a new 'life'[139,210], and potentially explain why clusters are more likely to proliferate and less likely undergo apoptosis[199]. These studies further support the results of our results where we found higher frequency of clusters and higher frequency of large sized clusters in higher staged prostate cancer samples.

Despite harboring supportive cells and molecules, the half-life of clusters (10 minutes) still remains less than that of a single CTC (30 minutes)[200]. However, it is the ability of a cluster to establish and continue supporting metastatic foci that makes them more dangerous. One possible reason that clusters have a shorter half-life is its propensity to be entrapped in the microvasculature and thus lost to detection devices[139,200]. When that happens, clusters can either passively traverse through a weakened vascular spot or actively traverse the endothelia of the vasculature[171]. Either way, the entrapment gives opportunity for a cluster streaming in the vasculature to have the chance to exist the microvessels at the site of entrapment and enter into the tissue where it can initiate a new or maintain a previously created metastatic foci. Given that cluster entrapment in organ microvasculature is a way to initiate micrometastasis, the shorter half-life of

clusters might after all be a misleading way of understanding their behavior and metastatic potentials.

Cancer cells' nuclear size increases at the expense of the cytoplasm, and hence the nuclear size would be a close resemblance of the actual cell size[202]. Currently, there is no literature that specifically links the size of a CTC with tumor aggressiveness. However, one study[151] which examined the speed of PCa CTCs in a microfluidic device noted that some CTCs might find it advantageous to be small in size, in reference to their ability to circulate faster than larger cells. In another study using microfluidics Nanovalcro chips (Ctulumina®), it was found that the larger the nuclear size the lower the tumor stage[220]. In order of ascending aggressiveness, they calculated the ratio between the specific nuclear size to the overall CTC counts and found 62% 'very large nuclei' in non-metastatic samples, 51% 'small nuclei' in non-visceral metastatic samples, and 65% 'very small nuclei' in visceral metastatic samples. In our experiment we found that the frequency of large CTCs is lower in higher stage PCa, which was consistent with other studies[220]. Given that larger CTCs show a slower passage in the microvasculature of organs[151], the added time increases their exposure to organ-specific monocytes (i.e. alveolar cells in the pulmonary system). Because single CTCs do not have the benefits that clusters have (concealed antigen, enhanced adherence ability, and supportive microenviroment of the cluster itself), they are more likely to be destroyed by the immune system and thus be forced into apoptosis[199].

We encountered another feature that has been observed in the past[203]. Some of our

high risk and metastatic samples included CTCs that did not stain for AR. The possible reasons for these findings include EMT, initial development of CRPC, and AR splice variants[73, 203]. Although some CTCs did not stain for AR, their detection and distinguishing features from leukocytes and other hematological cells is microscopically possible. RBCs (6-8μm) and platelets (2-5μm) are all drained during the initial filtration method. Even if there were leftover RBCs, they would have been lysed through the staining protocol. Additionally, mature RBC's have no nucleus and thus no DNA for DAPI to stain. As for the presence of leukocytes, one must rely on nuclear morphology to distinguish them from CTCs. Most of our CTCs were a minimum of 15μm in size. Most leukocytes (12-15μm) are smaller than CTCs. The morphology of leukocytes facilitates the researcher to distinguish them from CTCs. The most common leukocytes are polymorphonuclear (PMN) cells. PMNs are easily recognizable by their multilobular nuclei. Also, they are usually present in a ring around the edges of the filtration area[198]. Monocytes (15-20 μm) are large cells that could potentially be mistaken for CTCs. However, the kidney shaped nucleus is usually very apparent and thus distinguishes it from CTCs[198]. Lastly, lymphocytes (8-10μm) are distinguished from CTCs (>15μm) because of their small size[198]. Although most CTCs stained for AR, those that did not stain had to undergo further visual examination to exclude any potential mislabeling.

Based on our findings we have shown that size-based filtration is a consistently effective method for capturing CTCs in all risk groups of localized prostate cancer and metastatic disease. In addition, the captured cells were enumerated to validate this isolation method. Our automated and manual enumeration results produced

comparable data and thus show that the GenASIs program by ASI® is a reliable program to enumerate and count CTCs. The enumerated counts steadily increase between the low stage and metastatic disease. Finally, the physical characteristics of CTCs (size and ability to form clusters) has histopathological meanings because large CTCs and small clusters are more likely to be found in lower stage and high stage prostate cancer, respectively.

7. FINANCIAL DISCLOSURE

This project was funded by the University of Manitoba, CancerCare Manitoba, MITACS, and the Prostate Cancer Fight Foundation.

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