Comparative Pathology and Androgen Receptor Signaling of Prostate Cancer in Dogs

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“Well, first of all, through God all things are possible, so jot that down.”

-Mac

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ABSTRACT

Prostate cancer (PCa) is the most common cancer in men. While initial treatments involve androgen deprivation therapies and targeting the androgen receptor (AR), a subset of patients eventually develop aggressive forms of PCa resistant to these treatments, leaving them with limited therapeutic options. Because of this, there is a critical need to identify an animal model that recapitulates the advanced stages of human PCa to facilitate the development of novel, effective therapeutics. Dogs present a unique opportunity as they naturally develop aggressive AR null PCa, resembling advanced human PCa. However, to effectively leverage dogs as models, it is essential to discern which animals are suitable for preclinical trials ante-mortem and understand the pathophysiology of androgen receptor signaling in dog PCa. In this study, we examined the clinicopathologic features of PCa in dogs to identify characteristics indicative of prostatic adenocarcinoma (PRAD), the most prevalent subtype in humans, compared to other types such as prostatic transitional cell carcinoma (P-TCC) in order to identify animals appropriate for preclinical trials. Our findings revealed associations between specific clinicopathologic features and different PCa subtypes in dogs, including strong association of hypoalbuminemia with PRAD and the presence of Melamed-Wolinska bodies and necrosis on cytology specimens with P-TCC. Additionally, we explored the red blood cell distribution width to albumin (RAR) ratio as a potential diagnostic marker to differentiate between PRAD and P-TCC, demonstrating its utility when a certain cut-off ratio was applied. Furthermore, we conducted a proof-of-concept study to investigate the outcomes of reconstituting AR signaling in AR null PRAD dog cell lines. We aimed to determine whether restoring AR signaling in these dog cell lines attenuates aggressive behaviors, as observed in human PCa cell lines. Our results showed varied responses across different cell lines, with AR signaling leading to the abrogation of aggressive behaviors in some cell lines resembling androgen-dependent PCa, while inciting more aggressive behavior in others resembling castration-resistant prostate cancer (CRPC). Overall, this foundational research lays the groundwork for utilizing dogs as valuable models for studying human PCa progression and provides insights into selecting appropriate dog PCa cell lines for in vitro studies and identifying suitable dog PCa patients for preclinical trials for emerging therapeutics.

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ABBREVIATIONS AND ACRONYMS

ABCG2	ATP binding cassette subfamily G member 2
ADT	Androgen deprivation therapy
AF1	Activation function 1
AF2	Activation function 2
AIPC	Androgen-indifferent prostate cancer
AKT	AK strain transforming
ALB	Albumin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
APC	Adenomatous polyposis coli
AR	Androgen receptor
ARE	Androgen response element
ARI	Androgen receptor inhibitor
ARV7	Androgen receptor variant 7
ARSI	Androgen receptor signaling inhibitors
AST	Aspartate aminotransferase
AUC	Area under curve
AVPC	Aggressive variant prostate cancer
BPH	Benign prostatic hyperplasia
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
BRN2	Brain-specific homeobox/POU domain protein 2
BUN	Blood urea nitrogen
CBC	Complete blood cell count
CCL2	Chemokine (C-C motif) ligand 2
CCR2	Chemokine (C-C motif) receptor 2

CHGA	Chromogranin A
CHIP	E3 ubiquitin-protein ligase CHIP
CHOL	Cholesterol
CI	Confidence interval
CK	Creatinine kinase
CK20	Cytokeratin 20
CK7	Cytokeratin 7
c-Myc	Cellular Myelocytomatosis
CR-DNPC	Castration resistant double negative prostate cancer
CR-NEPC	Castration resistant neuroendocrine prostate cancer
CRPC	Castration resistant prostate cancer
CSC	Cancer stem cell
CTD	C-terminal domain
CYP60	Cytochrome 60
DBD	DNA binding domain
DDX5	DEAD box helicase 5
DHT	Dihydrotestosterone
DNPC	Double negative prostate cancer
eIF4E	Eukaryotic initiation factor 4E
eIF4E-BP1	Eukaryotic initiation factor 4E binding protein 1
EMT	Epithelial mesenchymal transition
ENO2	Neuron-specific enolase 2
ER	Estrogen receptor
ERG	E-26 transformation-specific (ETS)-related gene
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
FANCI	Fanconi anemia complementation group gene
FBS	Fetal bovine serum

FFPE	Formalin fixed paraffin embedded
FGF	Fibroblast growth factor
FKB52	FK506 binding protein 52
FKBP51	FK506 binding protein 51
FOLH1	Folate hydrolase 1
Fz/LRP	Frizzled / lipoprotein receptor-related protein complex
GGT	Gamma-glutamyl transferase
HCT	Hematocrit
HGB	Hemoglobin
HPF	High powered field
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HR	Hazard ratio
HRP	Horseradish peroxidase
HSP	Heat shock protein
IAP	Inhibitor of apoptosis protein
IHC	Immunohistochemistry
IL-6	Interleukin 6
ITGA6	Integrin subunit alpha 6
KLF4	Krüppel-like factor 4
LBD	Ligand binding domain
MAPK	Mitogen-activated protein kinase
MCH	Mean cell hemoglobin
MCHC	Mean cell hemoglobin concentration
MCM7	Minichromosome maintenance complex gene
mCRPC	Metastatic castration resistant prostate cancer
MCV	Mean cell volume
MPV	Mean platelet volume
MST	Median survival time

mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
MWB	Melamed-Wolinska bodies
NCAM1	Neural cell adhesion molecule 1
NE	Neuroendocrine
NEPC	Neuroendocrine prostate cancer
NLS	Nuclear localization sequence
nRBC	Nucleated red blood cell
NSAID	Non-steroidal anti-inflammatory drug
NTD	N-terminal domain
OCT 3/4	Octamer binding transcription factor 3/4
OD	Optical density
p-4EBP1	Phosphorylated eIF4E-binding protein 1
p70S6	P70 ribosomal S6 kinase
PAS	Period-acid Schiff
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline 0.1% Tween 20
PCa	Prostate cancer
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PLT	Platelet
p-mTOR	Phosphorylated mammalian target of rapamycin
PP5	Protein phosphatase 5
PRAD	Prostatic adenocarcinoma
PRC1	Polycomb repressor complex 1
PSA	Prostate specific antigen

PSAP	Prostatic-specific acid phosphatase
PSMA	Prostate specific membrane antigen
P-TCC	Prostatic transitional cell carcinoma
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
RAR	Red blood cell distribution width to albumin ratio
RB1	Retinoblastoma 1
RBC	Red blood cell
RDW	Red blood cell distribution width
RETIC	Reticulocyte
ROC	Receiver-operating characteristic
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SCPC	Small cell prostate cancer
SGTA	Small glutamine-rich tetratricopeptide repeat-containing protein α
Sn	Sensitivity
SNAIL	Drosophila embryonic protein snail
SOX2	Sex determining region Y-box 2
Sp	Specificity
SYP	Synaptophysin
TCC	Transitional cell carcinoma
TCF/LEF	T-cell specific factor/ lymphoid enhancer-binding factor
TGF-β	Transforming growth factor beta
TMPRSS2	Transmembrane serine protease 2
t-NEPC	Treatment-emergent neuroendocrine prostate cancer
TNF-α	Tumor necrosis factor alpha
TP	Total protein
TP53	Tumor protein p53
TPR	Tetratricopeptide repeats

TWIST1	Twist-related protein 1
UPKIII	Uroplakin 3
USG	Urine specific gravity
WBC	White blood cell
WNT	Wingless-related integration site
ZEB 1/2	Zinc finger E homeobox binding 1 and 2

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CHAPTER 1: MOLECULAR SIMILARITIES AND DIFFERENCES BETWEEN CANINE PROSTATE CANCER AND HUMAN PROSTATE CANCER VARIANTS

Abstract: Dogs are one of few species that naturally develop prostate cancer (PCa) spontaneously, which clinically resembles aggressive, advanced PCa in humans. Moreover, PCa tumor samples from dogs are often androgen receptor (AR) negative and may enrich our understanding of AR-indifferent PCa in humans, a highly lethal subset of PCa for which few treatment modalities are available. This narrative review will discuss molecular similarities between dog PCa and specific human PCa variants, underscoring the possibilities of using the dog as a novel pre-clinical animal model for human PCa resulting in new therapies and diagnostics that may benefit both species.

1.1 Introduction

The initiation and progression of prostate cancer (PCa) in humans is initially reliant on androgen receptor (AR) signaling [1–3]. Directly targeting androgen ligands with androgen inhibiting drugs (e.g., chemical castration) or decreasing their production via surgical castration has been utilized over the last 80 years in an attempt to suppress AR signaling and PCa tumor progression [4–6]. Unfortunately, androgen-deprivation therapies (ADT) eventually fails for a subset of patients, and despite castrate levels of andro-gens present, PCa progresses to an incurable form termed castration-resistant prostate cancer (CRPC) [7–9]. CRPC often continues AR signaling that is not reliant on androgen ligands (i.e., androgen independent) by way of multiple mechanisms, including mutations in the receptor or copy number variations, and is typically treated with androgen receptor inhibitors (ARIs) [10–13]. Eventually, new driver mutations develop in various genes in CRPC tumors that leads to the abandonment of AR signaling altogether. Once PCa progresses irrespective of AR signaling, it is termed androgen receptor-indifferent PCa, a highly aggressive lethal form of the disease with poor outcomes [14,15]. Recently, molecular characterization of CPRC and androgen-indifferent prostate cancer (AIPC) have improved our understanding of the drivers of these variants, which is critical for the identification of novel therapeutics. However, animal models for advanced forms of PCa are lacking and are wrought with limitations [16,17],

which may make approval of novel therapeutics challenging or translation of results inconsistent between species.

The prostate of the dog, unlike rodents, is morphologically, histologically, and physiologically similar to humans and is under androgen control via androgen receptor signaling [18–20]. Dogs also naturally develop other pathologic prostatic conditions with age, such as benign prostatic hyperplasia (BPH), in contrast to rodents which typically develop prostatic atrophy with age [21,22]. More importantly, dogs are one of the only animals that spontaneously develop PCa, and often present with highly aggressive metastatic disease. Neutering or castrating male dogs is common in developed countries and represents the majority of male dogs in the United States [23,24]. It has been shown that castration influences PCa disease progression as castrated dogs develop PCa at higher rates and experience more metastases than intact dogs [25–29].

This review aims to discuss the molecular similarities between dog and specific human PCa variants to further examine the use of dogs as a suitable animal research model.

1.2. Androgen-dependent PCa

AR is a ligand-dependent nuclear receptor transcription factor that mediates the action of androgen ligands (e.g., testosterone) in androgen-dependent tissues in health and plays an important role in prostate organogenesis and maintenance in adulthood [32–34]. Initially, PCa is reliant on androgens and AR signaling for tumor growth and progression and is characterized by a rising prostate specific antigen (PSA) level in humans, a marker not typically expressed in dog PCa [33,34]. This requirement for androgen-dependent growth is clinically exploited to combat advanced or recurrent PCa (following initial treatment with surgery and radiation) with the use of androgen deprivation therapies (ADT) (i.e., androgen ablation therapies, chemical castration) to inhibit the production or actions of androgens [35,36]. Invariably, PCa becomes resistant to ADT, and is then termed CRPC, where AR remains signaling irrespective of the presence of androgens [37,38]. Because of this continued signaling, AR targeting remains a valid treatment strategy using androgen signaling inhibitors (ASIs) (e.g., androgen synthesis inhibitors, androgen receptor inhibitors). Dog PCa is most often low or null for AR expression, as well as null for androgen-regulated proteins such as NKX3.1 [39,40] (**Table 1.1**). However, opposing reports

show some AR expression in intact dogs with PCa and cytoplasmic sequestration or loss of AR in castrated dogs with PCa [41,42], making intact dogs with PCa a potential model for androgen-dependent disease. As noted over 50 years ago in landmark studies performed by Huggins et al., dogs have similar pathophysiology with respect to androgen-dependent growth of the prostate and may still be of value to researchers [6,43].

Androgen Receptor Structure

The AR in humans is a protein of 919 amino acids consisting of several functional domains, including an N-terminal domain (NTD), DNA binding domain (DBD), and a ligand binding domain (LBD) at the C-terminus [44,45]. In the dog, AR is approximately 907 amino acids, and has a homologous DBD and LBD, which is highly conserved across evolution in various species and is activated upon binding to androgen ligands [45,46].

The NTD of AR is essential for function and the least evolutionarily conserved region of AR, however, there are still similarities between humans and dogs. The polyglutamine (i.e., polyQ, CAG) repeat region of the NTD has an average of 21-23 Qs in humans. Longer polyQ repeats is related to decreased AR transcriptional activity, while shorter polyQ repeats is related to increased AR transcriptional activity and is often associated with increased PCa risk [47–49]. This finding has also been recapitulated in dogs in vitro, where the introduction of AR with fewer polyQ repeats resulted in higher AR activity [50]. Though, the association between shorter polyQ repeats regions and PCa is unclear in dogs, while some studies reveal that a shorter polyQ length does not predispose dogs to PCa others show that certain breeds with a shorter polyQ length are pre-disposed to developing PCa [25,51–53].

Androgen Receptor Co-Chaperones

In the absence of agonist ligand, AR is bound to heat shock proteins (HSP40, HSP70 and HSP90) and other co-chaperone proteins in a complex known as the foldosome [54,55]. Many small co-chaperone proteins with tetratricopeptide repeats (TPR), like CYP60, PP5, FKBP51, FKB52, PP5, CHIP and SGTA, have been shown to interact with the AR-foldosome complex [56]. SGTA, or small glutamine–rich tetratricopeptide repeat containing protein α, is a co-chaperone of interest in PCa, and is known to

stabilize the apo-AR structure in the cytoplasm prior to ligand binding. In human PCa, small glutamine-rich tetratricopeptide repeat-containing protein α (SGTA), a steroid receptor molecular co-chaperone that influence hormone action, is known to regulate AR function. AR:SGTA ratio is increased when compared to patient-matched BPH and is also increased when metastatic PCa tumors are compared with their primary tumor [57,58]. It is hypothesized that AR thereby overwhelms the capacity for SGTA to limit AR response to ligands and ensure appropriate cellular localization of AR in vivo. In addition, in vitro work from this study showed that SGTA overexpression blunted AR's response to androgen ligands [58].

Table 1.1. Summary of Molecular Characteristics of Dog and Human PCa

PCa Variants	Marker of Pathway	Dog	Humans
Androgen-dependent			
	AR+	No	Yes
	NKX3.1 +	No	Yes
	PSA +	No	Yes
CRPC			
	PI3K-AKT-mTOR overexpression	Yes	Yes
	ERβ downregulation, ERα overexpression	No[ǂ]	Yes
	Markers of CSCs +	Yes	Yes
	Markers of EMT +	Yes	Yes
	Wnt/β-catenin overexpression	Yes	Yes
AIPC			
DNPC	AR (-)	Yes	Yes
	Markers of NE (-)	Yes	Yes
NEPC	Markers of NE +	No	Yes
AVPC	*TP53* (-) or mutated	No	Yes
	RB1 (-) or mutated	Unknown	Yes
	PTEN (-) or mutated	Yes	Yes

ǂ Estrogen receptor expression in dog PCa is present but is not identical to human PCa. Abbreviations: +, positive for expression; (-), negative for expression; AR, androgen receptor, PSA, prostate specific antigen; CSC, cancer stem cell; ER, estrogen receptor; EMT, epithelial mesenchymal transition; AIPC, androgen indifferent prostate cancer; DNPC, double negative prostate cancer; NE, neuroendocrine; NEPC, neuroendocrine prostate cancer; AVPC, aggressive variant prostate cancer

This concept has also been explored in multiple studies in dogs, which have shown that overexpression of SGTA in vitro abates AR signaling [59,60]. Because of this, androgen-independent disease has been hypothesized to be attributed to SGTA overexpression in dog PCa patient tumor samples

by some, who have also subsequently showed that interference with SGTA dimerization in vitro rescues AR signaling [61].

1.3. CRPC

CRPC is a hormone-independent but AR-dependent progression of PCa, where ADT has failed yet AR signaling axis is still functional and promotes growth and progression. Often, this stage is characterized by more aggressive tumor behavior, including initiation of cancer stem cell (CSC) signaling, epithelial-mesenchymal transition (EMT), and metastasis. A subset of patients develop CRPC second to uncontrolled AR signaling due to a mutation in the AR. Splice variants of the AR lacking the ligand-binding domain, such as ARV7, are responsible for this uncontrolled signaling in humans [120,121]. To date, splice variants have not been identified in dog PCa, and the use of dogs as a model for splice variant-driven CRPC is undetermined. Moreover, TMPRSS2-ERG fusion and its alternative splice variants have also not been investigated in dog PCa and their value as a model for this event is yet to be determined.

Additionally, mutations in AR-independent pathways become more frequent in CRPC and are thought to drive CRPC to become AR-indifferent PCa after ASI therapy. As such, AR presence and signaling coupled with new driver mutations in other pathways, EMT, and metastasis are hallmarks to this progression of PCa (metastatic CRPC [mCRPC]). Because dogs often present with low or null AR expression, are castrated at an early age, develop PCa without the influence of androgens, and often present with metastasis, it has been argued that dogs closely resemble CRPC in humans.

PI3K-AKT-mTOR and PTEN

PI3K-AKT-mTOR signaling axis has been shown to play a crucial role in the development and maintenance of CRPC [62–64]. This pathway integrates growth signals with downstream processes that promote cell survival and proliferation [64]. PI3K activation by growth factor transmembrane signaling leads to activation of AKT, and in turn, autophosphorylation and activation of mTOR [65]. From here, mTOR joins raptor (to form mTORC1) or rictor (to form mTORC2), the former implicated in cellular proliferation and protein synthesis and the latter implicated in cytoskeletal organization and cellular metabolism [66,67]. Upon activation, mTORC1 increases mRNA translation by phosphorylating eIF4E-

BP1 and p70S6, the former leading to the release of eIF4E from its hold and allowing it to join the translation initiation complex, and the latter leading to the enhanced translation of mRNA transcripts that encode for ribosomal proteins and insulin growth factor 2 [68]. This pathway is of great interest as overactivation of the PI3K-AKT-mTOR pathway has been implicated in cell survival of many cancer types, including PCa [69,70]. Many factors contribute to the aberrant activation of the PI3K-AKT-mTOR pathway, such as PI3K amplification, PTEN loss of function or deletion, and AKT, p-4EBP1 and eIF4E overexpression [69].

This entire pathway has also been observed to be upregulated in dog PCa tissue samples when compared to non-malignant prostatic tissue of normal intact dogs [40]. Moreover, another study found that p-mTOR and eIF4E were overexpressed in dog PCa tissues compared to normal prostate tissue, and this overexpression was correlated with a higher Gleason score in these dog PCa histology sections, similar to what has been reported in human PCa [71,72]. Deletion or loss-of-function PTEN has also been implicated in aberrant activation of this pathway in human PCa and has also been shown to be lost in dog PCa [73]. Because PI3K pathway inhibitors, as well as eIF4E inhibitors, are a class of emerging therapeutics in CRPC and are being used in current clinical trials [74–76], the dog may be a useful model in testing drugs targeting this pathway.

Estrogen Receptors

Estrogens have been linked to CRPC progression, and increased levels of estrogens have been correlated with more aggressive PCa [77–80]. Estrogens serve as a ligand for two nuclear receptor isoforms, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), where ERα has been implicated in oncogenic functions and increased proliferation of cancer cells, while ERβ has tumor suppressor functions because its loss leads to hyperplasia of the prostate and initiation of PCa [81–84]. Moreover, PCa has been shown to have increased expression of ERα and decreased expression of ERβ [85–87]. Although ERα expression is typically observed in the tumor stromal cells, one study has found that ERα is expressed in a small sections of patient's tumor epithelial cells as well, although this had no effect on clinical or biochemical recurrence of disease [88]. Estrogen receptor expression in dog PCa has been explored in one study, where ERα was strongly expressed in normal prostate epithelium and epithelium of BPH samples

but had decreased expression in PCa tumor epithelium and null expression in the tumor stroma [73]. This appears to be in contrast to what is found in the majority of human PCa, although additional studies are needed to confirm these findings.

Stem Cell Markers

Cancer stem cells (CSC), or tumor progenitor cells, have been linked to driving clonal evolution, tumor heterogeneity, growth, and progression via their ability to self-renew, resist apoptosis, and differentiate [89–91], and have been implicated in the development of CRPC [92]. During development, basal epithelial stem cells of the prostate differentiate into luminal epithelial cells under the influence of androgens and the AR [93]. These multipotent basal progenitor cells remain quiescent in the basal epithelial layer, acting only to replenish defunct or apoptotic luminal cells [92]. However, CSCs are believed to most likely arise from these basal epithelial cells in PCa, although there is evidence that suggests that the luminal cell population have their own lineage-restricted stem cells [92]. Classic markers of pluripotency include OCT3/4, SOX2, KLF4, c-Myc, and NANOG, which have been observed in PCa [94]. In addition, PCa CSC populations CD44+ CD20- and often express CD133 and ABCG2 in humans. These markers are often associated with more aggressive tumors, higher Gleason scores, metastasis and chemoresistance in humans [95–99].

Stem cell marker expression has also been explored in dog PCa [100,101]. In patient-derived dog PCa cell lines, one study found increased expression of CD44, CD133, ITGA6 and DDX5 [100]. Moreover, these findings were recapitulated and expanded on in another study using patient-derived tumor spheroids, which were found to express OCT3/4, Nestin, NANOG and CD44 [101]. Two additional factors, SOX9 and survivin, have also been associated with CSCs in PCa [91,102]. SOX9 is repressed by androgens and is often seen overexpressed in CRPC as a result, while survivin is an inhibitor of apoptosis protein (IAP) family member and is overexpressed in many cancers [103–106]. Both of these factors have also been observed to have increased expression in dog PCa [107]. Taken together, the dog appears to develop PCa with the presence of CSCs and may serve as a model for therapeutic targeting of these tumor progenitor cells.

Epithelial-Mesenchymal Transition (EMT) Markers

Epithelial cells can undergo EMT, a phenomenon that is hallmarked by phenotypic changes of the epithelial cells to appear more spindle-shaped like mesenchymal cells. Additionally, this phenotypic change is accompanied by biochemical changes, where epithelial markers associated with adhesion, like E-cadherin, are downregulated and mesenchymal lineage markers, like vimentin, become upregulated [108]. This event leads to higher metastatic potential of cancer cells and plays a critical role in mCRPC [109,110]. EMT induction has been associated with multiple molecular drivers, including TGF-β and the WNT signaling pathways [110–112]. This signaling induces expression of multiple transcription factors that are known as classic regulators of EMT, such as Snail, Twist1, and zinc-finger E homeobox-binding 1 and 2 (ZEB1/2) [110].

EMT has been shown to occur in dog PCa as well. One study showed that more undifferentiated appearing dog PCa tumors and metastatic lesions had increased TGF-β nuclear positivity on immunohistochemistry (IHC) [113]. E-cadherin has been shown to be decreased in dog PCa and associated with higher Gleason scores in dog PCa histology sections, and neoplastic luminal epithelial cells have been shown to have increased expression of vimentin when compared to non-neoplastic lesions [39,114,115]. Because dogs with PCa often have aggressive disease and metastatic lesions [114], EMT is an unsurprising occurrence in their PCa progression, and they may glean insight on this process in human PCa.

Canonical Wnt Signaling

The canonical Wnt signaling pathway is integral to EMT and has been shown to be important in the development of CRPC and proliferation of CSCs in the PCa microenvironment [116]. Briefly, Wnts are secreted glycoproteins that direct development, tissue homeostasis and stem cell proliferation. Classically, Wnts bind to the Frizzled and lipoprotein receptor-related protein (Fz/LRP) co-receptor complex of the cell membrane, which leads to the release of β-catenin from a group of regulatory proteins (i.e., destruction complex) that promote its ubiquitination and destruction in the proteosome. Once released, β-catenin increases in the cytosol, which allows for its migration to the nucleus where it regulates target gene expression by interacting with the T-cell specific factor (TCF)/lymphoid enhancer-binding factor (LEF) family

of transcription factors, ultimately leading to transcription of target genes promoting cell differentiation and proliferation [117].

Wnt signaling has been implicated in human PCa progression for decades and is critical for CRPC. Wnt signaling has been shown to increase proliferation and differentiation of prostate cells, and promote EMT, leading to more aggressive and invasive disease [118]. This pathway has also been shown to have cross talk with AR and is activated after ADT, encouraging and maintaining CRPC development and progression [119]. One study had found increased cytoplasmic β-catenin staining, but not nuclear, in pre-neoplastic and neoplastic lesions when compared to BPH lesions in dog PCa [114]. In contrast, a more recent study showed overexpression of nuclear β-catenin and loss of membranous β-catenin in dog PCa, with these findings exacerbated in metastatic lesions [120]. This was not due to hypermethylation of APC [120], as is found in human PCa, and a different mechanism is likely responsible for aberrant Wnt signaling in dog PCa. Coupled with E-cadherin, membranous downregulation and increased nuclear TGF-β expression were also observed in dog PCa [39,113–115], Wnt signaling is likely a key player in the progression of PCa in this species, and for that, dog PCa may be a good model for phases of human PCa that are reliant on this aberrant pathway (i.e., CRPC and mCRPC).

1.4 Androgen-indifferent PCa variants (AIPC) of mCRPC

Androgen-indifferent prostate cancer (AIPC) is a form of PCa with treatment-resistant phenotypes that do not rely on AR signaling and typically arise from mCRPC following treatment with ASIs [121,122], although they may arise de novo in treatment-naïve PCa [123,124]. They are associated with an aggressive clinical course and poor treatment outcomes [14,124]. AIPC variants of mCRPC include aggressive variant prostate cancer (AVPC), neuroendocrine prostate cancer (NEPC), and double-negative prostate cancer (DNPC), with each having their own distinct molecular signatures [14,125] (**Figure 1.1**). Multiple reports have shown that some dogs with PCa have molecular signatures similar to some AIPC variants which are outlined below.

DNPC

DNPC is characterized by loss of AR and neuroendocrine (NE) markers and is sometimes referred to as CRPC subtype DNPC (CR-DNPC) [126]. DNPC is frequently characterized by an increase in fibroblast growth factor (FGF) and mitogen-activated protein kinase (MAPK) signaling and has upregulated genes for EMT [122]. This has been shown to occur second to polycomb repressor complex 1 (PRC1) activity, a histone methylase, upregulating CCL2 and promoting stemness and metastasis in DNPC [127].

Similarly, dog PCa is often AR and NE negative with increased FGF and MAPK signaling [40] and has been shown to have upregulation of indirect markers of EMT [39,114,115]. As such, dog PCa may be considered molecularly similar to human DNPC. Whether DNPC in dog PCa is reliant on PRC1 and CCL2 signaling as it is in human PCa is yet to be explored.

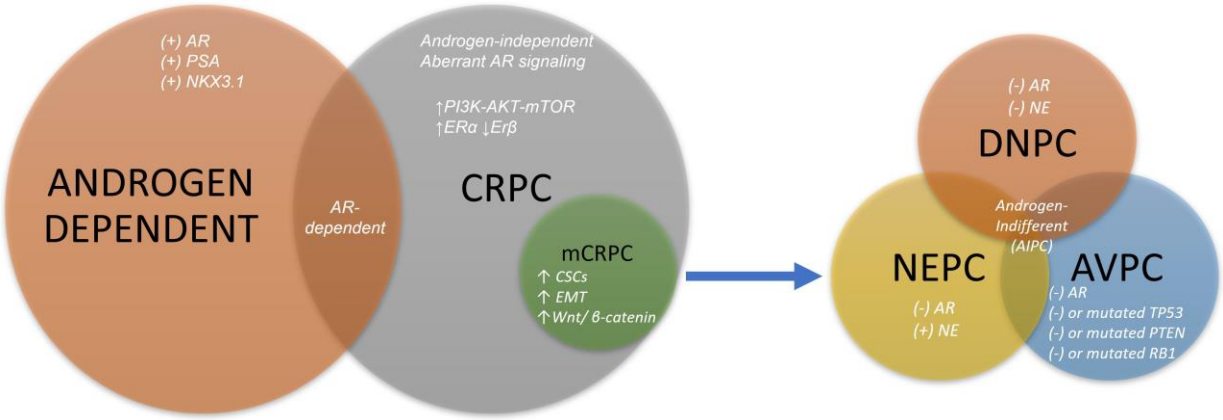

Figure 1.1. Molecular characteristics of each PCa variant or subtype. PCa is initially reliant on androgens binding to the AR and promoting growth. Genes commonly regulated and expressed second to this signaling include NKX3.1 and PSA. CRPC is also reliant on aberrant AR signaling, which does not require androgen ligands. CRPC also accumulates additional driver mutations in other genes and pathways, including PI3K-AKT-mTOR. A subtype of CRPC, mCRPC, is also known to acquire overexpression of Wnt/B-catenin signaling, as well as increased CSCs and evidence of EMT. Lastly, AIPC is a group of PCa that is androgen-indifferent and usually AR-indifferent as well and often arises from mCRPC. Subtypes of AIPC have specific molecular signatures. DNPC has loss of AR and NE marker expression; AVPC has loss of AR expression and mutation or loss in TP53, PTEN, or RB1; and, NEPC usually has loss of AR expression and gain of NE markers, like chromogranin A or synaptophysin. Abbreviations: AR, androgen receptor; PSA, prostate specific antigen; ER, estrogen receptor; CRPC, castration resistant prostate cancer; CSC, cancer stem cell; EMT, epithelial mesenchymal transition; NE, neuroendocrine; DNPC, double negative prostate cancer; NEPC, neuroendocrine prostate cancer; AVPC, aggressive variant prostate cancer.

NEPC

NEPC may occur de novo or as a consequence of selection pressures on CRPC tumors from ASIs, which is termed treatment-emergent NEPC (t-NEPC) or castration-resistant NEPC (CR-NEPC) [127,128]. These tumors often lack AR and AR-regulated genes and have increased expression of NE markers,

including pro-neural transcription factors, like BRN2, and immunohistochemical markers like chromogranin A (CHGA), synaptophysin (SYP), neuron-specific enolase 2 (ENO2) and neural cell adhesion molecule 1 (NCAM1, CD56) [128,129]. In contrast, dog PCa rarely expresses NE markers [130] and NEPC has not yet been reported. This may be in part because pharmaceutical androgen signaling therapies are not clinically used to treat dogs with PCa, thus avoiding the selection pressures that may influence CSCs to differentiate into NE cells or harbor a tumor microenvironment where NE or NE-like clones proliferate. Moreover, there are conflicting reports as to whether or not dogs contain NE cells in the prostate at all [131,132], likely rendering the dog an unrewarding model for de novo NEPC, as well as t-NEPC.

AVPC

AVPC criteria were created to identify patients predicted to have aggressive disease that is unlikely to respond to ASI therapy and may arise de novo or after pharmaceutical treatment. Any morphological variant of PCa (e.g., small cell prostate cancer [SCPC], adenocarcinoma, adenocarcinoma with NE differentiation) may qualify as AVPC if it has a particular molecular signature and/or meets criteria of an aggressive clinical course [132], including signs of visceral and lytic bone metastases, lymphadenopathy, and androgen-independent progression within 6 months of starting treatment [133]. AVPC is characterized by deleterious loss or mutation of at least two of the three molecular tumor suppressors, TP53, PTEN, and RB1, and is typically responsive to platinum drugs [125]. Moreover, a study on circulating tumor cells from patients with AVPC had shown that these cells not only have losses in PTEN, RB1, and TP53, but also losses in BRCA2 as well [134].

Dog PCa is highly aggressive and clinically similar to AVPC, but whether it shares the same molecular hallmarks as human AVPC is still unclear. One study showed decreased expression of p53 in dog PCa tissues when compared to normal and inflammatory prostatic lesions, but not complete loss [42], while another study showed no significant increase or decrease in P53 [40]. PTEN is either downregulated or completely lost according to a few studies in dog PCa, similar to AVPC in humans [42,73]. Interestingly, RB1 has not yet been studied in dog PCa. Loss of BRCA1 has been documented in dog PCa, but BRCA2 was not lost or downregulated according to another study utilizing RNA-sequencing [40,135]. Because ASIs

are not used in veterinary medicine, dog PCa may not completely match the molecular picture of human AVPC, but the molecular pictures is still incomplete and further investigation is warranted.

1.5. Conclusions

Undeniably there are molecular similarities between dog PCa and specific human PCa variants, although there are differences and areas that are understudied. Because the majority of dogs are neutered, present with advanced disease, and have AR null tumors, they are likely not feasible models for androgen-dependent PCa, with the exception of intact dogs in the early stages of the disease. Because of the lack of screening methods for PCa in dogs (i.e., PSA levels), establishing which intact dogs have early PCa would be challenging. Dog PCa clinically and molecularly is similar to CRPC and mCRPC with upregulation in the PI3K-AKT pathway, Wnt signaling, EMT, CSCs, and indifference to AR, although ER expression appears to be different. Moreover, the importance of splice variants is yet to be determined in dog PCa as well. Lastly, regarding AIPC, it has been shown that dogs molecularly model DNPC, and may also model AVPC, but are unlikely to recapitulate NEPC. Taken together, dogs are likely suitable models for certain variants of advanced PCa, but not all, and additional studies are warranted to further characterize the molecular characteristics of dog PCa.

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CHAPTER 2: CLINICOPATHOLOGIC CHARACTERIZATION OF PROSTATIC CANCER IN DOGS

Simple Abstract: Prostatic adenocarcinoma (PRAD) and prostatic transitional cell carcinoma (P-TCC) are the most common subtypes of prostate cancer (PCa) in dogs and differentiating them often requires an invasive tissue biopsy with histopathology. Routine laboratory data from blood work and minimally invasive tests, such as fine-needle aspiration cytology, have been overlooked as a tool for discerning between these tumors. This is the first study to utilize clinicopathologic and cytologic data to differentiate PRAD and P-TCC in dogs.

Abstract: Clinicopathologic data in dogs with prostate cancer (PCa) may aid in the differentiation between tumor types and subsequent treatment decisions; however, these data are often unreported. Demographic, clinicopathologic, cytologic, histologic, and survival data from dogs with primary prostatic adenocarcinoma (PRAD) (n = 56) and primary prostatic transitional cell carcinoma (P-TCC) (n = 74) were acquired from a tertiary veterinary teaching hospital from 1992 to 2022. Red blood cell distribution width (RDW) to albumin ratio (RAR) was evaluated for diagnostic utility in differentiating between PRAD and P-TCC. Sections from PRAD tumors (n = 50) were stained for androgen receptor (AR) expression, and laboratory data were compared between AR-positive (AR+) and AR-negative (AR−) groups. RDW was increased in PRAD, while albumin was decreased ($p < 0.05$). P-TCC was associated with Melamed-Wolinska bodies (MWB) and necrosis on cytology ($p < 0.05$). RAR had acceptable diagnostic utility in the differentiation of PCa tumors (AUC = 0.7; $p < 0.05$). Survival rates and metastases were equivocal. AR+ and AR− PRAD tumors did not differ in clinicopathologic data or survival ($p > 0.05$). In conclusion, hypoalbuminemia was significantly associated with PRAD and decreased survival, while MWB and necrosis were significantly associated with P-TCC on cytology. These clinicopathologic data may help clinicians differentiate between these tumors antemortem to guide appropriate treatment and intervention.

2.1 Introduction

Prostate cancer (PCa) in dogs has a poor prognosis and there is currently no consensus on standard-of-care treatment [1]. Prostatic transitional cell carcinoma (P-TCC) and prostatic

adenocarcinomas (PRAD) are the most prevalent PCa in dogs, and the use of nonsteroidal anti-inflammatory drugs (NSAIDs) is often recommended as a first-line treatment; however, this has been largely researched in dogs with tumors of transitional cell (i.e., urothelial) origins [2,3]. The effects of NSAIDs have some conflicting results in human PCa [4–6], which are often of glandular origin (i.e., adenocarcinoma) [7]. Though the effect of NSAIDs on dog PRAD specifically is currently unknown, it may be useful to differentiate between P-TCC and PRAD prior to initiating treatment, particularly if pathway-targeting therapeutics are used for pathways enriched in the carcinogenesis of one tumor type and not the other.

One signaling pathway that is often examined prior to the onset of treatment of PCa in humans is the androgen receptor (AR) signaling pathway. This is variably present in dog PCa; however, one study has shown that up to 40% of TCC in dogs have an AR presence and that revisiting this pathway may be warranted [8,9]. Disruption of this pathway is a mainstay in the initial treatment, management, and detection of human PCa, and determining whether AR signaling is present in tumors is conducted by measuring serum prostatic specific antigen (PSA) levels [10,11]. In dogs, however, early screening tests for PCa do not exist, and the gold standard for diagnosing and phenotyping PCa is biopsy with histopathology, an invasive procedure that incurs considerable cost and risk [1,12]. Because it is controversial whether dogs have detectable serum PSA, and no species-specific assays currently exist, evaluating other pre-existing routine laboratory data to decipher the AR status of dog tumors could be useful in guiding treatment [13,14].

An additional routine blood work parameter yet to be explored in dog PCa is the red blood cell width (RDW) and red blood cell distribution width to serum albumin ratio (RAR). RDW is a numerical parameter that represents the variation of erythrocyte volume, and an increased RDW alone has been shown as a positive predictor of tumor progression, decreased overall survival, and poor treatment outcomes in human PCa [15–18]. It has also been associated with unfavorable outcomes in the clinical course of other cancers, including breast cancer, hematologic cancers, and osteosarcoma [19–21]. RAR is the ratio of RDW to serum albumin, a negative acute phase protein and the most abundant protein in the blood [22]. High RAR ratios in humans have been associated with all-cause mortality in cancer patients, as well as other non-neoplastic morbidities, including poor prognosis in sepsis and aortic valve

replacement [23–25]. These data, which are also available on routine blood work in dogs, may help clinicians differentiate and prognosticate PCa in dogs.

The objectives of this study were to determine if discernment between PRAD and P-TCC, as well as PRAD AR+ versus PRAD AR−, could be accomplished with routine laboratory data, and minimally invasive fine needle aspiration (i.e., fine needle biopsy) and cytology, as well as RAR. The results of this study demonstrate that clinicopathologic and cytologic data are useful in differentiating PRAD from P-TCC in dogs.

2.2 Materials and Methods

Dog Selection and Demographic Data

A retrospective electronic medical record search was performed to identify dogs with prostatic cancer. Medical records from the University of California Davis Veterinary Medical Teaching Hospital from January 1992 to May 2022 were investigated. Medical records were searched for all visits by dogs that had a final diagnosis of primary prostate cancer with confirmation by histopathology (i.e., necropsy or biopsy). Cases were excluded if another cancer was present that invaded the prostate secondarily (e.g., primary bladder cancer), if the patient had any unrelated neoplasia in another location, or if the patient had neoplasia of the prostate other than prostatic adenocarcinoma (PRAD) or prostatic transitional cell carcinoma (P-TCC). Data abstracted from the medical records included signalment (i.e., age, castration status, breed), diagnostics performed, co-morbidities, treatment, survival time, and presence of metastases.

Clinicopathologic and Histopathologic Data

Histopathologic and immunohistologic data differentiating between PRAD and P-TCC were also abstracted from the medical records when available. Cases were excluded if the histologic report or immunohistologic results were unable to make a definitive diagnosis. Clinicopathologic data (i.e., complete blood count [CBC], serum biochemistry panel, urinalysis) were also abstracted and recorded from the medical record retrospectively. Hematology analyzers used during the study period included Baker Systems 9110 Plus (BioChem Immunosystems Inc., Allentown, PA, USA), ADVIA 120 and ADVIA

2120 (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA). Biochemistry analyzers used during the study period included a Hitachi 717C, Roche Hitachi 917, and Hitachi Cobas c501 (Roche Diagnostics Corporation, Indianapolis, IN, USA). Results were calibrated when instruments were upgraded to maintain consistency in results between analyzers. Semiquantitative urinalysis dipstick data were converted to their equivalent quantitative values per dipstick manufacturer instructions (e.g., "+1 or +2" protein was recorded as 75 mg/dL). Values recorded as "trace" were considered negative. Mean values were recorded when ranges were provided for components of the urine sediment examination (e.g., white blood cell [WBC] sediment counts recorded as "5– 9 WBC/field" were noted as 7 WBC/field). Cytologic data and specimens from May 2012 (which was the earliest year slides were available to re-examine) to May 2022 were reviewed and recorded by a board-certified veterinary clinical pathologist (D.M.V.), and observations of interest were imaged. These observations of interest were recorded as either present or absent, and they included Melamed–Wolinska bodies, necrosis, inflammation, mineralization, vacuolation of neo-plastic cells, mitotic figures, keratinization of neoplastic cells, and presence of hemosiderin. Receiver-operating characteristic (ROC) curves were performed to evaluate the diagnostic utility of the red blood cell distribution width to albumin ratio (RAR), and RDW and ALB were used as diagnostic tests to differentiate PRAD from P-TCC.

Immunohistochemistry Staining for Androgen Receptors and Laboratory Data Evaluation

FFPE sections from PRAD tumors were requested from January 1992 to May 2022 and sectioned. Immunohistochemistry was conducted as previously described [26], and AR (dilution 1:150; N-20, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) positivity expression and location (cytosolic vs. nuclear) was recorded. Positivity for expression was defined as staining in ≥ 10% of neoplastic cells [27]. Tumors were considered AR-positive (AR+) if the nuclei or cytoplasm were positive for expression. Normal prostate tissues from intact dogs were used as a positive control. Baseline laboratory data (i.e., CBC, serum biochemistry, and urinalysis) from dogs with AR+ PRAD tumors were compared to dogs with AR-negative (AR−) PRAD tumors to determine if any analyte was suitable as a biomarker for androgen receptor signaling ante mortem. Additionally, ROC curves were performed to evaluate the diagnostic utility of RAR as a test to differentiate AR+ from AR− PRAD tumors.

Statistical Analysis

Data was downloaded into a Microsoft Excel spreadsheet and analyzed in GraphPad Prism version 10.1.0. Descriptive statistics for categorical variables were reported as frequency or frequency with percentage, while continuous variables were reported as median with range (i.e., minimum-maximum). The association between categorical variables was assessed by chi-squared test or Fisher's exact test, pending sample size of each observation ($n < 5$, Fisher's exact test; $n \geq 5$, chi-squared test). Normality of laboratory data was determined by a Shapiro–Wilk test. Non-parametric data were compared with a Mann–Whitney test for data separated into two groups, or a Kruskal–Wallis test with a post-hoc Dunn's multiple comparison test when data were separated into three groups. Red blood cell distribution width to albumin ratio (RAR) diagnostic utility was determined by a receiver–operator curve (ROC), and test cut-off value was determined using the value closest to (0,1) criterion. The Youden index was also calculated to confirm diagnostic utility. Patient survival times were measured from onset of clinical signs (time = 0) until death unless otherwise noted. Kaplan–Meier analyses were compared by a Mantel–Cox log-rank test. A multivariate Cox proportional hazards regression model was used to compare RDW, ALB and RAR with survival data in PRAD and P-TCC with survival >1 day. A p-value of <0.05 was considered significant.

2.3 Results

Medical Record Inclusions and Exclusions

There were 150 records found within the study period that had a final diagnosis of primary prostatic cancer confirmed by histology (necropsy or biopsy). Eleven records were excluded for neoplasia (amelanotic melanoma [n = 1], anal sac gland adenocarcinoma [n = 3], anaplastic carcinoma [n = 5], colonic carcinoma [n = 1], and gastric carcinoma [n = 1]) other than P-TCC or PRAD in the prostate. Eight records were excluded because another neoplastic process was present concurrently and was the primary cause for presentation (anal sac gland adenocarcinoma [n = 1], nasal B-cell lymphoma [n = 1], chemodectoma [n = 1], oligodendroglioma [n = 1], melanoma [n = 1], rhabdomyosarcoma [n = 1], thyroid carcinoma [n = 1] and histiocytic sarcoma [n = 1]). One record was excluded because neoplasia was not

found in the prostate and a bladder mass was mistakenly believed to be a prostatic mass ante-mortem. Ultimately, 130 records of patients with primary prostate tumors that were confirmed by either biopsy (n = 21), or necropsy (n = 109) were included in this study. Seventy-one specimens (nine 9 biopsy, 62 necropsy) had immunohistochemistry performed to reaffirm the diagnosis (i.e., prostatic-specific acid phosphatase [PSAP], cytokeratin 7 [CK7], cytokeratin 20 [CK20], uroplakin 3 [UPKIII]). A diagnosis of PRAD was made if glandular epithelial cells were the neoplastic cells of origin or neoplastic cells were positive for expression of PSAP in on IHC for PSAP. A diagnosis of P-TCC was made if prostatic ductal epithelium or prostatic urethral epithelium were the neoplastic cells of origin or neoplastic cells stained were positive for expression of UPKIII, CK7 or CK20. If IHC staining was equivocal, a definitive diagnosis was made based on the pathologist's overall impression. Of the 130 records, 74/130 (56.9%) received a final histopathologic diagnosis of P-TCC, while 56/130 (43.1%) were deemed PRAD. Most dogs affected by PRAD or P-TCC were castrated, large mixed-breeds, and both groups had a median age of 10.1 years. German shepherds and American Pit Bull terriers were the most prevalent purebreds in PRAD, while Labrador retrievers and Scottish terriers were the most prevalent purebreds in P-TCC. Additional age, sex, and breed distributions for each tumor type were recorded (**Tables 2.1 and 2.2**).

Table 2.1. Age and neuter status of dogs with PRAD and P-TCC.

		PRAD (*n* = 56)	**P-TCC (*n* = 74)**
Age	Young Adult (0–5 years)	3 (5%)	2 (3%)
	Mature Adult (6–10 years)	26 (46%)	42 (57%)
	Geriatric (11–15 years)	21 (38%)	22 (30%)
	Unknown	6 (11%)	8 (11%)
Neuter Status	Intact	15 (27%)	12 (16%)
	Castrated	41 (73%)	62 (84%)

Table 2.2. Top 5 breeds of dogs with PRAD and P-TCC.

Tumor Type	Breeds	N (%)
PRAD (*n* = 56)	Large Mixed Breed (>50 lbs.)	8 (14%)
	German Shepherd	5 (9%)
	American Pit Bull Terrier	5 (9%)
	Labrador Retriever	3 (5%)
	Medium Mixed Breed (20–49 lbs.)	3 (5%)
P-TCC (*n* = 74)	Large Mixed Breed (>50 lbs.)	13 (18%)
	Labrador Retriever	7 (9%)
	Scottish Terrier	6 (8%)
	German Shepherd	4 (5%)
	Golden Retriever	4 (5%)

Clinicopathologic Data Results

Of the 56 PRAD dogs, 32 had a CBC performed, 29 had a serum biochemistry panel performed, 25 had a urinalysis performed, and 24 had fine-needle aspirate cytology performed at initial presentation. Three cytologies were excluded because they were from metastatic sites and not the primary tumor, resulting in a final number of 21 cytologies for PRAD. Of the 74 P-TCC dogs, 34 had a CBC performed, 31 had a serum biochemistry panel performed, 30 had a urinalysis performed and 25 had fine-needle aspirate cytology performed at initial presentation.

Complete Blood Count (CBC), Serum Biochemistry and Urinalysis

Regarding CBC, RDW% was significantly increased in PRAD compared to P-TCC but within reference range ($p = 0.02$) (**Table 2.3**), while on-serum biochemistry albumin was mildly decreased below the reference interval in PRAD compared to P-TCC ($p = 0.03$) (**Table 2.4**).

Table 2.3. Complete Blood Cell Count Findings for PRAD and P-TCC

Variable	PRAD (*n* = 32)	P-TCC (*n* = 34)	Ref. Int.	*p*-Value
RBC ($\times 10^6$/µL)	6.06 (2.97–7.57)	6.54 (4.22–7.8)	5.6–8	n.s.
HGB (g/dL)	14.2 (6.9–19.5)	15.3 (10.1–17.8)	14–19	n.s.
HCT (%)	41 (21.4–52.5)	43 (29.2–52.5)	40–55	n.s.
MCV (fL)	68.9 (59.9–75.6)	67.3 (58.3–73.9)	65–75	n.s.
MCH (pg)	23.8 (20.4–28.7)	23.4 (20.6–25.5)	22–26	n.s.
MCHC (g/dL)	34.5 (32.2–38.3)	34.6 (33–37.5)	33–36	n.s.
RDW (%) *	13.95 (11.3–19.7)	12.9 (11.5–15.7)	11–14	0.02
RETIC (/µL)	0 (0–155,400)	0 (0–129,800)	7000–65,000	n.s.
nRBC (/100 WBC)	0 (0–3)	0 (0–5)	RARE	n.s.

WBC (/µL)	13,717 (6610–34,772)	12,190 (2780–53,500)	6000–13,000	n.s.
Neutrophils (/µL)	11,228 (4158–31,295)	9935 (1918–48,632)	3000–10,500	n.s.
Immature Neutrophils (/µL)	0 (0–2576)	0 (0–1725)	RARE	n.s.
Lymphocytes (/µL)	1109 (409–7523)	1394 (417–2954)	1000–40,000	n.s.
Monocytes (/µL)	892 (258–3492)	864 (241–3549)	150–1200	n.s.
Eosinophils (/µL)	218 (0–959)	129 (0–1341)	0–1500	n.s.
Basophils (/µL)	0 (0–898)	0 (0–107)	0–50	n.s.
PLT ($\times 10^3$/µL)	277 (19–717)	295 (54–876)	150–400	n.s.
MPV (fL)	9.9 (7–17.6)	10 (7.6–16.7)	7.0–13.0	n.s.
TP (g/dL)	7.1 (6.1–8.9)	7.2 (5.5–9.1)	6.0–8.0	n.s.

Data are median (min–max). A Mann–Whitney test was used to compare differences in CBC data; ref. int., reference interval; n.s., no significant difference; *CBC interpretations that are significantly associated with PRAD.

Table 2.4. Serum Biochemistry Findings for PRAD and P-TCC.

Variable	PRAD (*n* = 29)	P-TCC (*n* = 31)	Ref. Int.	*p*-Value
Anion Gap (mmol/L)	21 (13–36)	21 (16–39) †	12–20	n.s.
Sodium (mmol/L)	146 (138–153)	147 (114–166) †	143–151	n.s.
Potassium (mmol/L)	4.3 (2.8–6.7)	4.4 (3.6–7.4) †	3.6–4.8	n.s.
Chloride (mmol/L)	111 (89–126)	110 (72–129) †	108–116	n.s.
Total CO_2 (mmol/L)	19 (11–29)	20 (10–26) †	20–29	n.s.
Phosphorus (mg/dL)	4.7 (2.4–14.0)	4.8 (2.7–44)	2.6–5.2	n.s.
Calcium (mg/dL)	10.2 (8.3–12.4)	10.2 (2.6–15.2)	9.6–11.2	n.s.
BUN (mg/dL)	19 (6–152)	19 (0.7–186)	11–33	n.s.
Creatinine (mg/dL)	1.1 (0.5–11.1)	0.9 (0.5–44.0)	0.8–1.5	n.s.
Glucose (mg/dL)	103 (1.8–170) ‡	107 (77–158)	86–118	n.s.
Total protein (g/dL)	6.2 (4.9–7.6)	6.3 (4.9–8.0)	5.4–6.9	n.s.
Albumin (g/dL) *	3.2 (1.7–4.0)	3.5 (2.0–4.6)	3.4–4.3	0.03
Globulins (g/dL)	3.1 (2.1–5.2)	2.9 (2.0–4.1)	1.7–3.1	n.s.
ALT (U/L)	42 (19–362) ‡	40 (9–183)	21–72	n.s.
AST (U/L)	31 (15–288) ‡	26 (14–113)	20–49	n.s.
CK (U/L)	199 (76–873) ‡	115 (72–591) †	55–257	n.s.
ALP (U/L)	103 (16–1395) ‡	89 (15–2531)	14–91	n.s.
GGT (U/L)	4 (0–20) ‡	2 (0–20)	0–5	n.s.
Cholesterol (mg/dL)	246 (117–398) ‡	245 (146–351)	139–353	n.s.
Total Bilirubin (mg/dL)	0.1 (0.0–22.1) ‡	0.1 (0.0–126.0)	0.0–0.2	n.s.
Magnesium (mg/dL)	2.1 (1.7–3.2) ‡	2.1 (1.7–2.5) †	1.9–2.5	n.s.

Data are median (min–max). A Mann–Whitney test was used to compare differences in biochemistry data between tumor types; ref. int., reference interval; n.s., no significant difference; * Biochemistry interpretations that are significantly associated with PRAD; † Fewer *n* were evaluated for these analytes in the P-TCC group: Anion Gap, Sodium, Potassium, Chloride, Total CO_2, *n* = 30; Creatinine Kinase, *n* = 19; Magnesium, *n* = 20; ‡ Fewer *n* were evaluated for these analytes in the PRAD group: Glucose, ALT, AST, ALP, CHOL, Total Bilirubin, *n* = 27; CK, *n* = 16; GGT, *n* = 24; Magnesium, *n* = 11.

The RDW to albumin ratio (RAR) was also evaluated, with PRAD having a significantly higher RAR value compared to P-TCC (p = 0.01). An ROC curve of RAR data to differentiate PRAD from P-TCC resulted in an AUC of 0.70 (95% CI: 0.56–0.85); p = 0.01); a cut-off score of >4.00 (sensitivity [Sn] = 60.0% [95% CI: 40.7–76.7%]; specificity [Sp] = 66.7% [95% CI: 48.8–80.8%]; likelihood ratio: 1.8) using the closest to (0,1) criterion; and had a Youden index of 0.27. The highest Youden index (0.35) in the RAR data resulted in a cut-off score of >4.850 (Sn = 48.0% [95% CI: 30.0–66.5%]; Sp = 86.7% [95% CI: 70.3–94.7%]; likelihood ratio: 3.6). In addition, ROC curves of ALB and RDW were also performed to examine their diagnostic utility individually in differentiating PRAD from P-TCC. An ROC curve of ALB data resulted in an AUC of 0.66 (95% CI: 0.53–0.80; p = 0.03), a cut-off score of <3.4 g/dL (Sn = 65.6% [95% CI: 47.4–80.1%]; Sp = 54.8% [95% CI: 37.8–70.8%]; likelihood ratio: 1.5); and a Youden index of 0.20. An ROC curve of RDW resulted in an AUC of 0.67 (95% CI: 0.54–0.81; p = 0.02), a cut-off score of >13.25% (Sn = 59.4% [95% CI: 42.3–74.5%]; Sp = 61.8% [95% CI: 45.0–76.1%]; likelihood ratio: 1.6); and a Youden index of 0.21 (**Figure S2.1**).

There were no significant differences in urinalysis interpretation or quantitative data between the groups, apart from isosthenuria being associated with P-TCC (p = 0.01) (**Tables 2.5 and 2.6**).

Table 2.5. Urinalysis quantitative results for PRAD and P-TCC.

Variable	PRAD (n = 25)	P-TCC (n = 30)	Ref. Int.	p-Value
pH	6.5 (5.0–8.5)	6.5 (5.0–9.0)	5.0–9.0	n.s.
USG	1.027 (1.010–1.049)	1.021 (1.002–1.070)	1.001–1.060	n.s.
Protein (mg/dL)	75 (0–500)	75 (0–500)	NEG	n.s.
Bilirubin (mg/dL)	1 (0–6)	1 (0–3)	NEG	n.s.
Hemoprotein (ery./µL)	250 (0–350)	250 (10–250)	NEG	n.s.
WBC (/HPF)	7 (0–500)	8 (0–100)	0–3	n.s.
RBC (/HPF)	45 (0–250)	39 (1–100)	0–2	n.s.

Data are median (min–max). A Mann–Whitney test was used to compare differences in urinalysis quantitative data between tumor types; ery., erythrocytes; ref. int., reference interval; NEG, negative; n.s., no significant difference. No animals had glucosuria or ketonuria.

Table 2.6. Frequency of urinalysis interpretation and association with PRAD and P-TCC.

Interpretation	PRAD (n = 25)	P-TCC (n = 30)	Ref. Int.	p-Value
Alkaluria	5 (20%)	5 (17%)	pH > 7.5	n.s.
Aciduria	2 (8%)	4 (13%)	pH < 6.0	n.s.

Adequate USG	23 (92%)	21 (70%)	1.014 < USG < 1.030	n.s.
Isosthenuria *	0 (0%)	7 (23%)	USG 1.007–1.013	0.01
Hyposthenuria	2 (8%)	2 (7%)	USG < 1.007	n.s.
Proteinuria	15 (60%)	22 (73%)	>75 mg/dL	n.s.
Bilirubinuria	15 (60%)	15 (50%)	>0 mg/dL	n.s.
Hematuria	22 (88%)	28 (93%)	>2 RBC/HPF	n.s.
Pyuria	14 (56%)	20 (67%)	>3 WBC/HPF	n.s.
Crystalluria	10 (40%)	5 (17%)	NONE	n.s.
Casts	8 (32%)	3 (10%)	NONE	n.s.
Epithelial cells	24 (96%)	28 (93%)	NONE	n.s.
Bacteriuria	5 (20%)	4 (13%)	NONE	n.s.

Data includes the number of animals (percentage of animals) with that urinalysis interpretation; the chi-squared test performed when all observations $n \geq 5$; Fisher's exact test performed when any observation $n < 5$; n.s., no significant difference; ref. int., reference interval. *Urinalysis interpretations that are significantly associated with P-TCC.

Fine-Needle Aspirate Cytology of PRAD and P-TCC

Cytology specimens from PRAD and P-TCC tumors were evaluated for features typically associated with urogenital tumors (e.g., necrosis, vacuolation, etc.) and enumerated for each specimen. Melamed–Wolinska bodies (MWB) ($p = 0.02$) and necrosis ($p = 0.03$) were significantly associated with P-TCC (**Figure 2.1**), while all other features were not significantly associated with either neoplasia (**Table 2.7**).

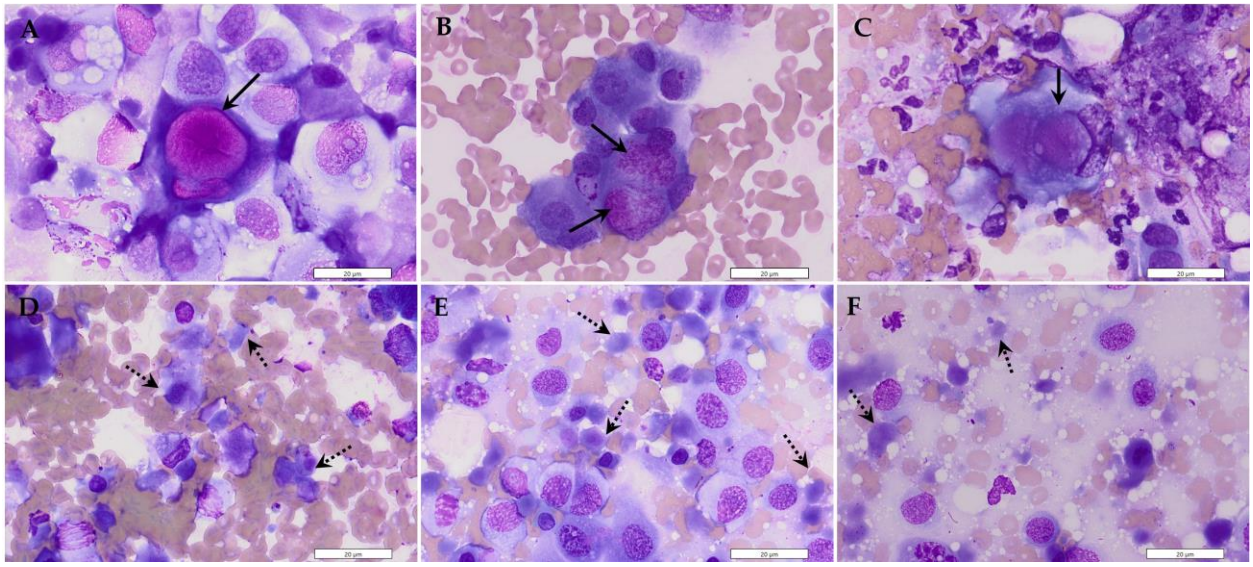

Figure 2.1. Cytologic features of P-TCC. (**A**–**C**) P-TCC was significantly associated with Melamed–Wolinska bodies, large eosinophilic globular inclusions in neoplastic cells (solid arrows). (**D**–**F**) P-TCC was also significantly associated with necrosis, which are grey–blue amorphous cellular remnants with deteriorated nuclei (dashed arrows). Panels (**A**–**F**), 100× oil objective.

Table 2.7. Cytologic features associated with PRAD and P-TCC

Cytologic Feature	PRAD (*n* = 18)	P-TCC (*n* = 22)	*p*-Value
Melamed-Wolinska bodies *	5 (28%)	14 (63%)	0.02
Necrosis *	3 (17%)	11 (50%)	0.03
Inflammation	3 (17%)	6 (27%)	n.s.
Mineralization	1 (6%)	2 (9%)	n.s.
Vacuolation	7 (39%)	6 (27%)	n.s.
Mitotic Figures	2 (11%)	3 (14%)	n.s.
Keratinization	3 (17%)	3 (14%)	n.s.
Hemosiderin	3 (17%)	1 (5%)	n.s.

Data includes the number of animals (percentage of animals) with that feature on their cytology specimen; chi-squared test performed when all observations n ≥ 5; Fisher's exact test performed when any observation n < 5; n.s., no significant difference; p < 0.05. *Cytological features that are significantly associated with P-TCC.

Androgen Receptor Immunohistochemistry and Clinicopathologic Data Results

Sections from 50 PRAD dogs were available for androgen receptor (AR) immunohistochemical staining. Six dogs did not have enough tissue left from their archived samples for sectioning. Seven dogs (14%) had tumors with AR-positive nuclei, 14 dogs (28%) had tumors with AR-positive cytoplasm, and 29 dogs (58%) were negative for AR staining in either location (**Figure 2.2**).

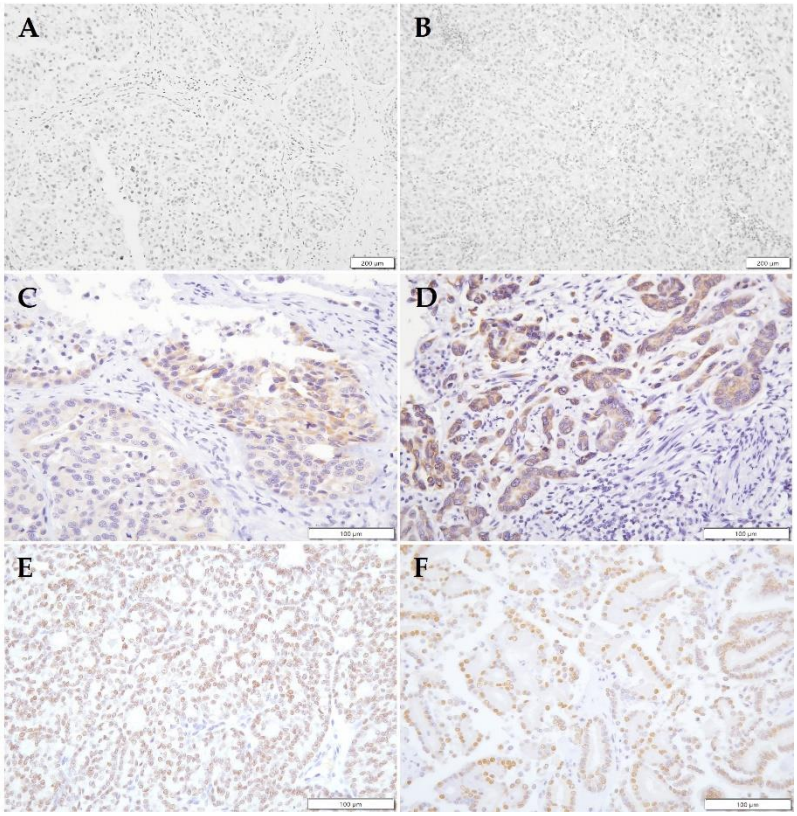

Figure 2.2. AR expression in PRAD tumors. (**A**,**B**) The majority of PRAD is negative for AR, with few tumors displaying AR cytoplasmic staining (**C**,**D**). Fewer cases of PRAD tumors have AR-positive nuclei (**E**,**F**), suggesting dog PRAD tumors do not often utilize canonical AR signaling. Panels (**A**,**B**), 20× objective; panels (**C**–**F**), 40× objective.

Of the 50 dogs with FFPE blocks, 30 had CBC performed, 28 had serum biochemistry performed, and 24 had urinalysis performed. The clinicopathologic data were not significant between AR+ (positive nuclear or cytoplasmic staining) and AR− (negative staining) tumor sections ($p > 0.05$), apart from higher-circulating monocytes ($p = 0.02$) and aspartate transaminase (AST) ($p = 0.01$) values in dogs with AR− tumors (**Tables S2.1–S2.4**), but these were not above the reference interval. The ROC curve of RAR data to differentiate between AR+ from AR− PRAD tumors was not significant ($p > 0.05$) (**Figure S2.2**).

Survival Times in Prostate Cancer

Survival times were not attainable for 15 records (8 PRAD, 7 P-TCC), with four necropsy specimens having no mention of a start date of symptoms in the medical records and 11 biopsy specimens having no mention of death or euthanasia because the patient was lost to follow-up. The majority. Nearly 40% of patients were euthanized less than 24 hours after diagnosis for both groups (**Table S2.5**). Notably, 48 PRAD and 67 P-TCC dogs were included in survival analysis, with a median survival time (MST) of 3 days for PRAD and 17 days for P-TCC. When animals that survived less than 24 hours were excluded and the MST of PRAD was increased to 35 days and the MST of P-TCC increased to 45 days for P-TCC, respectively. There was no significant difference in survival times between the groups ($p > 0.05$) (**Figure S2.3**). Moreover, there were no significant differences in survival times between dogs with AR+ and AR− PRAD tumors, and they featured an overall MST of 2 and 3.5 days, respectively ($p > 0.05$) (**Figure S4**). This increased when animals that survived less than 24 hours were excluded to 18.5 days and 35 days for AR+ and AR−, respectively. Additional information regarding survival times for PRAD and P-TCC is provided in Supplementary **Table S2.5**. Lastly, Cox regression multivariate modeling revealed hypoalbuminemia (hazard ratio [HR] 11.79 [95% CI: 1.99–102.9]; $p = 0.01$) as a risk factor for shorter survival in PRAD, but not increased RDW or RAR. These variables did not affect survival in P-TCC ($p > 0.05$) (**Table S2.6**).

2.4. Discussion

In this study, we found that hypoalbuminemia in serum biochemistry was significantly associated with PRAD, while MWB and necrosis were significantly associated with P-TCC on cytology. In addition to this, we found RDW was significantly increased in PRAD when compared to P-TCC, and that RAR could be used with acceptable diagnostic utility to differentiate PRAD from P-TCC when ratio values were >4.00. These findings have important implications for the use of routine, minimally invasive diagnostic tests to distinguish different subtypes of PCa in dogs in order to guide appropriate treatment and intervention.

Increased RDW, which represents anisocytosis in the erythrocyte population, is normally present in regenerative anemias due to the presence of reticulocytes, or in iron-deficiency anemias due to the presence of microcytes. It has also been observed in inflammatory states in humans where erythrocyte fragmentation, altered erythrocyte morphology, impaired erythrocyte maturation, or extended erythrocyte lifespan may be affected by pro-inflammatory cytokines, leading to increased heterogeneity in erythrocyte volume [18,24,28]. Though still under investigation, this has been supported by other studies showing a significant relationship between RDW and C-reactive protein and leukocyte counts in humans as well as dogs [29–33]. Because local and/or systemic inflammation is commonplace with PCa and other cancers, we suspect this is why the median RDW value was at the higher end of the reference range in dog PCa [34,35]. RDW values were significantly higher in PRAD ($p < 0.05$) than P-TCC, but median values were within the reference interval, thereby limiting the utility of this hematologic parameter in differentiating PRAD from P-TCC.

Additionally, RAR is a novel simple biomarker of inflammation that evaluates RDW and albumin, with high RAR values associated with increased RDW and decreased albumin [25]. This parameter is largely underutilized in veterinary medicine, and our study is one of the first to investigate its utility in dogs. Ultimately, the diagnostic benefit of RAR to discern PRAD from P-TCC is acceptable when the value is >4.00, but a value of >4.850 may be of better diagnostic use with a higher Youden index and specificity, though with a lower sensitivity. Moreover, RAR had a slightly better AUC and Youden index than ALB or RDW alone and may be more useful for differentiation of dog PCa. Taken together, these

data imply that PRAD has a greater effect on hematologic parameters and inflammation than P-TCC, which is supported by the significant hypoalbuminemia in PRAD. However, this assumption could benefit from prospective studies with additional confirmatory diagnostics, such as serum testing for C-reactive protein, interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) levels. Moreover, despite its use for prognostication in survival times for cancer in humans, RAR did not appear to significantly affect predict survival outcomes in PRAD or P-TCC in this study, though hypoalbuminemia significantly affected predicted survival times in PRAD, which has also been demonstrated in human studies [36–38]. Therefore, further studies investigating the utility of RAR to predict survival outcomes are warranted.

Hypoalbuminemia in dogs may be found through increased losses (e.g., protein-losing enteropathy, protein-losing nephropathy, hemorrhage), decreased production (i.e., liver failure, inflammation [negative acute phase protein]), increased sequestration or third-spacing (i.e., protein-rich body cavity effusions), and with a negative energy balance (i.e., catabolism) [39,40]. None of the medical records in this study or laboratory data indicated that protein-losing enteropathy, protein-losing nephropathy, body cavity effusions or liver failure were present in either the PRAD or P-TCC groups. Furthermore, there was no difference in hematuria or proteinuria between the groups. As such, hypo-albuminemia in PRAD suggests that it incites more inflammation than P-TCC, although CBC leukocyte values and cytology findings between the groups do not entirely support the latter. Future studies evaluating inflammatory cytokine levels between these groups may be necessary to support this suspicion.

Cytologic evaluation of prostatic tissue specimens via fine needle aspiration has a strong agreement with histopathologic diagnosis and is a less invasive diagnostic modality in diagnosing prostatic neoplasia [41,42]. In this study, P-TCC was significantly associated with the presence of MWB and necrosis on cytology, but other cytologic features (e.g., inflammation, vacuolation) did not significantly differ between the groups. MWB are intracytoplasmic inclusions found within degenerating benign and malignant urothelial cells, and they appear as large, pink-red, globular material on cytology without the need for special stains [43]. These structures have been predominantly associated with urothelial carcinoma in humans and dogs but are not pathognomonic [44,45]. In regard to histology, MWB usually appear as large clear vacuoles or vacuoles with faint pink stippling, but they can be highlighted with

Period-acid Schiff (PAS) special stain [45,46]. This finding in our study is supported by other accounts of MWB associated with urothelial carcinomas in dogs [47,48]. Necrosis is a non-specific and common finding in neoplasia, but it was more prevalent in P-TCC than PRAD in our study. This may be owing to the confined location of P-TCC arising from the centralized urethra and collecting ducts of the prostate, which may have caused tissue compression and subsequent ischemic necrosis, but additional histopathologic-based studies are needed to further evaluate this observation.

Limitations to our study include those associated with retrospective data collection, including missing data in charts, loss of follow-up, inherent selection bias, and lack of standardization of treatment or diagnostic investigation of patients [49]. Survival times in this study, for instance, may have been shortened compared to other studies in the literature, as the requirement for histopathologic diagnosis may have biased the case selection towards dogs that were euthanized and necropsied at a tertiary referral institution. Moreover, the majority of a large portion of patients were euthanized <24 h. Additionally, because the data was acquired from a tertiary referral hospital and may not represent the breadth of clinicopathologic parameters that patients with P-TCC and PRAD may have. In addition, retrospective laboratory data was not able to be confirmed by follow-up testing or pathologist review (e.g., confirm proteinuria, confirm RBCs per HPF on sediment examination). Furthermore, three different hematology and chemistry analyzers were utilized over the 30y-ear time span of the laboratory data, which may have introduced variability into the values reported. Moreover, the hypoalbuminemic values reported in this study may be considered within reference range at other institutions depending on their laboratory's instrument validation protocols and reference intervals. A limitation of the study was the small sample size used when comparing the laboratory data of AR+ to AR− PRAD patients, as well as the regression analysis of the effect of RAR, RDW and ALB on survival times, which may result in type II statistical errors. Lastly, P-TCC was not explored for AR status and association with blood work parameters, and so remains a viable opportunity for further exploration.

2.5 Conclusions

In summary, hypoalbuminemia was significantly associated with PRAD and decreased survival, while MWB and necrosis were significantly associated with P-TCC on cytology. RAR is modestly helpful

in differentiating PRAD from P-TCC. These clinicopathologic data may help clinicians differentiate between these tumors antemortem to guide appropriate treatment and intervention, especially if novel pathway inhibitors are being explored. Future studies evaluating the AR status of P-TCC and its clinicopathologic associations, as well as the ability of RAR to prognosticate outcomes for other tumor types in dogs, may be of interest to investigators and veterinary oncologists to help promote patient welfare.

2.6 Supplementary Tables and Figures

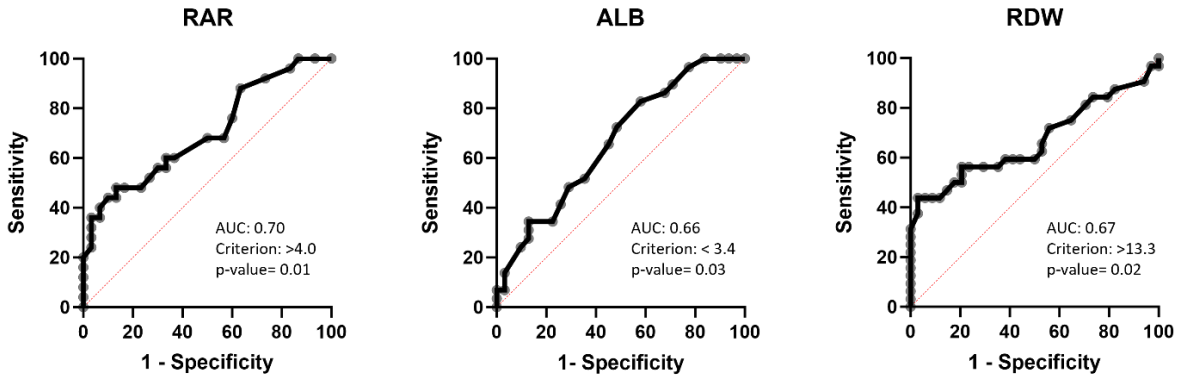

Figure S2.1: RAR is of acceptable diagnostic utility in distinguishing PRAD from P-TCC. Using an RAR cut-off value of >4.0 distinguishes PRAD from P-TCC using the (0,1) criterion, while a cut-off value of >4.850 is suggested when using the highest Youden index (0.35) for the ROC curve.

Variable	AR+ (n = 9)	AR- (n = 21)	Ref. Int.	*p*-Value
RBC ($\times 10^6$/µL)	6.0 (3.8-7.4)	6.1 (3.0-7.6)	5.6-8.0	n.s.
HGB (g/dL)	14.3 (9.6-18.0)	14.2 (6.9-19.5)	14-19	n.s.
HCT (%)	41.8 (25.4-50.7)	41.2 (21.4-52.5)	40-55	n.s.
MCV (fL)	67.7 (61.7-75.6)	69.0 (59.9-75.0)	65-75	n.s.
MCH (pg)	23.7 (21.5-26.5)	23.8 (20.4-28.7)	22-26	n.s.
MCHC (g/dL)	34.7 (32.4-38.2)	34.6 (32.2-38.3)	33-36	n.s.
RDW (%)	12.9 (11.8-19.7)	14.2 (11.30-18.7)	11-14	n.s.
RETIC (/µL)	0 (0-155400)	0 (0-154700)	7000-65000	n.s.
nRBC (/100 WBC)	0 (0-0)	0 (0-3)	RARE	n.s.
WBC (/µL)	10900 (6610-22230)	16500 (7970-34772)	6000-13000	n.s.
Neutrophils (/µL)	9156 (4158-18509)	12098 (5252-31295)	3000-10500	n.s.
Immature Neutrophils (/µL)	0 (0-446)	0 (0-2576)	RARE	n.s.
Lymphocytes (/µL)	1109 (503-2230)	975 (409-7523)	1000-40000	n.s.
Monocytes (/µL)	637 (258-1247)	1082 (409-3492)	150-1200	0.01
Eosinophils (/µL)	218 (101-483)	231 (0-959)	0-1500	n.s.
Basophils (/µL)	1 (0-101)	5 (0-898)	0-50	n.s.
PLT ($\times 10^3$/µL)	266 (163-546)	272 (19-717)	150-400	n.s.
MPV (fL)	10 (8.2-13.0)	9.9 (7.0-17.6)	7.0-13.0	n.s.
TP (g/dL)	6.7 (6.2-8.9)	7.1 (6.1-8.3)	6.0-8.0	n.s.

Table S2.1: Complete blood cell count findings for AR+ and AR- PRAD tumors. There were no significant differences in CBC parameters between the groups apart from slightly higher circulating monocytes in AR- group (p = 0.02).

Variable	AR+ (n = 8)	AR- (n = 20)	Ref. Int.	*p*-Value
Anion Gap (mmol/L)	21 (13-36)	20 (13-34)	12–20	n.s.
Sodium (mmol/L)	146 (142-152)	146 (138-153)	143–151	n.s.
Potassium (mmol/L)	4.2 (3.8-6.3)	4.4 (2.8-6.7)	3.6–4.8	n.s.
Chloride (mmol/L)	111 (103-115)	112 (89-126)	108–116	n.s.
Total CO_2 (mmol/L)	19 (11-22)	19 (11-29)	20–29	n.s.
Phosphorus (mg/dL)	6.3 (5.7-12.8)	4.5 (2.4-14.0)	2.6–5.2	n.s.
Calcium (mg/dL)	9.9 (8.3-12.4)	10.3 (8.5-11.6)	9.6–11.2	n.s.
BUN (mg/dL)	9 (6-152)	22 (6-93)	11–33	n.s.
Creatinine (mg/dL)	0.9 (0.5-11.1)	1.1 (0.5-4.6)	0.8–1.5	n.s.
Glucose (mg/dL)	104.5 (86-131)	103 (18-170)	86–118	n.s.
Total protein (g/dL)	5.95 (4.9-7.6)	6.3 (5.5-7.5)	5.4–6.9	n.s.
Albumin (g/dL)	3.1 (1.8-3.9)	3.3 (1.7-4.0)	3.4–4.3	n.s.
Globulins (g/dL)	3.4 (2.1-4.4)	3.0 (2.2-5.2)	1.7–3.1	n.s.
ALT (U/L)	41 (19-62)	43 (21-362)	21–72	n.s.
AST (U/L)	24 (15-33)	38 (19-288)	20–49	0.01
CK (U/L)	137 (76-152)	293 (119-873)	55–257	n.s.
ALP (U/L)	83 (16-365)	112 (17-1395)	14–91	n.s.
GGT (U/L)	4 (0-9)	5 (0-20)	0–5	n.s.
Cholesterol (mg/dL)	254 (163-336)	246 (117-398)	139–353	n.s.
Total Bilirubin (mg/dL)	0.2 (0.0-0.3)	0.1 (0.0-22.1)	0.0–0.2	n.s.
Magnesium (mg/dL)	2.7 (2.1-3.2)	2.1 (1.7-2.3)	1.9–2.5	n.s.

Table S2.2: Serum biochemistry findings for AR+ and AR- PRAD tumors. There were no significant differences in biochemistry parameters between the groups apart from a higher AST in AR- group ($p = 0.01$).

Variable	AR+ (n = 7)	AR- (n = 17)	Ref. Int.	*p*-Value
pH	6.0 (5.0-8.5)	7.0 (5.0-8.5)	5.0-9.0	n.s.
USG	1.020 (1.010-1.041)	1.027 (1.011-1.049)	1.001-1.060	n.s.
Protein (mg/dL)	25 (0-500)	150 (0-500)	NEG	n.s.
Bilirubin (mg/dL)	1 (0-1)	1 (0-6)	NEG	n.s.
Hemoprotein (ery./µL)	250 (150-250)	250 (0-350)	NEG	n.s.
WBC (/HPF)	5 (1-100)	7 (0-500)	0-3	n.s.
RBC (/HPF)	38 (10-100)	70 (0-250)	0-2	n.s.

Table S2.3: Quantitative urinalysis findings for AR+ and AR- PRAD tumors. There were no significant differences in urinalysis quantitative parameters between the groups.

Interpretation	AR+ (n = 7)	AR- (n = 17)	Ref. Int.	*p*-Value
Alkaluria	2 (29%)	3 (18%)	pH >7.5	n.s.
Aciduria	1 (14%)	1 (6%)	pH <6.0	n.s.
Adequate USG	6 (86%)	16 (94%)	1.014 < USG <1.030	n.s.
Isosthenuria	1 (14%)	1 (6%)	USG 1.007-1.013	n.s.
Hyposthenuria	0 (0%)	0 (0%)	USG <1.007	n.s.
Proteinuria	3 (43%)	11 (65%)	>75 mg/dL	n.s.
Bilirubinuria	5 (71%)	9 (53%)	>0 mg/dL	n.s.
Hematuria	7 (100%)	13 (76%)	>2 RBC/HPF	n.s.
Pyuria	4 (57%)	10 (59%)	>3 WBC/HPF	n.s.
Crystalluria	1 (14%)	9 (53%)	NONE	n.s.
Casts	2 (29%)	6 (35%)	NONE	n.s.
Epithelial cells	4 (57%)	9 (53%)	NONE	n.s.
Bacteriuria	2 (29%)	2 (12%)	NONE	n.s.

Table S2.4: Urinalysis interpretations and associations for AR+ and AR- PRAD tumors. There were no significant associations between urinalysis interpretation and AR status of PRAD tumors.

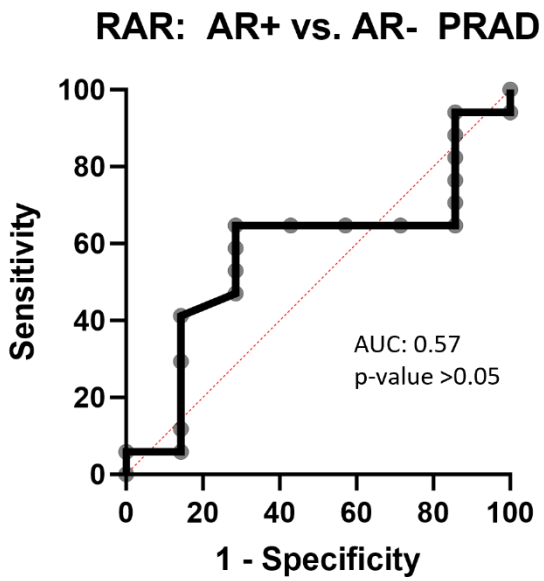

Figure S2.2: RAR is not significant in distinguishing AR+ from AR- PRAD.

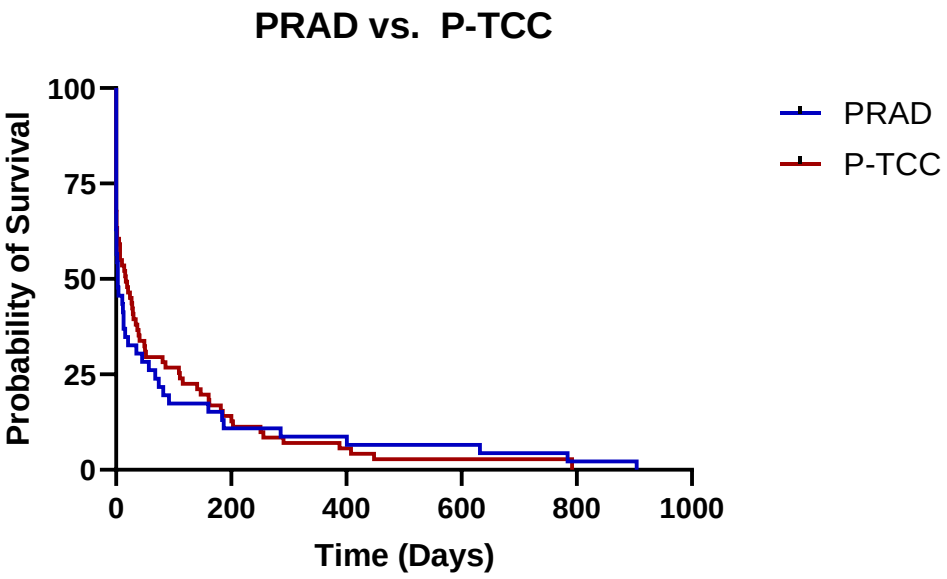

Figure S2.3: Survival times do not significantly differ between PRAD and P-TCC.

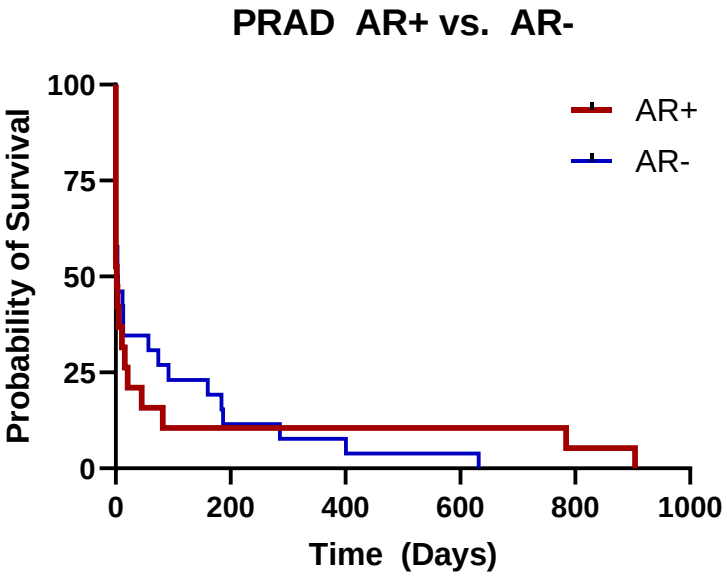

Figure S2.4: Survival times do not significantly differ between AR+ and AR- PRAD tumors. Overall MST for AR+ tumors was 2 days, while AR- tumors was 3.5 days.

Survival Time	PRAD (n = 48)	P-TCC (n = 67)
0 Days	18/48 (38%)	25/67 (37%)
1-30 Days	15/48 (31%)	15/67 (22%)
31-60 Days	3/48 (6%)	7/67 (10%)
61-90 Days	3/48 (6%)	2/67 (3%)
91-180 Days	3/48 (6%)	7/67 (10%)
181-365 Days	3/48 (6%)	7/67 (10%)
>1 Year	2/48 (4%)	3/67 (5%)
>2 Years	1/48 (2%)	1/67 (2%)
>3 Years	0/48 (0%)	0/67 (0%)

Table S2.5: Ranges of survival times in PRAD and P-TCC dogs. Many animals were euthanized the same day as diagnosis.

PCa Type	Variable	Hazard Ratio (95% CI)	*p*-Value
PRAD	RAR (High, >4.11)	0.49 (0.06-2.95)	0.46
	RDW (High, >14)	0.714 (0.11-5.76)	0.73
	ALB (Low, < 3.4)	11.79 (1.99-102.93)	0.01
P-TCC	RAR (High, >4.11)	1.00 (Undetermined)	>0.99
	RDW (High, >14)	7.9×10^{-13} (Undetermined)	>0.99
	ALB (Low, < 3.4)	1.00 (Undetermined)	>0.99

Table S2.6: Multivariate survival analysis of 13 PRAD and 20 P-TCC dogs surviving >1 day.

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CHAPTER 3: ANDROGEN RECEPTOR SIGNALING RESTORATION IN CANINE PROSTATE CANCER CELL LINES ATTENUATES AGGRESSIVE BEHAVIOR

Abstract: Revival of androgen receptor (AR) signaling in human prostate cancer (PCa) has been shown to abrogate aggressive behaviors and may be a novel gene therapeutic. Dogs are a naturally occurring animal model for human PCa, but AR revival has been unexplored in dog PCa. Three canine PCa cell lines (1508, Leo, 1258) were transfected with canine wild-type AR and treated with dihydrotestosterone (DHT). Downstream assays measuring changes in proliferation, metabolism, migration, and invasion were then performed and compared to controls. All cell lines were successfully transfected with AR. When AR signaling was restored, proliferation decreased in 1508 and Leo (p = 0.001; p = 0.04), but not 1258. Metabolism decreased in 1258 (p = 0.04) but was unchanged in 1508 and Leo. Migration decreased in 1508 (p = 0.006) but was unchanged in Leo and 1258. Invasion decreased in 1508 (p = 0.01), was unchanged in Leo, and increased in 1258 (p = 0.006). Overall, 1508 had the majority of aggressive behaviors attenuated and is most similar to androgen-dependent PCa, while Leo remains AR-indifferent, and 1258 increases aggressive behaviors similar to castration-resistant PCa. PCa is a heterogeneous disease in dogs and AR-abrogation of aggressive behavior is cell line-dependent.

3.1 Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer in men and ranks second in cancer-related deaths worldwide [1]. Despite its prevalence, PCa has very few animal models that adequately recapitulate the breadth of the disease, which is required for the preclinical testing and approval of novel therapies [2]. Dogs (i.e., *Canis lupus familiaris*; “canines”) are one of the few species that spontaneously develops PCa, but much remains unknown about the signaling cascades involved in their disease initiation and progression [3,4]. Moreover, little is known about the hallmark androgen receptor (AR) signaling pathway in dogs, the principal pathway targeted by therapeutics in PCa of men and is important to explore when investigating the use of dogs as an animal model for novel therapeutics [5–7].

PCa initially progresses through the canonical genomic androgen receptor (AR) signaling cascade, where AR remains sequestered by heat shock proteins (HSP) in the cytosol of cells until it meets its cognate

androgen ligand (e.g., testosterone or dihydro-testosterone [DHT]) before dimerizing and moving to the nucleus to act as a transcription factor. Here, AR promotes expression of target genes by binding to their localization sequences called androgen-responsive elements (ARE) and drives proliferation and differentiation. Furthermore, the actions of AR can be modulated by co-activators and co-repressors that serve to enhance or dampen target gene expression (**Figure 3.1**) [8,9]. This cascade is initially suppressed in PCa with androgen-deprivation therapies (ADT) or chemical castration, which blocks androgen production or androgen binding to AR, and tumors that respond to this therapy are called androgen-sensitive or hormone-sensitive PCa [10].

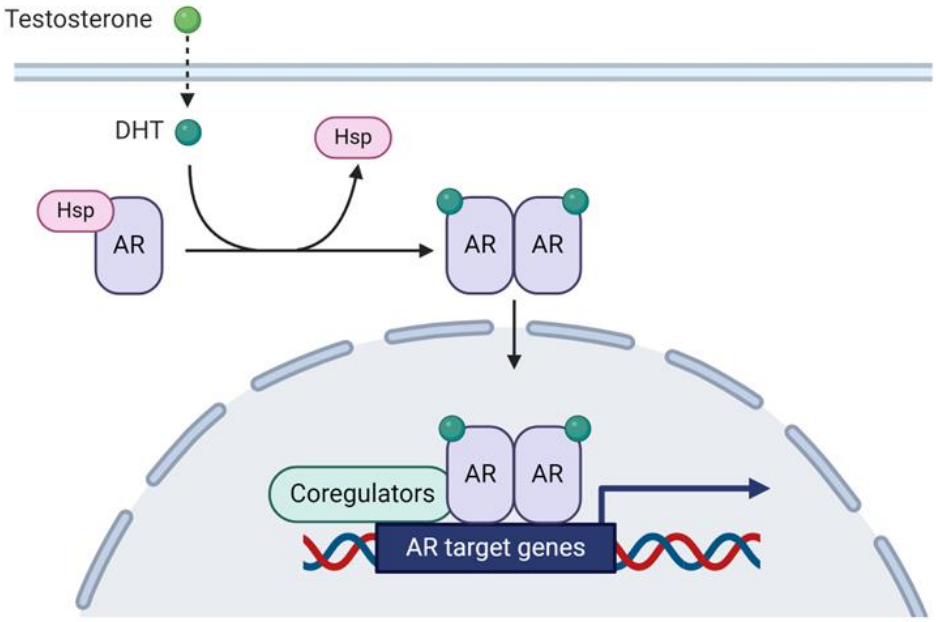

Figure 3.1. Canonical Genomic Androgen Receptor Signaling. Androgen receptor (AR) remains cytosolically bound by heat shock proteins (HSP) in the absence of androgens. Once bound to an androgen ligand, such as testosterone or dihydrotestosterone (DHT), AR dimerizes and localizes to the nucleus where it acts as a transcription factor where it promotes cell growth and differentiation. Co-regulators of AR (co-activators or co-repressors) can enhance or dampen the actions of AR on gene expression.

Upon prolonged treatment with ADT, a subset of PCa becomes resistant to this treatment (castration-resistant prostate cancer [CRPC]); however, AR signaling continues irrespective of non-response to ADT by various means, including gain-of-function mutations in AR, copy number variations of AR, mutations in co-repressors or co-activators of AR, and others [11]. Treatment for CRPC includes the chemotherapeutic agent docetaxel and androgen-receptor signaling inhibitors (ARSI) which include the

androgen synthesis inhibitor abiraterone acetate and the AR inhibitors enzalutamide, apalutamide and darolutamide [7]. Following prolonged treatment with ARSIs, a smaller subset of patients with CRPC go on to develop other driver mutations and no longer rely on AR signaling, a subcategory of highly lethal PCa variants termed androgen-indifferent prostate cancer (AIPC) where effective treatments are lacking [12]. Since the discovery and mainstream use of ARSI therapies, researchers have investigated the possibility that AR targeting has led to more aggressive, untreatable disease in a subset of patients and that targeting of other pathways and even the reconstitution of AR signaling may be worth exploring [13,14].

Although AR is known to promote PCa growth, it also has tumor suppressor properties and has been shown to target genes that decrease DNA replication, repair, and synthesis [15]. Because of this, researchers have explored whether the revival of AR signaling in AR-indifferent or AR null human PCa cell lines would abrogate aggressive behavior which may have broader implications for gene therapies in men with refractory disease [14,16,17]. Because dogs often have AR null PCa, they may serve as a suitable model for this potential gene therapy. The aims of this study were to evaluate if AR reconstitution in AR null canine PCa cell lines would abrogate aggressive behaviors in order to show that canine PCa utilizing this pathway in a similar way and may serve as a novel animal model for potential gene therapy in PCa. Because PCa is a highly heterogeneous disease in humans as well as dogs, we hypothesize that AR revival will have varying effects on canine PCa that are cell line-dependent.

3.2 Materials and Methods

Cell Lines and Cell Culture Materials

Canine prostate cancer cell lines 1508 and 1258 were generated by co-authors (E.M.P. and H.M.E.) from the University of Veterinary Medicine Hannover (TiHo), Hannover, Germany; canine prostate cancer cell line Leo was purchased from Applied Biological Materials, Inc. (Cat. No. T8278). All cell lines used were canine prostatic adenocarcinomas. All cells tested negative for *Mycoplasma* bacterial contamination. Cell lines were cultured in Roswell Park Memorial Institute (RPMI) cell culture medium 1640 (Invitrogen/Gibco, Carlsbad, CA, USA) and supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Omega Scientific, Inc., Tarzana, CA) and 100 U/mL penicillin-100 µg/mL streptomycin (Invitrogen/Gibco, Carlsbad, CA). Treated cells had the aforementioned cell culture media supplemented

with physiologic levels (1 nM) of dihydrotestosterone (DHT) (Cat. No. 521-18-6; Sigma Aldrich). Cells were kept at 37 °C in a humidified environment of 5% CO2 in air.

Androgen Receptor Plasmid Construction, Transfection, and Sequence Alignment

DNA sequence encoding full-length canine androgen receptor from reference genome Dog10K_Boxer_Tasha was obtained from Ensembl database (ENSCAFG00000016656.4; NCBI gene 403588) then synthesized and cloned between restriction sites BamH1 and Not1 in a pcDNA3.1(+)-C-HA vector (*nom. nov.* pcDNA3.1-AR_{can}) (GenScript USA Inc., Piscataway, NJ) [37]. Cell lines were transiently transfected for 8 hours with the AR-containing plasmid using jetPrime® DNA transfection reagent (Polyplus) according to the manufacturer's instructions. Mock transfection was performed with an empty pcDNA3.1(+) vector (Cat. No. V79020; Thermo Fisher Scientific, Inc.). Groups transfected with AR plasmid and then treated with 1nM DHT for 24 hours were considered the treatment group (+AR+DHT); groups transfected with the AR plasmid without DHT treatment were considered the plasmid group (+AR); and lastly, groups mock transfected with an empty vector were considered the control group (Ctrl).

Protein sequence alignment was performed using open access sequence alignment tool (EMBL-EBI, EMBOSS Water Pairwise Sequence Alignment) [38] pairing wild-type full-length 907 amino acid canine androgen receptor (ENSCAFG00000016656.4; NCBI gene 403588) to canonical wild-type full-length 920 amino acid human androgen receptor (ENSG00000169083; NCBI gene 9606).

Cell Lysate and Protein Immunoblotting

Protein was extracted from cells grown for 3 days in RPMI-supplemented media using 2X loading buffer (100 mM Tris-Cl pH 6.8; 4% (w/v) SDS; 0.2% (w/v) bromophenol blue; 20% (v/v) glycerol; 200 mM β-mercaptoethanol) [39]. Protein was quantitated by BCA assay (Pierce™ BCA Protein Assay Kit; Cat. No. 23225; Thermo Fisher Scientific, Inc.) and separated on 10% SDS-PAGE gels at 130V for 1 hour using minivertical electrophoresis cells (Mini-PROTEAN 3 Electrophoresis Cell, Bio-Rad, Hercules, CA). Protein was transferred to 0.2 μM nitrocellulose membranes with the Trans-Blot Turbo transfer system (Bio-Rad, Hercules, CA) for 30 minutes and then blocked with 5% nonfat dry milk in phosphate-buffered saline and 0.1% Tween 20 (PBST) for 1 hour. Membranes were cut prior to incubation with primary

antibody overnight at 4C (uncut membranes provided in the supplementary materials). The following antibodies were used: AR (N-20; 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA); lamin A/C (Cat. No. 2032; 1:1000; Cell Signaling Technology, Danvers, MA). The next day the membranes were washed with phosphate-buffered saline 0.1% Tween 20 (PBST) three times for 10 minutes each and then incubated with a secondary antibody conjugated to horseradish peroxidase (HRP) for 2 hours. Development was performed using chemiluminescence (Pierce™ ECL Western Blotting Substrate; Cat. No. 32106; Thermo Fisher Scientific, Inc.) and was imaged using GE Amersham™ Imager 680 (GE Healthcare Life Sciences, Chicago, IL). Gel loading was assessed by housekeeping protein lamin A/C (Cat. No. 2032; 1:1000; Cell Signaling Technology, Danvers, MA).

Differential Gene Expression (RT-qPCR)

Total cellular RNA was prepared using RNeasy kit (Cat. No. 47104; QIAGEN, Inc.). cDNA was synthesized from 1 mg RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA) from three biological replicates. Real-time PCR was performed in triplicate using PowerUp™ SYBR™ Green Master Mix (Cat. No. A25741; Thermo Fisher Scientific). All aforementioned steps were performed according to the manufacturer's instructions. HPRT1 was used as the endogenous expression standard. Data were collected on an Applied Biosystems 7500 Fast machine and analyzed using the relative standard curve method. Differential expression of various genes was compared between the control (Ctrl) and the transfected groups (+AR and +AR+DHT) of each cell line. Primers for each gene evaluated are provided in **Table 3.1**.

Table 3.1. Primers used in this study for target gene amplification.

Gene	Sequence (5'—3')
AR[a]	F: CGCCCCTGACCTGGTTT R: GGCTGTACATCCGGGACTTG
NKX3.1[a]	F: TGAGGTGGTTGGAGGTTTGC R: TTTCATTGGCCCATCACTGA
FOLH1[b]	F: GTGTTTGGTGGCATTGACC R: TTCTGCATCCCAGCTTGC
Vimentin[c]	F: TACGCCAGCAATATGAAAGCG R: AGGGCATCATTGTTCCGGTTA
N-cadherin[c]	F: AGCACCCTCCTCAGTCAACG R: TGTCAACATGGTCCCAGCA
SNAIL1[d]	F: ACTGCAGCCGTGCCTTTG R: AAGGTTCGGGAACAGGTCTTG
HPRT1[a]	F: AGCTTGCTGGTGAAAAGGAC R: TTATAGTCAAGGGCATATCC

Primers are from the following publications: a, Calderon, et al. [40]; b, Lai, et al. [5]; c, Yu, et al. [41]; d, Sammarco, et al [42].

Immunofluorescence

Transfected cell lines were seeded at 10,000 cells per coverslip and were incubated for 24 hours in media in a 37°C CO2 incubator. Cells were then treated with vehicle or DHT for 24 hours. After, cells were rinsed with PBST and then fixed to the coverslip with ice-cold methanol for 10 minutes on ice. Coverslips were then washed three times with PBST and then blocked with 10% goat serum for one hour at room temperature. Primary antibody (AR) was diluted 1:100 in 10% goat serum, applied to the coverslips and then incubated at 4C overnight in a humidity chamber. The next day, coverslips were washed three times with PBST and had anti-rabbit secondary antibody conjugated to rhodamine (1:500 in PBST; Life Technologies, Carlsbad, CA) applied. Coverslips were then incubated in secondary antibody for 1 hour at room temperature in the dark. After, coverslips were washed three times with cold PBST, and coverslips were inverted and mounted onto uncharged glass slides with antifade mounting medium plus DAPI (Life Technologies, Carlsbad, CA).

MTT Assay

Cells were grown in triplicate in 24 well plates at 50,000 cells per well and transfected and treated as abovementioned. Following treatment, each well was incubated with 25 µL of 3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium bromide (MTT; 5 mg/ml) (Sigma Aldrich) for 1 hour in an incubator (37°C, 5% CO2). Next, 500 µL of DMSO was added to each well and rocked at RT for 20 minutes. Then, 100 µL of each well was pipetted into its own well in a 96-well plate and inserted into the plate reader (BioTek Cytation 5). The optical density (OD; 590 nm) was compared between control (Ctrl) and the transfected groups (+AR and +AR+DHT) of each cell line.

Clonogenic Assay

Clonogenic assays were prepared as previously described [43]. In short, cells were transfected and treated as abovementioned then plated in triplicate in a 6-well plate at 1,000 cells per well. Media or media supplemented with DHT was refreshed every 48 hours and all cells were allowed to grow to 14 days. Colonies were fixed and stained with 0.5% crystal violet. Total colony area (µm2) per well was measured to combat the tendency of some cell lines to make few large colonies versus others that make many smaller colonies. Colonies were measured and imaged with BioTek Cytation 5 cell imaging multimode plate reader (Agilent) and the average area of 50 cells was calculated and set as a minimum threshold of detection. The total colony area was then compared between the control (Ctrl) and the transfected groups (+AR and +AR+DHT) of each cell line.

Flow Cytometry for Apoptosis

Cells were grown in 12-well plates at 100,000 cells per well in triplicate and transfected and treated as abovementioned. Cells were conjugated to Annexin V and propidium iodide per manufacturer's instructions (FITC Annexin V/Dead Cell Apoptosis Kit; Cat. No. V13242; Thermo Fisher Scientific, Inc.). Flow cytometry was then performed on FACSAria (Becton Dickinson Immunocytometry Systems, San Jose, CA) for cell lines 1508 and Leo and FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) for cell line 1258. Cells were illuminated with 200 mW of 488 nm light or 635 nm light. Fluorescence was detected through a 630/22nm (for PI) or 661/16 nm (for Annexin V-Alexa Fluor 647)

band-pass filter. Frequency histograms were collected from 20,000 events and analyzed in FlowJo software version 10.8.1 (TreeStar, FlowJo LLC., Ashland, OR).

Migration Assay

Migration assays were performed as previously described [44]. In brief, cells were grown in 6-well plates at 150,000 cells per well in triplicate and transfected and treated as abovementioned. Wells were at 100% confluency after the 24-hour DHT or vehicle treatment. The monolayer was then linearly scratched with a p200 pipette tip. Wells were then washed with PBS and then media or media supplemented with DHT was added to the well. Time-lapse microscopy was used to acquire images every hour from the same field automatically over 24 hours by a multimode plate reader (37°C, 5% CO2; BioTek Cytation 5; Agilent). A masking algorithm was used to determine the wound confluency at baseline and at 20 hours relative to the original scratch wound's diameter to combat variability in scratch wound diameters between replicates and cell lines.

Invasion Assay

Boyden chamber invasion assay was performed as previously described [45]. Briefly, cells were grown in a 24-well plate in triplicate at 50,000 cells per well and transfected and treated as aforementioned. Then, cells were serum-starved for an additional 24 hours. Transwell chambers (Cat. No. PTEP24H48; 0.8 µM pore size; Millicell® 24-well hanging cell culture inserts; Millipore-Sigma) were placed in a 24-well plate on ice, coated with 20 µL of Matrigel (2mg/mL) with a p200 pipette tip, then incubated for 1 hour (37°C, 5% CO2). After, 100,000 cells from treatment and control groups were seeded in the upper chamber of the transwell in serum-starved media, while the lower chamber contained complete media. Cells were then allowed to migrate for 24 hours. Inserts were decanted and the transwell was immersed in 4% paraformaldehyde (PFA) for 10 minutes at room temperature (RT). The excess PFA was then decanted and the transwells were then immersed in methanol for 10 minutes at RT. After, inserts were gently washed with PBS and then immersed in 0.5% crystal violet for 30 minutes at RT. Inserts were then gently washed with water and the upper side of the transwell membrane was gently brushed with a cotton swab. Filters were allowed to dry overnight and then the underside of the filter was imaged. Five

fields at low magnification were imaged and cells were then quantified and averaged single-blindly by a veterinary pathologist (D.M.V.). The average number of cells that invaded were then compared between the control (Ctrl) and the transfected groups (+AR and +AR+DHT) of each cell line.

Statistical Analysis

Data was analyzed in GraphPad Prism version 10.1.0. Normality was determined by Shapiro-Wilk. Differential gene expression was evaluated by Student's t-test or ANOVA with Dunnett's multiple comparison test. Clonogenic formation area, MTT assay OD, migration assay end wound confluence, and invasion assay cellularity were compared between control and transfected groups with ANOVA with Dunnett's multiple comparison test. A p-value of < 0.05 was considered significant.

3.3 Results

Androgen Receptor Gene is Highly Conserved between Canines and Humans

Gene alignment between canines and humans resulted in 89.7% gene homology overall. There was 100% sequence conservation of the N-terminal domain (NTD) motif 23FQNLF27, which is required for binding the NTD to the activation function 2 (AF2) region of the ligand binding domain (LBD) as well as co-activators and allowing dimerization of AR [18]. There was 100% sequence conservation of NTD motif ^{437}WHTLF^{450}, which stabilizes ligand binding to AR. Moreover, there was 100% sequence conservation of the hinge region and DNA-binding domain (DBD), which interacts with androgen receptor elements (ARE) of target genes [8]. The DBD also contains the nuclear localization sequence (NLS; ^{629}RKLKKL^{634}), which had 100% sequence conservation between the species. In addition, there was also conservation of a coactivator binding site in the C-terminal domain ("LxxLL" motifs) [19]. The NTD glutamine repeats (amino acid abbreviation Q; DNA codon "CAG" or "CAA") were less homologous between the species and in slightly different NTD locations, which is a finding between different ethnicities of humans (**Figure 3.2**; **Figure S3.1**) [20].

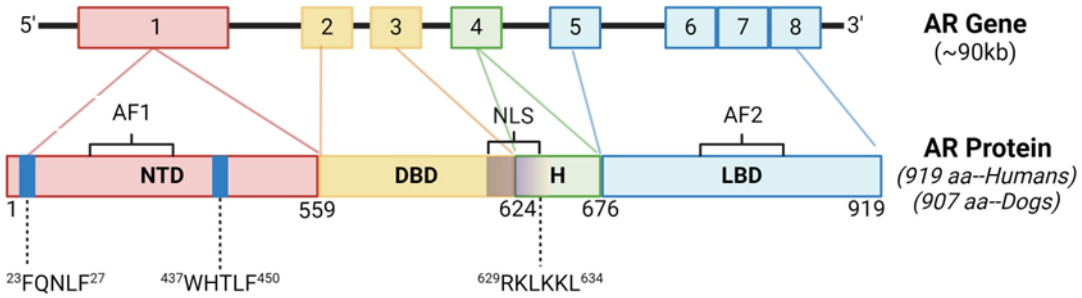

Figure 3.2. Androgen Receptor Gene Structure and Homology Between Canines and Humans. Androgen receptor (AR) is comprised of 8 exons. The N-terminal domain (NTD) contains sequences imperative for C-terminal binding during conformational changes in AR after ligand binding, as well as ligand stabilization, and are 100% conserved between the species. The DNA binding domain (DBD) and nuclear localization sequence (NLS) are also 100% conserved between the species. AF1, activation function 1; AF2, activation function 2; H, hinge region; LBD, ligand binding domain.

Androgen Receptor Transfection and Treatment with DHT results in Nuclear Localization and Expression of Downstream Target Genes in Canines

To examine the similarity of AR signaling in dogs compared to humans we transfected three AR null canine PCa cell lines (1508, Leo and 1258) with a pcDNA3.1(+) plasmid containing wild-type canine AR (pcDNA3.1-AR_{can}). Then, the pcDNA3.1-AR_{can} transfected experimental groups were either left untreated (+AR) or treated with 1nM DHT (+AR+DHT) while control groups were mock transfected. All three canine PCa cell lines showed successful protein expression of AR (**Figure 3.3A**) when compared to cell line-matched controls. Moreover, all three canine PCa cell lines showed successful translocation of AR to the nucleus with DHT treatment (**Figure 3.3B-D**, *right* column, *bottom image*) and not in plasmid-transfected only groups treated with a vehicle (**Figure 3.3B-D**, *right* column, *top image*). DHT treatment did not affect AR levels significantly in any cell line (**Figure 3.3E**). AR target gene *NKX3.1* was upregulated in cell lines 1508 ($p = 0.001$) and Leo ($p = 0.02$), but overall downregulated in cell line 1258 ($p > 0.05$) (**Figure 3.3F**). *FOLH1* (folate hydrolase 1), the canine gene analogous to human prostatic specific membrane antigen (PSMA), was unchanged with AR restoration in 1508 and 1258 ($p > 0.05$) but decreased when treated with DHT in cell line Leo ($p < 0.01$) (**Figure 3.3G**). Thus, AR transfection enabled its transcriptional activity as well.

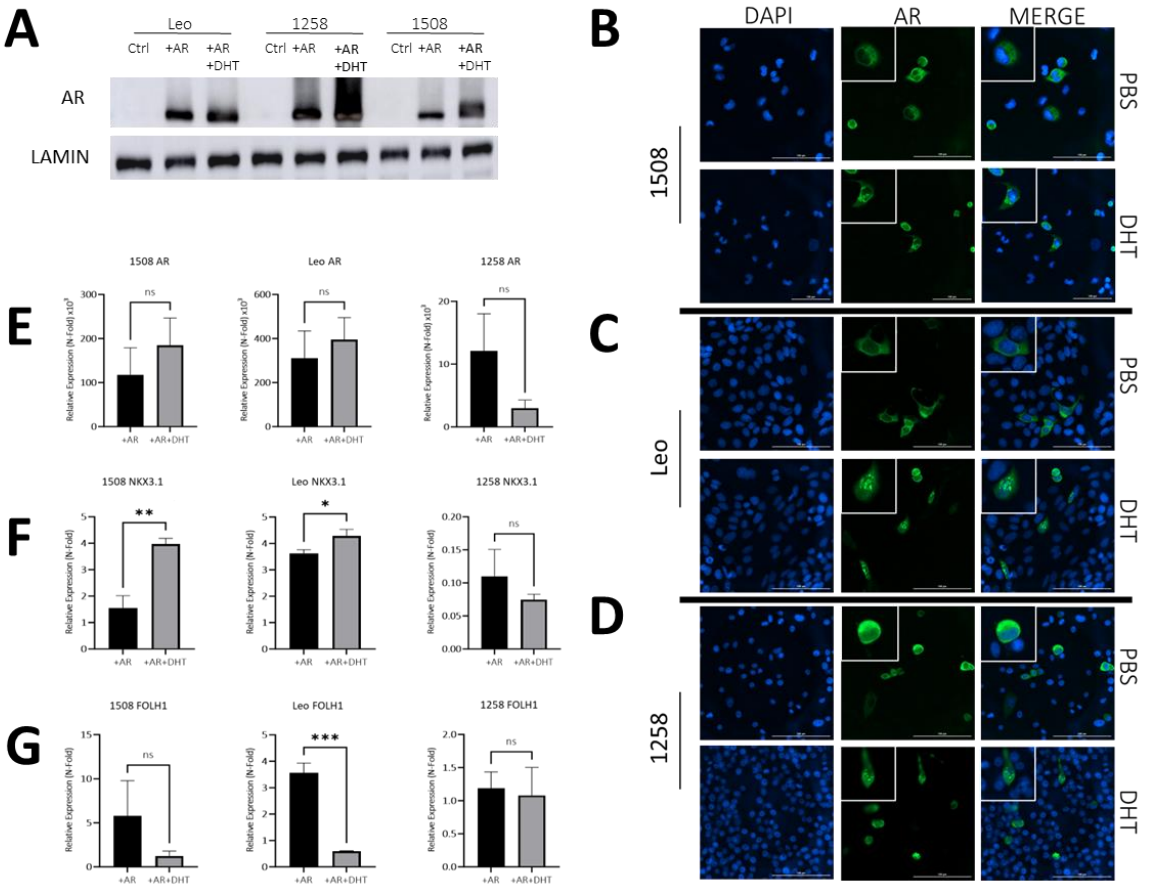

Figure 3.3. AR signaling restoration alters expression of downstream AR targets. (**A**) Protein immunoblot confirmed successful transfection and protein expression of canine AR. (**B-D**) AR transfection and DHT treatment leads to AR translocation to the nucleus, while without DHT treatment AR remains cytosolically sequestered. (**E**) qPCR shows successful AR gene overexpression after transfection in experimental groups relative to control groups. (**F**) NKX3.1 is a downstream target of AR and was successfully upregulated via qPCR in cell lines 1508 ($p = 0.001$) and Leo ($p = 0.01$) but appears absent in cell line 1258. (**G**) FOLH1 is downregulated in the presence of AR, which occurred in cell line Leo ($p = 0.0002$) via qPCR but not in cell lines 1508 or 1258 ($p > 0.05$).

AR Signaling Restoration Decreases Proliferation in Certain Canine PCa Cell Lines and Decreases Metabolic Activity in Others

Clonogenic assays were performed to determine whether AR restoration abrogates proliferation in canine PCa lines as it has been shown to do in human PCa cell lines [14,15]. Restoration of AR signaling attenuated growth in cell lines 1508 (**Figure 3.4A**; $p = 0.03$) and Leo (**Figure 3.4B**; $p = 0.04$) but did not have an effect on proliferation in cell line 1258 (**Figure 3.4C**). Notably, 1258 had visibly increased proliferation with DHT treatment, though not significantly ($p > 0.05$). The effect of AR restoration on metabolism was investigated with MTT assays for all cell lines and all control and experimental groups.

There was no significant difference in metabolism between any groups in cell lines 1508 (**Figure 3.4D**) and Leo (**Figure 3.4E**) ($p > 0.05$), but there was a decrease in metabolic activity in the treated group of cell line 1258 compared to controls (**Figure 3.4F**; $p = 0.04$). Flow cytometric assays for apoptosis and cell death were performed on all experimental groups for all three cell lines to determine whether the decrease in proliferation was due to transfection reagents or DHT treatment. There was no apoptosis detected in any of the control or experimental groups for any cell line (**Figures 3.4G-I**, quadrant 3 [Q3]). Though some cell death occurred in all groups, there was no substantial difference between the control groups and the experimental groups (**Figures 3.4G-I**, quadrant 1 [Q1]; $p > 0.05$).

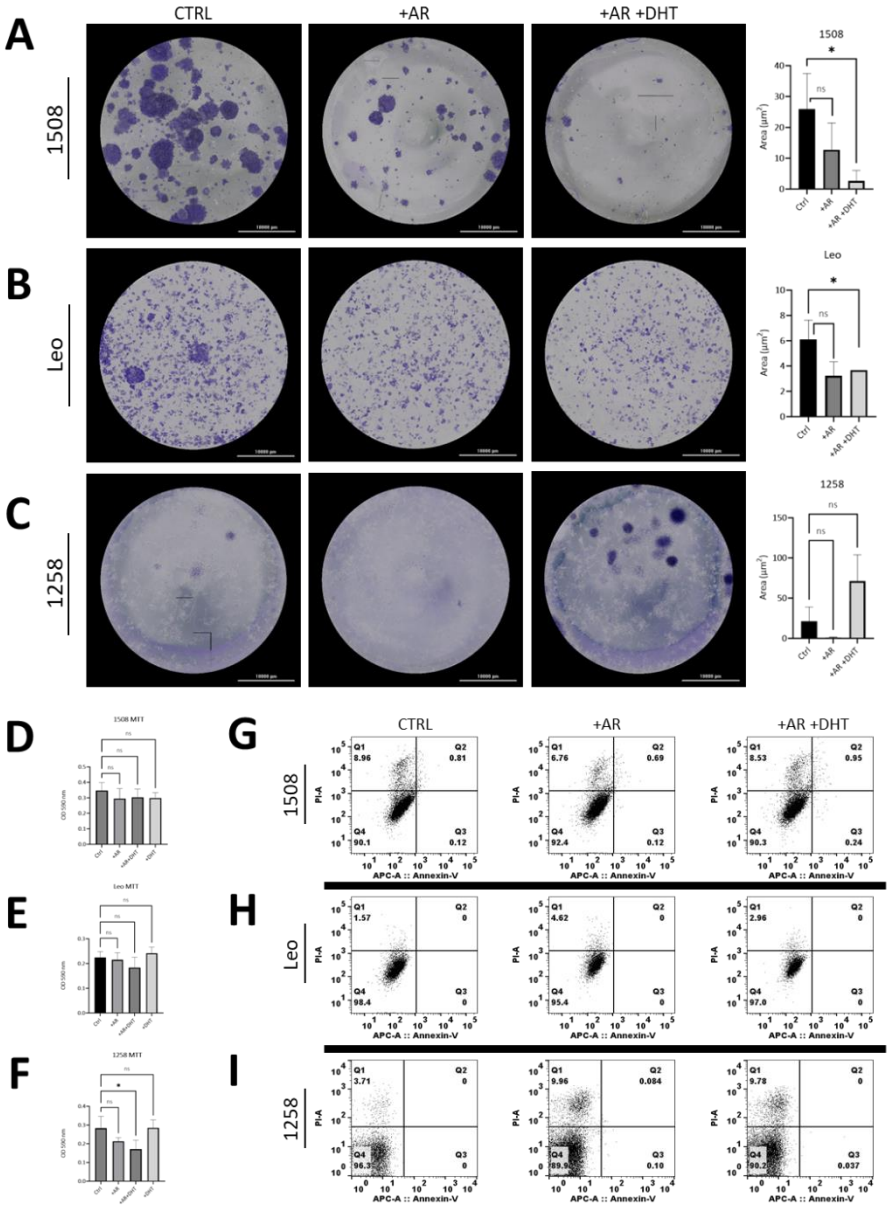

Figure 3.4. AR signaling restoration affects proliferation and metabolism. (**A-C**) AR signaling revival attenuates proliferation in Leo (p = 0.04) and 1508 (p = 0.03) and appears to increase proliferation in 1258 but not significantly (p > 0.05). (**D,E**) Metabolism is unchanged with AR signaling revival in cell lines 1508 and Leo (p > 0.05) but decreased in cell line 1258 (**F**; p = 0.04). (**G-I**) Changes in proliferation assays was not secondary to cell death or apoptosis.

AR Signaling Restoration Decreases Migration in One Canine PCa Cell Line but Not Others

To investigate whether restored AR signaling in canine PCa cell lines attenuates migration as has been shown in human PCa cell lines, wound closure assays were performed [21].

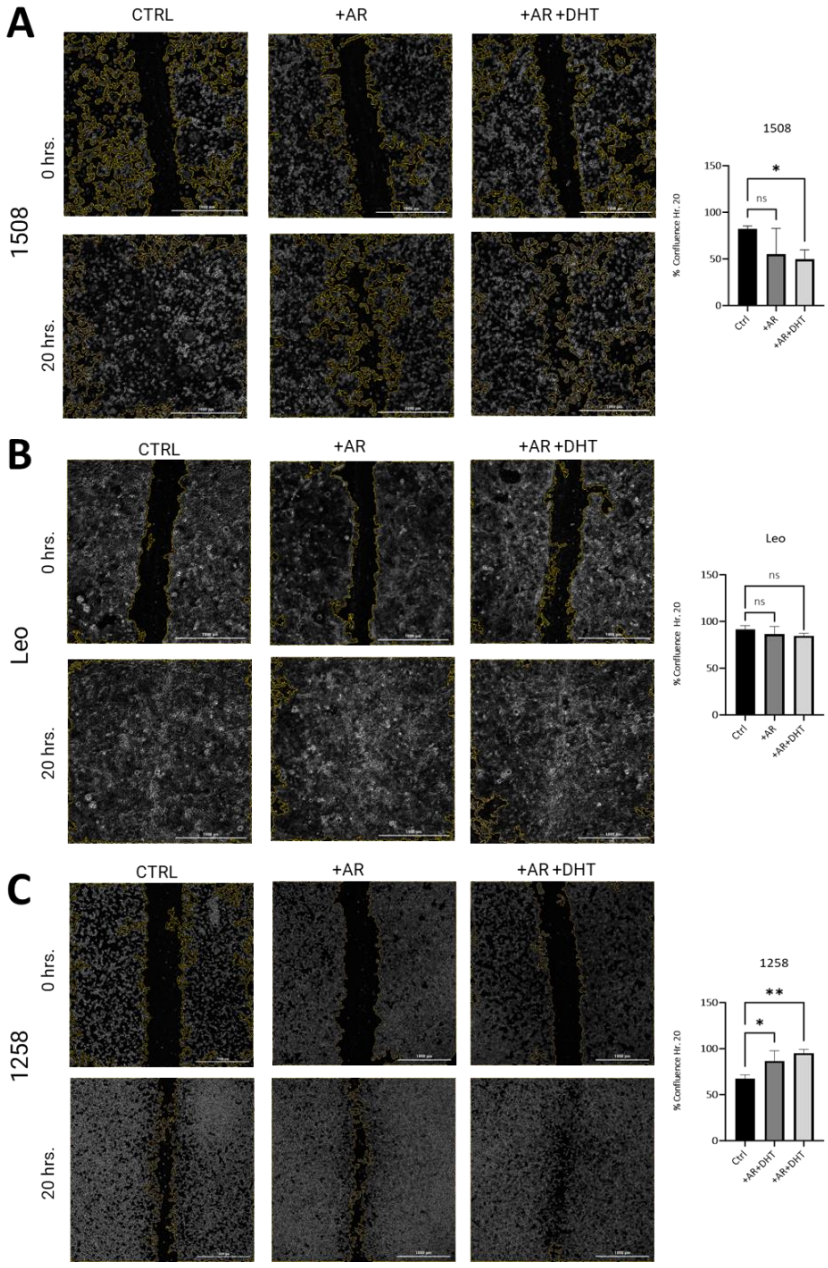

Figure 3.5. AR signaling restoration affects migration. (**A**) AR signaling revival attenuates migration in cell line 1508 ($p = 0.03$) but is unchanged in cell line Leo ($p > 0.05$). Migration increases in 1258 when AR alone is restored ($p = 0.03$), and even further when AR signaling is restored ($p = 0.006$), suggestive of a CRPC phenotype.

Cell line 1508 had decreased migration with restored AR signaling (**Figure 3.5A**, *right* column; $p = 0.006$), while Leo had visibly decreased migration (**Figure 3.5B** *right* column) but not significantly ($p > 0.05$). Contrastingly, cell line 1258 had increased migration with AR transfection (**Figure 3.5C**, *center* column; $p = 0.03$) and AR signaling (**Figure 3.5C**, *right* column; $p = 0.006$). An unmasked version of Figure 3.5 is provided in supplementary materials (**Figure S3.2**).

AR Signaling Restoration Decreases Invasion and Markers of EMT in Some Canine PCa Cell Lines but Increases in Others

Boyden chamber invasion assays were performed to investigate if AR restoration in canine PCa cell lines attenuates invasion as has been demonstrated in human PCa cell lines [22]. Invasion was decreased in transfected and treated groups for cell line 1508 ($p = 0.01$) when compared to the control group (**Figure 3.6A** *center* column, *right* column).

While there was a slight decrease in invasion in transfected and treated groups for cell line Leo it was not significantly different than the control group ($p > 0.05$) (**Figure 3.6B**). Cell line 1258 had decreased invasion with the presence of the AR plasmid ($p = 0.01$) (**Figure 3.6C**, *center* column), however with DHT treatment and restoration of AR signaling, invasion increased compared to the control group ($p = 0.03$) (**Figure 3.6C**, *right* column).

All experimental groups were evaluated for changes in the expression of EMT markers (i.e., *SNAIL1*, *Vimentin*, *N-cadherin*) and compared to their respective control group. *Vimentin* was significantly upregulated with AR signaling restoration in cell lines Leo ($p = 0.03$) and 1258 ($p = 0.03$), but not in cell line 1508. *N-cadherin* expression was also upregulated in both Leo ($p = 0.01$) and 1258 ($p = 0.01$), but not in cell line 1508 ($p > 0.05$). Lastly, *SNAIL1* was significantly upregulated in cell line 1258 as well ($p < 0.0001$), but unchanged in cell lines Leo and 1508 ($p > 0.05$). A summary of the results is available in **Table 3.2**.

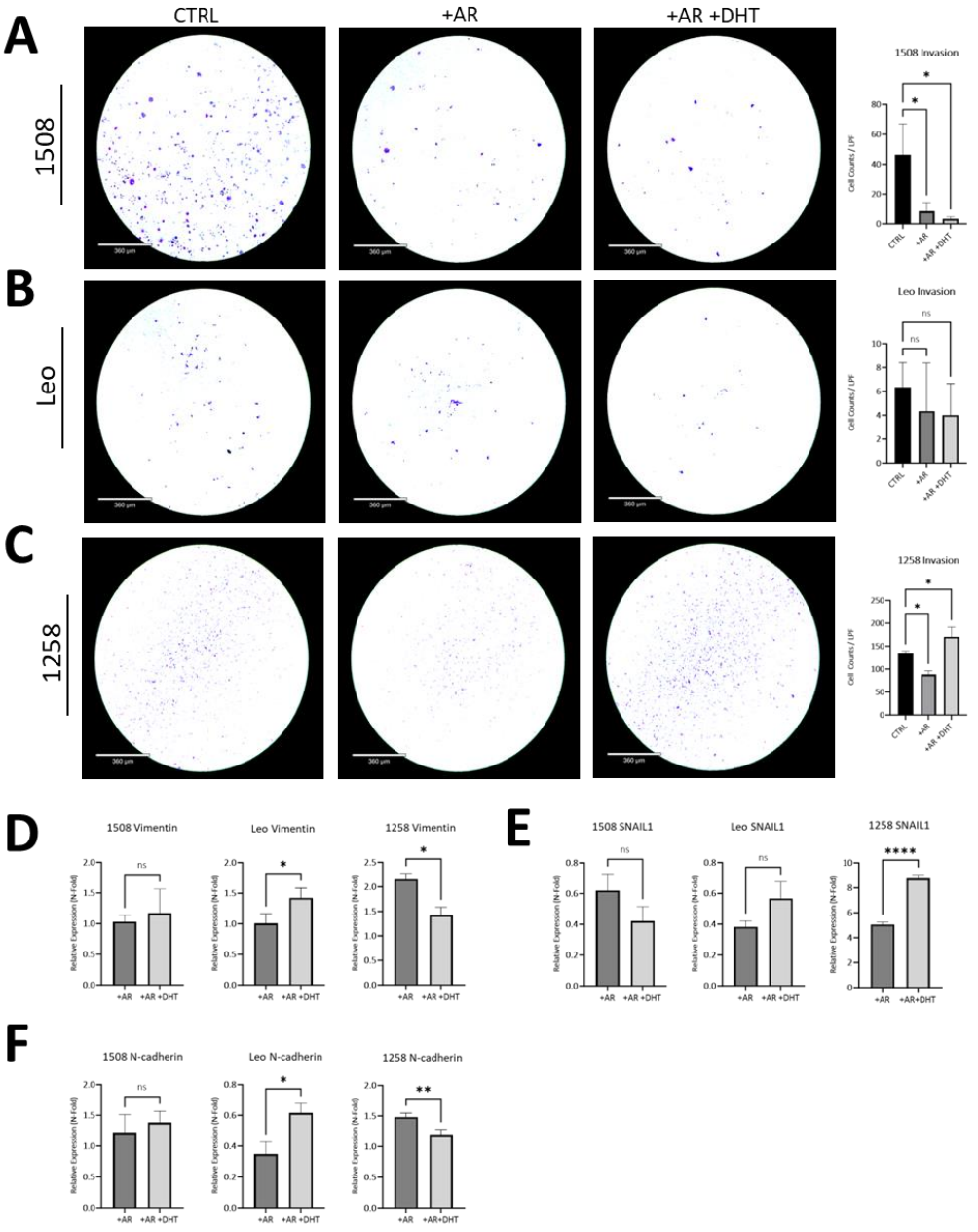

Figure 3.6. AR signaling restoration affects invasion and markers of EMT. (**A**) AR signaling revival attenuates invasion in 1508 (p = 0.01), though EMT markers do not appear affected (**D, E, F**, *left*). **B)** AR signaling restoration did not change the invasiveness of cell line Leo (**B**; $p > 0.05$) but did increase EMT markers *Vimentin* (***D***, *center*) and *N-cadherin* (**F**, *center*). Invasiveness increased with restored AR signaling in cell line 1258 (**F**, *center*, *right*) and all markers of EMT increased significantly (**D**, *right*, p = 0.03; **E**, *right*, $p < 0.0001$; **F**, *right*, p = 0.01)

Table 3.2. Summary of findings in canine PCa cell lines after AR signaling restoration

Aggressive Behavior	1508	Leo	1258
Proliferation	↓	↓	No Change
Oxidative Metabolism	No Change	No Change	↓
Migration	↓	No Change	No Change
Invasion	↓	No Change	↑
EMT Marker Expression	No Change	↑	↓↑[1]

[1] Some markers of EMT increased while others decreased.

3.4 Discussion

In this study, we explored restoration of AR signaling in canine PCa cell lines to determine the similarity of this pathway between canines and humans as well as the potential use of canines as animal models for novel therapies in human PCa. We successfully transfected AR into AR null canine PCa lines and recreated AR signaling in all three cell lines in this study. We found that cell line 1508 had multiple aggressive behaviors (i.e., proliferation, migration, invasion) abrogated by AR signaling revival, while cell line Leo had fewer aggressive behaviors (i.e., proliferation) attenuated with this signaling restoration. In contrast, we found that cell line 1258 became more aggressive with restoration of AR signaling, with increases in proliferation, aberrant migration, increased invasion, and increase in the expression of multiple EMT markers. These findings are important as they show the heterogeneity of physiology and response to AR restoration in canine PCa cell lines, which recapitulates the variabilities found in the physiology of different variants of human PCa and supports their use as a model for this dynamic disease.

Restoration of AR signaling was accomplished through transfection with a canine wildtype AR plasmid and treatment with a physiologic dose of DHT. All three canine PCa cell lines were initially AR null, but AR was successfully expressed in the experimental groups (+AR or +AR +DHT) after transfection. Moreover, AR was shown to be sequestered to the cytoplasm when without androgenic stimulation (+AR) but translocated to the nucleus with DHT treatment (+AR +DHT), a hallmark feature of all nuclear transcription factor signaling pathways when cognate ligands are present [8,23]. The downstream target gene of AR, NKX3.1, was upregulated when AR signaling was restored in cell lines 1508 and Leo ($p = 0.001$; $p = 0.01$), but appeared nearly absent in cell line 1258, which is likely to be a specific feature of that cell line [24,25]. Studies have shown that staining for NKX3.1 protein is positive in the majority of primary prostatic adenocarcinomas, downregulated in many high-grade prostate cancers

and completely lost int eh majority of metastatic prostate cancers. Another AR target, *FOLH1,* has been reported in dogs to be analogous to human PSMA, a gene non-canonically repressed by AR [26–28]. There was decreased expression of FOLH1 with restoration of AR signaling in cell line Leo ($p = 0.0002$), insignificant decrease in expression in cell line 1508 but not significantly ($p > 0.05$), and no difference in expression in cell line 1258 ($p > 0.05$). Overall, cell lines 1508 and Leo displayed expected changes to AR targets with restoration of AR signaling, but not cell line 1258. Reasons for this may vary widely, including aberrant activity of AR second to mutations in its coactivators or corepressors, as well as mutations or aberrations in either of these two target genes. Further genomic, transcriptomic, and proteomic exploration of cell line 1258 is needed to evaluate these possibilities.

Abrogation of aggressive behaviors, including proliferation, has been shown to occur in human PCa cell lines with AR signaling restoration and this was also explored in our study in three canine PCa cell lines [14,15]. Although AR can promote PCa growth, it has also been demonstrated to be a potent tumor suppressor that inhibits proliferation by acting on numerous genes that influence DNA replication, synthesis, modification, and repair (e.g., MCM7 [minichromosome maintenance complex gene], FANCI [Fanconi anemia complementation group gene],) by way of retinoblastoma protein (RB) recruitment, particularly when DHT is present [15]. AR signaling attenuated proliferation in two cell lines, 1508 and Leo ($p = 0.03$; $p = 0.04$), but appeared to increase proliferation in 1258, though this was not significant ($p > 0.05$). Moreover, to ensure that differences in proliferation were not due to cell death or apoptosis, flow cytometry was performed for annexin V (apoptosis) and propidium iodide (cell death) for all cell lines and no difference was found between control and experimental groups ($p > 0.05$). MTT assays were performed to evaluate whether AR restoration changes oxidoreductase metabolism (i.e., cell viability, cell activity) [29]. Metabolism was unchanged between control and experimental groups in cell lines 1508 and Leo ($p > 0.05$) but was decreased with AR signaling restoration in cell line 1258 ($p = 0.04$). This was an interesting discovery as AR-mediated PCa metabolism reduces glycolysis, enhances mitochondrial oxidative phosphorylation and lipogenic metabolism compared to non-cancerous prostate tissue [15,30,31] and decrease in metabolism was not due to cell death as demonstrated by the aforementioned flow cytometric analyses. Nonetheless, MTT assays more accurately represent cytosolic oxidoreduction by NADH and NADPH mechanisms and restoration of AR signaling in cell line 1258 may have affected

cytosolic metabolic activity in unknown ways, including through non-genomic signaling [32,33]. Again, cell line 1258 represents an interesting outlier as a cell line to model AR signaling restoration and needs further investigation of its underlying signaling pathways.

In addition to the attenuation of proliferation observed in human PCa cell lines with AR signaling restoration, others have found that AR revival also leads to decreased migration of human PCa by way of negatively regulating chemokines involved with migration, including several C-C motif ligand (CCL) and C-C motif receptors (CCR), like CCL2-CCR2 [21]. We found that only cell line 1508 had decreased migration ($p = 0.006$) upon AR signaling restoration. Cell line Leo did not have a significant change in migration ($p > 0.05$) with AR re-constitution while cell line 1258 had significantly increased migration when AR signaling was reconstituted ($p = 0.006$). Furthermore, the transfected group (+AR) also had increased migration compared to the control. One possible cause for this radically different outcome in 1258 compared to the other two lines is that there are mutations in AR targets (since there are no mutations in the AR itself) which may result in differential effects of AR signaling in the different lines [33]. This possibility warrants further investigation. Moreover, the increased aggressiveness, as demonstrated by increased migration, in cell line 1258 with restored AR signaling is a feature of CRPC where the presence of AR leads to more aggressive disease [10].

AR signaling restoration has been shown to decrease invasion of human PCa cell lines in numerous ways, including by way of reducing adhesion to the extracellular matrix (ECM) and modulation of genes involved in metastasis [14,34]. Investigation of AR signaling restoration in canine PCa revealed variable results in attenuation of invasion and was cell line-dependent. Invasion was abrogated in cell line 1508 ($p = 0.01$) with AR signaling restoration, although expression of EMT markers (i.e., *SNAIL1*, *Vimentin*, *N-cadherin*) was not significantly changed ($p > 0.05$) which may be due to other mechanisms driving metastasis in that cell line apart from those explored in this study. Cell line Leo did not have a significant change in invasion with AR signaling restoration, but interestingly had an increase in *Vimentin* ($p = 0.03$) and *N-cadherin* ($p = 0.01$) expression with AR signaling revival. This increase in *N-cadherin* expression is part of a dysregulated Wingless/Integrated (Wnt) signaling pathway, which has been shown to be further advanced by AR in castration-resistant prostate cancer (CRPC) [35]. Thus, Leo may have pathway aberrations that reflect a cell line more similar to CRPC than androgen-dependent PCa when AR

signaling is revived. However, though the EMT markers are increased the Boyden chamber assays did not show an increase in invasiveness, which is speculated to be due to the short time period the assay was performed (24 hours) to avoid confusion from cell replication. Additional invasion assays with longer invasion times could be performed to exclude this possibility. Cell line 1258 not only showed increased invasion with restored AR signaling (**Figure 5C**) but also showed increased expression of *SNAIL1* (p <0.0001), but not *Vimentin* or *N-cadherin*. *SNAIL1* is a downstream target of activated PI3K/AKT/mTOR signaling pathway, which was shown to be overexpressed in multiple canine PCa cell lines and may be this cell line's primary mechanism of promoting EMT [36]. Similar to Leo, this increase in invasiveness with restoration of AR signaling in cell line 1258 is most compatible with a cell line that resembles CRPC.

3.5 Conclusions

Cell line 1258 had increased proliferation, increased migration, increased invasion, and increased expression of EMT markers when AR signaling was restored. Because of this, cell line 1258 most closely fits a CRPC cell line, where AR signaling leads to more aggressive behavior, although the exact underlying mechanisms for how this occurs have yet to be explored in this cell line. Leo had decreased proliferation, had unchanged migration, unchanged invasion but some upregulation of EMT markers when AR signaling was restored, and as such, has qualities most similar to an AR-indifferent cell line. Lastly, cell line 1508 had decreased proliferation, decreased migration, and decreased invasion with AR signaling restoration, and most closely resembles an androgen-dependent cell line where restoration of AR signaling abrogates aggressive behaviors. Though canine PCa may serve as a model for human PCa, it is important to remember that it is a heterogeneous disease in canines as it is in humans, and each cell line may reflect different stages of PCa carcinogenesis.

3.5 Supplementary Tables and Figures

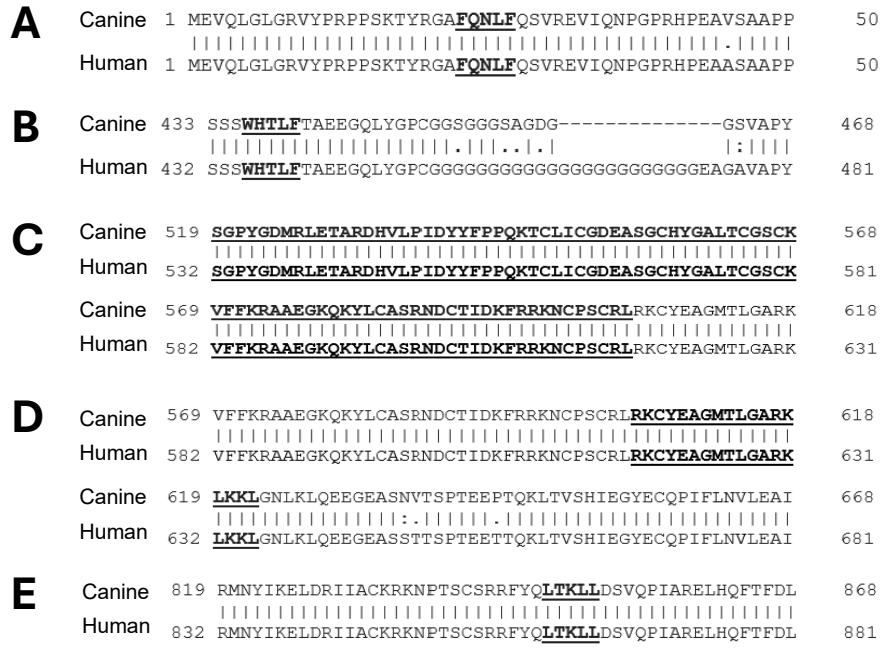

Figure S3.1. AR gene homology between canines and humans. (**A, B**) Sequences “FQNLF” and “WHTLF” are NTD sequences that interact with the C-terminal domain after ligand binding and are conserved between the species. (**C**) DNA binding domain of AR to AREs in the genome is conserved between the species. (**D**) The NLS of AR is also conserved. (**E**) Co-activator sequences in the C-terminal domain follow a “LxxLL” motif, and there is evidence of conserved co-activator elements between the species.

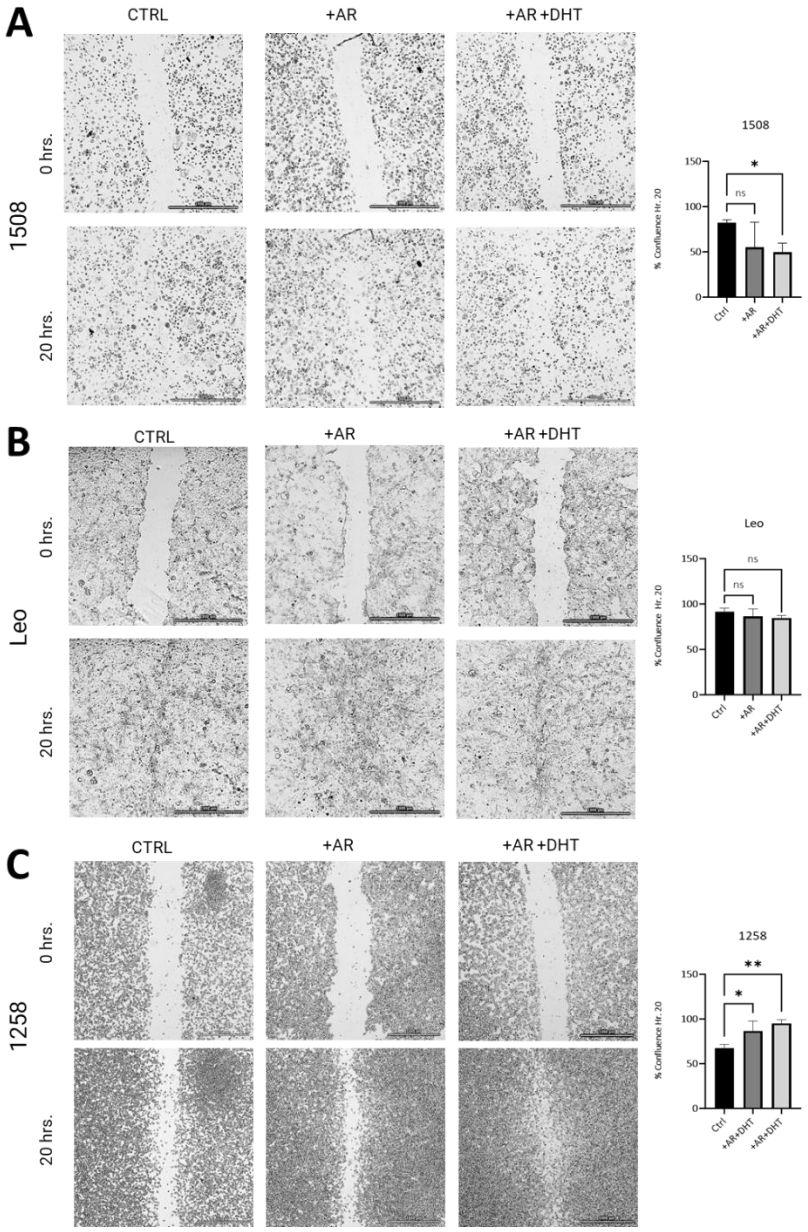

Figure S3.2. **AR signaling restoration affects migration (unmasked version).**

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