

Monitoring Prostate Cancer Heterogeneity Through Circulating Genes

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ABSTRACT

Patient stratification remains an obstacle for the optimal treatment of prostate cancer (PCa). At diagnosis, it is not always clear which patients are at risk of recurrence after curative treatments. The main therapies for recurrent disease target the androgen receptor (AR), killing the majority of AR+ luminal-like cells. Subsets of AR+ cells may develop resistance, while AR-negative neuroendocrine and stem-like populations can emerge. AR remains the main target in metastatic and castration resistant (mCRPC) stages of disease. AR inhibitors and taxanes prolong survival but inevitably fail. Drugs targeting other pathways have been tested in clinical trials, but few have entered clinical practice. However, some have shown efficacy in small subsets of unselected patients. This highlights the need for better stratification to provide effective personalized treatments. Stratification based on markers in primary tumours may show limited relevance after years of treatments. Access to biopsies of metastases would be ideal for identifying prognostic markers, but such invasive procedures are not part of clinical practice. Blood is an attractive alternative, being accessible non-invasively and longitudinally to provide a global view of molecular changes. We hypothesise that intra-tumoural cell heterogeneity underlies both androgen-dependent and -independent PCa progression and can be monitored through liquid biopsies.

In **Manuscript 1**, cellular heterogeneity was explored by cell-by-cell quantification of AR in the prostate and PCa. While AR+ cancer cells were predominant, AR- cells increased with grade and hormone treatment. Hormone treatment increased AR expression in the remaining AR+ cells. AR- cells were interspersed among AR+ cells and expressed neuroendocrine and stemness markers. Fibroblastic AR was lost with high grade and upon hormone treatment. An AR index predicted recurrence and further stratified intermediate risk patients.

In **Manuscript 2**, we assessed intratumoural heterogeneity through 14 genes of prostate epithelial cell subtypes (luminal, neuroendocrine, and stem cells) in whole blood RNA from 40 mCRPC patients and 40 controls. We demonstrated the clinical relevance of gene patterns with treatments and progression. To further develop this approach, we expanded the panel with additional genes representing cell-subtypes, encoding drug targets, or relevant to AR inhibitor or taxane resistance. This new 67 gene panel was tested in 26 controls, 16 patients prior to surgery, and 36 samples from 25 metastatic cases. Results in **Manuscript 3** reveal that patients with intraductal carcinoma displayed more circulating genes pre-surgery, including more neuroendocrine and stemness genes. In metastatic patients, signatures of each subtype were associated with progressive disease. PCa-specific luminal genes were associated with shorter overall survival, while targetable neuroendocrine genes came up in cases with initial radiation therapy, and treatment resistance genes with lines of treatment.

Finally, we validated this approach in the DPC-1 canine model of aggressive PCa. In **Manuscript 4**, a prostate-specific gene (*FOLH1*) was detected in all blood samples post- DPC-1 cell inoculation, but not pre-inoculation. The DPC-1 model resembles both naturally occurring canine PCa and human mCRPC by its mesenchymal and stemness features.

Altogether, AR expression in prostate cell subtypes reflects concomitant mechanisms of selection and adaptation of these cell subtypes, explaining therapeutic resistance. Monitoring these cell types through genes in liquid biopsies at different stages of disease revealed phenotypic and functional differences in patterns according to pathological features, treatments, and progression. This may allow for clinical trials adopting this approach to stratify patients and predict response to therapies throughout their entire trajectory. This may lead to personalized treatments impacting on this unpredictable and lethal disease.

RÉSUMÉ

La stratification de patients est nécessaire pour traitement optimal du cancer de la prostate (CaP). Au diagnostic, il n'est pas toujours clair quels patients présentent un risque de récurrence après des traitements curatifs. Les traitements principaux après une récurrence ciblent le récepteur des androgènes (RA) et tuent ainsi la majorité des cellules de type luminal RA+. Cependant, certaines cellules RA+ peuvent développer une résistance, et des populations neuroendocrines et souches RA- peuvent émerger. Le RA reste la cible principale dans les stades métastatiques et résistants à la castration (mCRPC). Les inhibiteurs du RA et les taxanes présentent une efficacité limitée. Des médicaments ciblant d'autres voies de signalisation ont été testés dans le cadre d'essais cliniques, mais peu d'entre eux sont entrés en pratique. Cependant, certains ont montré une réponse importante dans de sous-groupes de patients non sélectionnés. Cela met en évidence le besoin d'une meilleure stratification pour fournir des traitements personnalisés efficaces. La stratification basée sur des marqueurs dans les tumeurs primaires est limitée après des années de traitements. Des biopsies de métastases seraient idéal pour identifier des marqueurs pronostiques, mais ne font pas partie de la pratique clinique. Le sang est une alternative intéressante présentant une vue globale des changements moléculaires et étant accessible de manière non-invasive et longitudinale. Nous proposons que l'hétérogénéité cellulaire intra-tumorale est à la base de la progression du CaP dépendante et indépendante des androgènes et peut être surveillée par des biopsies liquides.

Dans le **Manuscrit 1**, l'hétérogénéité cellulaire a été explorée par quantification du RA cellule-par-cellule dans la prostate et le CaP. Alors que les cellules cancéreuses RA+ étaient prédominantes, les cellules RA- augmentaient avec le grade et le traitement hormonal. Le traitement hormonal a augmenté l'expression du RA dans les cellules RA+ restantes. Les cellules RA- étaient intercalées parmi les cellules RA+ et exprimaient des marqueurs neuroendocriniens et

souches. Le RA fibroblastique est perdu à haut grade ou suivant un traitement hormonal. Un « AR Score » prédit la récurrence et stratifie davantage les patients à risque intermédiaire.

Dans le **Manuscrit 2**, l'hétérogénéité intratumorale a été évaluée par 14 gènes représentant les sous-types de cellules épithéliales de la prostate (luminales, neuroendocrines, et souches) dans l'ARN du sang total de 40 patients mCRPC et 40 contrôles sains. Nous avons démontré la pertinence clinique des gènes avec les traitements et la progression. Pour développer davantage cette approche, nous avons rajouté des gènes représentant les sous-types cellulaires, codant pour des cibles médicamenteuses, ou reliés à la résistance aux inhibiteurs du RA ou aux taxanes. Ce nouveau panel de 67 gènes a été testé dans le sang de 26 contrôles, 16 patients avant la chirurgie et 36 échantillons provenant de 25 cas métastatiques. Les résultats du **Manuscrit 3** révèlent que les patients atteints d'un carcinome intracanalairé présentent davantage de gènes circulants avant la chirurgie, notamment de gènes neuroendocriniens et souches. Chez les patients métastatiques, les signatures de chaque sous-type étaient associées à une maladie progressive. Les gènes luminaux spécifiques au CaP étaient associés à une survie plus courte, tandis que des gènes neuroendocriniens ciblables sont apparus dans les cas de radiothérapie initiale et des gènes de résistance avec des traitements.

Enfin, cette approche a été validée dans le modèle canin DPC-1 de CaP agressif. Dans le **Manuscrit 4**, un gène spécifique à la prostate (*FOLH1*) a été détecté dans tous les échantillons de sang après l'implantation des cellules DPC-1. Le modèle DPC-1 reproduit le CaP canin naturel et ressemble au mCRPC humain par ses phénotypes mésenchymateux et souches.

En conclusion, l'expression du RA dans les sous-types de cellules de la prostate reflète des mécanismes concomitants de sélection et d'adaptation de ces sous-types de cellules, expliquant la résistance thérapeutique. La surveillance de ces types de cellules grâce à des gènes dans le sang à

différents stades de la maladie révèle des différences phénotypiques et fonctionnelles en fonction des caractéristiques pathologiques, des traitements, et de la progression. Cela pourrait permettre des essais cliniques adoptant cette approche pour stratifier les patients et prédire la réponse aux thérapies tout au long de leur trajectoire. Cela pourrait conduire à des traitements personnalisés ayant un impact sur cette maladie imprévisible et mortelle.

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CONTRIBUTION TO ORIGINAL KNOWLEDGE

Manuscript 1:

The clinical relevance of androgen receptor (AR) expression patterns was determined by a cell-by-cell quantification of its expression in the prostate and prostate cancer (PCa)

- Demonstration that the proportion of AR-negative cancer cells and surrounding fibroblasts increase with grade and upon hormone treatment, accompanied by a compensatory increase in AR expression in the remaining AR-positive cancer cells.
- Definition of an “AR Score” based on AR expression patterns in cancer cells and fibroblasts, and demonstration of its association with recurrence in a cohort of radical prostatectomy patients, including further stratification of CAPRA-S intermediate risk patients.
- Identification of AR-negative cancer cells as neuroendocrine and stem-like cells intermingled among the majority of AR-positive cells in primary tumours of hormone-treated patients with advanced PCa.

Manuscript 2:

The traceability and clinical relevance cell-subtype specific genes (luminal, neuroendocrine, stem) in whole blood RNA was demonstrated.

- Generation of a panel of 14 PCa cell-subtype specific genes representing luminal, neuroendocrine, and stem-like cells. The mining of transcriptomic datasets of diverse cohorts of patients proved the relevance of the chosen genes in primary tumours of high-grade cancers and metastases from patients at advanced stages.

- Demonstration that testing of this panel by RT-qPCR in whole blood RNA of 40 mCRPC patients and 40 healthy controls showed no correlation with age or patients' white blood cells, instead correlating with the disease.
- Demonstration that circulating gene patterns are associated with clinical features, treatments, and outcomes. This non-invasive approach may be meaningful to ascertain the evolution of PCa during the course of the disease.

Manuscript 3:

The suitability of the approach in Manuscript 2 was further proven by expanding on the gene panel and including different categories of patients.

- Implementation of strict inclusion and exclusion criteria for choosing additional PCa-relevant genes to study in whole blood RNA.
- Establishment of a panel of 64 genes based on cell subtypes, resistance to AR inhibitors or taxanes, and promising drug targets tested in PCa clinical trials.
- Demonstration of improved methodology, including both technical and biological validation of assays and showing high reproducibility of results.
- Demonstration that circulating genes are traceable in blood prior to curative therapies in patients with aggressive pathological features.
- Confirmation of earlier findings (Manuscript 2) and further validation with the extended gene panel in patients with advanced disease; signatures of cell subtypes and treatment resistance showed phenotypic and functional changes in blood with treatments received and predicted further progression.

- Identification of genes for which treatments are available for other indications, showing that overexpression patterns may help identify patients who are likely to respond to the relevant treatments.

Manuscript 4:

The relevance of canine PCa and the DPC-1 model was proven through transcriptomic analyses, highlighting important similarities to human mCRPC and suggesting treatments that could be relevant to both canine and advanced human PCa.

- Identification of consistent differences between canine PCa cell lines vs. tumours and between orthotopic vs. subcutaneous DPC-1 model.
- Demonstration of the heterogeneity among DPC-1 tumours and its relevance to naturally occurring canine PCa.
- Identification of potential targets for canine PCa which are also pertinent to advanced human disease.
- Demonstration that canine PCa closely resembles a subset of human mCRPC presenting mesenchymal and stem-like features and shows a distinct neuroendocrine signature.
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LIST OF ABBREVIATIONS

ADT: Androgen deprivation therapy	DNPC: Double-negative prostate cancer
ALDH1A1: Aldehyde dehydrogenase 1family member 1A	DPC-1: Dog prostate cancer 1
AR: Androgen receptor	EGFR: Epidermal growth factor receptor
ARI: Androgen receptor inhibitor	EMT: Epithelial to mesenchymal transition
ARPC: Androgen receptor driven prostate cancer	ENO2: Neuron specific enolase
ARV12: Androgen receptor variant 12	EPCAM: Epithelial cell adhesion molecule
ARV7: Androgen receptor variant 7	ESR1: Estrogen receptor 1 (ER-alpha)
ARV: Androgen receptor variant	ERG: ETS Transcription Factor ERG
AURKA: Aurora A kinase	EV: Extracellular vesicle
BCR: Biochemical recurrence	EZH2: Enhancer of Zeste Homolog 2
BPH: Benign prostatic hyperplasia	FFPE: Formaline-fixed paraffin-embedded
CAPRA-S: Cancer of the Prostate Risk Assessment Post-Surgical score	FOLH1: Folate hydrolase 1
CfDNA: Cell-free DNA	FPKM: Fragments per kilobase million
CHGA: Chromogranin A	GG: Gleason grade
CK: Cytokeratin	GRPR: Gastrin releasing peptide receptor
CI: Confidence interval	GS: Gleason score
CRPC: Castration resistant prostate cancer	GSEA: Gene set enrichment analysis
CTC: Circulating tumour cell	GSVA: Gene set variation analysis
DHT: Dihydrotestosterone	HR: Hazard ratio
	IDC: Intraductal carcinoma
	Ki-67: Marker Of Proliferation Ki-67
	KLK3: Kallikrein 3

LN: Lymph node	PSMA: Prostate specific membrane antigen
mCRPC: Metastatic castration resistance prostate cancer\	PTEN: Phosphatase and tensin homolog
MET: Hepatocyte growth factor receptor	RIN: RNA integrity number
MSPC: Mesenchymal and stem-like prostate cancer	RNA: Ribonucleic acid
NCAM1: Neural cell adhesion molecule	RP: Radical prostatectomy
NE: Neuroendocrine	RT: Radiation therapy
NED: Neuroendocrine differentiation	RT-qPCR: Quantitative reverse transcription polymerase chain reaction
NEPC: Neuroendocrine prostate cancer	SC: Stem cell
NHT: Neoadjuvant hormone therapy	SD: Standard deviation
OCT4: Octamer-binding transcription factor 4	SOX2: SRY/Sex determining region Y-box 2
OR: Odds ratio	SSTR: Somatostatin receptor
ORA: Over-representation analysis	SYP: Synaptophysin
PBMC: peripheral blood mononuclear cell	TMA: Tissue microarray
PCa: Prostate cancer	TP53: Tumor protein P53
PCA: Principal component análisis	TPM: Transcripts per million
PET: Positron emission tomography	TURP: Trans-urethral resection of the prostate
POU5F1: POU Class 5 Homeobox 1	VEGFA: Vascular endothelial growth factor A
PSA: Prostate specific antigen	VIM: Vimentin
PSCA: Prostate stem cell antigen	WBC: White blood cell
	WGS: Whole genome sequencing

INTRODUCTION

1. The Prostate

a. Prostate anatomy and function

The prostate is an accessory gland of the male reproductive system responsible for producing components of semen necessary for sperm cell function. It is located at the base of the bladder, surrounding the prostatic urethra. It is composed of transitional, central, and peripheral zones, which are histologically and anatomically distinct. While benign prostatic hyperplasia (BPH) occurs mainly in the transitional zone, prostate cancer (PCa) can arise in the peripheral (~75%) or transitional (~25%) zone.^{1,2}

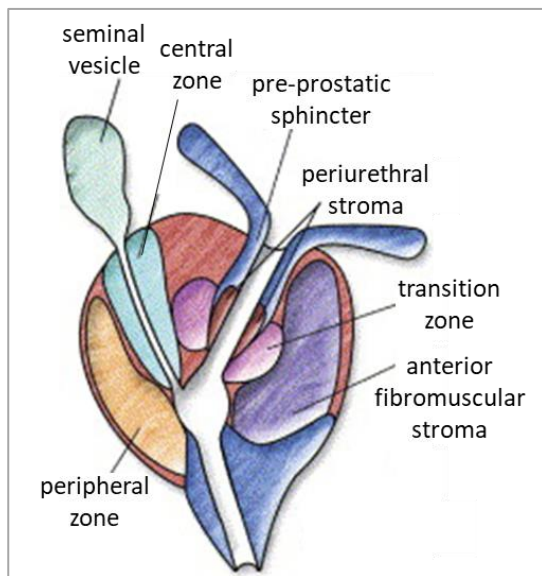


Figure 1: Anatomy of the human prostate. Adapted from McLaughlin et al. 2004.³

b. Androgen dependence in prostate development

Prostate development and homeostasis are reliant on androgen signaling through the androgen receptor (AR). The prostate starts to develop in the fetus from the urogenital sinus (UGS). Briefly, circulating testosterone is converted to the more active dihydrotestosterone (DHT) by 5 α -reductase in the UGS, stimulating prostatic development through AR activation.⁴ This leads to the outgrowth

of epithelial buds into the mesenchyme, branching morphogenesis, and canalization to reorganize the epithelium into distinct basal and luminal layers.⁴ Recombination studies have shown that while mesenchymal AR is necessary for fetal prostate morphogenesis, epithelial AR is important for the final stages of morphogenesis, specifically luminal differentiation and secretory function.^{5, 6} Thus, mesenchymal AR is necessary for prostate development and growth, as well as playing a role in fibroblast differentiation and proliferation through regulation and secretion of various growth factors; epithelial AR is necessary for prostate homeostasis, including epithelial cell growth, differentiation, and secretory function.^{7, 8}

AR is a member of the nuclear hormone receptor family. It is comprised of an N-terminal domain, a DNA binding domain, a hinge region containing a nuclear localization signal, and a ligand binding domain.⁹ In the absence of androgens, AR remains bound to Heat Shock Protein 90 (HSP90), keeping it inactive in the cytoplasm.⁹ In the prostatic epithelium, testosterone is converted to DHT. Both can bind to the AR ligand-binding domain, causing its release from HSP90 and nuclear translocation. In the nucleus, AR dimers bind to androgen response elements to act as transcription factors regulating expression of androgen-mediated AR target genes.¹⁰

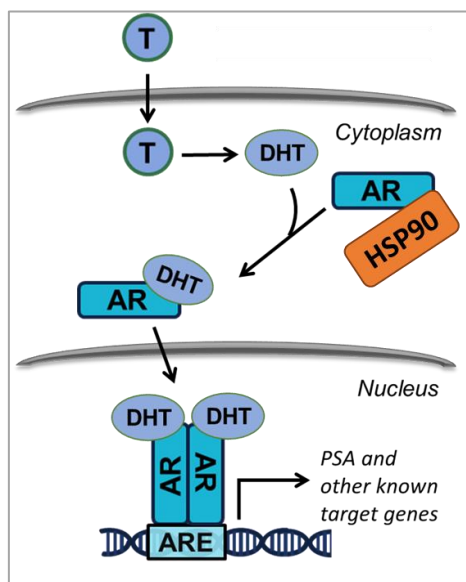


Figure 2: Canonical androgen/androgen receptor signaling.

T: testosterone; DHT: dihydrotestosterone; AR: Androgen receptor; HSP90: Heat shock protein 90; ARE: androgen response element; PSA: Prostate specific antigen.

c. Epithelial cell subtypes in the mature prostate

The mature prostate is comprised of a glandular epithelium surrounded by a fibromuscular stroma composed of fibroblasts, blood vessels, and immune cells. The epithelium is composed of a continuous basal layer that includes rare stem cells, a luminal layer, and rare neuroendocrine (NE) cells. Basal cells have been reported to be AR negative, while luminal cells express AR. Common markers include cytokeratin (CK)8, CK18, and PSA for luminal cells, and CK5, CK14, and P63 for basal cells.

Stem cells in the basal epithelium can either self-renew or give rise to transit amplifying cells, which in turn differentiate into basal and luminal cells.^{11, 12} Luminal cell differentiation and survival are androgen-dependent; castration leads to prostate involution upon apoptosis of luminal cells, whereas subsequent androgen supplementation allows the reconstitution of the luminal layer.¹³⁻¹⁵

NE cells are AR-negative cells making up ~1% of the prostate epithelium, interspersed among cells of the luminal and basal layers. They can be visualized by immunohistochemistry for markers such as chromogranin A (CHGA), synaptophysin (SYP), neuron specific enolase (NSE), and neural cell adhesion molecule (NCAM1). There are two hypotheses for the cellular origin of NE cells in the prostate. The first suggests that NE progenitor cells migrate to the prostate epithelium from the neural crest.^{16, 17} Others suggest that these cells arise from prostate basal/stem cell neuroendocrine differentiation (NED), which is more in line with NED seen in PCa.^{18, 19} Indeed, normal prostate basal cells showing features of stemness can regenerate all three cell populations.²⁰

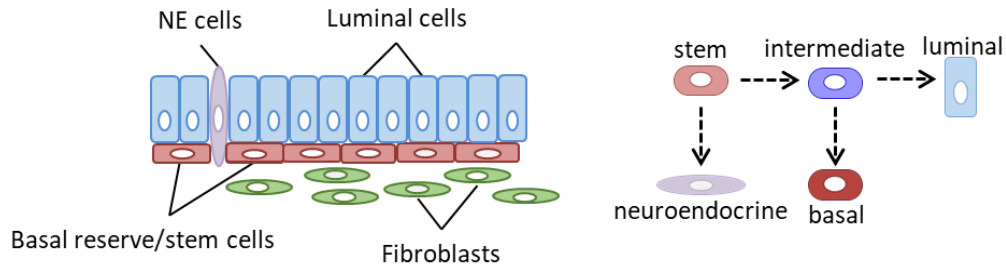


Figure 3: Schematic representation of the normal prostate epithelium (left) and differentiation of basal stem cells into all epithelial cell subtypes of the prostate (right).

NE cells are thought to play a role in regulating growth, differentiation, and secretory function of the prostate epithelium.^{21, 22} Some NE cells can receive only basal stimuli (closed cells), while others can also receive luminal/secretory stimuli through microvilli reaching the lumen (open cells).²² They secrete a variety of neuroproducts, such as chromogranins, synaptophysin, serotonin, histamine, vascular endothelial growth factor (VEGF), gastrin-releasing peptide (GRP), calcitonin, and somatostatins.^{21, 22} These are generally angiogenic and growth/motility promoting factors, in line with the proximity of NE cells to highly proliferative cells.^{21, 23} The receptors for these products can be expressed by luminal cells or other NE cells, suggesting endocrine and paracrine functions.

2. Prostate cancer

a. Statistics

PCa is the second most common cancer worldwide, and the most common for Canadian men.^{24, 25} It is the leading cause of cancer mortality among men in 48 countries, accounting for approximately 375 000 deaths in 2020.²⁴ Based on estimates for 2023 in Canada, PCa accounted for 25,900 new diagnoses and 4,900 deaths.²⁶ There is great variation in incidence and mortality worldwide, mainly attributed to availability of screening and access to healthcare, but also to genetic and lifestyle factors.²⁴ The major risk factors for PCa include age, family history, ethnicity, and lifestyle habits including obesity/diet, alcohol, smoking, and certain occupational chemical exposures.²⁴

b. Detection and diagnosis

Screening for PCa involves blood PSA (prostate specific antigen) tests and digital rectal examination. Histopathological results of biopsy specimens, in combination with clinical and imaging results, can be used to determine the best course of treatment for each patient.

PSA is a serine protease encoded by *KLK3* (kallikrein 3) and is a canonical AR target gene produced and secreted by luminal cells into the glandular lumen. It increases sperm motility by liquefying seminal fluid through proteolysis of semenogelin and fibronectin.²⁷ PSA is liberated in the blood of patients with various prostatic diseases, including PCa, BPH, and prostatitis.²⁸ As PSA is not cancer-specific, all diagnoses are confirmed by biopsy. Additional tissue, blood, urine, and imaging tests have been developed in recent years for PCa diagnosis and prognostication with increased accuracy compared to PSA testing, but these are not yet widely used in clinical practice. These tests will be reviewed in the section on “Novel approaches for patient stratification”.

c. PCa prognostication: grade and stage

Grading for PCa follows the Gleason system first established in 1966.²⁹ This system defines five patterns of tumours resulting in a Gleason score determined by the two most common patterns (ranging from 1+1 = 2 to 5+5 = 10). It is applied to both biopsy and prostatectomy samples. The Gleason system has been modified throughout the years to account for new findings in the field, the current version being that of the 2019 International Society of Urological Pathology (ISUP) consensus conference.³⁰

Table 1: Characteristics of Gleason patterns.

Gleason pattern	Characteristics
1	circumscribed, closely packed but separate uniform glands, with no infiltration into the stroma
2	similar to pattern 1, with minimal infiltration
3	discrete glands smaller than patterns 1 and 2, with infiltration among benign acini and variation in size and shape
4	fused microacinar glands, ill-defined glands with poorly formed glandular lumina, and cribriform or glomeruloid features
5	lacks glandular differentiation; composed of solid sheets, cords, or single cells

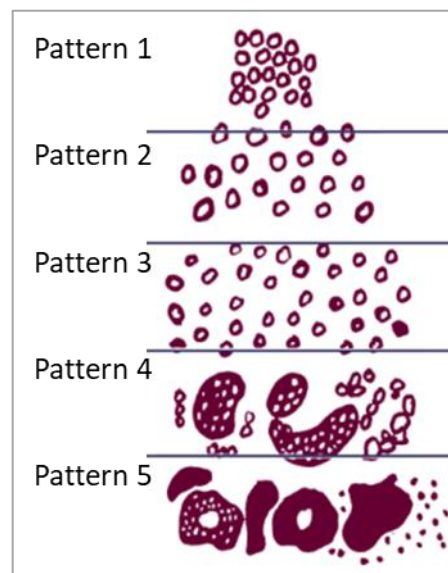


Figure 4: Schematic depiction of Gleason patterns. Adapted from Sunassee et al., 2019.³¹

Gleason scores < 6 are not recommended to report on biopsies, due to the high likelihood of higher-grade cancer being missed; the main limitation is that the entire edge cannot be seen to determine whether it is circumscribed.³² True cases of Gleason < 6 are no longer considered malignant. A new grading system was derived in ISUP 2014, denoting Grade Groups (GG) based on prognosis

(Table 2).³³ This was especially important for the distinction of Gleason score 7 into 3+4 vs. 4+3, the later showing significantly worse prognosis.³³

Table 2: Gleason Groups.

Gleason Group	Gleason Score
1	≤ 6
2	3+4
3	4+3
4	8
5	9-10

Intraductal carcinoma (IDC) is defined by the World Health Organization as an “intra-acinar and/or intraductal neoplastic epithelial proliferation that has some features of high grade prostatic intraepithelial neoplasia (HGPIN) but exhibits much greater architectural and/or cytological atypia, associated with high grade or high stage prostate carcinoma”.³⁵ It is associated with aggressive disease and predictive of poor prognosis, including worse progression-free survival (PFS) and overall survival (OS).³⁶ In ISUP 2019, the incorporation of IDC into the Gleason score was suggested, whereby cribriform IDC may be considered as pattern 4, and solid pattern IDC or IDC with comedonecrosis as pattern 5.³⁰ However, the consensus remains to report IDC in addition to the Gleason grade. For instance, cribriform morphology (consisting of solid sheets of cancer cells with glandular lumens and no stroma) is more important in invasive cribriform cancer than in IDC.³⁷ As mentioned, these can be differentiated by staining for basal cells. Furthermore, IDC should not to be confused with ductal carcinoma, which is composed of large glands lined by tall columnar cells with pseudostratified nuclei, which can have cribriform, papillary, and solid patterns, classifying most as Gleason patterns 4-5.

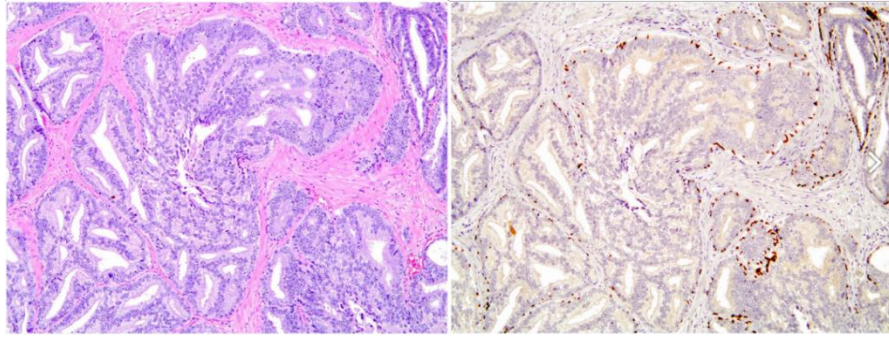


Figure 5: Intraductal carcinoma with cribriform pattern. H&E (left) and immunohistochemical p63 staining (right) at 100x magnification. Courtesy of PathologyOutlines.com, contributed by Erica Vormittag-Nocito, M.D.

PCa staging is determined using the American Joint Committee on Cancer (AJCC) tumour-node-metastasis (TNM) system. Briefly, tumours can be localized (T1/T2), show extraprostatic extension (T3), or spread to other adjacent structures (T4); lymph nodes can be negative (N0), positive (N1), or not assessed (NX); and patients can be non-metastatic (M0) or metastatic (M1).³⁷

Table 3: Clinical staging for PCa.

T1a	Incidental cancer <5% of prostate		T3a	Cancer in one side with extracapsular extension
T1b	Incidental cancer >5% of prostate		T3b	Cancer in both sides with extracapsular extension
T1c	Cancer detected only by high PSA		T3c	Seminal vesical invasion
T2a	Cancer in <50% of one side		T4a	Extra-prostatic extension to bladder neck, rectum, or external sphincter
T2b	Cancer in >50% of one side		T4b	Involvement of other adjacent structures
T2c	Cancer in both sides			

d. Risk stratification models and current treatment options for localized disease

Multiple risk stratification systems have been described for localized PCa, mainly based on PSA, Gleason Grade, and T stage. Current models are presented in Table 4.

Adding to these models is the CAPRA (Cancer of the Prostate Risk Assessment) score, which also includes age and % positive biopsies.^{38, 39} A surgical CAPRA score (CAPRA-S) accounts for PSA, grade, surgical margins, seminal vesicle invasion, extracapsular extension, and LN invasion.⁴⁰ Because it requires pathological data from prostatectomy samples, it is relevant for estimating risk of recurrence and guiding the use of adjuvant treatments accordingly.

Table 4: Risk stratification models for localized PCa.

Model	Low risk		Intermediate risk		High risk	
NICE, D'Amico, and EUA	PSA < 10 and GG 1 and T1-T2a		PSA 10-20 or GG 2-3 or T2b		PSA > 20 or GG 4-5 or ≥ T2c	
GUROC	PSA ≤10 and GG 1 and T1-T2a		PSA ≤20 and GG 2-3 and T1-T2		PSA > 20 or GG 4-5 or ≥ T3a	
AUA	PSA < 10 and GG 1 and T1c-T2a (Very Low Risk if <34% positive cores, no cores >50% cancer, and PSA density <0.15)		Favorable: (GG1 and PSA 10-20) or (GG2 and PSA <10)	Unfavorable: (GG2 and PSA 10-20 or T2b-T2c) or (GG 3 and PSA <20)	PSA >20 or GG 4-5 or ≥ T3	
CPG	CPG1: PSA <10 and GG 1 and T1-T2	CPG2: PSA 10-20 or GG 2 and T1-T2	CPG3: (PSA 10-20 and GG 2 and T1-T2) or (GG 3 and T1-T2)		CPG4: PSA >20 or GG 4 or T3	CPG5: (two of PSA > 20, GG 4, or T3) or (GG 5 or T4)

Adapted from the National Institute for Health and Care Excellence report on

“Prostate cancer: diagnosis and management”, 2021.⁴¹ Units for PSA are in ng/mL.

NICE: National Institute for Health and Care Excellence; EUA: European Association

of Urology; GUROC: Genitourinary Radiation Oncologists of Canada; AUA:

American Urological Association; CPG: Cambridge Prognostic Groups.

Treatment options may depend on risk classification, with radical prostatectomy (RP) and radiation therapy (RT) being the curative options for localized disease. While RP seems to show a slight

benefit for OS and PFS compared to RT, large-scale and long-term prospective studies by risk group are needed for a definitive answer to this question.⁴² There are also differences in adverse effects to consider between treatment modalities: RP is associated with urinary incontinence and erectile dysfunction, while RT is associated with bowel symptoms.⁴³

Alternatively, low-risk patients can remain closely monitored on active surveillance in lieu of curative treatment. While this approach prevents needless treatment of indolent disease and avoids the side effects of RP or RT, there remains a possibility that the disease is more advanced than expected, which can lead to progression and metastatic spread before administration of curative treatments.⁴⁴ Focal therapies have been developed to target cancer foci within the prostate, thus reducing side effects compared to RP and RT.^{45, 46} These include high-intensity focused ultrasound, transurethral ultrasound ablation, focal cryotherapy, and photodynamic therapy. While these are effective short-term, more long-term data is necessary before such treatments can enter standard practice.^{45, 46}

Due to the likelihood of early recurrence in high-risk cases, they are generally treated more aggressively by a combination of RT and hormone treatment. Although RP +/- RT can also be considered, it is not as common since the disease is likely to already have spread and formed micro-metastases.⁴⁷ However, it may still be a viable option for reducing disease burden and it does show comparable long-term outcomes in retrospective studies.⁴⁷ Various systemic therapies currently used to control metastatic disease, such as AR inhibitors and PARP inhibitors, are also being investigated for more aggressive treatment of such cases at earlier points in their trajectory.⁴⁷⁻⁴⁹

Intermediate-risk cases are the most heterogeneous in terms of outcomes, needing further sub-classification such as those proposed by the NCCN (favorable vs. unfavorable intermediate risk).⁵⁰

To complicate the matter, treatments for these cases include active surveillance, experimental focal therapies, RT, and RP. This is in line with the important differences between Gleason 3+4 vs. 4+3 cases, most of whom would be considered intermediate risk by current stratification models. Indeed, recent advancements such as the use of MRI (magnetic resonance imaging)-targeted biopsy leads to upstaging of 21% of cases from 3+4 to 4+3 or 4+4 compared to systematic biopsies.⁵¹ Overall, there is a risk up- or down-grading when comparing biopsy vs. RP specimens, although this risk is diminished with incorporation of targeted biopsies.⁵²⁻⁵⁴ This demonstrates the importance of accurate stratification, which would entail significant differences in disease management.

e. Tissue-based molecular models for risk stratification

Multiple tissue-based biomarker tests are commercially available for risk stratification of PCa.⁵⁵ Decipher, Prolaris, Oncotype DX, or ProMark are useful for stratifying patients, including intermediate risk cases, as recommended by the NCCN.⁵⁶ The relevant markers are in Table 5.

Decipher is a 22-gene genomic classifier based on gene expression microarray data.⁵⁷ It measures expression of mRNAs involved in AR signaling, cell proliferation, differentiation, motility, and immune modulation. This test is applicable in formalin-fixed paraffin-embedded (FFPE) biopsy tissues to stratify low- vs high-risk patients and determine the optimal treatment and treatment intensity (or active surveillance), or in RP tissue to determine whether adjuvant or salvage treatment may be useful. A systematic review found that Decipher results are predictive of pathological features, biochemical recurrence, metastasis, cancer-specific survival, and OS.⁵⁸

The Prolaris cell cycle progression (CCP) score measures mRNA expression of 31 cell cycle progression genes and 15 housekeeping genes in FFPE tissue from biopsy or prostatectomy.⁵⁹ It is

useful for determining the 10-year risk of metastasis after curative treatment and for deciding on whether adjuvant ADT is warranted for RT cases.^{60, 61}

The Oncotype Dx Genomic Prostate Score (GPS) is an RT-qPCR based test for 12 mRNAs related to proliferation, androgen signaling, cellular organization, and stromal response and five reference genes in biopsy tissues only.⁶² It predicts adverse features such as Grade Group ≥ 3 or extracapsular extension to identify cases suited for surveillance.⁶²

ProMark measures expression of eight proteins in biopsy tissue and uses automated image recognition to identify Grade Group ≥ 2 or stage $\geq T3b$.⁶³ The proteins were chosen among 12 markers previously shown to predict aggressive disease.⁶⁴

Table 5: Commercially available tissue-based tests for PCa risk stratification.

Classifier	Marker type	Method	Tissue type	Markers
Decipher ⁵⁷	mRNA	Microarray	FFPE biopsy or RP	<i>LASPI, IQGAP3, NFIB, SIPR4, THBS2, ANO7, PCDH7, MYBPC1, EPPK1, TSBP, PBX1, NUSAP1, ZWILCH, UBE2C, CAMK2N1, RABGAP1, PCAT-32, GLYATL1P4, TNFRSF19</i>
Prolaris ⁵⁹	mRNA	RT-qPCR	FFPE biopsy or RP	<i>ASPM, CDC2, CDCA8, CDKN3, DTL, FOXM1, KIAA0101, NUSAP1, PRC1, TK1, ASF1B, BIRC5, BUB1B, C18orf24, CDC20, CDCA3, CENPF, CENPM, CEP55, DLGAP5, KIF11, KIF20A, MCM10, ORC6L, PBK, PLK1, PTTG1, RAD51, RRM2, TOP2A</i>
Oncotype Dx ⁶²	mRNA	RT-qPCR	FFPE biopsy	<i>AZGP1, BGN, COL1A1, FAM13C1, FLNC, GSN, GSTM2, KLK2, SFRP4, SRD5A2, TPM2, TPX2</i>
ProMark ⁶³	Protein	Immunofluorescence	FFPE biopsy	SMAD4, PDSS2, HSPA9, FUS, YBOX1, DERL1, CUL2, pS6

These are not the only molecular stratification models that have shown relevance in PCa; three additional approaches are summarized here. Lapointe et al. classified primary tumours as three subgroups based on gene expression profiles relevant to tumour aggressiveness, and later found that these subtypes show distinct profiles of copy number alterations.^{65, 66} They also used such copy number alterations to establish a multiplex ligation-dependent probe amplification (MLPA)-based six-gene classifier with prognostic value for low and intermediate risk cases, compatible with small amounts of DNA from FFPE.⁶⁷ In the Cambridge and Stockholm cohorts, Ross-Adams et al. identified five subgroups with different outcomes based on 100 genomic and transcriptomic features.⁶⁸ The Cancer Genome Atlas (TCGA) used genomic and transcriptomic data to identify seven subtypes of PCa, characterized by various drivers: gene fusions in ETS family genes (*ERG*, *ETV1*, *ETV4*, or *FLI1*), or mutations in *SPOP*, *FOXA1*, or *IDH1*.⁶⁹ These showed differences in AR activity and epigenetic profiles between subtypes, identified actionable MAPK and PI3K mutations in 25% of cases, and DNA repair genes in 19%.

f. Treatments upon recurrence after curative therapies

Approximately 30% of cases who received potentially curative treatments eventually develop a recurrence. This is often detected by a consistent rise in PSA >0.2 ng/ml for RP or >2 ng/ml for RT cases. PCa remains an androgen-regulated cancer, as evidenced by the importance of PSA for monitoring disease. Accordingly, surgical castration was initially shown to be effective for the treatment of PCa by the Canadian Nobel laureate, Dr Huggins.⁷⁰ This has now been replaced by pharmacological castration, termed androgen deprivation therapy (ADT). ADT consists of luteinizing hormone/gonadotropin-releasing hormone (LHRH/GnRH) agonists or antagonists which inhibit testosterone production by Leydig cells in the testes. Serum testosterone levels are

reduced to under 0.5 ng/mL, which is too low to activate AR signaling. 5 α -reductase inhibitors are also used to inhibit conversion of testosterone to DHT in prostate cells.

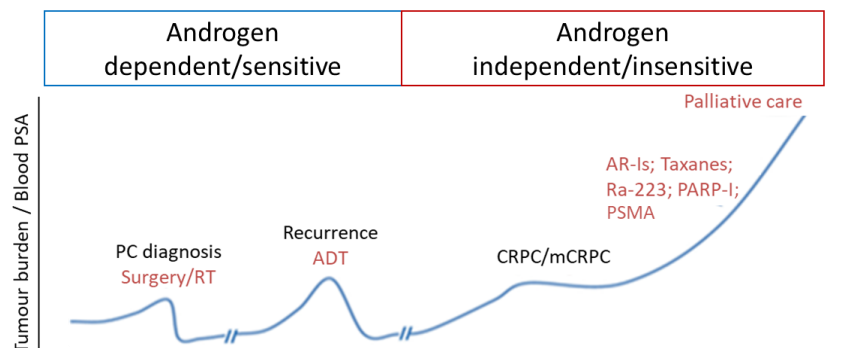


Figure 6: PCa trajectory and treatments.

While response to ADT can last for years, a subset of resistant cells eventually gives rise to metastatic castration-resistant disease (mCRPC). The main treatments for CRPC still target the androgen/AR signaling axis using AR inhibitors (AR-Is). The two main AR-Is used today are abiraterone and enzalutamide. Abiraterone inhibits the synthesis of various hormones, including testosterone, by inhibition of CYP17.⁷¹ Enzalutamide directly binds the AR ligand-binding domain, thus inhibiting its binding to androgens and conformational changes necessary for its transcriptional activation.⁷² Taxanes, especially docetaxel and cabazitaxel, are used, often after AR-I resistance. Taxanes are microtubule stabilizing agents which block cell cycle progression, leading to apoptosis. Interestingly, in PCa, taxanes also affect AR signaling directly by inhibiting its nuclear translocation and subsequent transcriptional activity.⁷³ Unfortunately, resistance to all of these treatments eventually arises.

Few other treatments are available for mCRPC. Targeted radiation can be used to treat metastatic lesions. Radium-223 is an alpha-emitting radionuclide that acts as a calcium mimetic, delivering targeted radiation to bone lesions.⁷⁴ Prostate specific membrane antigen (PSMA)-targeting radioligands show great promise for imaging and treatment of mCRPC.⁷⁵ Finally, the poly (ADP-Ribose) polymerase (PARP) inhibitor olaparib has now been approved in Canada for treatment of

mCRPC with germline or somatic *BRCA1/2* or *ATM* mutations; in these cases with deficient homologous recombination, PARP inhibitors inhibit alternate pathways of DNA repair, resulting in synthetic lethality.⁷⁶

g. Mechanisms leading to castration resistant phenotypes

Mechanisms of castration resistance can be classified as AR-dependent or AR-independent. A subset of AR positive (+) PCa cells can develop androgen-independence resulting from various mechanisms, including AR amplification and overexpression, mutations leading to promiscuous ligand binding, and expression of splice variants (ARVs).^{9, 77} This can also entail androgen-independent AR reactivation through crosstalk with various other signaling pathways relevant in CRPC (e.g. interleukin 6, insulin-like growth factor 1, epithelial growth factor, or gastrin-releasing peptide signaling).⁷⁸⁻⁸¹ Most ARVs conserve the N-terminal domain and DNA binding domain, but lack part or all of the AR C-terminal domain which consists of the ligand binding domain and hinge region, making them constitutively active. This is the case for ARV7, a constitutively active AR variant predictive of AR-I resistance.⁸²

AR-independent mechanisms of castration resistance can involve activation of other survival and growth pathways, but also selection for or (de)differentiation towards NE and stemness phenotypes. This is expected, as NE and stem-like cells lack AR expression and are thus not targeted by ADT or AR-Is. Such treatments first induce stemness phenotypes, with NED requiring lineage plasticity, induced by genetic (*RB1* and *TP53* loss, *MYCN* amplification) or epigenetic (*EZH2* overexpression) changes.⁸³ NED upon ADT is now well established, and it is clear that AR-Is are not effective against this cell type, as they are AR-negative.⁸⁴⁻⁸⁸ However, NED can also be induced by RT by increasing nuclear phosphorylated CREB and sequestering ATF2 in the cytoplasm.⁸⁹⁻⁹³

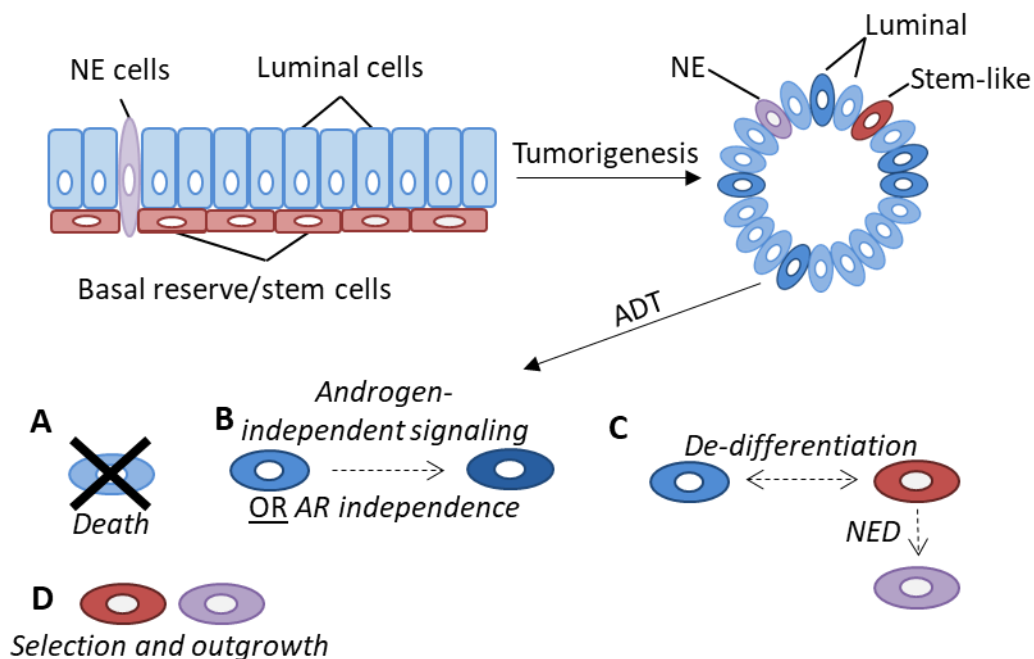


Figure 7: Mechanisms of castration resistance by cell phenotype. (A) *Death of AR+ androgen dependent luminal-like cancer cells.* (B) *Adaptation of a subset of luminal-like cancer cells to androgen independence, either remaining AR dependent (AR mutation, amplification, alternative splicing, crosstalks with other pathways) or independent.* (C-D) *De-differentiation or selection and outgrowth of stem-like and NE cells.*

Efforts to identify molecular subtypes of mCRPC have revolved around these phenotypes. Aggarwal et al. categorized cases as luminal or basal, with cases of small cell NE PCa being enriched in the basal subtype.⁹⁴ Luminal, but not basal, cases treated with AR-Is showed better OS. Liang et al. identified four subtypes: luminal, basal, NE, and homologous recombination repair (HRR). Luminal cases showed high AR, while HRR and NE cases showed high HRR pathway suggesting potential PARP inhibitor resistance.⁹⁵ HRR cases also showed higher immune infiltration, and basal cases showed higher TMPRSS2-ERG fusion.⁹⁵ Bluemn et al. categorized

mCRPC into three subtypes: AR pathways active (ARPC), NE (NEPC), and “double negative” (DNPC; no AR activation and no NED) cases showing elevated FGF and MAPK activity.⁹⁶

Han et al. further examined this DNPC variant, reclassifying them as mesenchymal and stem-like (MSPC).⁹⁷ MSPC showed features of epithelial to mesenchymal transition, as well as signatures identified in bi-lineage or progenitor cells, i.e. the equivalent of “intermediate” cells in the normal prostate.^{97, 98} Features of MSPC were also found in ARPC and NEPC cases. Interestingly, they showed that MSPC arises from ARPC as a result of therapy-induced lineage plasticity, and that both may be treated with the addition of HER2/3 and/or mTORC inhibition in addition to AR-Is (tested *in vitro* and *in vivo*). It is noteworthy that many cases showed “mixed phenotypes”, in line with intra-tumoural heterogeneity.

Among other features, NEPC shows enrichment in *RB1* loss, *TP53* mutation/deletion, *AURKA* and *MYCN* amplification, *EZH2* overexpression, upregulation of transcription factors such as *BRN2* and *SOX2*, and a lack of alterations in known AR co-activators compared to other non-NE CRPC cases.^{83, 99, 100} Cases with small cell NEPC, also referred to as AVPC (aggressive variant PCa) or SCPC (small cell PCa), show combined alterations in *RB1*, *TP53*, and/or *PTEN*; these tumours respond to platinum-based chemotherapy.^{101, 102} These findings are summarized in Figure 8.

All of these resistance mechanisms lead to phenotypes not currently targetable based on available treatments for PCa. Most importantly, this cellular heterogeneity, including NED, stemness features, and AR-dependent resistance phenotypes, is not currently assessed in clinical practice to aid in decision-making.

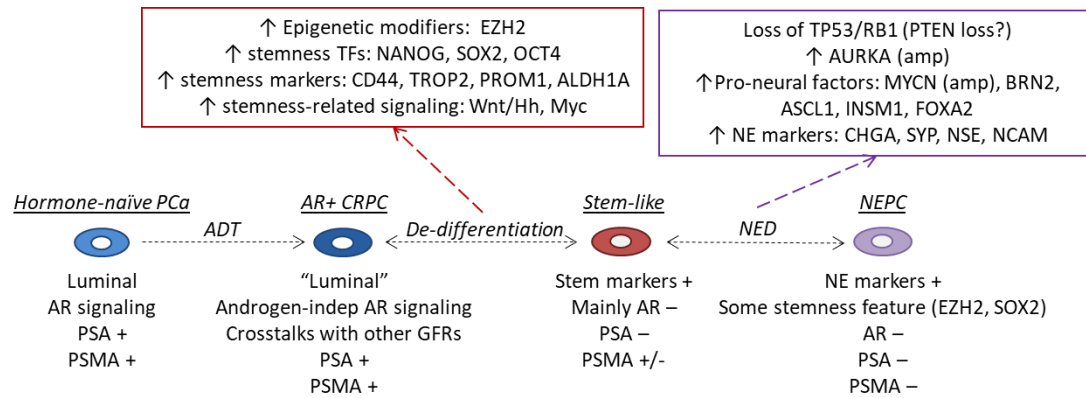


Figure 8: Key features of PCa cell types in advanced disease.

3. Alternative treatments for mCRPC in clinical trials: Moving towards stratification?

Of the novel targets and pathways being tested as possible therapeutic options for PCa, a select few are described here in relation to their roles in AR-independent disease and crosstalks with AR.

a. Aurora A kinase inhibitors for NEPC

Aurora A kinase (AURKA) is a serine/threonine kinase crucial for cell cycle control. It associates with the centrosome and spindle microtubules where it is involved in multiple roles including spindle assembly and centrosome duplication, separation, and maturation.¹⁰³ The importance of AURKA in CRPC was first apparent when it was found to be amplified in 40-65% of NEPC vs. 5% of non-NEPC cases; approximately 70% of these cases showed concurrent amplification of MYCN.^{104, 105} MYCN was previously linked to AURKA in neuroblastoma, where AURKA is essential for stabilizing MYCN by inhibiting its degradation.¹⁰⁶ MYCN is now well established as a driver of NEPC differentiation with sensitivity to AURKA inhibitors.^{104, 107-109}

In a phase 2 study (NCT00766324), danusertib showed minimal efficacy in non-selected docetaxel-refractory mCRPC patients, but 13/38 (34%) patients on the higher dose showed stable disease by tumour assessment, of which 7 showed stable disease ≥ 6 months.¹¹⁰ Unfortunately, the primary endpoint was PSA response, which was not met as NE tumours do not produce PSA. However, the relation of AURKA to NE disease was not known at the time of this study.

Alisertib was tested in a phase 2 study in CRPC cases with NE features (at least one of: small cells NE morphology, >50% NE marker expression, liver metastases with no PSA progression, or serum NE markers; NCT01799278).¹¹¹ Even with these stricter criteria, responders were limited, likely because of the low number of cases with AURKA (n=5) and MYCN (n=6) amplification in this limited cohort of 60 patients. AURKA amplification was associated with longer OS, and among

AURKA/MYCN overactivated cases, four men showed exceptional response. This included two patients with resolution of liver metastases and two with stable disease for 14 months and 3.8 years. This calls for further studies with more strict inclusion criteria, limited to the cases most likely to respond to AURKA inhibition, and complemented with other therapies to treat non-NE tumours in the same patient.

b. Enhancer of Zeste Homologue 2 inhibitors in AR-independent CRPC

Enhancer of Zeste Homologue 2 (EZH2) is the catalytic subunit of Polycomb Repressive Complex 2 (PRC2), allowing methylation of histone H3 lysine 27 (H3K27me) which represses transcription. It is associated with aggressive PCa in the hormone-naïve setting.¹¹²⁻¹¹⁴ In PCa, EZH2 is involved in epigenetic silencing of tumour suppressor genes and induction of cell plasticity and stemness features.¹¹⁵⁻¹¹⁷ EZH2 is also important for NED, including via the AURKA/MYCN axis, suggesting that these cases would be sensitized to EZH2 inhibition.^{100, 107, 108, 118} EZH2 can have non-histone substrates, including transcription factors such as GATA4, STAT3, and FOXA1, resulting in their repression, activation, and stabilization, respectively.¹¹⁵ It has also been shown to bind to AR as a transcriptional coactivator driving CRPC-specific AR signaling to stem and NE gene networks.^{119, 120} EZH2 depletion or inhibition of its methyltransferase activity may be useful for overcoming AR-I and taxane resistance *in vitro* and *in vivo*.¹²¹⁻¹²⁴

Clinical studies are ongoing for EZH2 inhibitors in CRPC in combination with other novel or current treatments (NCT04846478, NCT04179864, NCT03741712, NCT03460977, NCT03480646), but only one has moved on with specific molecular selection criteria: the NCT04388852 study targets specifically patients with features of AVPC (i.e. mutations or loss of at least two of *TP53*, *RB1*, and *PTEN*).

c. Theranostics for AR-dependent and -independent disease: PSMA, SSTRs, GRPR

Theranostic treatments are examples of therapies for which there is an easy pre-selection of patients using complementary imaging radionuclides. Theranostics (“Therapeutic” and “Diagnostic”) combines a specific diagnostic imaging test with its therapeutic counterpart linked to a more radioactive tracer.

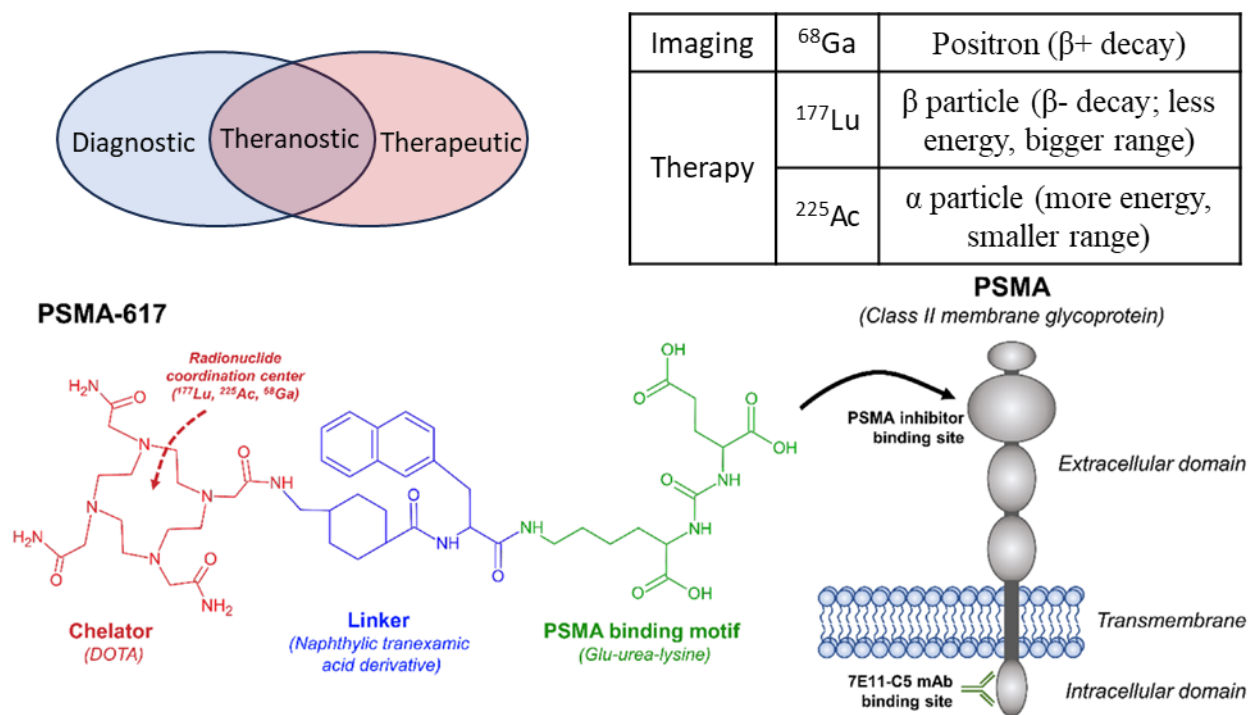


Figure 9: PSMA-617 as example of a theranostic agent. *Adapted from Plichta et al., 2021.*

The main molecular imaging target for PCa in recent years has been PSMA. PSMA is a cell surface protein highly expressed in prostate luminal cells.¹²⁵ Although its role in the prostate is not yet clear, it is likely involved in folate metabolism, as shown by an increase in proliferation of PSMA+ PCa cells in the presence of high folate.^{126, 127} PSMA is overexpressed in PCa and further increases with higher pathologic grade and with ADT.^{125, 128-131} It has become of interest because of its common overexpression and high specificity to PCa.¹³²

PSMA imaging started with ProstaScint, an Indium labelled antibody against an intracellular epitope of PSMA.¹³³ Although this was FDA-approved for imaging of both patients considered high-risk at diagnosis and those who have a recurrence, there was great room for improvement in sensitivity. The second generation of anti-PSMA antibodies targeted to the extracellular domain of PSMA.^{134, 135} The current radiotracers are not antibodies, but radiolabeled small molecule inhibitors of PSMA, which show better distribution and kinetics, with higher tumour-to-background ratio.^{136, 137}

With PSMA-targeting theranostics now becoming available, the problem remains that PSMA is not expressed in all lesions, especially in AR-independent NE or highly undifferentiated tumours. Other targets are currently being studied.

Gastrin releasing peptide receptor (GRPR) is another novel target for both AR-dependent and -independent tumours. Although its ligand, GRP, is a neuroproduct released by NE cells, the receptor itself is expressed more broadly in PCa, with its activation leading to growth and motility.¹³⁸⁻¹⁴⁰ GRPR radioligands have higher binding in primary tumours than in CRPC, whereas the opposite is observed for PSMA; nevertheless, GRPR remains of interest even at the CRPC stage, where GRPR+/PSMA- lesions have been detected.¹⁴¹ There are currently two studies underway for two different theranostic radioligands of GRPR. The phase 1 study NCT05283330 is testing the safety and tolerability of ²¹²Pb-DOTAM-GRPR1 in patients with recurrent or metastatic tumours expressing GRPR, including mCRPC with 51-80% GRPR+ cells at moderate intensity of staining. A phase 1/2 study of ⁶⁷Cu-SAR-BBN is recruiting mCRPC patients negative by PSMA imaging but positive by GRPR (⁶⁴Cu-SAR-BBN) imaging (NCT05633160).

Somatostatin receptors (SSTRs) are easily targetable NE markers, including NEPC, with approved theranostic ligands currently used for the treatment of gastroenteropancreatic NE tumours (¹⁷⁷Lu-

DOTATATE). A trial is currently underway to assess the safety and effectiveness of ^{177}Lu -DOTATATE for the treatment of metastatic PCa (NCT05691465). Inclusion criteria include the presence of NED as determined by (1) small cell or NE histology of primary tumour or metastasis biopsy; (2) show features of AVPC (at least two of *PTEN*, *TP53*, or *RB* loss); (3) progressive visceral lesions with no increase in PSA; or (4) high serum CHGA or NSE.

d. Integrin $\alpha\text{v}\beta 3$ and focal adhesion kinase signaling

The $\alpha\text{v}\beta 3$ integrin complex is involved in growth, differentiation, motility, and survival of PCa mediated by focal adhesion kinase (FAK) signaling.^{142, 143} Its ligands include vitronectin (VTN), fibronectin, and osteopontin. Integrin $\alpha\text{v}\beta 3$ is highly expressed in PCa metastases, being involved in PCa bone metastasis through its role in adhesion and migration on osteopontin and vitronectin and has recently been linked to stemness and NED.¹⁴⁴⁻¹⁴⁷ Integrin $\alpha\text{v}\beta 3$ is also involved in GRP-induced motility through FAK.^{140, 148}

Abituzumab is a monoclonal antibody against integrin αv which was tested in a phase 2 trial for unselected asymptomatic or mildly symptomatic mCRPC patients (NCT01360840). While there was no difference in PFS vs. ADT alone, there was a significant decrease in bone lesion progression.¹⁴⁹ Intetumumab is also a monoclonal antibody against integrin αv , tested in a phase 2 trial for mCRPC in combination with docetaxel, but was outperformed by docetaxel alone (NCT00537381).¹⁵⁰ Cilengitide is a small molecule inhibitor of $\alpha\text{v}\beta 3$ and $\alpha\text{v}\beta 5$ signaling, which showed no clinical activity in unselected non-metastatic CRPC cases (NCT00121238). Novel approaches are still being developed to target this pathway in PCa.^{142, 146, 151} Interestingly, dual-specificity imaging targets for GRPR and integrin $\alpha\text{v}\beta 3$ are now being tested in clinical trials for PCa (NCT05549024, NCT02747290), and may inform on the relevance of patient selection in cases with bone metastases.

FAK integrates signals not only from integrins, but also growth factor receptors; it is mostly related to cell adhesion and movement, but also plays a role in proliferation, EMT, and angiogenesis. While many FAK kinase inhibitors are available, only PF-00562271 has been tested in a phase 1 trial including PCa patients (NCT00666926); results for the three mCRPC cases in the dose expansion cohort were mixed (one stable disease, one progression, and one showing a decrease in all lesions from baseline), suggesting a need for better patient selection. The MATCH trial (NCT02465060) which uses genetic testing results to offer personalized treatments to patients with refractory solid tumours includes the FAK inhibitor defactinib for patients with inactivating mutations of the *NF2* (Neurofibromin 2) tumour suppressor. While this is a rare mutation in PCa (~1% of primary tumours and ~2% of metastases based on cBioportal), it may be relevant depending on the results of the trial.

e. Receptor and non-receptor tyrosine kinases

Growth factor receptors each play various roles in different aspects of PCa progression, including cell survival, proliferation, migration, invasion, metastasis, and androgen-independence. These signaling cascades can be especially difficult to block due to redundancy between pathways. The epidermal growth factor receptor (EGFR) and HER2 are mostly involved in androgen-independent survival and EMT, including AR-dependent and -independent pathways.¹⁵² Vascular endothelial growth factor receptors (VEGFRs) are more involved in angiogenesis and motility, while the hepatocyte growth factor receptor (MET) is most relevant to androgen-independence and is highest in bone metastases.^{153, 154}

Various EGFR/HER2 kinase inhibitors and monoclonal antibodies have been tested in PCa. The EGFR kinase inhibitor erlotinib in chemo-naïve CRPC resulted in partial response and stable disease in 2/29 and 5/29 patients, respectively, and two patients showed no progression up to 12

months (phase 2; NCT00272038).¹⁵⁵ Cetuximab is a monoclonal antibody against EGFR which showed better PFS for patients with EGFR overexpression and persistent PTEN activity compared to other patients (phase 2; NCT00728663).¹⁵⁶ One trial aiming to test the anti-HER2 antibody trastuzumab in selected mCRPC cases found infrequent HER2+ samples in archival biopsies of their cohort (phase 2; NCT00005857).¹⁵⁷ They did not move forward with the study, suggesting a need to assess metastatic lesions. Many drugs for these targets are now included in the MATCH trial: afatinib (EGFR/HER2 kinase inhibitor) for EGFR or HER2 activating mutations; pertuzumab and trastuzumab (anti-HER2 monoclonal antibodies) for HER2 amplified tumours. The DETERMINE trial is also assessing these antibodies specifically in selected cases (NCT05786716). One other phase 2 trial is currently ongoing for these antibodies in mCRPC; while they are collecting biopsy samples, molecular selection criteria were not specified (NCT05730712). Lastly, the anti-HER2/HER3 bi-specific antibody zenocutuzumab is now being tested in mCRPC (NCT05588609).

VEGFR and VEGF targeting drugs have been assessed in multiple phase 3 trials for PCa. Aflibercept (soluble decoy receptor VEGFR1/2) in pre-chemo mCRPC showed significantly more tumour responses but no improvement in OS when added to docetaxel (NCT00519285).¹⁵⁸ Bevacizumab (mAb against VEGF) combined to docetaxel in CRPC showed improved PFS but not OS (NCT00110214).¹⁵⁹ Sunitinib (kinase inhibitor of VEGFRs, PDGFRalpha/beta, Kit, FLT-3, CSF-1R) post-docetaxel showed improvement in PFS but was poorly tolerated by patients, suggesting other similar inhibitors or other combinations of drugs may be useful (NCT00676650).¹⁶⁰

MET has also been targeted in PCa. Tivantinib is a small molecule MET inhibitor which showed significantly improved PFS compared to ADT alone in asymptomatic or minimally symptomatic

mCRPC (phase 2, NCT01519414). Crizotinib (ALK, MET, and ROS1 inhibitor) is in the MATCH trial for cases with *MET* amplification, *MET* exon 14 deletion or mutation, *ALK* translocation, or *ROS1* translocation or inversion (NCT02465060). However, the most promising has been cabozantinib, an inhibitor of VEGFRs, MET, and AXL. Results in Phase 1/2 showed responses in unselected subsets of patients (NCT00940225, NCT01630590, NCT01834651); phase 3 (NCT01605227) in heavily pre-treated mCRPC showed improved bone biomarkers, bone scan responses, and improved radiographic PFS, but not PSA-PFS or OS. This led to the early termination of many ongoing cabozantinib studies in PCa.

More recently, cabozantinib has reentered clinical trials for testing in combination with immunotherapies. The combination of abiraterone, cabozantinib, and nivolumab (anti-PD1) for metastatic hormone-sensitive PCa (mHSPC) showed 4/7 partial responses and 3/7 stable disease.¹⁶¹ Cabozantinib and nivolumab are now being tested in mCRPC (NCT05502315). Studies are ongoing in combination with ipilimumab (anti-CTLA-4) in *de novo* small cell NEPC (NCT03866382). The most promising has been the combination of atezolizumab (anti-PDL1) and cabozantinib. In CRPC cases who progressed on AR-Is, 2/44 showed complete response, 12 showed partial response, and 21 stable disease (NCT03170960).¹⁶² For the ongoing phase 3 trial in mHSPC and mCRPC post-AR-I compared to AR-Is, an interim analysis showed a trend towards improved OS (NCT04446117).¹⁶³ This effect is thought to be due to the direct action of cabozantinib on TAM family kinases, which are overexpressed in PCa and are known to downregulate inflammation and promote an immunosuppressive microenvironment.¹⁶⁴

Various downstream effectors of these pathways have also been targeted, including Src family kinases (SFKs). Dasatinib is a kinase inhibitor of SFKs and ABL. A phase 2 trial in chemo-naïve mCRPC showed no progression at 24 weeks for 9/41 patients, with evidence of bone response

(NCT00385580).¹⁶⁵ Post-chemo, results were more limited, but one patient showed prolonged benefit with partial response for 284 days (NCT00570700).¹⁶⁶ With no pre-selection of cases, the phase 3 trial of dasatinib + docetaxel vs. docetaxel alone showed no improvement in OS (NCT00744497).¹⁶⁷ Dasatinib is now being tested to see if it alters PSMA expression when used in combination with the AR-I darolutamide (NCT04925648). Another phase 2 study in combination with enzalutamide is also underway (NCT01990196).

Several other inhibitors have been tested in phase 1/2 trials for PCa with different selectivity for various kinases, and many show tumour responses in subsets of unselected cases. There is a clear need to include prognostic biomarkers to move towards precision oncology.

4. Novel approaches for patient stratification

Having identified a handful of relevant phenotypic and functional features that may be useful for patient stratification, we must next consider the options for non-invasive longitudinal testing of molecular evolution of the disease.

a. Tissue biopsies

Despite the importance of previously defined tissue-based signatures, there remain significant disadvantages to such approaches, mainly for metastatic disease. While tissue biopsies would be the best option for identification of molecular drivers of disease for each case, offering also spatial information about the tumours, they are usually only available at diagnosis. Considering the inter- and intra-tumoural heterogeneity of PCa, multiple biopsies would be required to assess oligometastatic disease, which is not feasible. Even in cases where such material is available, longitudinal collections would be extremely unlikely. Given our current knowledge of molecular markers in PCa as derived from previous tissue-based approaches, it is timely to develop non-invasive and longitudinal strategies for monitoring patients at all stages of disease.

b. Imaging

Molecular and metabolic imaging by positron emission tomography (PET) using radionuclide-bound tracers is of interest for longitudinal monitoring of treatment responses. Metabolic agents include ^{18}F -FDG (fluorodeoxyglucose; a glucose analogue), $^{11}\text{C}/^{18}\text{F}$ -choline, ^{11}C -acetate, and ^{18}F -fluciclovine (leucine analogue). These have different specificities and sensitivities in different scenarios.¹⁶⁸ For example, FDG marks tumours with high glycolysis, which is relevant in aggressive PCa and mCRPC but not in indolent PCa.¹⁶⁹ Indeed, it is useful mainly for detection of metastases with high glycolytic activity but not for assessing localized disease at diagnosis.¹⁶⁸ FDG

is also of interest for NE tumours, which have higher glycolysis than other mCRPC tumours.^{170,}

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PSMA imaging has now been proven useful for PCa staging, guiding targeted biopsy, and detection of recurrence.¹⁷² However, as previously mentioned, it is not expressed in NEPC and must be used in combination with other imaging modalities; these cases may be positive for FDG uptake. Other imaging targets such as SSTRs may further be used to identify such NE tumours. SST analogue radioligands are currently used in imaging and theranostics of grade 1 and 2 gastroenteropancreatic NE tumours as a first line of therapy with or without cytoreductive surgery, radiation, and additional targeted therapies.¹⁷³ SST analogues have been tested in PCa for imaging and theranostics for NEPC showing promising results, and are now being tested in phase 2 clinical trial (NCT05691465).^{174, 175} A recent report by Pouliot et al. denoted Triple-Tracer strategy against Metastatic PrOstate cancer (3TMPO) suggests “the non-invasive detection of inter-metastatic heterogeneity and transdifferentiation to neuroendocrine prostate cancer by using a multi-tracer PET/CT strategy to further personalise the care of mCRPC patients.”¹⁷⁶

Multiparametric magnetic resonance imaging (mpMRI) is now becoming a second step to identify cases with clinically significant cancer after detection of high PSA, as opposed to those with low-risk disease who would not benefit from a biopsy.^{177, 178} However, mpMRI may have a 5-10% false negative rate.¹⁷⁸ Overall, the main difficulty with imaging modalities for non-invasive molecular profiling is the small number of ligands available to study such a heterogeneous disease.

c. Liquid biopsies

Liquid biopsies are useful for following patients longitudinally, predicting outcomes, and monitoring response to treatments. Blood has been the most popular in cancer research, where

different methods have been described to analyze cancer cells and cancer-related macromolecules non-invasively. Urine has also been considered extensively for urologic cancers.

Commercially available tests concentrate on early diagnosis; they are mainly useful for informing on the need for biopsy and, in cases of negative biopsy, informing on the need for a repeat biopsy. These are mainly RNA-based assays in urine and PSA related assays in blood (Table 5).^{179, 180}

Table 6: Commercially available liquid biopsy tests for PCa diagnosis and prognostication.

Biomarker	Marker	Source
PCA3 (Progenisa)	PCa antigen 3 (<i>PCA3</i>) mRNA	urine
ExoDx (Intelliscore)	Exosomal <i>PCA3</i> and <i>ERG</i> mRNA	urine
MiPS (MLabs)	<i>TMPRSS2-ERG</i> and <i>PCA3</i> mRNA (+ serum PSA)	urine
SelectMDx (MDx Health)	HOXC6 and DLX1 mRNA	urine
4Kscore (OPKO Lab)	Total PSA, free PSA, intact PSA, and hK2 (encoded by <i>KLK2</i>)	blood
PHI (Beckman Coulter)	Total PSA, free PSA, proPSA	blood
Stockholm3	Total PSA, free PSA, hK2, MIC1, MSMB, and 254 single nucleotide polymorphisms	blood

Additional studies have been carried out in whole blood, white blood cells (WBCs), peripheral blood mononuclear cells (PBMCs), plasma, and serum, as well as specific fractions of circulating tumour cells (CTCs), extracellular vesicles (EVs), and circulating cell-free nucleic acids (cfDNA/RNA). These include work on proteins, metabolites, DNA (mutations, genomic alterations, methylation, fragmentation patterns), and RNA (mRNAs, lncRNAs, miRNAs,

circRNAs, RNA modifications). Their relevance is becoming increasingly evident for the diagnosis, prognostication, and monitoring of disease.

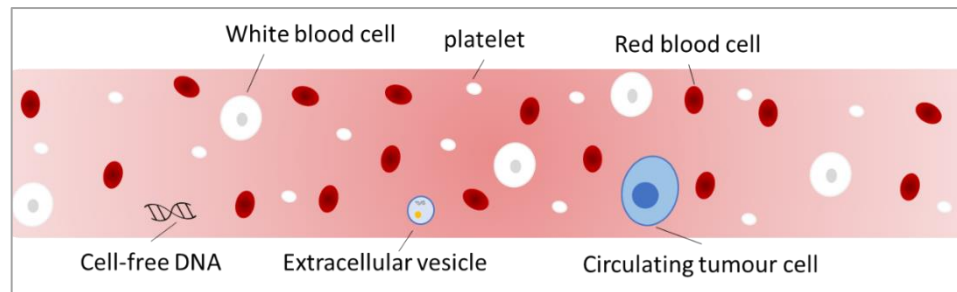


Figure 10: Blood components in cancer patients.

d. Circulating tumour cells

While enumeration and characterization of CTCs is of interest at all stages of disease, results are limited at early stages before detection of metastatic lesions. At diagnosis, CTC detection varied by methodology, from 7.5% to 73% of cases.¹⁸¹ However, detection of CTCs with EMT features has been associated with extraprostatic extension.¹⁸²

Results are more clear at metastatic stages of disease, likely due to the higher number of CTCs. A phase 3 trial showed that CTC enumeration outperformed PSA for prediction of OS and should be considered as a response measure for early-phase mCRPC trials.¹⁸³ Others have also shown the relevance of CTC enumeration with PFS and OS in mHSPC and mCRPC.¹⁸⁴⁻¹⁸⁸ The presence of ARV7 positive CTCs in mCRPC cases is associated with resistance to AR-Is, and patients may instead benefit from taxane treatment.¹⁸⁹⁻¹⁹¹ CTCs in cases with and without NED show different morphological characteristics, which may be useful for treatment decision-making.¹⁹² Additional studies have assessed specific markers on the cell surface, cell viability for functional studies, morphological features, as well as specific proteins, RNAs, and genomic alterations in CTC.¹⁹³⁻¹⁹⁷

While studies in CTCs are promising, there remains a major limitation especially due to their rarity and considerations in isolation methods when trying to assess tumour heterogeneity. Most CTC isolation approaches rely on positive selection, usually based on EpCAM or cytokeratins, although not all CTCs express these markers.¹⁹⁸⁻²⁰⁰ Size-based selection methods are attractive, but would exclude small CTCs, as identified for subsets of NE cells in PCa and in patients with visceral metastases.^{198, 201-204} Negative-selection based approaches (CD45 depletion) are available, but they may exclude circulating hybrid cells and CTCs that cluster with WBCs.^{36, 205} Thus, combinations of multiple methodologies or multiple surface markers to detect all CTCs would be ideal.

e. Cell-free DNA

Cell-free DNA are degraded fragments of DNA released from cells. Circulating tumour DNA (ctDNA) refers to cfDNA released from tumour cells, reflecting their genomic alterations. Analyses of cfDNA can be achieved by targeted PCR or sequencing and low-pass or deep whole genome sequencing (WGS). While targeted approaches can provide specific actionable targets and outcome measures, low-pass WGS can be used to estimate the tumour fraction, and deep WGS can identify genomic alterations in cases with enough ctDNA. Methylation and fragment size and patterns are also of interest.²⁰⁶⁻²⁰⁸

CfDNA amounts, ctDNA fraction, and specific alterations detected in ctDNA are relevant in diagnosis and prognosis of PCa, longitudinal monitoring of minimal residual disease, outcomes at metastatic stages, and development of CRPC.^{195-197, 209-212} Targeted sequencing approaches have shown 80% concordance between cfDNA and matched tissue biopsy at diagnosis of *de novo* mHSPC, while all genomic alterations identified in metastatic tissue biopsies were also identified in matched cfDNA samples.^{213, 214} Specific genomic and epigenomic alterations related to NED have also been found in CRPC ctDNA and confirmed in tissue biopsy.²¹⁵ Concurrent genomic

alteration of AR in cfDNA and expression of ARV7 in cfRNA was related to shorter PFS on AR-Is and taxanes, and shorter OS.²¹⁶ Interestingly, ctDNA fraction increases post-biopsy for PCa, suggesting that this would be opportune timing for ctDNA analysis to identify molecular features and guide treatments.²¹⁷ Chemotherapy also induces rapid increase in cfDNA, correlating with tumour activity on PET/CT.^{218, 219}

f. Extracellular vesicles

EVs are lipid bound vesicles released from cells, which can circulate and be uptaken by other cells as a form of cell-cell communication. Most studies on EVs concentrate on their extensive and complex functions, including roles in PCa progression, formation of the ‘pre-metastatic niche’, angiogenesis, immune response, lineage plasticity, and drug resistance.^{220, 221} The relevance of their specific cargo as biomarkers has also been considered, with the major focus being on proteins and miRNAs. These are summarized in various reviews, but almost all findings need further validation, having been considered only in single studies and not in the setting of clinical trials. The main limitations remaining to be overcome for the clinical use of EV-based biomarkers and therapeutics are the standardization of methods for their isolation, storage, and identification of EVs from cancer cells vs. normal EVs whenever applicable.²²⁰

5. Whole blood RNA as a proxy of molecular changes in patients' tumours

a. Overview of whole blood RNA in cancer

RNAs can be studied in the blood fractions mentioned above, each with their advantages and limitations. While all are informative, whole blood RNA can provide a global view of molecular changes in each patient throughout their disease trajectory, not only representing systemic immunity, but also mirroring changes in the evolving tumours. Testing for cancer-specific genes in whole blood started in the 1990s, usually with article titles referencing CTC detection in blood. These studies used reverse-transcription (RT)-PCR to detect cancer- or tissue-specific genes in blood of cancer patients, focusing on markers completely absent in the blood of healthy individuals. Studies eventually switched to RT-qPCR (real-time quantitative PCR), and later included gene expression microarrays and RNA-sequencing.

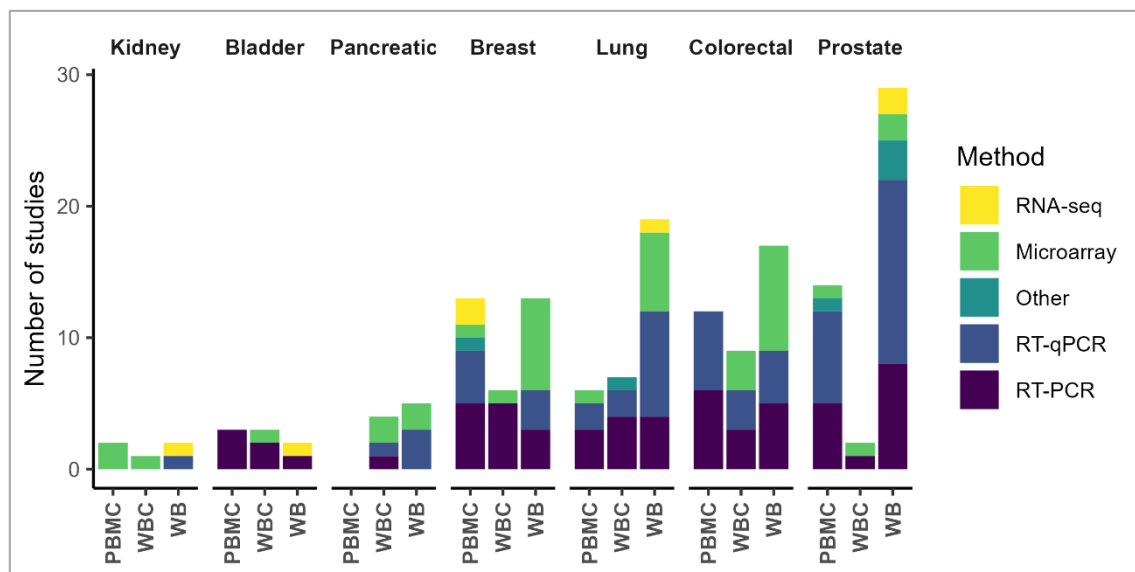


Figure 11: Number of studies in blood RNA by cancer type, blood fraction, and methodology. “Other” methods include four studies by digital droplet PCR and one using a NanoString nCounter panel. PBMC: peripheral blood mononuclear cells; WBC: white blood cells/buffy coat; WB: whole blood.

One of the biggest advantages of whole blood RNA is that it requires no immediate processing; it is usually collected in PaxGene or Tempus tubes containing RNA stabilizing agents. Even with storage up to seven days at room temperature, both types of tubes yield RNA of stable quantity and quality.²²² RNA is stable with up to 7 years of cryopreservation in PaxGene tubes, making it optimal for long-term storage and retrospective longitudinal studies.²²³ Some studies have focused specifically on PBMCs or WBCs instead of whole blood, but these need to be processed immediately upon collection as the cells are not resistant to *ex vivo* changes.^{224, 225}

b. Prostate specific genes in blood

Early molecular studies in the blood of PCa patients used RT-PCR or nested RT-PCR and primarily focused on *KLK3* (encoding PSA), with some interest in *FOLH1* (encoding PSMA) and *PSCA* (prostate stem cell antigen). These genes were popular due to their specificity to PCa and lack of expression in blood of controls.²²⁶⁻²²⁹ Questions were raised about assay specificity, noting the importance of including control blood to rule out false positives.²³⁰ Of the eight articles studying *KLK3* by RT-PCR, three included no controls,²³¹⁻²³³ three included blood of healthy men and women,^{228, 229, 234} and four included cases with BPH.²²⁶⁻²²⁹ The most complete study showed expression of *KLK3*, *FOLH1*, and *PSCA* in the blood of patients with LN and/or distant metastases, with high specificity compared to patients with localized cancer or BPH.²²⁶ The question of false-positive results post-biopsy was also raised, which remains relevant in all cancers.²²⁷

c. Quantitative assessments and new circulating genes as prostate specific markers

With the introduction of quantitative approaches such as RT-qPCR, *KLK3* remained the most common gene studied in whole blood, often included in PCa-specific panels. With the increased sensitivity of quantitative methods, *KLK3* remained consistently negative in controls. At diagnosis, a few cases of BPH were found to express *KLK3* in their PBMCs, but at levels 10-1000x lower

than in *KLK3*+ PCa cases.²³⁵ Few cases have been found positive for whole blood *KLK2* or *KLK3* at diagnosis of localized disease, with no apparent association with unfavorable disease features.^{236, 237} These genes are much more commonly expressed in whole blood of mCRPC cases, with different studies detecting 39-59% positivity for *KLK2* and 32-72% for *KLK3*.²³⁶⁻²⁴³ Both have been associated with CTC counts and OS.^{236, 243} Studies in PBMC RNA also showed a relation of *KLK3* expression with shorter PFS in mCRPC patients on AR inhibitors.²⁴⁴ A longitudinal study of *KLK3* in patients starting first-line abiraterone showed that pre-treatment expression is predictive of PFS; levels decrease with treatment, disappearing in responders but not in all non-responders.²⁴² However, other studies found no significant correlation with PFS or OS for *KLKs*, specifically in advanced cases starting AR-Is or chemotherapy.^{240, 241} Overall, the prognostic and predictive value of *KLK3* as a prostate-specific marker in blood remain to be validated, especially in relation to PSA, and ideally in long-term longitudinal studies. *PCA3*^{242, 245, 246} and *PSCA*^{236, 237} have been considered, showing that they are detectable in blood of PCa patients, but additional studies are needed to understand their prognostic significance and predictive value.

Several groups turned to studying constitutively active ARVs in blood of CRPC cases. In whole blood RNA, *ARV7* has been tested along with *AR* and other variants (*ARV1*, *ARV3*, *ARV9*, *ARV12*).^{240-243, 247-252} One study found that high *AR* and *ARV1* predicted shorter PFS and OS in mCRPC cases starting abiraterone.²⁵⁰ *ARV7* and *ARV12* were found negative in controls and in PCa patients with no evidence of disease, but positive in a subset of CRPC cases, especially in those treated with AR-Is vs. ADT alone.²⁴⁷ Results for *ARV7* have been mixed in CRPC, with some finding it associated with OS and PFS, while others found no significance.^{240-242, 244, 249, 250} Interestingly, there seem to be differences in sensitivity between studies; some found controls to

be completely negative for *ARV7*,^{242, 247, 249, 252} while others found low basal levels of *ARV7* in control blood.^{241, 248, 250} It is thus necessary to add such controls in all studies to set a threshold for overexpression in patients. Altogether, considering only studies that included control blood, *ARV7* has been detected in 11-23% of CRPC cases.^{241-243, 249, 250, 252} Thus, the clinical relevance of *AR* and its variants, especially *ARV7*, remains to be confirmed.

The most common PCa fusion gene, *TMPRSS2-ERG*, has also been studied in blood RNA as a highly specific marker. Despite representing an early alteration in PCa, it has only been studied in mCRPC so far, showing expression in 16-81% for various cohorts.^{240, 241, 246, 253, 254} In patients starting docetaxel, it was found predictive of PFS.²⁵³ Interestingly, all patients positive for *TMPRSS2-ERG* were also positive for *KLK3* in this study. Similar trends were seen for cabazitaxel treated patients. In a follow-up study in patients on taxanes, *TMPRSS2-ERG* status in blood was found predictive of PFS and OS, but only in cases with no prior AR-I treatment; this could be related to epithelial to mesenchymal transition (EMT) or NED in line with AR-I resistance mechanisms, making *TMPRSS2-ERG* status less relevant to disease progression at this stage.²⁵⁴

Considering the heterogeneity of cancer, the inclusion of multiple genes can increase sensitivity and specificity in biomarker studies. For example, in mCRPC cases, expression of *KLK2* and/or *TMPRSS2* was associated with worse OS, with a loss of their expression post-treatment being associated with improved PFS and OS.²⁵⁵

Danila et al. came up with a gene panel based on genes expressed in PCa metastases but negative in control blood and other tissues, ensuring high specificity.²³⁸ They identified a 5 gene panel (*KLK2*, *KLK3*, *HOXB13*, *GRHL2*, *FOXA1*) with 94-100% specificity for progressive mCRPC vs. controls. Kwan et al. expanded on this 5 gene panel, adding *FOLH1*, *NPY*, *TMPRSS2*, *ARV7*, and

ARV9, to address the predictive value of these genes in mCRPC.²⁴⁰ They found that the number of genes overexpressed among *ARV7*, *GRHL2*, *HOXB13*, and *FOXA1* was associated with shorter OS and PFS for patients starting chemotherapy or AR-Is. In a follow-up study, they discovered that *GRHL2* in mHSPC patients was associated with shorter time to CRPC.²⁵⁶ *GRHL2* was also associated with shorter PFS and OS in mCRPC cases on AR-Is, but not those on taxanes; this may be attributable to its role in enhancing AR and ARV transcriptional activity. Todenhöfer et al. also based their study on the initial panel from Danila et al., adding *ARV7* and *TMPRSS2-ERG*.²⁴¹ In mCRPC, having more than 2 positive markers was significantly associated with shorter PFS and OS, but did not remain significant in multivariate analyses. Overall, these studies support the further exploration of prostate- and PCa-specific genes in whole blood RNA as prognostic and predictive markers in advanced PCa cohorts.

Ross et al. took a different approach to identify genes of interest. They chose 168 genes related to immunity or to PCa and studied these by RT-qPCR in whole blood RNA.²⁵⁷ They came up with a signature including three downregulated genes related to cellular and humoral immunity and three upregulated genes related to monocyte differentiation into macrophages. Their six-gene model was predictive of survival in CRPC cases. It is not surprising that none of their chosen PCa-specific genes were included in the final signature, as they intentionally excluded genes that were negative in the blood of normal volunteers.

d. Whole transcriptome approaches in PCa; A focus on systemic immune response

Instead of curating gene lists related to PCa, other groups have used discovery-based approaches, identifying disease-relevant signatures using gene expression microarrays or RNA-sequencing. These are often validated by RT-qPCR. A microarray study comparing whole blood of CRPC vs. PCa cases under active surveillance was able to stratify CRPC cases into two categories, one of

which had significantly worse OS.²⁵⁸ This category overexpressed genes of CD71+ early erythroid cells and under-expressed T-cell function and B-cell related genes. Early erythroid cells are immature red blood cells making up 0.1-1% of PBMCs in healthy adults, but increased in cancer, anemia, infection, and pregnancy; they are immunosuppressive.²⁵⁹ Interestingly, these cases also had higher levels of *ERG*, *AR*, and *KLK3*. Based on these results, they came up with a 9 gene classifier validated by RT-qPCR.²⁵⁸ In line with previous findings, Wang et al. found an increase in myeloid genes and a decrease in lymphocyte genes in CRPC cases with worse outcomes.²⁶⁰ As a result, a new 4 gene model was derived, outperforming both of these previous signatures.

A microarray study of PBMC RNA compared CRPC patients with <5 vs. >5 CTCs.²⁶¹ They identified genes related to cell movement, transport, metabolism, and signaling, including many previously found overexpressed in PCa tissues, such as *ERG*. Some of the identified genes may be from CTCs (e.g. *SELENBP1*), but many also have known functions in PBMCs (e.g. *MMP9*, highly expressed in neutrophils).

A whole blood RNA-sequencing study in CRPC cases pre- and post-chemotherapy compared responders to non-responders.²⁶² They identified genes differentially expressed pre- and post-treatment, which they validated by targeted sequencing. The main genes identified were alpha-defensins (*DEFA1*, *DEFA1B*, *DEFA3*), which were higher in responders than non-responders and increased in responders post chemotherapy. Defensins are mainly expressed in neutrophils and have anti-microbial and anti-tumour roles.²⁶³ However, they can also promote tumour growth in some conditions, and have been found overexpressed in certain cancers.²⁶³ Interestingly, *KLK2* and *KLK3* were also found higher in non-responders pre-treatment, in line with RT-qPCR data showing their expression in blood with progressive disease.

Overall, a combination of PCa-specific and immunity-related genes would be useful as diagnostic, prognostic, and possibly predictive biomarkers.

e. Highlights of whole blood RNA based research in other cancers

Studies in whole blood RNA have also been reported in other cancers, including colorectal, lung, breast, and bladder cancers. Cytokeratins such as *KRT20* were often studied in colorectal cancer, *KRT19* in breast and lung, uroplakins in bladder, and carcino-embryogenic antigen (*CEA*) in various malignancies.²⁶⁴⁻²⁸⁴ A few interesting studies from other cancer types are summarized here.

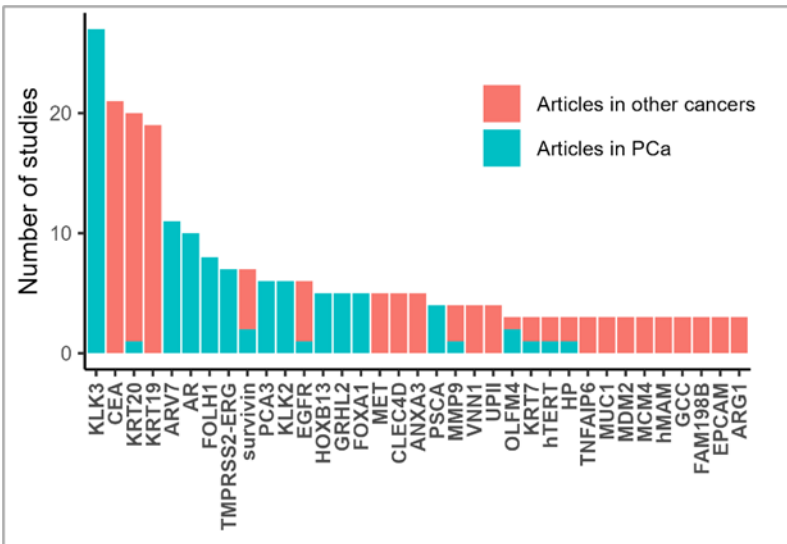


Figure 12: Breakdown of the most commonly studied genes in whole blood RNA by PCa vs. other cancers.

A study of blood RNA-sequencing of metastatic urothelial carcinoma patients identified a DNA replication and cell cycle regulation signature linked to T cell proliferation, which predicted the cases likely to respond to anti-PD1 (Programmed cell death protein 1) checkpoint inhibitors.²⁸⁵ In metastatic clear cell renal cell carcinoma, immune-related genes predictive of shorter OS were identified by RNA-sequencing.²⁸⁶

In breast cancer, common changes were identified in blood related to distinct cancer subtype-specific immune activity at the tumour site.²⁸⁷ A distinct signature was identified in blood of basal-

like breast cancer with low immune activity at the tumour site, along with several immune-suppressive signals in blood for those with 8q amplification. Another study focused on patients up to two years pre-diagnosis of breast cancer compared to controls.²⁸⁸ Genes related to excess risk of metastasis were identified, including: upregulation of plasmacytoid dendritic cell function involved in antigen presentation and coordination of immune responses; upregulation of glutamine metabolism necessary for proliferation and various functions of WBCs; and downregulation of several members of the SLC22 family of transporters (various roles in metabolism and inter-organ signaling).

Whole blood expression profiles specific to NSCLC (non small cell lung cancer) compared to chronic obstructive pulmonary disease (COPD) were enriched mainly in immune-associated genes, and able to differentiate even at stage 1.²⁸⁹ An RNA-sequencing study in NSCLC patients treated with nivolumab (anti-PD1) compared responders vs. non-responders showed that pre-treatment blood of responders was enriched in type 1/2 interferon signaling (involved in antitumour immune response in the tumour microenvironment) and antigen processing and presentation (APP).²⁹⁰ The APP signature was further increased post-treatment in responders, demonstrating the lasting effect of immunotherapy. Interestingly, these were not seen in their tumours pre-treatment, reflecting the immunosuppressive microenvironment.

Few groups have studied whole blood RNA in pancreatic cancers, despite its frequently late diagnosis and subsequent lethality. A microarray study in whole blood aimed to identify genes specific to pancreatic cancer cases with diabetes vs. non-cancer cases with diabetes.²⁹¹ This is of interest, as 68% of pancreatic cancer cases are diabetic,²⁹² such that this approach may ameliorate early pancreatic cancer detection for a subset of patients. The best combination of genes discriminating diabetes in pancreatic cancer vs. non-cancer cases was *MMP9* and *VNN1*

upregulation.²⁹¹ Increased *VNN1* has since been shown to play a role in pancreatic cancer-associated new-onset diabetes, possibly through increasing oxidative stress which leads to islet dysfunction.²⁹³ Although most studies found that *MMP9* is overexpressed in pancreatic cancer, it seems to play both pro- and anti-tumour roles based on studies in patients and animal models.²⁹⁴ Among three microarray studies by different groups in different populations, two identified and chose to validate *VNN1* among their overexpressed genes, and two chose *ANKRD22*. *ANKRD22* is associated with poor survival in pancreatic tumour metastases.²⁹⁵ Further work on these genes and the dysregulated pathways identified could be of interest for early detection of pancreatic cancer.

Various neuroendocrine tumours (NETs) have been studied using the NETest, which was developed in the context of bronchopulmonary and gastroenteropancreatic NETs.²⁹⁶ NETest consists of RT-qPCR based detection of 51 genes in whole blood RNA, aiming to diagnose NETs, monitor progression, and predict therapeutic response, specifically to somatostatin receptor (SSTR) targeting theranostics.

RATIONALE AND OBJECTIVES

There is a clear need for improved patient stratification for the optimal treatment of PCa patients throughout the disease trajectory. Understanding the cell subtypes represented in PCa, the variability within subtypes, and inherent intra- and inter-patient heterogeneity is essential for developing suitable tests to reach this central goal. Furthermore, non-invasive methods are necessary for longitudinal disease monitoring to offer specific treatments accordingly. While liquid biopsies have shown promise for such monitoring, whole blood RNA has not been fully studied in terms of molecular characterization of patients' disease; studies in PCa have focused on genes related to AR-driven luminal-like phenotypes or immune signatures.

Thus, we hypothesise that intra-tumoural cell heterogeneity underlies evolving androgen-dependent and -independent mechanisms of PCa progression and can be traceable in whole blood RNA. Specific objectives are presented in four manuscripts:

Manuscript 1- To explore the phenotypic heterogeneity of PCa through a cell-by-cell quantification of AR to better understand the relationship between epithelial cell subtypes present at different stages of disease.

Manuscript 2- To verify whether intratumoural heterogeneity of PCa can be assessed through genes representing prostate epithelial cell subtypes in whole blood RNA of mCRPC patients compared to healthy controls, and whether this has clinical relevance.

Manuscript 3- To determine whether our strategy can be expanded to include genes encoding drug targets and genes relevant to treatment resistance, and whether it is applicable at diagnosis of localized disease.

Manuscript 4- To verify if the canine DPC-1 model of aggressive PCa is relevant to study the human disease through phenotypic and functional comparisons.

MANUSCRIPT 1: Cell-by-cell quantification of the androgen receptor in benign and malignant prostate leads to a better understanding of changes linked to cancer initiation and progression

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PREFACE

To better understand the contribution of intratumoural cell heterogeneity to clinical heterogeneity in patients, it is imperative to first have a clear understanding of the cell subtypes present in primary tumours. This is especially important in the context of ADT once curative therapies have failed. This was achieved by studying AR expression in the prostate as a whole.

NE cells are rare in the normal prostate, and their number remains relatively low in PCa. Stem cells are believed to be housed in the basal layer of normal glands, but their identification remains elusive. Stem-like cells are present in PCa and remain difficult to identify. Most cancer cells have a differentiated luminal-like phenotype and are AR-positive, as demonstrated by the effectiveness of ADT.

However, despite this crucial role of AR in the normal prostate and in PCa, the literature on its expression patterns is heavily inconsistent, often seeming contradictory. While some suggest that the proportion of AR-positive cells and/or AR expression level increase in PCa compared to the normal prostate, others suggest the opposite or no difference. AR remains the main therapeutic

target at all stages of the disease. Thus, it is essential to better understand its expression patterns to discern why such discordant results exist in the literature.

Manuscript 1 presents a thorough literature review on AR expression and a comprehensive quantification of AR protein expression in the prostate and PCa. This includes epithelial cells of the benign and malignant prostate and surrounding fibroblasts in tissues from different categories of PCa patients. A comparison between AR and its constitutively active variant (ARV7) were included, as well as a comparison between detection of all forms of AR (N-terminal) or only full-length AR (C-terminal). The clinical relevance of AR expression patterns was assessed and confirmed through a newly defined “AR Score”. AR-negative cancer cells were characterized by further staining with markers of NE and stem cells.

CONTRIBUTION OF AUTHORS

Seta Derderian: Performed immunostaining and cell-by-cell quantification of AR N-terminal and C-terminal in cases where staining by both antibodies was compared (a TMA of paired benign and cancer tissue and true BPH and section of ADT treated cases). Performed statistical analyses of AR expression patterns and defined of an “AR Score”. Analyzed AR expression in relation to patients’ clinical data for the McGill RP cohort. Optimized and stained tissue from ADT-treated cases using antibodies for ARV7 and NE and stemness markers for identification of AR- cells. Performed bioinformatic analyses for ARV7 expression in PCa datasets. Wrote the manuscript and participated in all steps up to publication.

Tarik Benidir (McGill MSc student): Performed optimization, immunostaining, and cell-by-cell quantification of AR N-terminal on the McGill RP TMAs and section from other categories of patients. Analyzed results as a function of grade and treatments. Edited the manuscript.

Eleonora Scarlata: Studied prostate tissue sections to generate TMAs. Analyzed stained sections and counting of cells to validate data of T. Benidir. Analyzed cancer cells stained for NE and stem cell markers. Reviewed images for publication. Edited the manuscript.

Turki Altaylouni (McGill MSc student): Optimized AR N-terminal and C-terminal with dilutions of antibodies to determine the best concentration enabling optimal staining over a range of concentrations.

Lucie Hamel: Periodically updated patients' clinical data.

Fatima Zahra Zouanat: Cut tissue sections and optimized staining methods.

Fadi Brimo, McGill Professor and Genito-urinary pathologist at the MUHC: Analyzed stained sections to confirm the identity of cells under study. Edited of the manuscript.

Armen Aprikian, McGill Professor and Uro-oncologist at the MUHC: Analyzed and confirmed patients' data. Edited the manuscript.

Simone Chevalier, Senior Scientist at the Research Institute of the MUHC: Planned and directed the research project and supervised Seta Derderian, Dr Tarik Benidir, and Dr Turki Altaylouni. Participated in all steps of the project, including setting up methods and determining clinical relevance of the results and their value in the field of PCa. Edited the manuscript and participated in all steps up to publication.

TITLE PAGE

Cell-by-cell quantification of the androgen receptor in benign and malignant prostate leads to a better understanding of changes linked to cancer initiation and progression

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ABSTRACT

The androgen receptor (AR) plays a crucial role in the development and homeostasis of the prostate and is a key therapeutic target in prostate cancer (PCa). The gold standard therapy for advanced PCa is androgen deprivation therapy (ADT), which targets androgen production and AR signaling. However, resistance to ADT develops via AR-dependent and -independent mechanisms. As reports on AR expression patterns in PCa have been conflicting, we performed a cell-by-cell AR quantification by immunohistochemistry in the benign and malignant prostate to monitor changes with disease development, progression, and hormonal treatment. Prostates from radical prostatectomy (RP) cases, both hormone-naïve and hormone-treated, prostate tissues from patients on palliative ADT, and bone metastases were included. In the normal prostate, AR is expressed in >99% of luminal cells, 51% of basal cells, and 61% of fibroblasts. An increase in %AR- cancer cells along with a gradual loss of fibroblastic AR were observed with increasing Gleason grade and hormonal treatment. This was accompanied by a parallel increase in staining intensity of AR+ cells under ADT. Staining AR with N- and C-terminal antibodies yielded similar results. The combination of %AR- cancer cells, %AR- fibroblasts and AR intensity score led to the definition of an AR Index, which was predictive of biochemical recurrence in the RP cohort and further stratified patients of intermediate risk. Lastly, ARV7+ cells and AR- cells expressing neuroendocrine and stem markers were interspersed among a majority of AR+ cells in ADT cases. Altogether, the comprehensive quantification of AR expression in the prostate reveals concomitant changes in tumour cell subtypes and fibroblasts, emphasizing the significance of AR- cells with disease progression and palliative ADT.

INTRODUCTION

Prostate cancer (PCa) is the most common non-cutaneous cancer in North American men and among leading causes of cancer mortality. Androgen deprivation therapy (ADT) is administered upon recurrence following potentially curative treatments and in patients with advanced disease at diagnosis. Despite meaningful advances with the use of ADT and second-generation androgen receptor inhibitors (ARIs), this signaling axis remains the main therapeutic target for the systemic control of disease progression. Unfortunately, these therapies inevitably fail, and patients die from metastatic castration-resistant PCa (mCRPC). Several AR-dependent mechanisms contribute to ARI resistance, such as mutations in the AR gene and genomic amplification, the expression of constitutively active splice variants (ARVs) not responsive to androgens (e.g. ARV7), and androgen-independent activation of AR downstream of growth factor- and cytokine- signaling pathways.¹⁻⁸

Previous reports on AR protein expression in PCa have been conflicting. Most studies show nuclear localization in the normal and malignant prostate, with negligible cytoplasmic staining. Authors have reported increases⁹⁻¹¹ or decreases¹²⁻¹⁵ in expression in PCa compared to normal glands. While in some instances, high AR expression correlates with unfavorable clinicopathological parameters and predicts disease progression or PCa specific death,^{14,16,17} the opposite^{10,18} or no prognostic significance^{9,11,15} were also demonstrated. Moreover, reports on percentage of AR positive/negative (%AR+/-) cells in PCa are contradictory, with various groups showing %AR- cells decreases^{11,19} or increases^{12,20} compared to normal luminal cells. The %AR- cells may be predictive^{20,21} or not^{12,22} of earlier BCR and disease aggressiveness²³. These discrepancies may be explained by differences in cohort size, quantitative vs. semi-quantitative methods assessing AR expression, and epitopes recognized by AR antibodies.

Reports on AR expression in fibroblasts showed that fibroblastic AR regulates the production of several growth factors fulfilling multiple functions in the prostate, including the maintenance of homeostasis by controlling epithelial cell proliferation and differentiation.²⁴⁻²⁷ The altered stromal microenvironment also allows for malignant cell growth and invasion.^{28,29} Indeed, loss of fibroblastic AR occurs concomitantly with glandular breakdown, the inability of cancer cells to differentiate or further dedifferentiation.³⁰⁻³²

The present investigation aimed to perform a quantitative analysis of AR expression in all cell subtypes of the prostate in men with benign prostatic hyperplasia (BPH) or PCa and in bone metastases. It led to the definition of an AR index (using %AR- cancer cells, AR staining intensity, and %AR- fibroblasts) which was applied to study a radical prostatectomy (RP) cohort. Moreover, we compared the detection of AR full-length and ARV7 on consecutive sections. Finally, we assessed the presence of AR- neuroendocrine and stem cells through specific markers. Such a comprehensive cell-by-cell approach with multiple markers is worth considering to better understand progression based on AR expression throughout disease states.

METHODS

Inclusion of patients

The main cohort comprised 332 consenting patients who underwent RP between 1993-2008 at the McGill University Health Center. Their clinicopathological characteristics are summarized in Table 1. Biochemical recurrence (BCR) following RP was defined as two consecutive rises in blood prostate specific antigen (PSA) >0.2 ng/ml. Patients (n=35) were excluded from survival analyses if they had received neoadjuvant hormone therapy (NHT), if no prostate PSA data was

available at follow-up, or if they had failed surgery (PSA detectable post-surgery). The final RP cohort consisted of 297 cases.

This study also included the following categories of cases: transurethral resection of the prostate (TURP) for symptomatic relief of urinary obstruction attributed to benign prostatic hyperplasia (BPH; n=10); cases having received 3 months of NHT prior to RP (n=11); advanced TURP cases under palliative ADT (n=26); and bone metastases (n=13) from patients with advanced disease.

Tissue Processing and Immunohistochemistry (IHC)

Formaldehyde-fixed paraffin-embedded (FFPE) blocks were retrieved from the Pathology department at the MUHC. Tissue microarrays (TMAs) were built for primary tumours of de-identified RP cases and bone metastases. Tumours were represented by 1mm duplicate cores. Benign cores were included for 62 hormone-naïve RP cases. Details on the TMAs are available in Supplemental Methods.

IHC was performed using the following antibodies: AR N-terminal (aa 299-315; Thermo Fisher Scientific; clone 441; 1/250), AR C-terminal (aa 900-920; Abcam; ab227678; 1/250), ARV7 (Revmab, 31-1109-00; 1/500), SOX2 (SRY-box 2; Cell Signaling Technologies; 3579; 1/2000), Chromogranin A (CHGA; Dako; DAK-A3; 1/300), and Synaptophysin (SYP; Abcam; ab32127; 1/1600). The IHC protocol is detailed in Supplemental Methods. Antibodies were first tested at several dilutions on consecutive sections. For double staining (DoubleStain IHC Kit: M&R on human tissue; Abcam), sections were stained with AR N-terminal or CHGA (DAB) antibodies, scanned, and re-stained with SYP or AR C-terminal (AR/Red) antibodies.

Quantitative Scoring

Staining was assessed at 40x magnification using an inverted microscope (Olympus IX-81). Stained slides were scanned using an Aperio image scanner system for further visualization and image acquisition. Additional details are provided in Supplemental Methods. Benign foci from hormone-naïve RP cases were noted as benign “far” in cores with no tumour cells (>1mm) or “close” for benign areas in tumour cores (<1mm) obtained from different blocks. Fibroblasts were counted as positive/negative. Nuclei from luminal, basal, and cancer cells were counted as positive or negative. Positive nuclei were scored by intensity (1=weak, 2=moderate, 3=strong) to derive a score differing from H score (%AR- cells was not included) where i represents the staining intensity (1-3):

$$\sum_{i=1}^3 i * \left(\frac{\# \text{ cells at intensity } i}{\text{total } \# \text{ positive cells}} \right) = \text{Intensity Score}$$

Statistical Analyses

AR expression was analyzed using Mann-Whitney U test or Kruskal-Wallis H Test and Dunn-Bonferroni post hoc test. Survival analyses (Kaplan-Meier, Cox uni- and multivariable, Harrel’s C-index) were used to determine clinical significance. Best cutoffs for dichotomizing AR expression were identified using Cutoff Finder.³³ Validation methods for cutoff selection and Cox analyses are described in Supplemental Methods. Markers were compared to clinicopathological parameters using Fisher’s exact test and unpaired t-test. The Cancer of the Prostate Risk Assessment Post-Surgical score (CAPRA-S) was calculated.⁽³⁴⁾ All statistical analyses were carried out in R.

Bioinformatic analyses

Gene expression microarrays were accessed through Gene Expression Omnibus for the Stanford (GSE3933), Cambridge (GSE70770), and MSKCC (GSE21032) PCa datasets. RNA-sequencing results were authorized and accessed through dbGaP for SU2C (phs000915.v2.p2) and GDC for TCGA (phs000178.v11.p8). Details are presented in Supplemental Methods.

RESULTS

Quantification of AR expression

Previous studies on AR expression by IHC have shown conflicting findings, which are summarized in Supplementary Table (S)1. Authors using the AR clone 441 antibody tend to show an increased %AR+ cells and AR staining intensity in PCa compared to benign. Several studies showed different ranges for %AR+ luminal cells,^{11,19,35} others grouped luminal and basal cells,^{9,36} and some found completely negative benign areas in PCa.³⁷ As such discrepancies may be due to differences in IHC protocols, AR staining was first optimized by antibody titration. Consecutive sections from tumour tissues of three PCa patients (one GS7 and two ADT) were stained at different dilutions with an AR N-terminal antibody detecting full-length protein and most variants. As shown for an ADT case in Figure 1A, staining intensity increased with higher antibody concentrations. Similar trends were observed for the three cases. We chose to use the lowest concentration at which the %AR- nuclei remained constant (1/250), while minimizing background and allowing for optimal AR detection at varying staining intensities for accurate quantification. These conditions were next applied to sections of all abovementioned tissues. Results are summarized in Table S2, with representative images presented in Figure 1B-H.

AR negative cancer cells and fibroblasts increase with grade and hormonal therapies

The %AR+/- cells in the epithelium was first assessed in BPH and benign foci from prostate of the RP cohort. Approximately 50% of basal and >99% of luminal cells were AR+, with no difference between true BPH and benign foci from cancer (Fig. 2A, B).

The %AR- cancer cells in this cohort was higher compared to benign luminal cells ($p=2.00E-16$), increasing gradually with Gleason score (GS) up to 6.4% ($p\leq 0.003$ for 3+3/3+4 vs. ≥ 8 ; Fig. 2B). Interestingly, hormone-treated cases showed further increase up to 8.1% ($p=8.1E-10$ vs. treatment-naïve). Finally, the %AR- cancer cells in bone metastases resembled that of hormone-treated rather than hormone-naïve patients ($p=1.20E-03$ vs. hormone-naïve; Fig. 2B). Notably, 3/13 bone metastases consisted of >30% AR- cancer cells.

In the prostatic stroma, inflammatory cells were negative for AR (Fig.1G). Approximately 5% of endothelial cells appeared to be AR+ (Fig. 1H), with heterogeneous staining in the same vessel, showing no clinical relevance (data not shown). However, there were significant changes in fibroblastic AR (Fig. 2C). The %AR- fibroblasts increased from BPH to benign far from cancer ($p=5.16E-03$). This was higher in cancer compared to benign ($p=1.40E-06$), increasing gradually with GS up to 84% ($p\leq 2.94E-03$ for 3+3/3+4 vs. ≥ 8). Loss of fibroblastic AR was prominent upon hormonal treatment (91%, $p=9.70E-10$ vs. RP cohort). Interestingly, only 6/23 ADT cases had >5% AR+ fibroblasts.

Overall, the %AR- epithelial cells gradually increased from benign luminal to cancer cells, increasing with GS, and highest in primary tumours from hormone-treated cases and bone metastases. AR+ fibroblasts are predominant in benign tissue, decreasing in cancer, and often lost upon hormonal treatment.

Cancer cell AR staining intensity increases upon hormonal treatment

We next analyzed nuclear AR staining by intensity scores, calculated from epithelial cells at 1+ to 3+, as described in Methods. Expression patterns for basal cells showed a trend towards more cells with intense staining in cancer cases but not significant in intensity scores (Fig. 2D, E). Luminal cells showed mainly 1+ staining, with more cells at intense staining in benign foci close to cancer, resulting in higher intensity scores compared to benign far ($p=0.006$; Fig. 2F, G).

Cancer cells in the RP cohort resembled benign luminal cells close to cancer in terms of staining patterns and intensity scores, irrespective of GS (Fig. 2F, G). More cancer cells were stained at 3+ in hormone-treated cases, resulting in higher intensity scores compared to hormone-naïve cases ($p=4.6E-06$). Bone metastases were distinct, with a high proportion of cells at 2+ and an intensity score higher than hormone-naïve primary cases, but lower than treated cases ($p\leq 0.015$).

These findings on nuclear AR protein levels were next compared to AR gene expression in five PCa transcriptomic datasets (Fig. 2H). Whereas transcript levels were comparable to benign cases in most RP cases, they were significantly increased in a few cases of $GS \geq 8$, TURP from advanced cases ($p=0.071$), and in metastases, particularly in mCRPC metastases of the SU2C cohort. Taken together, there is an increase %AR- cancer cells, accompanied by an increased staining intensity of AR+ cancer cells and a gradual loss of fibroblastic AR during PCa progression and hormonal treatments. The elevated AR protein levels in cancer cells are in line with overexpressed AR transcripts in advanced disease.

Full-length AR and AR variants are detected in cancer cells

ARVs appear to contribute to PCa progression, especially upon hormone treatment.⁷ Several variants lack the C-terminal domain.⁽³⁸⁾ We thus questioned whether AR C-terminal antibodies

detecting full-length AR display expression patterns similar to N-terminal antibodies recognizing full-length AR and most ARVs, except AR45.³⁸ Following titration experiments (Fig. 3A), the 1/250 dilution of the C-terminal antibody was chosen to stain consecutive cancer sections from a subset of cases. Representative images in Figure 3 (B-E) show nuclear AR in normal and malignant cells and some positive fibroblasts. Cytoplasmic staining was seen but relatively low and constant across cases, therefore the focus was on nuclear AR (Table S2).

Analyses of concordance between N- and C-terminal staining showed similar results for fibroblasts and basal cells for all samples tested (Fig. 3F, G). The %AR- luminal and cancer cells were also similar for most cases (Fig. 3H), except for one bone metastasis which contained a higher %AR- C-terminal compared to N-terminal (45% vs 31%). This suggests that ~15% of these cancer cells express high ARVs levels with lesser full-length AR. Intensity scores were also concordant for most cases (Fig. 3I). No noteworthy differences were observed between categories of cases.

To get insights into expression of ARVs, we opted to investigate the most well-studied transcript ARV7. In transcriptomic datasets, we found that ARV7 is rarely and minimally detected in RP cases, higher in $GS \geq 8$, and common in mCRPC metastases (Fig. 3J). We next compared AR and ARV7 expression for three ADT cases with >10% AR- cells. An example of ARV7+ cancer cells is shown in a tumour area filled with AR+ cells, highly stained with C-terminal and moderately with N-terminal antibodies (Fig. 3K). Another area of the same tissue showing morphologically different AR+ cancer cells was negative for ARV7. Thus, cancer cells expressing ARV7 may represent cell subsets. Altogether, these findings demonstrate similar patterns of AR+ cells detected with AR N- and C-terminal antibodies, which are not suitable to study ARV7. Cancer foci are highly heterogeneous, with AR- cells being scattered among AR+ cells, including subsets expressing ARV7.

Clinical relevance of AR expression

To ascertain the clinical significance of AR expression in cancer, survival analyses were performed in the RP cohort. Optimal cutoffs were determined for dichotomization of variables and were further validated using resampling methods (Fig. S1). Kaplan-Meier analyses showed that high %AR- cancer cells (>5.5%) and high %AR- fibroblasts (>87%) were each predictive of earlier BCR ($p=0.035$; $p=0.00028$; Fig 4A, B). The %AR- cancer cells and fibroblasts correlated with T stage/extraprostatic extension and GS, respectively (Table S3). High AR intensity score showed a trend for worse outcomes but was not significant (Fig. 4C).

Interestingly, these three measures of AR expression were not correlated (Fig. S2). Accordingly, they were combined to reflect changes in AR expression with progression and hormonal treatments. Cases showing at least two of these unfavorable features (high %AR- cancer cells, high %AR- fibroblasts, high AR intensity score) had shorter time to BCR in Kaplan-Meier analyses ($p<0.0001$; Fig. 4D). Thus, we defined an “AR index”, considered “high” for cases with at least two of these unfavorable features, and otherwise considered “low”. A high AR index correlated with high Gleason grade, T-stage, and extraprostatic extension (Table 2). The AR index was significant in univariable Cox analyses and remained significant in multivariable analyses when combined with standard clinicopathological parameters (preoperative PSA, age, Gleason grade, T-stage, surgical margins) or CAPRA-S risk groups as covariates (Table 3). Finally, we found that the AR index further stratifies patients in the intermediate and high CAPRA-S risk groups, but not the low-risk group (Figs 4E, S3m Table 3). The results of these Cox analyses were validated using a bootstrap method (Table 3). In addition, by comparing the C-index for each measure of AR expression, we found that the CAPRA-S score and standard clinical parameters performed better when combined with the AR index (Fig. 4F).

To further understand biological relevance, we analyzed the AR expression patterns of the additional categories of cases included in this study (Fig. 4G). All benign samples had a low AR index, while 82% of NHT and 96% of ADT cases had a high AR index. Notably, only 4% for untreated RP cases scored high for all three unfavorable parameters, compared to 58% of hormone-treated cases.

Altogether, a high %AR- cancer cells, high %AR- fibroblasts, and high nuclear AR expression in AR+ cancer cells are associated with clinical parameters, predict recurrence, and can further stratify patients with intermediate CAPRA-S scores.

Neuroendocrine and stem cells are scattered among AR expressing cancer cells

As AR- cancer cells are clinically significant and increases upon hormone-treatment, we investigated whether they display neuroendocrine or stem cell features. Consecutive sections from ADT cases with >10% AR- cells were double-stained for AR/CHGA, AR/SYP, or for SOX2 alone. Representative images are shown in Figure 5. Various expression patterns were observed, often in adjacent areas of the same tissue. In Figure 5A, a few CHGA+, SOX2+, and SYP+ cells were scattered in a vast area of predominantly AR+ cells. Heterogeneity was noticed within cells of this subtype. Another area with a higher %AR- cells concurrently displayed several CHGA+ and SOX2+ cells (Fig. 5B) but no SYP+ cells in the same field (data not shown).

In another ADT case, adjacent areas in the same piece of prostate tissue displayed strikingly different expression profiles regarding these markers (Fig. 5C). Area 1 contains several SYP+, CHGA+, and SOX2+ cells interspersed between AR+ cells. In the adjacent field (Area 2), no cells are positive for the tested neuroendocrine or stem cell markers. In a third area of this tissue (Fig.

5D), almost half AR⁺ cancer cells formed layers, intermingled with AR⁻ layers of cells uniformly expressing neuroendocrine or stem cell markers. This implies that the three cell subtypes co-exist within the same tumour foci. Collectively, these findings support that the increase in %AR⁻ cells in hormone-treated cases is associated with an increase of neuroendocrine and stem cells scattered among luminal-like AR⁺ cells.

DISCUSSION

IHC studies of AR in the last 30 years have led to contradictory results, some of which are reconciled by the present cell-by-cell quantification of the protein in benign and malignant prostates of patients at different stages of disease and under different hormonal environments. Our findings on nuclear AR levels revealed significant changes in expression during PCa initiation and progression, affected by hormonal treatment. To our knowledge, no such detailed analysis of AR in all cell types has previously been performed. A complete quantitative approach is necessary to understand the relevance of AR, given its expression in various cell types and the use of ADT/ARIs throughout disease progression. The importance of staining intensity and the proportion of AR⁺/⁻ cancer cells would have been missed if results were analyzed solely by H-score. Furthermore, quantification was only possible at high magnification since AR⁺ cells remain prominent even in advanced cases, with variable patterns of AR⁻ cancer cells scattered among them. The low and constant cytoplasmic AR staining was not linked to progression, as previously reported.¹⁰ This does not rule out the importance of cytoplasmic post-translational AR modifications by phosphorylation or interactions with other proteins.³⁹

Optimization of staining protocols was of utmost importance to ensure reliability of expression levels. This minimized background staining, reaching a plateau on the %AR- cells and providing a range of scoring intensities. Indeed, the %AR+/- cells varies drastically in the literature. This may be partly attributed to experimental conditions. Three studies reported %AR+/- cells similar to ours,^{11,23,40} one of which found that %AR+ cells decreases with grade.²³ A decrease in AR+ cells with progression was reported, but overall %AR+ cells was low.^{12,14,20,32} Reports on AR in basal cells range from AR-, <1% AR+, to mainly AR+.^{12,23,32,41} In this study, we detected low levels of AR in most luminal cells in BPH and near half basal cells and fibroblasts (illustrated in Figure 6). AR- cells in the basal layer are comprised of few “true” stem cells, quiescent basal/reserve cells, and few neuroendocrine cells.⁴²⁻⁴⁵ AR in basal cells is likely necessary to regenerate the epithelium through a rapid androgenic response leading to their maturation and differentiation. Fibroblastic AR is consistent with androgens regulating their production of growth factors fulfilling functions such as epithelial homeostasis.²⁵ The situation was different in benign glands of cancer cases, where luminal cells close to cancer exhibited higher AR staining intensity. It is possible that “normal” cells undergo molecular changes not yet detectable phenotypically, as reported in spatially resolved copy number alterations.⁴⁶ This may also be due to factors released by tumour or stromal cells,²⁸ which explains why benign cells farther from cancer are less affected. Although stromal AR is important for carcinogenesis,⁴⁷ it is unclear how or why fibroblasts are progressively AR-. It was reported that AR expression in fibroblasts inhibits their proliferation, which may allow selection for AR- fibroblasts during progression.⁴⁸

In cancer cells of RP cases, we found no change in AR intensity, but a gradual increase in AR- cancer cells with GS. A decrease in AR+ fibroblasts was also observed with progression, in agreement with previous reports.³⁰⁻³² This may play a role in the decrease of AR+ cancer cells,

resulting from the decrease in fibroblastic AR-mediated secretion of paracrine factors controlling PCa cell growth, and leading to reduced luminal differentiation rather than de-differentiation,^{28, 49} affecting the balance of AR+/AR- cancer cells. The lack of correlation between AR intensity, %AR- cancer cells, and %AR- fibroblasts suggests that these are independent, although all contribute to progression.

To date, studies on the clinical relevance of AR expression in PCa were often inconclusive and contradictory. The present findings on AR expression led to an AR index with significant predictive power, regrouping the staining intensity in AR+ cells, the prevalence of AR- cells, and fibroblastic AR loss. Kaplan-Meier, multivariate Cox analysis, and Harrel's C-index demonstrated a strong association of the AR index with earlier BCR and allowed further stratification of CAPRA-S intermediate-risk cases. Further studies are required to verify if its predictive value may be improved by adding other markers, such as AR post-translational modifications, AR interactors, or other factors such as Ki-67, p53, PTEN, MYC, or ERG status.⁵⁰

Mechanisms of AR- cell selection and AR+ cell adaptation in cancer are promoted by ADT, where the proportion of AR- cells increases, AR+ fibroblasts are lost, and AR intensity increases. Figure 6 illustrates how ADT eliminates the fully differentiated luminal-like AR+ cells requiring androgens for survival, leaving behind AR+ cells that progressively adapt and become androgen-independent. This is observed clinically with PSA fluctuations under intermittent ADT until castration-resistance.⁵¹ These AR+ cells grow under the influence of non-androgenic growth-factors, resulting in activated pathways that may be androgen-independent, although cells are not necessarily totally androgen-insensitive or AR-independent, in line with AR crosstalks in diverse signaling pathways.^{1,52} In patients, AR remains targetable in advanced disease, as seen by the

temporary response to ARIs.⁸ Accordingly, it would be of interest to assess whether the AR index predicts response to palliative ARIs.

Our observation of increased nuclear AR intensity under NHT compared to hormone-naïve cases is in line with reports on higher AR and ARV7.^{53,54} This was substantiated in palliative ADT cases, pointing to enhanced expression of AR and possibly its variants, and also demonstrated for AR in bone metastases. This is supported by our analysis of AR and ARV7 transcripts in most advanced PCa compared to benign and primary tumours of several datasets. Of note are reports on approximately 30% of CRPC cases showing AR amplification⁵⁵ and high AR expression in bone metastases being associated with poor prognosis.⁵⁶ Preliminary IHC results on ARV7 in ADT cases show its nuclear expression in subsets of cancer cells within tumour foci comprised of more AR+ cells (N- and C-terminal), implying that differences in staining with the N- and C-terminal antibodies used cannot account for ARV7 expression. Variant-specific antibodies, RNA in-situ hybridization, and spatial profiling would be most informative in this regard.

Our findings on elevated %AR- cancer cells after ADT and in metastases are of particular interest considering the non-curative ADT and ARI therapies. These cells display neuroendocrine and stem-like features, according to CHGA, SYP, and SOX2 expression. Their distribution in tumour foci supports a non-clonal growth of AR- cells, as they are interspersed among AR+ cells. It is unclear if AR+/- cancer cells are resulting from a lack of differentiation, trans-differentiation, or dedifferentiation.⁵⁷ Neuroendocrine cell heterogeneity was noted, suggesting that multiple markers are necessary to study this subpopulation.⁵⁸ As cell patterns were shown to differ between adjacent foci, choosing tumour areas to build TMAs or dissecting cancer cells for further analysis may have substantial consequences. Nonetheless, our observation of different cell subtype patterns within a same patient and among patients is aligned with our recent report highlighting these cell subtypes

in PCa transcriptomic datasets and the traceability and clinical relevance of PCa cell-subtype genes in liquid biopsies of mCRPC patients.⁵⁹

In conclusion, the comprehensive cell-by-cell quantification of AR expression in the prostate, PCa, and bone metastases revealed concomitant changes in epithelial cells and fibroblasts throughout cancer initiation, progression, and treatment pressures. We defined for the first time an AR index which was associated with recurrence and further stratified intermediate-risk patients. Altogether, our findings confirmed the heterogeneity of AR expression in line with the clinical heterogeneity of this disease, further highlighting difficulties encountered with currently available treatments.

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AUTHOR CONTRIBUTIONS

SC design the project and was involved in all steps. SD, TB, ES, TA, and FZ optimized methods, carried out experiments, and analyzed staining data. LH updated clinical data. FB and AA reviewed and confirmed the histopathology of sections and clinical data analyses, respectively. All authors were involved in the writing/revising the manuscript and approved the final version.

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FIGURES

FIGURE 1

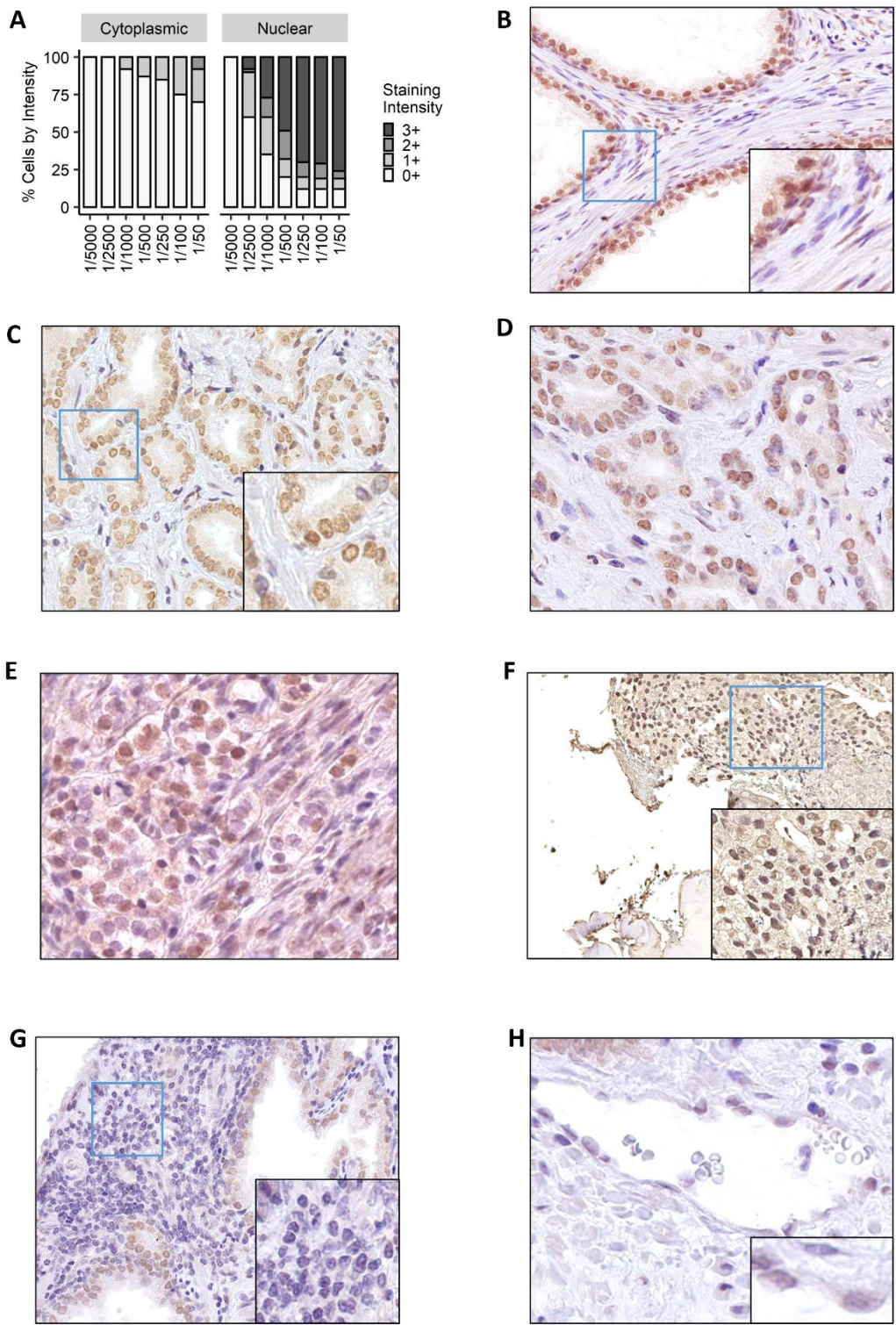


Figure 1: AR N-terminal staining optimization protocol and representative images of different cell types. (A) Stacked bar graph of the percentage of cancer cells at different staining intensities (0-3+) in the cytoplasm and nucleus for increasing concentrations of AR N-terminal antibody. (B-H) Representative images of AR N-terminal staining: (B) BPH (20x, insert at 40x), showing AR+ luminal cells and AR+ and AR- basal cells and fibroblasts; (C) Cancer case (Gleason score 3+4) from the RP cohort showing a majority of AR+ cancer cells with few AR- cancer cells, along with AR- fibroblasts (20x, insert at 40x); (D) Cancer case (Gleason score 4+3) from the RP cohort showing a higher proportion of AR- cancer cells and fibroblasts (40x); (E) Cancer cells in an advanced ADT case with a high proportion of AR- cells interspersed among AR+ cells (40x); (F) Bone metastasis showing AR+ and AR- cancer cells (40x); (G) AR- inflammatory cells in a benign area of a case from the RP cohort (20x, insert at 40x); and (H) AR+ and AR- endothelial cells in a blood vessel of a case from the RP cohort (40x, insert at 80x).

FIGURE 2

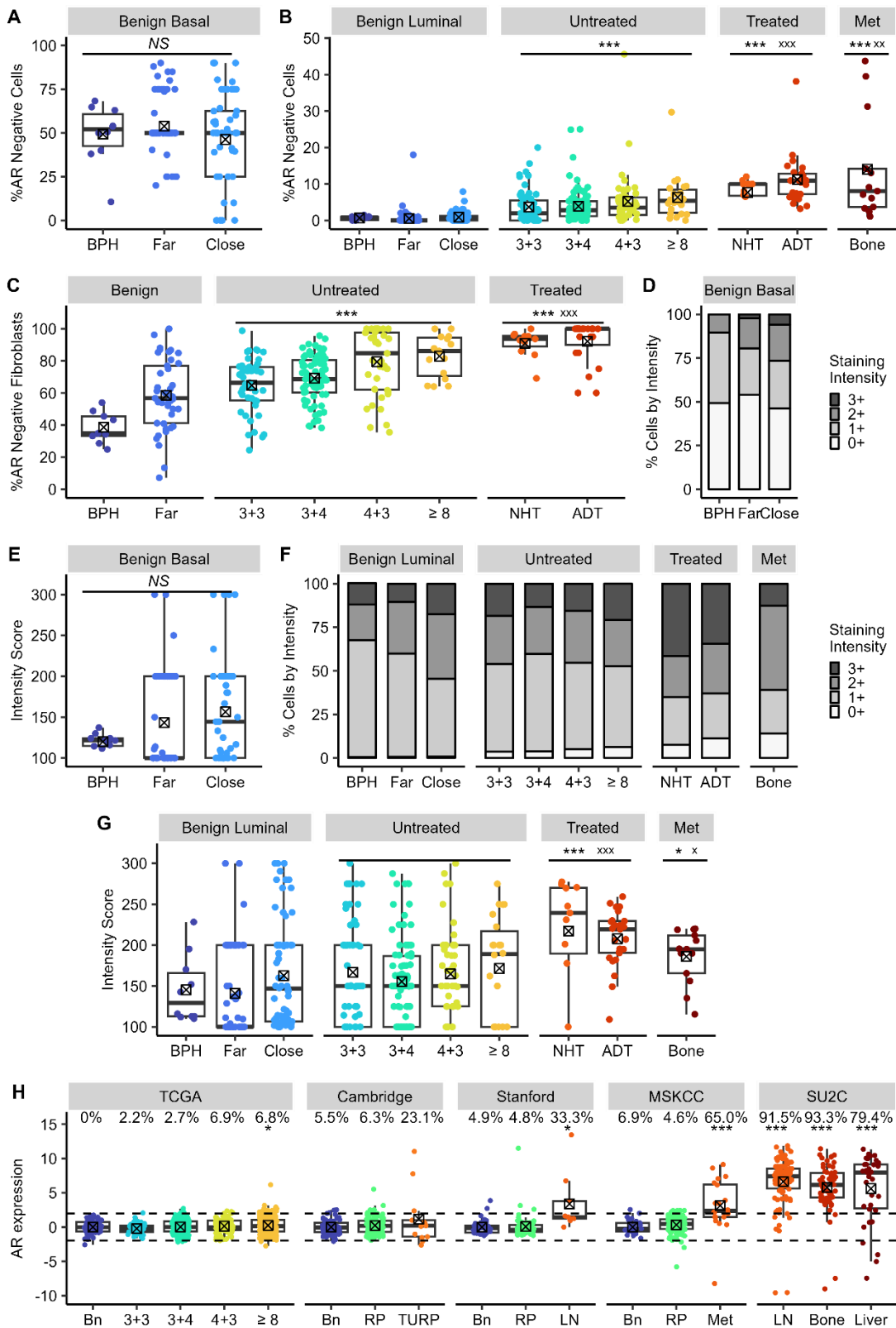


Figure 2: Quantification of AR staining in the normal and malignant prostate. AR N-terminal antibody staining quantified as (A, B) %AR- epithelial/cancer cells, (C) %AR- fibroblasts, and (D-G) AR intensity score (calculated as explained in Methods). Benign areas from cancer cases are separated in far vs. close to cancer foci. Cancer patients are referred to by cases from the RP cohort (hormone-naïve/untreated) further separated by Gleason Score (GS); hormone-treated prior to RP and referred to as NHT and palliative ADT, and bone metastases at advanced stage. (A-C, E, G) Box plots for %AR- (A) basal cells, (B) luminal/cancer cells, and (C) fibroblasts, as well as (G) AR intensity scores for luminal/cancer cells are shown for the different categories of patients. The number of fibroblasts did not change between the different categories of samples studied. All pairwise comparisons are given in these figures for overarching categories (i.e. benign vs. hormone-naïve vs. hormone-treated vs. bone metastases). For p values, * represents significant increase compared to benign, while x represents significant increase compared to hormone-naïve cancer. Significance levels are represented by */x for $p < 0.05$; **/xx for $p < 0.01$; ***/xxx for $p < 0.001$. Stacked bar graphs of the percentage of cells at different staining intensities (0-3+) in (D) basal and (F) luminal/cancer cells in the different categories of patients. (H) Box plots of AR gene expression z-scores in transcriptomic datasets (TCGA, Cambridge, Stanford, MSKCC, and SU2C). Overexpression was defined as +1.96 standard deviations over the mean expression in benign samples from the same cohort (dashed line), as indicated in Methods. For the SU2C dataset, gene expression was compared to benign samples from the TCGA dataset. Denoted above each graph is the percentage of cases overexpressing AR and the Fisher's exact test results comparing the proportion of cases overexpressing AR vs. benign cases (* for $p < 0.05$; *** for $p < 0.001$).

FIGURE 3

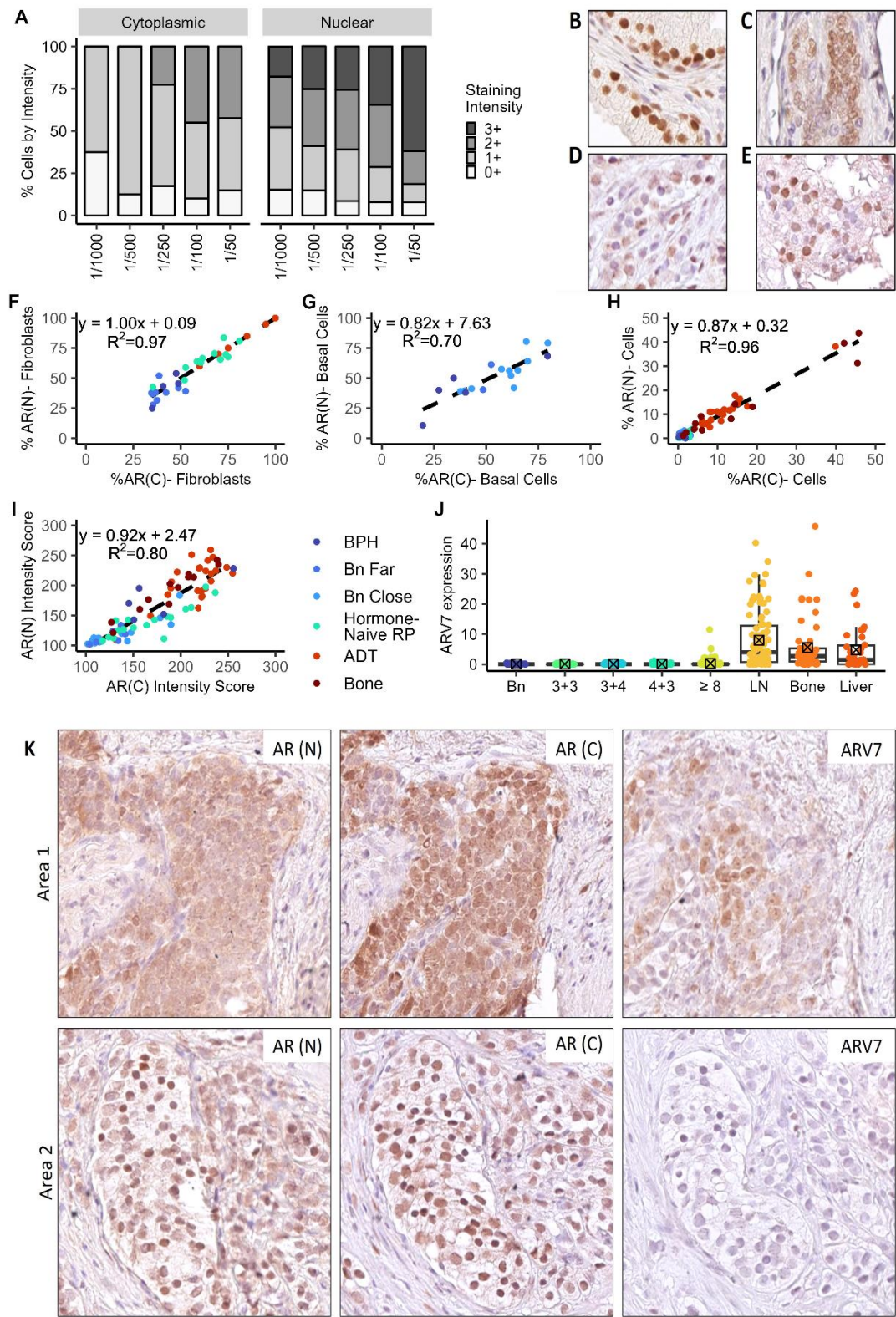


Figure 3: AR staining with N- and C- terminal and ARV7 antibodies. (A) Stacked bar graph of the percentage of cancer cells at different staining intensities (0-3+) in the cytoplasm and nucleus for increasing concentrations of AR C-terminal antibody. (B-E) Representative images of AR C-terminal staining (40x) in: (B) BPH; (C) Cancer from case of the RP cohort; (D) Palliative ADT; (E) Bone metastasis. The color legend is the same for (B-E). (F-I) Correlation between AR N- and C- terminal for: (F) %AR- fibroblasts; (G) %AR- basal cells; (H) %AR- luminal/cancer cells; (I) AR intensity score; (J) Box plots of *ARV7* gene expression (fragments per kilobase of exon per million mapped fragments / FPKM) from the TCGA and SU2C PCa cohorts. (K) Images of two cancer foci from an ADT case showing staining with AR N-terminal, C-terminal, and ARV7 antibodies on consecutive slides (20x).

FIGURE 4

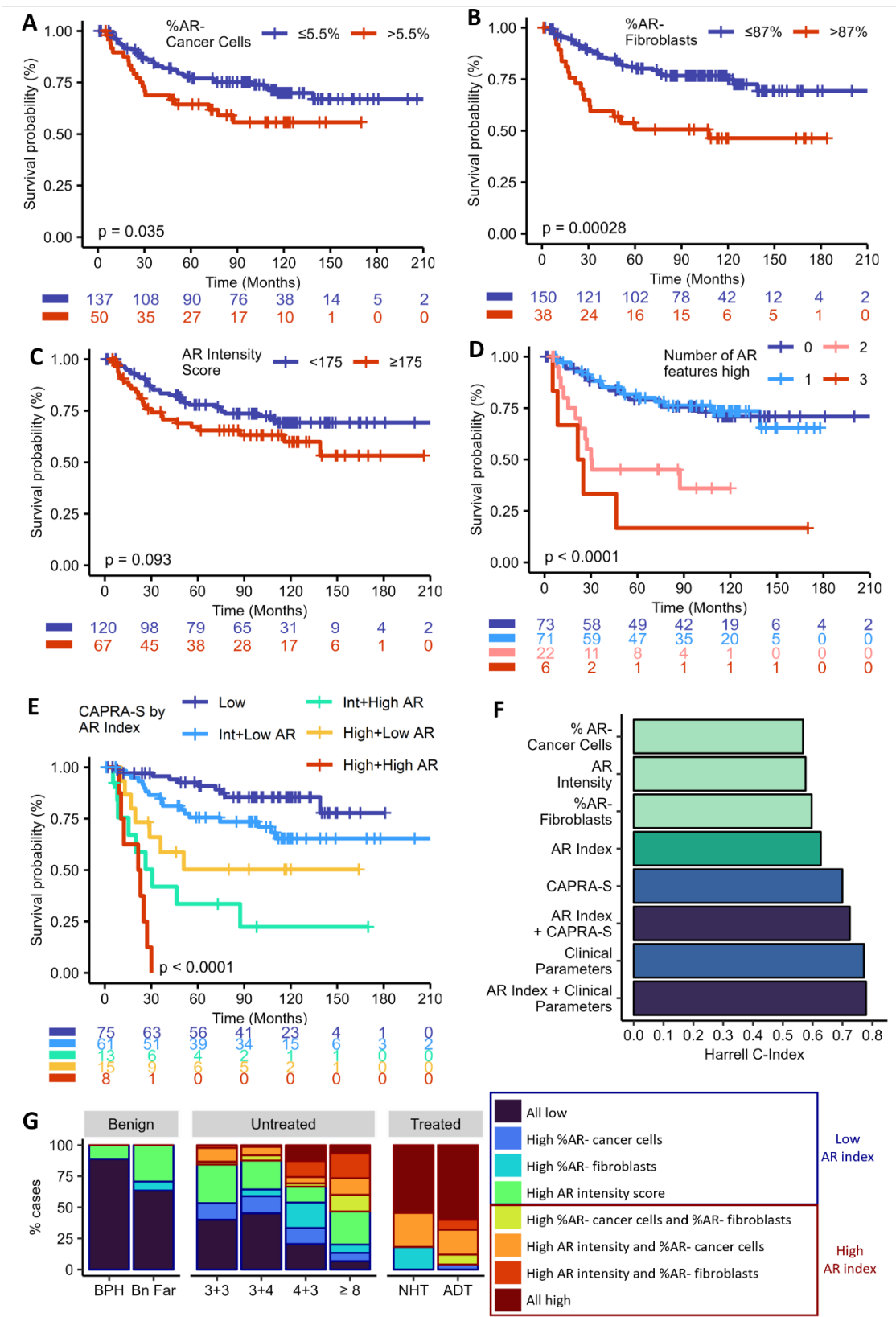


Figure 4: AR is predictive of biochemical recurrence. (A-E) Kaplan-Meier curves with risk Tables for BCR in the RP cohort. (A) %AR- cancer cells $\leq 5.5\%$ vs. $> 5.5\%$. (B) %AR- fibroblasts $\leq 87\%$ vs. $> 87\%$. (C) AR intensity score < 175 vs. ≥ 175 , as described in Methods. (D) Combination of markers: low %AR- cancer cells and low %AR- fibroblasts and low AR intensity score (dark blue); only one marker high (light blue); two markers high (pink); all markers high (red). These were combined into an AR index, where having at least two of these markers high (pink and red curves) represents a high AR index. (E) Combination of AR index and CAPRA-S risk score as: (dark blue) low risk; (light blue) intermediate (int) risk and low AR index; (green) intermediate risk and high AR index; (yellow) high risk and low AR index; or (red) high risk and high AR index. (F) Harrel C-index for (light green) individual measures of AR expression: %AR- Cancer cells, AR intensity, %AR- Fibroblasts; (dark green) AR index; (blue) CAPRA-S risk score, based on combination of clinical parameters: preoperative PSA, age, Gleason grade, T-stage, surgical margins; and (navy blue) AR index in combination with CAPRA-S risk score or clinical parameters. (G) Stacked bar graph of AR expression patterns in all categories of cases. Expression patterns were described as high vs. low %AR- cancer cells, %AR- fibroblasts, and AR intensity, using the optimal cutoffs described above for the RP cohort.

FIGURE 5

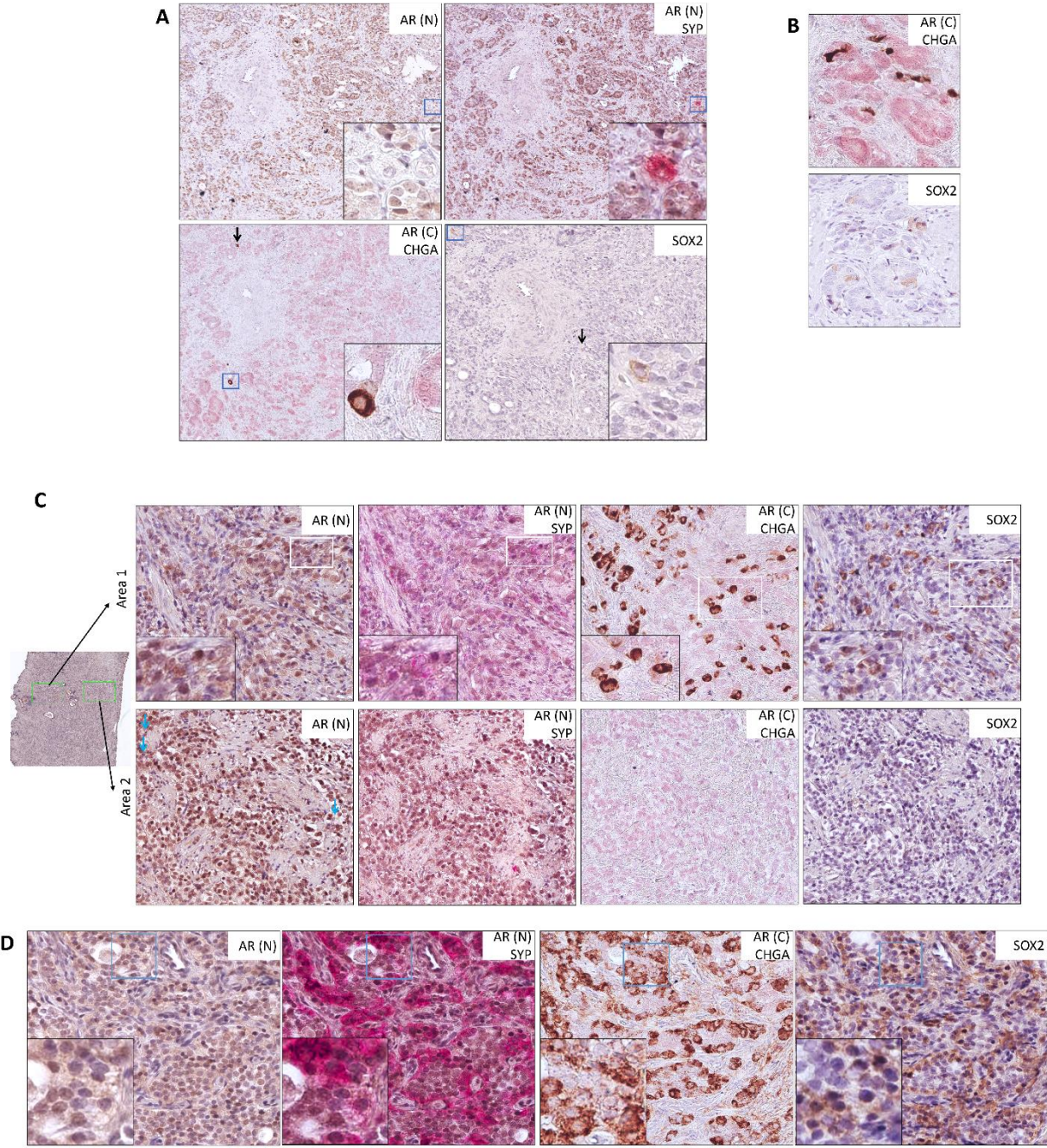


Figure 5: Neuroendocrine and stem cells scattered among AR+ luminal-like cancer cells.

Images of tumour foci on consecutive slides of advanced ADT cases, either dually stained with AR N-terminal (brown) + SYP (red) or AR C-terminal (red) + CHGA (brown), and for SOX2 (brown). (A) Tumour area with high AR cell staining with a few neuroendocrine/stem cells (10x; inserts 40x) within the same field. AR N- and C- terminal antibodies stained most cancer cells. (Top left) An AR N-terminal negative cell (blue box, magnified in insert, 40x), co-stained and positive for SYP (Top right). (Bottom left) Two CHGA positive cells (arrow and one magnified in insert, 40x) and (Bottom right) two SOX2 positive cells (arrow and one magnified in insert, 40x). Overall, these five AR- cells are each positive for only one of the neuroendocrine/stem markers tested. (B) Another tumour area of the same case showing more CHGA stained neuroendocrine cells and SOX2 stained stem cells among AR+ cells (20x). SYP was negative in this tumor foci (not shown). (C) Sections of a second ADT case were similarly stained (20x; inserts 40x). Images of two adjacent areas are compared. Area 1 (top) contains AR- cells, and a high proportion of SYP+ and CHGA+ (neuroendocrine) and SOX2+ (stem) cells among AR+ cells. In contrast, the adjacent Area 2 (bottom) shows only AR+ cells and no SYP, CGHA, or SOX2 staining, despite the presence of a few AR- cells (arrows in left panel). (D) In the same case, another area showed layers of AR+ cells, separated by layers of highly stained CHGA+, SYP+, and SOX2+ cells (20x; inserts 40x).

FIGURE 6

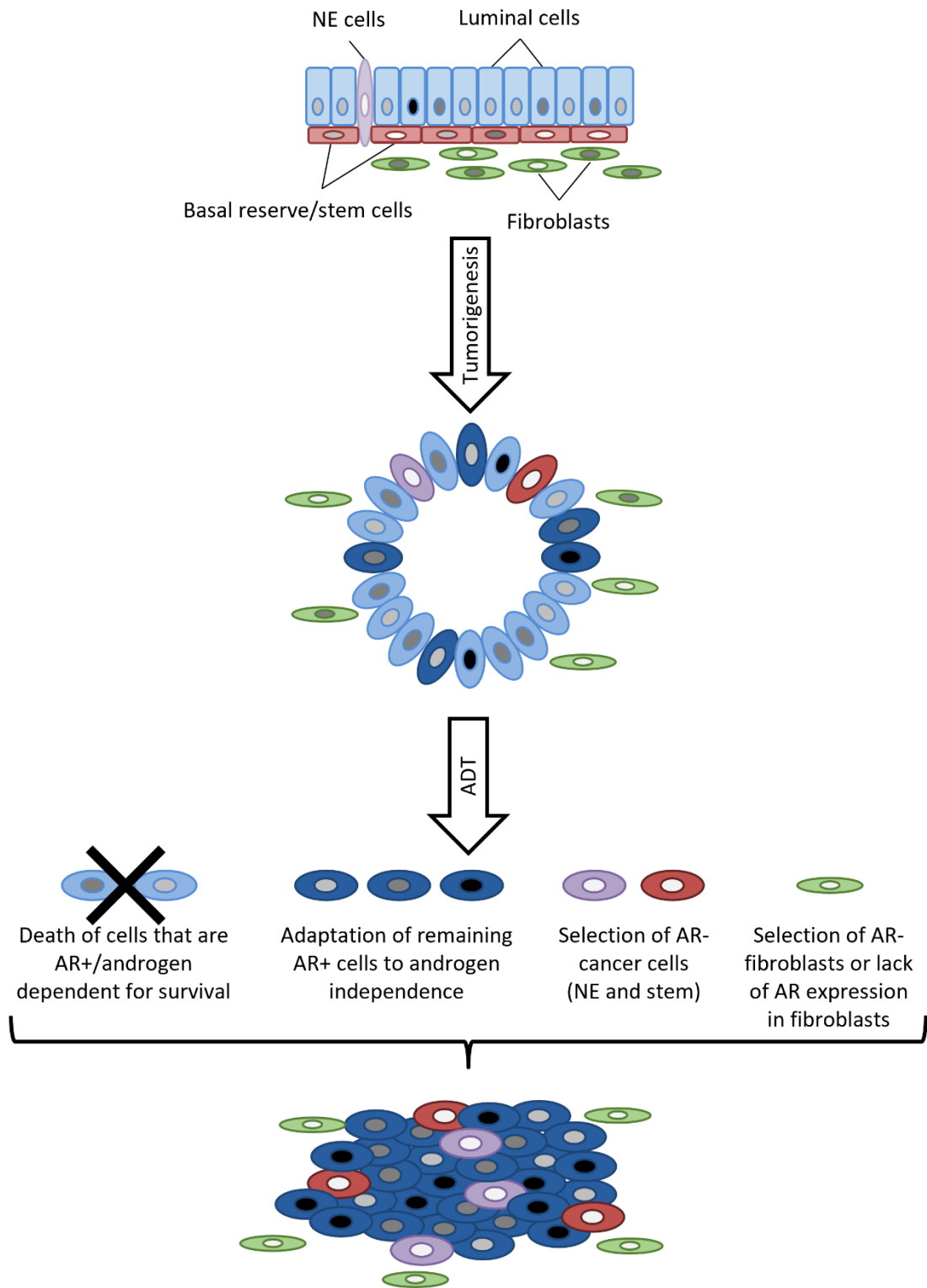


Figure 6: Schematic representation of cells differentially expressing AR in the normal and malignant prostate with changes observed upon progression and hormonal treatments. AR expression is denoted by the color of the nuclei: white = 0+, light grey = 1+, dark grey = 2+, black = 3+. Cell subtypes are denoted by the color of the cytoplasm. (Top) The benign prostatic epithelium is composed of luminal cells (blue), rare neuroendocrine cells (purple), and basal reserve cells (red), including rare AR- stem cells. Fibroblasts (green) are presented in the stroma. Luminal cells AR+, ~50% of basal cells are AR+, while neuroendocrine and stem cells are AR-. Both AR+ and AR- fibroblasts are present in the normal prostate. (Middle) Upon tumorigenesis, the proportion of AR- cells increases and a higher proportion of fibroblasts are AR-. AR+ cancer cells are prominent. (Bottom) With ADT, there is a loss of luminal-like cells that are AR+ and depend on androgens for survival, explaining the observed decrease of blood PSA in patients. Meanwhile, other luminal-like AR+ cells (dark blue) adapt to progressively become less sensitive to androgens and eventually androgen-independent while maintaining AR expression, along with emergence of ARV expression. AR- neuroendocrine and stem-like cells are selected for, while fibroblastic AR is gradually lost. These changes lead to tumour foci composed of varying proportions of cancer cells of the luminal, neuroendocrine, and stem subtypes, as illustrated in ADT cases of Figure 5.

TABLES

Table 1. Clinical Characteristics of the radical prostatectomy cohort.

Parameter	n total	n
Age (years) ^a	332	61 (43-73)
Preoperative PSA ^b	326	8.66 ± 8.28
Gleason grade	332	
Group 1 (GS 3+3)		72 (22%)
Group 2 (GS 3+4)		154 (46%)
Group 3 (GS 4+3)		75 (26%)
Group 4 (GS ≥ 8)		30 (9%)
T stage	332	
T2..		180 (54%)
T2+		40 (12%)
T3a		90 (27%)
T3b		22 (7%)
Positive surgical margin	332	94 (28%)
Extraprostatic extension	332	109 (33%)
Seminal vesicle invasion	332	22 (7%)
Follow-up (months) ^a	297	95 (1-222)
Biochemical recurrence	297	81 (27%)
Distant Metastases	321	18 (6%)

^aMedian (minimum-maximum); ^bMean (± standard deviation). GS: Gleason score; PSA: Prostate Specific Antigen.

Table 2. Correlation of AR index with clinicopathological parameters.

Parameter	Low AR index (n=145)	High AR index (n=28)	p
Age (years) mean ^a	60.5 (±4.95)	61.4 (±3.96)	0.3225
Preoperative PSA mean ^a	7.56 (±4.95)	9.12 (±4.90)	0.1302
Gleason grade			0.0023
Group 1 (GS 3+3)	32 (22%)	3 (11%)	
Group 2 (GS 3+4)	70 (48%)	8 (29%)	
Group 3 (GS 4+3)	35 (35%)	10 (36%)	
Group 4 (GS ≥ 8)	7 (5%)	7 (53%)	
T stage			0.0350
T2..	98 (68%)	13 (46%)	
T3a	40 (28%)	11 (39%)	
T3b	6 (4%)	4 (14%)	
Positive surgical margin	33 (23%)	11 (39%)	0.0949
Extraprostatic extension	45 (31%)	15 (54%)	0.0296
Seminal vesicle invasion	6 (4%)	4 (14%)	0.0580

Categorical variables analyzed by Fisher's exact test. ^aContinuous variables analyzed by unpaired t-test. GS: Gleason score; PSA: Prostate Specific Antigen.

Table 3. Uni- and multivariable Cox analyses of AR expression in the radical prostatectomy cohort

Variable		Univariable		Multivariable		Validation
		HR (95% CI)	p	HR (95% CI)	p	mean HR (95% CI)
<i>Clinical parameters and AR Index</i>						
AR Index		4.24 (2.36-7.62)	1.41E-06	2.87 (1.49-5.48)	0.0015	3.04 (1.44-6.43)
Age ^a		1.02 (0.97-1.08)	0.346	1.00 (0.94-1.05)	0.938	1.00 (0.94-1.06)
Preoperative PSA ^a		1.06 (1.03-1.09)	3.23E-05	1.03 (0.98-1.08)	0.2267	1.03 (0.96-1.11)
Gleason	Group 1-2 (ref)	--	--	--	--	--
	Group 3	3.84 (2.08-7.09)	1.67E-05	2.81 (1.45-5.46)	0.0022	3.01 (1.29-7.04)
	Group 4	8.05 (3.66-17.70)	2.08E-07	3.16 (1.17-8.51)	0.0229	3.52 (0.79-15.63)
T stage	pT2 (ref)	--	--	--	--	--
	pT2+	1.73 (0.64-4.66)	0.2795	1.17 (0.33-4.14)	0.8127	1.07 (0.08-14.62)
	pT3a	2.52 (1.34-4.73)	0.0042	1.36 (0.63-2.89)	0.4241	1.38 (0.55-3.49)
	pT3b	12.41 (5.10-30.21)	2.82E-08	2.88 (0.78-10.57)	0.1118	3.00 (0.52-17.50)
Surgical margin		2.08 (1.18-3.67)	0.0112	1.41 (0.64-3.10)	0.3906	1.47 (0.61-3.56)
<i>CAPRA-S risk score and AR Index</i>						
AR Index		4.24 (2.36-7.62)	1.41E-06	3.74 (2.05-6.82)	1.74E-05	3.90 (2.19-6.95)
CAPRA-S	Low risk (ref)	--	--	--	--	--
	Intermediate risk	3.01 (1.46-6.23)	0.0029	2.92 (1.41-6.04)	0.0038	3.08 (1.36-7.01)
	High risk	8.03 (3.58-18.02)	4.28E-07	7.15 (3.14-16.27)	2.79E-06	7.69 (2.99-19.81)
<i>CAPRA-S stratified by AR Index</i>						
Low risk (ref)		--	--			--
Intermediate risk + low index		2.26 (1.04-4.90)	0.0388			2.31 (1.02-5.22)
Intermediate risk + high index		9.97 (4.03-24.68)	6.55E-07			11.04 (3.89-31.34)
High risk + low index		4.72 (1.79-12.42)	0.0017			4.90 (1.57-15.26)
High risk + high index		29.06(10.60-79.64)	5.79E-11			32.77 (13.35-80.44)

Cox analyses of AR index adjusted for standard clinical parameters or CAPRA-S risk score. AR Index was also used to further stratify CAPRA-S risk groups. Validation results are presented as the mean HR and 95% CI of 300 multivariable analyses. ^aAge and PSA were analyzed as continuous variables. HR: Hazard ratio; CI: Confidence interval; PSA: Prostate Specific Antigen. CAPRA-S score: Cancer of the Prostate Risk Assessment Post-Surgical score.

SUPPLEMENTAL MATERIALS

FIGURE S1

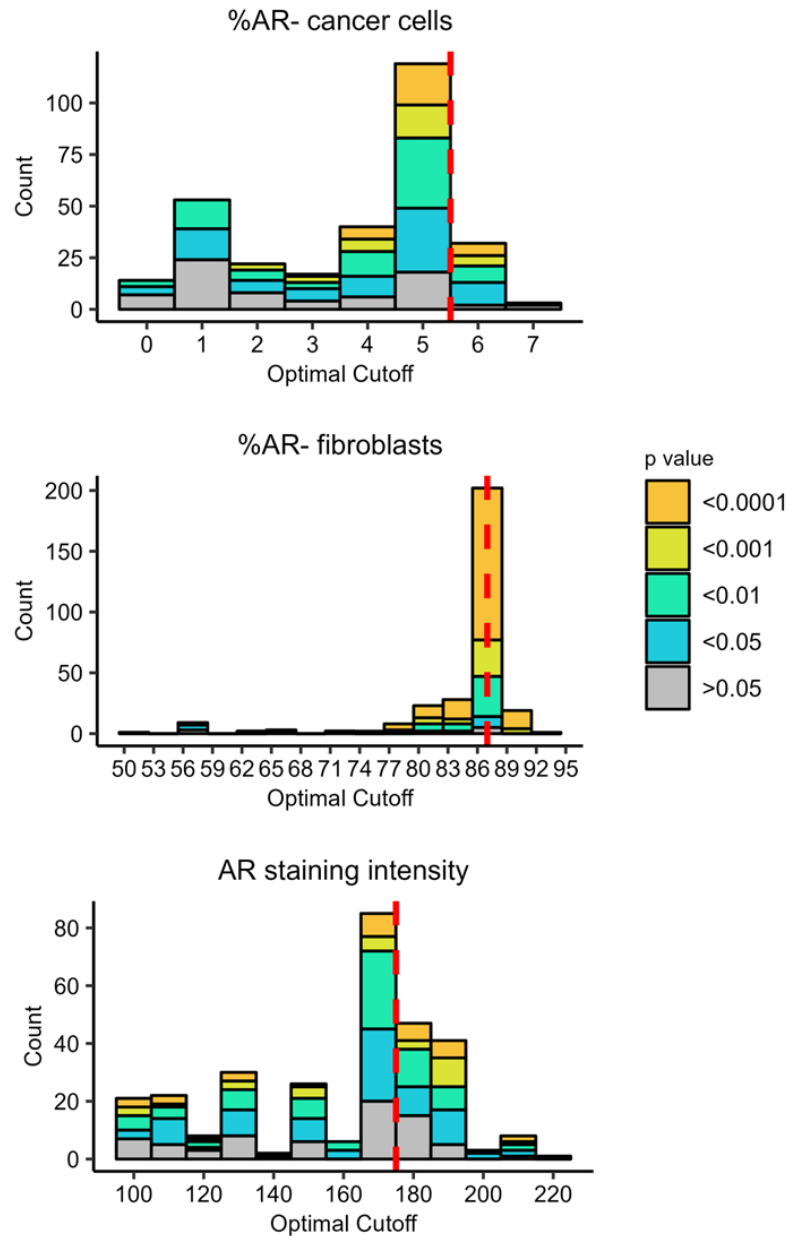


Figure S1: Statistical validation of cutoff points for AR expression. Histograms of optimal cutoff points determined for 300 bootstrap datasets stratified by log-rank p value for %AR- cancer cells, %AR- fibroblasts, and AR intensity score. Red lines represent the chosen cutoff points.

FIGURE S2

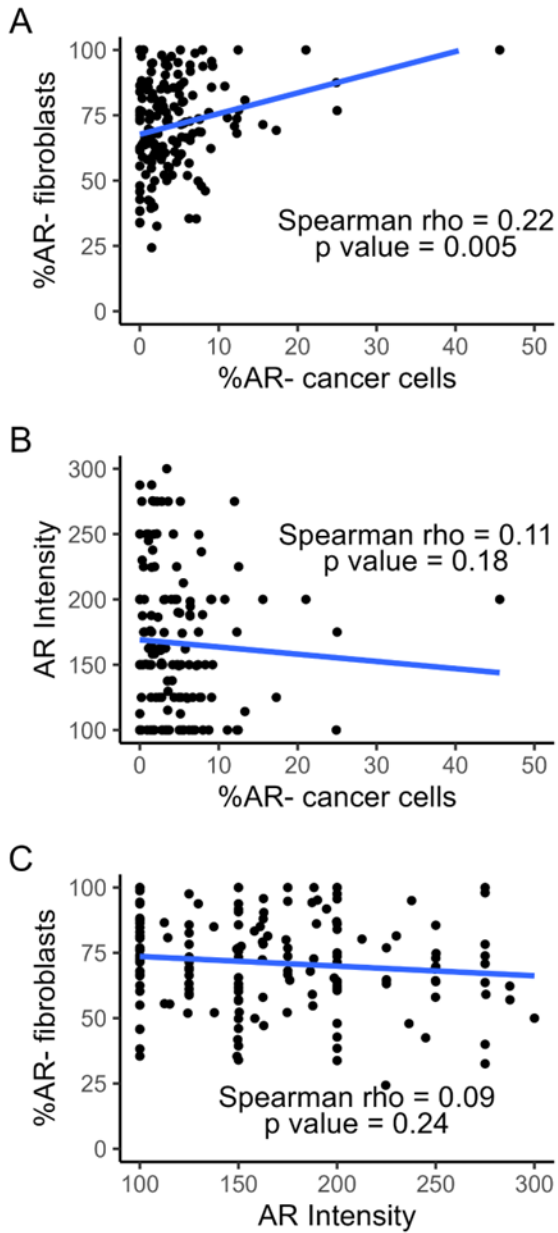


Figure S2: Independence of AR expression features in the RP cohort. Correlations between (A) %AR- cancer cells and %AR- fibroblasts, (B)%AR- cancer cells and AR intensity, and (C) AR intensity and %AR- fibroblasts in the radical prostatectomy cohort

FIGURE S3

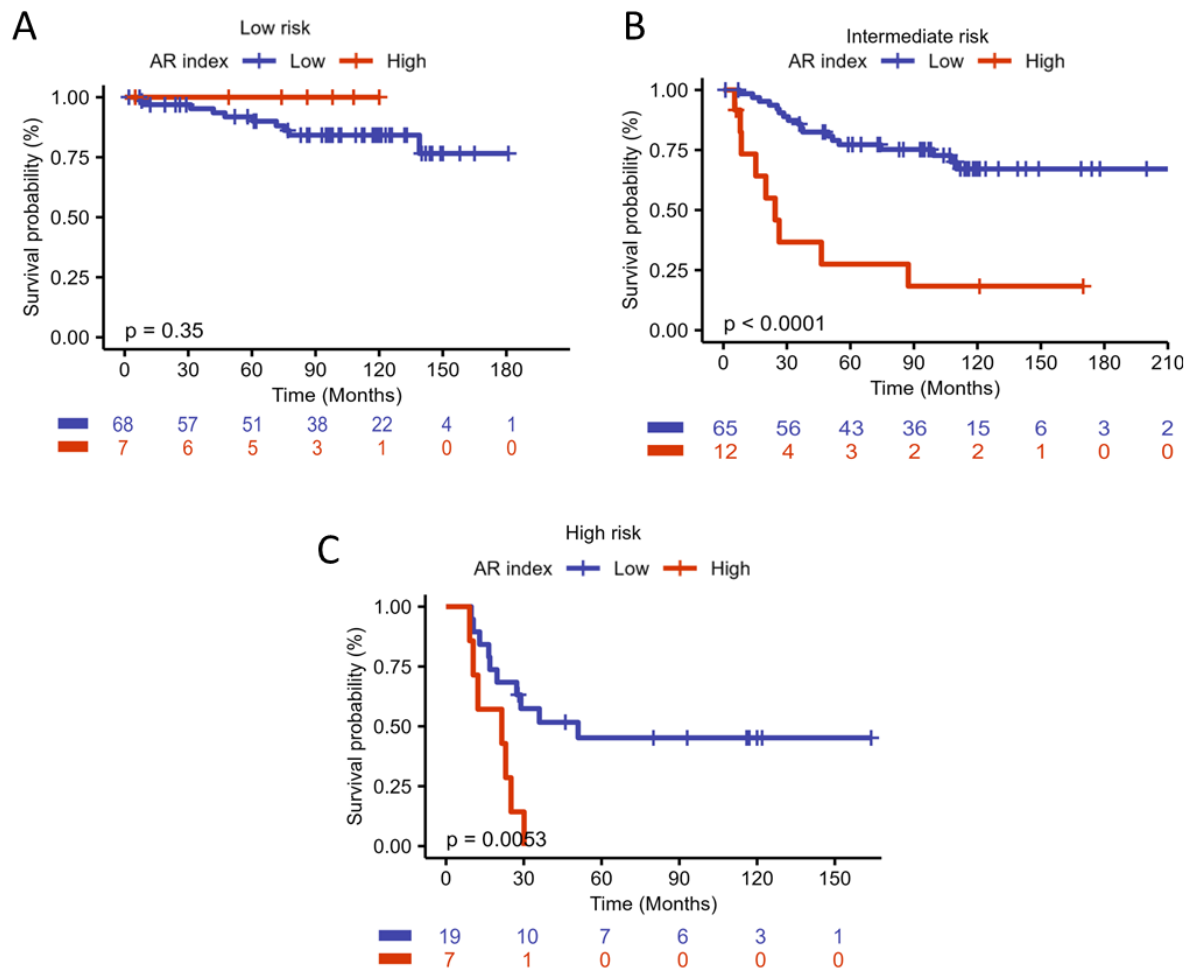


Figure S3: The AR Index further stratifies intermediate and high CAPRA-S risk groups.

Kaplan-Meier curves for biochemical recurrence in the radical prostatectomy cohort stratified by AR index for (A) low, (B) intermediate, and (C) high CAPRA-S risk groups.

TABLE S2. Summary of the cell-by-cell quantification results for AR N-terminal and C-terminal nuclear staining intensity in different categories of patients.

AR N-terminal staining			Nuclear						Fibroblast	
			Patients (N)	% cells by staining intensity				Intensity score	Patients (N)	Negative cells (%)
				0+	1+	2+	3+			
Benign	Basal	Pure BPH	10	49.3	40.2	10.5	0.0	120±10	NA	NA
		Benign Far	74	53.9	26.7	17.1	2.3	143±55	NA	NA
		Benign Close	59	46.2	27.2	20.6	6.0	155±60	NA	NA
	Luminal	Pure BPH	10	0.7	66.9	20.5	12.2	145±39	10	39±9
		Benign Far	74	0.5	60.0	28.8	10.6	150±62	41	59±22
		Benign Close	59	0.7	44.7	37.1	17.5	172±66	NA	NA
Primary tumour	Gleason Grade group 1 (GS 3+3)		59	3.7	50.3	27.5	18.5	167±62	45	65±17
	Gleason Grade group 2 (GS 3+4)		84	3.9	55.9	26.8	13.4	156±51	73	68±14
	Gleason Grade group 3(GS 4+3)		46	5.2	49.4	29.8	15.6	165±54	39	78±20
	Gleason Grade group 4 (GS ≥8)		22	6.4	46.3	26.5	20.9	172±61	15	84±14
	NHT		11	7.7	27.4	23.5	41.5	217±66	11	91±8
	ADT		24	10.1	24.6	29.4	35.9	208±34	24	91±13
Metastatic	Bone		13	14.0	25.1	48.1	12.7	186±32	NA	NA

AR C-terminal staining			Nuclear						Fibroblast	
			Patients (N)	% cells by staining intensity				Intensity score	Patients (N)	Negative cells (%)
				0+	1+	2+	3+			
Benign	Basal	Pure BPH	5	40.2	47.2	12.6	0.0	118±13	NA	NA
		Benign Far	7	55.1	34.8	10.1	0.0	120±17	NA	NA
		Benign Close	17	58.0	36.0	6.0	0.0	112±13	NA	NA
	Luminal	Pure BPH	5	0.9	43.1	34.8	21.3	178±41	4	43±6
		Benign Far	7	1.2	76.1	22.0	0.8	124±14	3	48±7
		Benign Close	17	1.3	75.4	18.2	5.2	129±31	NA	NA
Primary tumour	Hormone-Naïve		17	1.4	45.1	40.6	13.0	167±36	14	65±13
	ADT		26	12.1	23.3	26.6	37.9	215±26	25	92±12
Metastatic	Bone		14	15.3	27.9	42.4	14.4	185±40	NA	NA

BPH: Benign prostatic hyperplasia; GS: Gleason score; NHT: Neoadjuvant hormone-treated prostatectomy cases; ADT: Hormone-treated trans-urethral resection of advanced cases.

TABLE S3: Correlation of %AR- cells, %AR- fibroblasts, and AR intensity score in cancer cells with various clinicopathological parameters.

%AR- cancer cells			
Parameter	Low (n=137)	High (n=50)	p
Age (years) mean	60.3 (± 5.6)	61.7 (± 4.5)	0.0797
Preoperative PSA mean	7.81 (± 5.13)	9.00 (± 7.70)	0.3204
Gleason Grade Group			0.3255
1 (GS 3+3)	30 (22%)	7 (14%)	
2 (GS 3+4)	61 (45%)	22 (44%)	
3 (GS 4+3)	37 (27%)	14 (28%)	
4 (GS ≥ 8)	9 (7%)	7 (14%)	
T stage			0.0433
T2	95 (69%)	25 (50%)	
T3A	35 (26%)	21 (42%)	
T3B	7 (5%)	4 (8%)	
Positive surgical margin	35 (26%)	17 (34%)	0.2720
Extraprostatic extension	41 (30%)	25 (50%)	0.0152
Seminal vesicle invasion	7 (5%)	4 (8%)	0.4884

AR Intensity Score of cancer cells			
Parameter	Low (n=120)	High (n=67)	p
Age (years) mean	60.8 (± 5.6)	60.4 (± 5.1)	0.6423
Preoperative PSA mean	8.41 (± 6.68)	7.61 (± 4.31)	0.3253
Gleason Grade Group			0.3248
1 (GS 3+3)	21 (18%)	16 (24%)	
2 (GS 3+4)	59 (49%)	24 (36%)	
3 (GS 4+3)	31 (26%)	20 (30%)	
4 (GS ≥ 8)	9 (8%)	7 (10%)	
T stage			0.7379
T2	77 (64%)	43 (64%)	
T3A	37 (31%)	19 (28%)	
T3B	6 (5%)	5 (7%)	
Positive surgical margin	32 (27%)	20 (30%)	0.7339
Extraprostatic extension	42 (35%)	24 (36%)	1.0000
Seminal vesicle invasion	6 (5%)	5 (7%)	0.5270

%AR- fibroblasts			
Parameter	Low (n=150)	High (n=38)	p
Age (years) mean	60.4 (± 5.6)	61.6 (± 4.8)	0.2108
Preoperative PSA mean	7.40 (± 5.05)	9.74 (± 6.34)	0.0423 2.30E-07
Gleason Grade Group			
1 (GS 3+3)	41 (27%)	2 (5%)	
2 (GS 3+4)	75 (50%)	9 (24%)	
3 (GS 4+3)	27 (18%)	19 (50%)	
4 (GS ≥ 8)	7 (5%)	8 (21%)	
T stage			0.0900
T2	104 (69%)	20 (53%)	
T3A	41 (27%)	15 (39%)	
T3B	5 (3%)	3 (8%)	
Positive surgical margin	35 (23%)	13 (34%)	0.2110
Extraprostatic extension	46 (31%)	17 (45%)	0.1240
Seminal vesicle invasion	5 (3%)	3 (8%)	0.2042

Categorical variables analyzed by Fisher's exact test. Age and PSA analyzed by unpaired t-test.

GS: Gleason score; PSA: Prostate Specific Antigen.

SUPPLEMENTAL METHODS

Tissue Processing and Immunohistochemistry (IHC)

Formaldehyde-fixed paraffin-embedded (FFPE) archival blocks (1981- 2009) of prostate and bone metastases were collected from the Pathology Department. The Gleason score and tumor stage of each case were reassessed according to the latest International Society of Urological Pathology/World Health Organization recommendations.(1) In tissue microarrays (TMAs), tumors were represented by 1mm duplicate cores extracted from the dominant tumor nodule. A dominant nodule was defined as generally the largest nodule. In cases in which a smaller nodule was considered to be prognostically more significant (higher grade or stage), this smaller nodule was considered to be dominant. Sixty-two cases (18%) also had corresponding benign tissues from a different block represented on the TMAs. TMAs from bone metastases consisted of 0.6 mm duplicate cores.

FFPE blocks were sliced to obtain 4µm sections, deparaffinized in xylene, and rehydrated progressively in ethanol (100%, 90%, 70%) followed by water. Slides were washed in phosphate buffered saline (PBS) between subsequent steps. Antigen retrieval was carried out using 0.2M citrate buffer (pH 6.0) in a pressure cooker for 15 minutes. Endogenous peroxidase blocking was performed using a 3% hydrogen peroxide solution (Leica Biosystems). Slides were then immersed in a protein blocking reagent consisting of normal goat serum in PBS for one hour (Chemicon Millipore) before incubation overnight at 4°C with primary antibodies. This was followed by a biotinylated secondary antibody and streptavidin horseradish peroxidase (HRP) enhancer (Chemicon International, Millipore). The immunoreactivity was visualized by 3,3'-diaminobenzidine tetrahydrochloride peroxidase chromogen (DAB; Nichirei Biosciences). Sections were counterstained with hematoxylin. As a positive control, previously established

hormone-treated specimens with high AR expression were included. Negative controls (no primary antibody) were included on RP specimens.

For FFPE blocks from advanced TURP cases, a few pieces were completely negative for AR and three other markers tested independently (not included in this study). They were considered invalid for assessment due to denaturing effects intrinsic to TURP.

Quantitative Scoring of AR

Assessments were performed blindly by one individual counting a minimum of 50 cells per TMA core and 1500 cells from several fields per TURP case. All cells were counted in each TMA core, including in cores with heterogeneous staining. TMA cores with fewer than 50 cells were not considered. For TURP slices, multiple fields were chosen in which all cells were counted. Randomly selected sections and cores representing 10% of cases for each category were evaluated by a pathology-trained research associate who reached the same conclusions regarding the presence, intensity, and distribution of staining, with confirmation through FB, genito-urinary pathologist of the department of Pathology, at the MUHC.

Validation of Cutoff Values and Survival Analyses

To validate cutoff points for AR intensity score, %AR- cancer cells, and %AR- fibroblasts, 300 bootstrap sample datasets were generated randomly by resampling with replacement. For each bootstrap dataset, the optimal cutoffs were determined by Cutoff Finder (2) along with their log-rank p values. These results were consistent with the initial cutoffs chosen.

For validation of multivariable Cox proportional hazards models, 300 bootstrap sample datasets were generated randomly. Results were presented as the mean and 95% confidence interval of hazard ratios for the 300 sample sets.

Bioinformatic Analyses

For the Stanford dataset, pre-processed data were normalized as initially reported.(3) For the MSKCC and Cambridge datasets, pre-processed data were normalized using LIMMA Bioconductor package in R.(4) For the SU2C and TCGA datasets, fastq and BAM files were processed using the Canadian Centre for Computational Genomics GenPipes RNA-seq pipeline, and count normalization using the DESeq2 and LIMMA Bioconductor packages in R.(4-6) Overexpression was defined as 1.96 standard deviations (95% confidence interval) over the expression in benign samples from the same dataset. For SU2C, overexpression was defined compared to TCGA benign samples after normalization and batch correction. ARV7 expression in SU2C and TCGA datasets was assessed using SGSeq.(7) Fisher's exact test was used to compare AR overexpression by categories of cases for each dataset.

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MANUSCRIPT 2: Liquid biopsy-based gene screening highlights tumor cell subtypes in patients with advanced prostate cancer

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PREFACE

Manuscript 1 confirmed the clinical value of AR expression patterns and showed promising results for further patient stratification at diagnosis. The additional information provided by studying epithelial cell subtypes in early disease and upon hormone treatment substantiates the notion of intratumoural heterogeneity. It shows the adaptation of AR+ cells and emergence of AR- cells as populations evolving with disease progression, and also accounts for their spatial distribution.

As previously discussed, non-invasive approaches are necessary for stratification of patients, especially at advanced stages. Blood collection is non-invasive and can be tested longitudinally to provide a global view of evolving molecular changes in each patient.

In Manuscript 2, we considered whether these cell subtypes are traceable through genes in liquid biopsies. Briefly, we aimed to determine the traceability and clinical relevance of cell-subtype specific genes in whole blood RNA of mCRPC patients. We defined a 14-gene panel representative of luminal, NE, and stem-like cells. We studied the clinical relevance of these genes in whole blood of patients, assessed by determining gene overexpression compared to healthy controls. We addressed obstacles to overcome for testing such markers in blood: the choice of genes and their

relevance to PCa; normal variability between individuals; the choice of cut-off point based on basal expression in control blood; effects of age, white blood cell counts, and biological sex on gene expression to patients' blood. Lastly, the gene panel was validated in various transcriptomic PCa datasets, including samples from different stages of disease.

CONTRIBUTION OF AUTHORS

Seta Derderian: Banked blood from healthy controls. Extracted RNA and performed RT-qPCR of selected genes in blood of patients and healthy controls. Performed statistical analyses of gene expression results compared to healthy controls and in relation to patients' age, white blood cells, clinical parameters, treatments, and outcomes. Performed bioinformatic analyses of the 14 gene panel in PCa transcriptomic datasets. Wrote of the manuscript and participated in all steps up to publication.

Quentin Vesval (Master 2, French system): Participated in the Ethical review process of the project. Enrolled patients and healthy controls and banked blood. Selected genes of interest to test in blood RNA based on cell subtypes. Optimized RT-qPCR assays in cell lines and spiked control blood. Extracted RNA and performed RT-qPCR of genes in blood of patients and healthy controls. Quantified gene overexpression and performed preliminary analysis of data. Collected patients' clinical information. Wrote a first version of a manuscript, re-written by Ms Derderian, and edited the manuscript.

Michel Wissing (McGill post-doctoral fellow): Performed statistical analysis of RT-qPCR results in relation to clinical parameters and outcomes. Wrote and edited the manuscript, particularly in sections relating to biostatistics.

Lucie Hamel: Directly supervised Dr Vesval in the lab for all steps from primer design, blood banking, RNA extraction, and RT-qPCR. Provided PCa cell lines to establish the expression of the 14 genes panel. Performed RNA extraction of samples and establishing sample quality. Periodically updated patients' clinical data.

Nathalie Côté: Enrolled participants and periodically updated patients' clinical data.

Marie Vanhuyse: Oncologist seeing PCa patients at the advanced stage at the MUHC and involved in analysis of clinical data.

Cristiano Ferrario: Oncologist seeing PCa patients at the advanced stage at the Jewish General hospital (JGH) and involved in analysis of clinical data.

Franck Bladou: Urologist following patients under study at the JGH and involved in analysis of clinical data.

Armen Aprikian: Urologist following MUHC patients enrolled in the study and participating in the project as co-investigator. Involved in analysis of clinical data and editing the manuscript.

Simone Chevalier: Senior Scientist at the Research Institute of the MUHC. Planned and directed the research project; supervised Seta Derderian and Dr Q. Vesval. Participated in all steps of the project, from setting up methods to analyzing results, and determining their meaning and value in the field of PCa. Edited the manuscripts from the first version written by Drs Vesval and Wissing and re-writing of a second version by Ms Derderian after addition of new data, up to the publication.

TITLE PAGE

Liquid biopsy-based targeted gene screening highlights tumour cell subtypes in patients with advanced prostate cancer

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ABSTRACT

Prostate cancer (PCa) clinical heterogeneity underscores tumour heterogeneity, which may be best defined by cell subtypes. To test if cell subtypes contributing to progression can be assessed non-invasively, we investigated whether 14 genes representing luminal, neuroendocrine, and stem cells are detectable in whole blood RNA of patients with advanced PCa. For each gene, RT-qPCR assays were first validated using RNA from PCa cell lines, and their traceability in blood was assessed in cell spiking experiments. These were next tested in blood RNA of 40 advanced PCa cases and 40 healthy controls. Expression in controls, which was low or negative, was used to define stringent thresholds for gene overexpression in patients to account for normal variation in white blood cells. 35/40 patients overexpressed at least one gene. Patients with more genes overexpressed had a higher risk of death (Hazard ratio (HR) 1.42, range 1.12-1.77). Progression on androgen receptor inhibitors was associated with overexpression of stem (odds ratio (OR) 7.74, range 1.68-35.61) and neuroendocrine (OR 13.10, range 1.24-142.34) genes, while luminal genes were associated with taxanes (OR 2.7, range 1.07-6.82). Analyses in PCa transcriptomic datasets revealed that this gene panel was most prominent in metastases of advanced disease, with diversity among patients. Collectively, these findings support the contribution of the prostate cell subtypes to disease progression. Cell-subtype specific genes are traceable in blood RNA of advanced patients and are associated with clinically relevant endpoints. This opens the door to minimally invasive liquid biopsies for better management of this deadly disease.

INTRODUCTION

Despite recent therapeutic improvements, prostate cancer (PCa) remains among leading causes of cancer mortality worldwide, with virtually all deaths occurring at the metastatic castration-resistant (mCRPC) stage.¹ Tumour cells surviving androgen-deprivation therapy (ADT) continue to grow despite inhibition of steroid synthesis and androgen receptor (AR) signaling.² Resistance may involve the emergence of constitutively active AR variants (AR-Vs) and/or cell plasticity, a concept based on the relationship between reserve stem, neuroendocrine, and luminal cells in glandular acini, which remain relevant in cancer.^{2,3} PCa is initially defined as well-differentiated adenocarcinomas with predominantly AR-positive luminal-like cells. Neuroendocrine differentiation frequently emerges under ADT and AR inhibitors (ARIs), giving rise to AR-independence/androgen-insensitivity.^{4,5} ADT also favours cells expressing AR-Vs which are linked to epithelial-mesenchymal transition and stemness.^{6,7} Accordingly, cell plasticity and trans-differentiation resulting from treatment failure support the emergence of cancer cells displaying neuroendocrine- and stem-like properties, alongside altered luminal-like cells.

Identifying biomarkers of intra-tumoral cell diversity is critical for offering and developing optimal therapies. A major limitation of testing markers in advanced disease remains the invasiveness of biopsies of metastases. This can be overcome by liquid biopsies, particularly blood which contains extracellular vesicles (EVs), circulating tumour cells (CTCs), and cell-free nucleic acids.^{8,9} CTCs can be isolated by various methods, including antigen-based positive-selection or separation based on their physical properties.^{10,11} However, this often leads to an underestimation of CTCs due to loss of certain subpopulations, as highlighted in PCa.¹⁰⁻¹⁵ Whole blood RNA has been used as an attractive alternative to define gene expression profiles related to progression for diverse cancers, including PCa.¹⁶⁻²¹

In this pilot study, we assessed the (over)expression of 14 genes representing prostate cell subtypes in whole blood RNA of 40 patients with advanced PCa and 40 controls. We observed a variety of gene patterns in blood samples, also validated in PCa transcriptomic datasets. Circulating genes were associated with disease stage, treatment, and survival. Thus, this minimally invasive approach may potentially predict treatment resistance and outcome and refine therapeutic options.

MATERIALS AND METHODS

PCa cell lines

Human PCa cell lines LNCaP (ATCC CRL-1740), DU145 (ATCC HTB-81) and PC-3 (ATCC CRL-1435) were from ATCC (Manassas, VA, USA). NCI-H660 (ATCC CRL-5813) and 22Rv1 (ATCC CRL-2505) were generous gifts from Dr. A. Zoubeidi (Prostate Centre, Vancouver, BC, Canada) and Dr. M. Tremblay (McGill Goodman Cancer Institute, Montreal, QC, Canada), respectively. Cells were cultured according to ATCC recommendations for no more than passage 30, with routine mycoplasma testing (MycroFluor Mycoplasma kit; Thermo Scientific, Waltham, MA, USA).

Patients and healthy volunteers

This study (MP-37-2017-3158) was approved by the Ethics Review Board of the McGill University Health Center (MUHC) and accepted by the Ethic Review Board of the Jewish General Hospital (JGH). Participants voluntarily signed an informed consent. Blood was drawn from 40 CRPC patients at follow-up visits with oncologists (MV, CF), and 40 controls: 16 males below 50 years old, 15 males older than 50, and 9 females recruited among students, personnel, and patients

with no prostate-related disease or cancer. A pseudonymized database was generated from patient medical records.

Blood processing and RNA extraction

Peripheral blood was collected in PAXgene tubes (Qiagen; Germantown, MD, USA), kept at room temperature for 2 hours for cell lysis, frozen overnight at -20°C, and long-term storage at -80°C. RNA was extracted using PAXGene RNA Extraction kits and QIAcube following Qiagen protocols. RNA integrity (RIN) and concentration were determined by BioAnalyzer 2100 (Agilent, Millcreek, ON, Canada) and Nanodrop ND-1000 (Thermo Scientific, Waltham, MA, USA), respectively (RIN average 8.8 for patients and controls). RNA was stored at -80°C.

RT-qPCR

Total RNA (1 µg) was reverse transcribed using SuperScript™ First Strand Synthesis kit (Thermo Scientific, Waltham, MA, USA) with dNTPs (10 mM), oligo(dT)₁₂₋₁₈ (0.5 µg/µL), and water (total volume of 20 µL). The cDNA was stored at -20°C. Primers were obtained from OriGene (Rockville, MD, USA), according to best designed sequences, except *ARV7* for which primers were based on literature (Supplementary Table (S)1).²² Specificity was determined using Primer-BLAST.²³

Each qPCR reaction contained: 10 µL of SsoAdvanced SYBRGreen Master Mix (Bio-Rad, Mississauga, ON, Canada), 0.5 µL each of reverse and forward primers (10 µM), 4 µL of diluted cDNA (1:4) and 5 µL of water. Reactions were run in triplicate in 96-well plates, using CFX96 Touch™ (Bio-Rad, Mississauga, ON, Canada). The program was: 30s initiation at 95°C, 15s

denaturation at 95°C, 15s annealing and elongation at 60°C (58°C for *ARV7*) for 40 cycles. Amplicon length was verified by running qPCR reactions on agarose gels.

The CFX Manager software (v3.1) was used to quantify gene expression. Relative normalized gene expression was determined by the $2^{-\Delta C_t}$ method. Three reference genes were chosen based on previous literature and inter-individual stability verified using GeNorm.^{24,25} RNA from PCa cell lines mixed in equal proportions was used as positive controls and inter-run calibrator for all genes in each plate. Water was used as a negative control. Examples of qPCR amplification are shown (Fig. S1).

Statistical analyses

Overexpression was defined as 2.58 standard deviations (SD) above the mean expression of each gene in blood of volunteers, ensuring with 99.5% certainty that expression in controls was lower than this threshold.

To detect whether patient characteristics or treatments were associated with overexpression of individual genes, all characteristics were dichotomized, and differences evaluated using Chi-square tests in 2x2 tables. PSA levels were compared to *KLK3* expression using the Wilcoxon rank-sum test, due to the non-normal distribution of PSA levels (evaluated using a Shapiro-Wilk test). To adjust for repeated measurements within individuals, multi-level, mixed effects logistic regression was used to identify variables associated with overexpression of luminal, neuroendocrine, and stem cell genes. Survival analyses for disease progression and death were performed using the Cox proportional hazards model. If no event had occurred, patients were censored at the last date they were known to be alive and/or without disease progression, either clinical (worsening of symptoms: fractures, pain), biological (PSA rising in two subsequent

measurements), or radiological (new lesion or increased size of existing lesions). Results with $p < 0.05$ were considered significant. All statistical analyses were performed using Stata v15.0.

Datasets and bioinformatic analyses

Cell line RT-qPCR results were compared to RNA-sequencing from the Cancer Cell Line Encyclopedia Expression Atlas.²⁶ Gene expression data of white blood cells (WBCs) was obtained from the consensus dataset from the Human Protein Atlas (v20.proteinatlas.org).²⁷ Gene expression microarrays were accessed through Gene Expression Omnibus for the Stanford (GSE3933), Cambridge (GSE70770), and MSKCC (GSE21032) datasets. RNA-sequencing results were authorized and accessed through dbGaP for SU2C (phs000915.v2.p2) and GDC for TCGA (phs000178.v11.p8). For the Stanford dataset, pre-processed data were normalized as initially reported.²⁸ For the MSKCC and Cambridge datasets, pre-processed data were normalized using the LIMMA Bioconductor package in R.²⁹ For the SU2C and TCGA datasets, fastq and BAM files were processed using the Canadian Centre for Computational Genomics GenPipes RNA-seq pipeline, followed by count normalization using the DESeq2 and LIMMA Bioconductor packages in R.²⁹⁻³¹ Overexpression was defined as 2.58 standard deviations over the expression in benign samples from the same dataset, except for SU2C which was compared to TCGA benign samples after normalization and batch correction. Fisher's exact test was used to compare gene overexpression by categories of cases for each dataset. Cochrane-Armitage test for trends was used to analyze gene overexpression by Gleason score (GS). Kaplan-Meier survival analysis and uni- and multivariable Cox analyses were performed for MSKCC and TCGA datasets.

RESULTS

Prostate cell-subtype genes are differentially expressed in PCa cell lines and are traceable in blood

Cell-subtype specific genes were chosen based on a literature review encompassing prostate luminal, neuroendocrine (neuroendocrine differentiation), and stem cells. The luminal genes were: *KLK3* (PSA), *ARV7*, *FOLH1* (prostate-specific membrane antigen/PSMA), and *AR*.³² For the neuroendocrine subtype, *SYP* (Synaptophysin), *ENO2* (Neuron Specific Enolase), *VEGFA* (Vascular Endothelial Growth Factor-A), and *NCAM1* (Neural Cell Adhesion Molecule/CD56) were chosen.^{4,33} For stem cells, *ALDH1A1* (Aldehyde Dehydrogenase), *CD44*, *EZH2* (Enhancer of Zeste Homolog 2), *NANOG*, *POU5F1* (Octamer-binding Transcription factor/OCT4) and *SOX2* (SRY/Sex Determining region Y-box 2) were included.³⁴

These genes were first assayed in five PCa cell lines (Fig. 1A). Luminal genes were expressed in 22Rv1 and LNCaP (except 22Rv1-specific *ARV7*) and negative in the AR-negative PC-3 and DU145, whereas NCI-H660 showed low *AR* and *FOLH1* expression. Among neuroendocrine genes, *NCAM1* was mainly expressed in NCI-H660 with low expression in DU145, whereas *SYP*, *ENO2* and *VEGFA* were detected in all cell lines, notably in the neuroendocrine/NCI-H660 model. Similarly, stem cell genes were highest in NCI-H660, but also detected in other cell lines. These results are in line with CCLE RNA-sequencing data (Fig. S2). Altogether, each PCa cell line display some but not all features of pure cell subtypes.

To mimic the situation in patients, fresh control blood was spiked with 10-50 PCa cells (Fig. 1B). *KLK3*, *FOLH1*, and *ARV7* were detected upon spiking with LNCaP or 22Rv1 cells but not in controls, while other genes showed baseline expression in controls which increased upon spiking with 22RV1 (for *AR*) or NCI-H660 cells (for neuroendocrine and stem genes). Higher gene

expression upon spiking blood with few PCa cells suggested that cell-subtype genes may be traceable in blood.

Patient cohort and features

Forty patients with advanced PCa were enrolled in this pilot study; baseline characteristics are displayed in Table S2. Patients were intermediate or high-risk at diagnosis, with 52.5% having metastatic disease at diagnosis. At enrolment, all but one were mCRPC, for a median duration of 19 months. More than half of patients were progressing and 12.5% were on end-of-life palliative care. Ninety percent had received systemic therapy for mCRPC, mainly ARIs and taxanes. Median follow-up was 7 months, during which 12 patients died.

Cell-subtype genes are not related to age or WBCs but to clinical features

Our gene panel was tested in the blood of 40 patients and 40 controls. Analysis of expression in controls showed no differences between males and females (Fig. 1C). Comparisons by age showed increased *CD44* expression in older vs. younger men, with no difference for other genes, despite an increased age-related risk of benign prostatic hyperplasia. Accordingly, stringent thresholds were defined for gene overexpression in patients at the 99.5% confidence interval of expression in 40 controls (Table S3), except for *CD44* whose threshold was set using the 15 older males.

Results of patients and controls are shown in box plots (Fig. 1D). The accuracy, sensitivity, and specificity of identifying patients vs. controls by overexpression of at least one gene were 88.8%, 87.5%, and 90%, respectively. As expected from Figure 1C, there was no signal for *KLK3*, *FOLH1*, and *ARV7* in controls and basal expression for other genes. The mean expression in patients was

significantly higher than controls for 6/14 genes. Nonetheless, 12/14 genes were overexpressed in subsets of cases. *ENO2* and *NCAM1* were not overexpressed in any patients.

As baseline levels of 11 genes in controls suggest expression in WBCs, we investigated these genes in RNA-sequencing data from normal WBC lineages (Fig. 2A). Most genes were negative or minimally expressed, except for *CD44* found at varying levels in all lineages, *ALDH1A1* in monocytes, and *ENO2* and *EZH2* in subsets of lymphocytes.

Patients' WBCs (Fig. S3A) were next analyzed in relation to gene expression in blood. Most genes were not significantly associated to proportions of different WBC lineages, except *SYP* with neutrophils and *CD44* with monocytes (Fig. 2B). However, when genes showing any correlation with WBCs were represented in scatter plots (Fig. S3B), not all patients with high neutrophil or monocyte counts overexpressed *SYP* or *CD44*, respectively. These findings further strengthen the use of stringent thresholds when assessing overexpression in patients' blood. In contrast, 9/14 genes were related to patients' clinical features (Fig. 2C), such as PSA, visceral metastases, ECOG score, line of therapy, progression, surgery, and past or current mCRPC therapies. For instance, *KLK3* expression was associated with higher PSA levels (Fig. 2D) as well as disease progression and death in survival analyses (Table 1). Overexpression of *AR*, *VEGFA*, or *CD44* was also associated with increased risk of death. Altogether, cell-subtype genes are overexpressed in liquid biopsies of advanced PCa patients and gene patterns are associated with clinical features.

Cell-subtype genes in liquid biopsies support phenotypic diversity

The diversity of gene expression in blood among patients is illustrated in Figure 3A by a heatmap. 35/40 patients (87.5%) overexpressed at least one gene. Luminal genes were the most prevalent (62.5%), followed by stem (45%) and neuroendocrine (15%) genes. Overall, 26 unique patterns

were identified, with up to 11 genes in P09. No patient overexpressed all markers of a category, supporting diversity within subtypes. Correlations between genes showed positive trends among luminal genes (Fig. 3B). For neuroendocrine genes, positive correlations were found between *VEGFA*, *SYP*, and *NCAM1*, while *ENO2* displayed a distinct pattern. Among stem cell genes, positive correlations were observed between *SOX2*, *NANOG*, *POU5F1* (co-overexpressed in 22.5% of cases; Fig. 3A) and *CD44*, while *EZH2* was negatively correlated. Interestingly, neuroendocrine and stem cell genes were positively correlated (Fig. 3B).

Classifying patients by cell subtypes and combinations thereof highlighted phenotypic diversity in 8 subcategories (Fig. 3C). In survival analyses (Table 1), patients overexpressing a higher number of genes, regardless of cell subtype, had increased risk of death. This was also true when excluding *CD44* and *SOX2* which were co-overexpressed with *NANOG* and *POU5F1* in several patients. The total number of luminal genes, overexpression of neuroendocrine or stem cell genes, or overexpression of genes from more than one subtype also influenced overall survival.

Further cell-subtype analyses (Table 2) showed that luminal genes were significantly associated with higher PSA (also true for neuroendocrine), higher ECOG score, and progressive disease at inclusion. Thus, analyses of genes individually (Table 1) or by subtype showed similar results. Patients currently on ARIs were less likely to overexpress luminal genes, while patients not on mCRPC therapies were more likely to overexpress these genes. Moreover, patients who previously progressed on ARIs overexpress neuroendocrine or stem cell genes, while those who progressed on taxanes overexpressed luminal genes. Stem cell genes were also more common in patients who received surgery as a curative therapy, and less common in those who received non-curative therapies at diagnosis. These findings illustrate the wide diversity of gene patterns in patient's blood, with all cell subtypes being associated with treatments.

Prostate cell-subtype genes are overexpressed in advanced disease and metastases

To relate our findings to phenotypic changes at different stages of PCa, we analyzed our gene panel in five PCa transcriptomic datasets (TCGA, Stanford, Cambridge, MSKCC, SU2C). Results presented as heatmaps (Figs. S4A-E) show that these genes were not commonly overexpressed in primary tumours of hormone-naïve radical prostatectomy (RP) but increased with GS and progression, such as in lymph nodes (LN) metastases collected at RP, primary tumours from hormone-treated cases (TURP/trans-urethral resection of prostate), and in various metastases.

Looking at the number of genes overexpressed overall in primary tumours (Fig. 4 A-D), we observed that in hormone-naïve RP samples the number of cases overexpressing at least two genes overall increased significantly with GS in all studies, except the MSKCC cohort ($p=0.061$; Fig. 4D). This was also true for the number of luminal or stem cell genes for most studies. Further analyses in TCGA and MSKCC cohorts (Fig. S5) showed trends towards predicting earlier biochemical recurrence for the total number of genes overexpressed and for certain subtypes, but significance was lost in multivariable analyses.

Regarding more advanced disease, the likelihood of overexpressing at least 2 genes overall was significantly higher than in RP samples (Fig. 4B-D). LN metastases at RP overexpressed more stem genes than primary tumours (Fig. 4B), while metastases in the MSKCC cohort showed more cases with luminal and stem genes (Fig. 4D). Hormone-treated TURP resembled high GS cases of the hormone-naïve cohort, but with a significant increase in neuroendocrine genes (Fig. 4C). Samples with the most genes overexpressed were metastases from mCRPC cases (Fig. 4E). Compared to LN and bone, liver metastases overexpressed more genes overall, more neuroendocrine and stem cell genes, and a trend towards fewer luminal genes. Altogether, an

increasing proportion of cases overexpress these genes with further disease progression, while gene patterns remain heterogeneous among patients.

This diversity and evolution of gene patterns by cell subtype with progression is illustrated in Figure 5. Of interest, the pattern of circulating genes in the 40 advanced patients in our pilot study resembled the heterogeneity of advanced disease cases, mostly metastatic. The number of cell subtypes represented in each cohort also increased with more aggressive disease and in liver metastases compared to LN or bone, supporting diversified cell populations in the same tumour. These findings reinforce the relevance of the studied genes in the blood of advanced PCa patients.

DISCUSSION

This proof-of-concept study demonstrates for the first time the clinical relevance of testing PCa cell-subtype genes in whole blood of patients with advanced disease, setting the stage for studies on larger cohorts to trace circulating gene patterns longitudinally.

Several studies on cancer-related transcripts in blood have focused on CTCs. Despite reports on limitations in isolating such heterogeneous cells, most authors use positive selection-based isolation, often by EPCAM or CKs which are not present on all CTCs, or size-based methods which may miss small CTCs.^{9-11,15} Working in blood RNA avoids selecting only specific CTC subsets, while also detecting cell-free RNAs in EVs.³⁵ Cancer-specific genes were identified in whole blood.^{16-21,36-41} The sensitivity of RT-qPCR assays comparing gene expression directly in blood of PCa patients vs. CTCs was also reported to be similar.^{18,42} The central limitation remains genes expressed in WBCs, making gene selection a key step for success. Tracing immune signatures in parallel may provide useful information but was beyond the scope of this study.⁴³

To establish the reliability and specificity of our assays, we first tested each gene in cell lines and spiked control blood. Luminal genes were specific to luminal-like cells, whereas neuroendocrine and stem cell genes were highest in the neuroendocrine NCI-H660 cells. In spiking experiments, luminal genes were negative in control blood, except for *AR* as previously reported.^{36,44} All neuroendocrine and stem genes were detected at baseline levels. Similar expression patterns were also observed using probe-based TaqMan assays (not shown), implying that baseline expression was not due to non-specific amplification. Overall, expression of all genes increased upon spiking with a low number of PCa cells, suggesting their potential detection in patients' blood. *CHGA*, *VIM*, *EPCAM*, *PSCA*, *CD133*, and *ARV12* were considered but excluded due to issues of primer specificity and limitation in RNA quantity from banked blood.

The varying proportions of different WBC lineages in blood of patients showed mostly no or low correlation, except for *CD44* and *SYP*. However, not all patients with high levels of the relevant WBCs showed these genes overexpressed. *CD44* and *SYP* were always overexpressed with other genes of the same cell subtype. These observations highlight the importance of including blood RNA from healthy donors to account for variations in WBCs and accurately identify overexpression related to the patient's cancer by establishing stringent thresholds. For cases where overexpression is close to the threshold, we would recommend testing a second blood sample to assess clinical value.

We observed that 87.5% of CRPC patients (35/40) overexpressed one or more of the target genes in their blood. Cases with no genes overexpressed may be explained by a low number of CTCs and cancer-derived EVs due to less advanced disease or successful therapeutic elimination of cancer cells. RNA quality or quantity was not an issue, being similar across samples. Expanding our gene panel may lead to significant findings in these patients as well. Overall, patients showed

a wide phenotypic diversity in line with the clinical heterogeneity of advanced cases and reported for circulating genes in breast, colorectal, lung, and prostate cancers.^{16-21,37-39}

Among luminal genes, *KLK3* expression was significantly associated with high levels of PSA in blood, suggesting that PSA reflects tumour burden, while circulating *KLK3* would originate from CTCs or EVs. Of note, *KLK3* expression in 16/40 (40%) of mCRPC cases is not paralleled by *AR* overexpression. Testing *AR* amplification in circulating tumour DNA, expression of other ARVs, or additional *AR* target genes may be alternatives to assessing *AR* reactivation. *ARV7* was found in 8/40 (20%) of cases, in line with reported values ranging from 11-68%.^{16-18,36,45} This is clinically meaningful since taxanes may be more effective than ARIs in such patients.^{18,46,47} Other *AR* variants would be interesting to add, given their clinical relevance.^{18,36}

Among stem cell genes, *NANOG*, *POU5F1*, and *SOX2* were often, but not always, co-overexpressed. These genes have been studied in whole blood of small-cell lung cancer, where *SOX2* was identified as a promising prognostic marker.³⁹ In our PCa series, circulating *NANOG* and *POU5F1* were more often overexpressed than *SOX2*.

Neuroendocrine genes were infrequent and always found with other subtypes, consistent with the rarity of pure neuroendocrine prostate tumours.⁴ *VEGFA* is particularly interesting as a neuro-product promoting metastasis by enhancing PCa cell motility and being targetable in advanced disease.^{33,48} *ENO2* was not overexpressed. *NCAM1* was not overexpressed in patients' blood, possibly due to the diversity of splice variants.⁴⁹ Nevertheless, these neuroendocrine genes deserve attention as CD56+ (*NCAM1*) and SYP+ CTCs were reported in PCa patients.^{14,50}

Although patterns of individual genes are interesting, they are each overexpressed in a minority of patients, making it difficult to interpret gene-by-gene analysis results. For this reason, we consider analyses by cell subtype more relevant and informative. The contribution of different cell subtypes

to tumour development and metastatic progression is important in the context of therapeutic resistance. This was corroborated in our series, despite the low number of patients. Patients treated with ARIs expressed fewer circulating luminal genes, while those previously treated with ARIs overexpressed several stem and neuroendocrine genes. This is in line with hormonal therapies targeting differentiated AR-positive luminal cells and promoting neuroendocrine differentiation and stemness features.⁴⁻⁷ Moreover, the number of genes and cell subtypes represented were associated with an increased risk of death. This suggests less advanced disease or better disease control in patients expressing fewer genes or only luminal genes, while patients with more cell subtypes may be more difficult to treat with single agent therapies. Further studies are needed to evaluate whether this gene panel is of prognostic utility in mCRPC patients.

Bioinformatic analyses in PCa transcriptomic datasets validate our choice of genes and support our clinical findings. Results were consistent among RP cohorts, showing few cases overexpressing the selected genes. They were found more often in advanced cases, especially in mCRPC. Moreover, cell-subtype patterns differed between categories of cases, as well as by metastatic site. Furthermore, the circulating gene patterns of our 40 patients with advanced PCa resemble genes patterns in metastases. Altogether, these findings establish that genes representing epithelial cell subtypes contribute to progression, emphasizing the increasing importance of tumour cell diversity in PCa patients.

The main limitation of this pilot study remains that patients were at various stages of treatments, although they were all CRPC. Comparing circulating gene patterns with the corresponding transcriptomic data of primary tumours may be informative, and more so if adjacent LN metastases are harvested. Unfortunately, these advanced cases were not necessarily treated at our institutions early in their trajectory and no tissue samples were available for this study. Nonetheless, the gene

patterns defined in blood are related to patients' characteristics, previous treatments, and treatment response at time of blood draw, despite the low number of cases tested. This justifies further validation in larger, independent cohorts before translation in clinical practice. We anticipate conducting a study with serial blood sampling to demonstrate whether assessing prostate cell subtypes through liquid biopsies will predict therapeutic response and clinical outcome.

CONCLUSIONS

This pilot study supports the premise that prostate cell-subtype genes are traceable in blood of patients with advanced disease. The diversity among patients is relevant to the clinical heterogeneity of PCa. Our findings revealed associations of cell subtype genes in patients' blood with therapeutic regimens and clinical outcome. Bioinformatic analyses in transcriptomic data from over 1,000 patients confirmed that our selection of genes and overexpression patterns in the blood of 35/40 patients are best represented in metastases, thereby reinforcing the clinical relevance of our findings. Further studies, particularly longitudinal assessments, will determine whether this approach can guide individualized treatments.

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AUTHORS' CONTRIBUTIONS

S.C. designed the research; S.D., Q.V., and L.H. performed the research; S.D., Q.V., M.W., M.V., C.F., and F.B. analyzed the data; N.C. contributed reagents/analytical tools; S.C., S.D., M.W., Q.V., and A.A. wrote and edit the manuscript, also reviewed by all co-authors.

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FIGURES

FIGURE 1

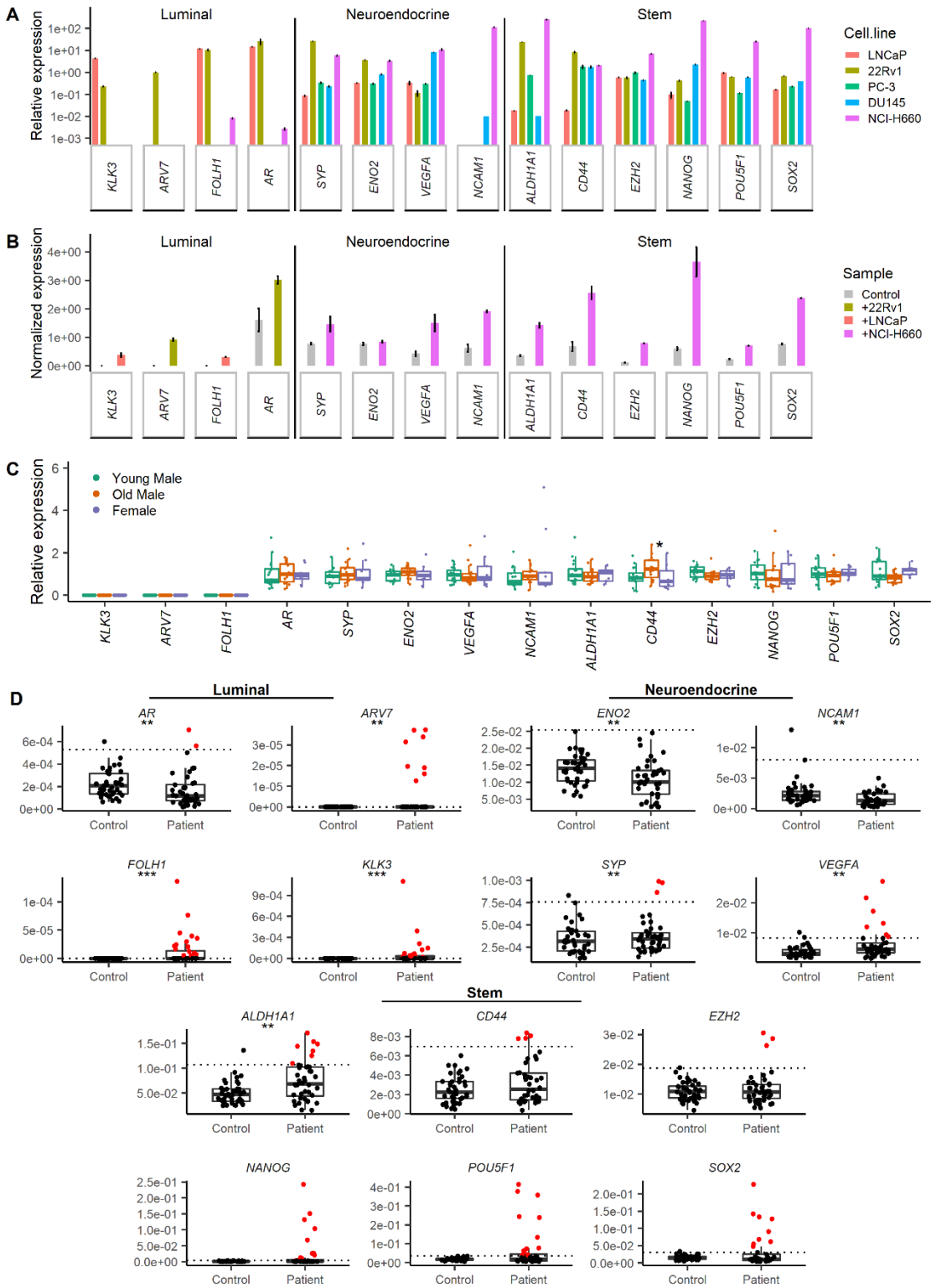


Figure 1. Genes representing prostatic cell subtypes are differentially expressed in PCa cell lines and are traceable in blood. Gene expression assays were optimized in PCa cell lines and tested in control blood spiked with PCa cells before testing in 40 patients and 40 controls. **(A)** RNA was extracted from five human PCa cell lines (LNCaP, 22Rv1, PC-3, DU145, NCI-H660) and reverse transcribed to test our panel of genes by RT-qPCR. Results were expressed for each gene as mean normalized relative quantities on a logarithmic scale. **(B)** Blood drawn from a healthy male in PAXgene tubes (2.5 mL) was spiked with LNCaP, 22Rv1, or NCI-H660 cells. For LNCaP and 22Rv1, 10 cells were added to the blood (equivalent of 4 CTCs per mL of blood). NCI-H660 cells grow as clumps in suspension, therefore we estimated that approximately 50 cells were added to the control blood. Genes were tested in control vs. spiked blood. Results are presented on a linear scale. **(C)** Genes were tested in control blood and compared by age and sex. Relative normalized gene expression is shown in box plots for each gene in male controls by age (< 50 vs. > 50 years old) and female controls (25-70 years old; no difference by age). Wilcoxon rank-sum test is significant ($p < 0.05$) for *CD44* between young vs. older males (*). No significant differences were detected by sex. Similar analysis of gene expression with age of patients also showed no correlation (data not shown). **(D)** Box plots of results in controls and patients for luminal, neuroendocrine, and stem cell genes. Red dots for patients represent overexpression, as defined by values greater than means of controls + 2.58SD (Table S3). Black dots in patients represent expression below cut-off values for each gene (dashed line). Some genes (*KLK3*, *ARV7* and *FOLH1*) are not detected in controls and in subsets of patients. Wilcoxon rank-sum test p-values comparing patients and controls are denoted by * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$ (under gene names).

FIGURE 2

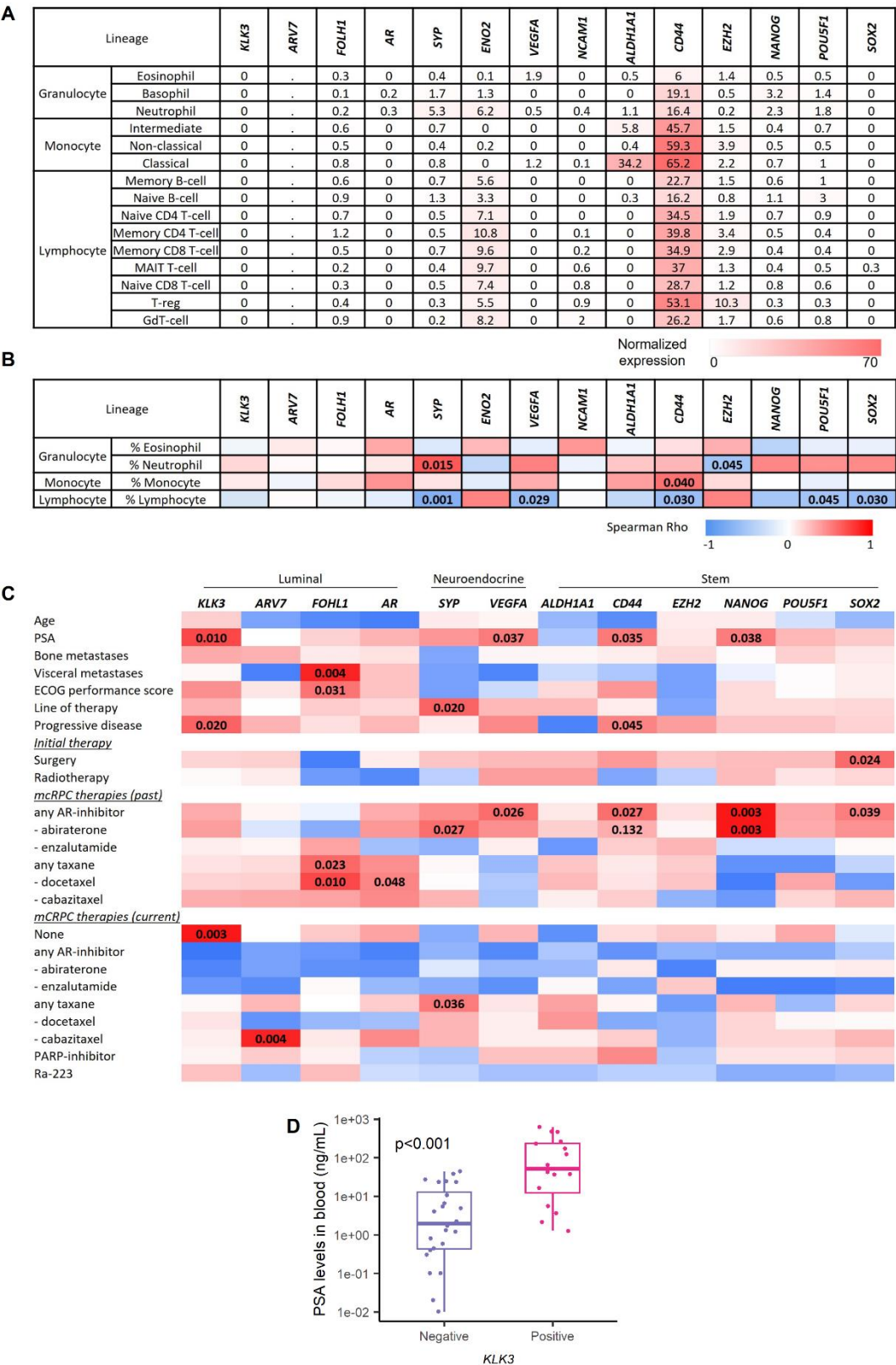


Figure 2. Cell-subtype genes are not related to WBCs but to clinical features of PCa patients.

In (A) are bioinformatic analyses of the 14 genes in normal WBC lineages in consensus data from the Human Protein Atlas (v20.proteinatlas.org), as described in Methods. Normalized expression from 0 (white) to 70 (red) is shown. (B) Spearman correlation matrix between gene expression in the 40 mCRPC patients and their WBC counts at the time of blood draw. Blue represents a negative association, red a positive association. P-values are entered when correlations were significant ($p < 0.05$), and at least > 0.4 or < -0.4 . (C) Heatmap depicting associations between patients and treatment characteristics and overexpression of individual genes. Blue represents a negative association, red a positive correlation. The darker the color, the stronger the association between gene overexpression and patients' characteristics. P-values are entered when the patient characteristic was significantly associated with overexpression of that gene ($p < 0.05$). (D) Association between the detection of the *KLK3* transcript in the blood of patients and PSA levels (logarithmic scale) at time of blood draw. The p-value was calculated using a Wilcoxon rank-sum test.

FIGURE 3

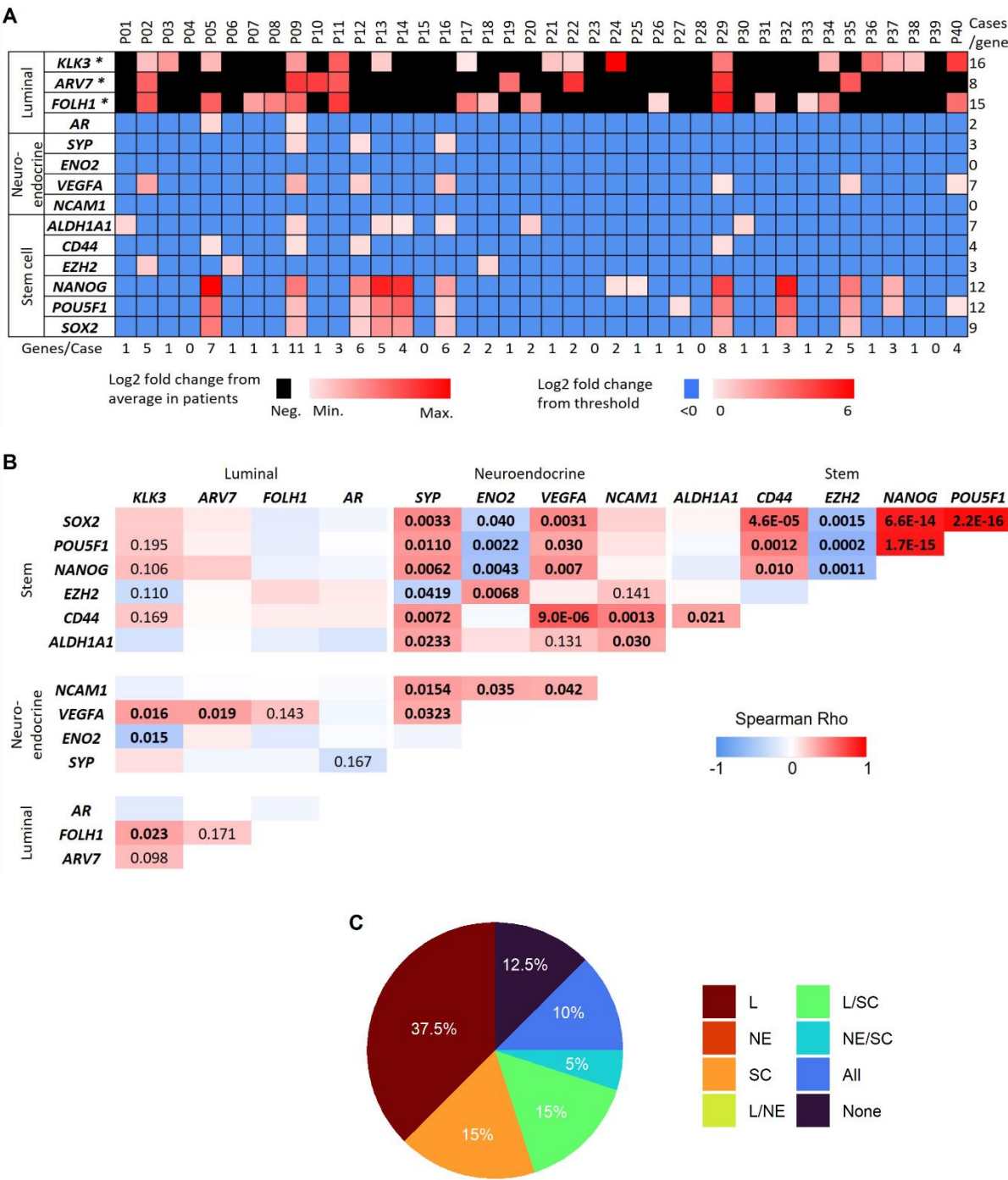


Figure 3. Cell-subtype genes in liquid biopsies support phenotypic diversity. (A) Heatmap of gene expression in 40 patients, with black squares representing no signal, blue squares for baseline expression, and white to red squares for overexpression. For *KLK3*, *FOLH1*, and *ARV7* which are completely negative in control samples, results are presented as log2 fold change from the average expression in positive patients. For the remaining genes, results are presented as log2 fold change from the overexpression thresholds calculated from 40 controls, except for *CD44*, whose threshold was established from older male controls. (B) Spearman correlation matrix between the 14 genes in mCRPC patients. Blue and red represent negative and positive associations, respectively. P-values are entered when the correlation was significant ($p < 0.05$). (C) Pie chart representing phenotypic diversity by cell subtypes (L: luminal, NE: neuroendocrine, SC: stem cell) and combinations thereof, as indicated on the right.

FIGURE 4

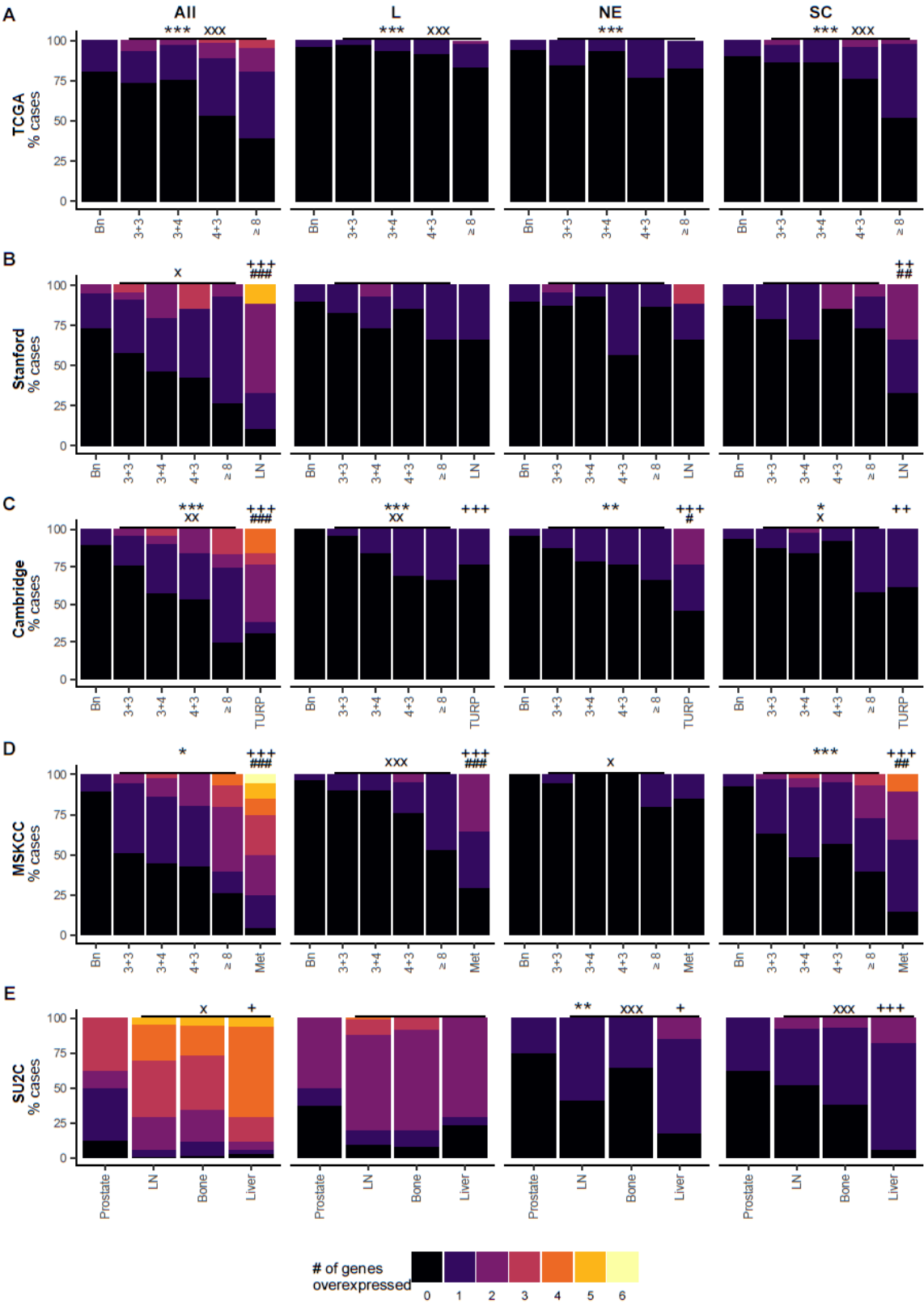


Figure 4. Prostate cell-subtype specific genes are overexpressed in PCa tissues of patients at different stages of disease. Stacked bar graphs representing the number of genes overexpressed (color-coded) overall or by cell subtype in different categories of patients from the (A) TCGA, (B) Stanford, (C) Cambridge, (D) MSKCC, and (E) SU2C datasets. Overexpression was defined as +2.58 standard deviations over the mean expression in benign samples from the same cohort, as indicated in Methods. For the SU2C dataset, gene expression was compared to benign samples from the TCGA dataset. (A-E) Denoted above each graph are the Fisher's exact test results comparing the number of genes overexpressed by categories of cases (0 vs. ≥ 1 gene overexpressed for individual subtypes, or 0-1 vs. ≥ 2 genes overexpressed overall, up to 6 genes). (A-D) Significant differences are shown between benign vs. RP (denoted by *), benign vs. advanced cases from the same cohort (LN, TURP, metastases; denoted by +), or RP vs. advanced cases (#), as well as the Cochran-Armitage test for trends comparing RP cases by Gleason score (x). (E) In the SU2C dataset, Fisher's exact test results denote difference between LN vs. bone (*), LN vs. liver (x), and bone vs. liver metastases (+). (A-E) The level of significance is denoted as: * / + / # / x for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$. L: luminal; NE: neuroendocrine; SC: stem cell; Bn: benign; LN: lymph node; TURP: primary tumours removed by transurethral resection from hormone-treated patients; Met: metastasis.

FIGURE 5

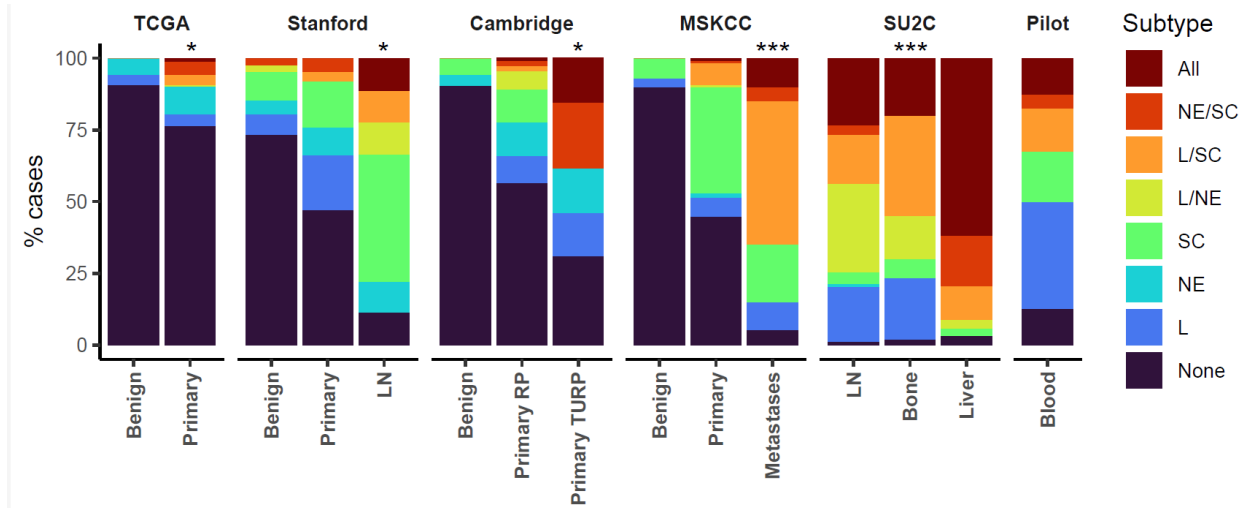


Figure 5. Cell subtype patterns in PCa tissues and metastases evolve with disease progression. Stacked bar graphs of cell-subtypes (L: luminal, NE: neuroendocrine, SC: stem cell) and combinations thereof (color-coded on the right) for different categories of patients in the TCGA, Stanford, Cambridge, MSKCC, and SU2C datasets. The number of cell subtypes represented in different categories of patients are compared by Fisher's exact test. Significance levels are represented by * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$ for benign vs. primary (TCGA), primary vs. advanced (LN/TURP/Metastases in Stanford/Cambridge/MSKCC), and between metastatic sites (SU2C). The results in blood RNA from our pilot study were added on the right for comparison purposes, mostly resembling metastases and differing from primary and benign prostate tissues.

TABLES

Table 1: Overexpression of circulating genes is associated with disease progression and patient death.

Genes and cell subtypes	Disease progression	Death
<i>KLK3</i>	2.37 (1.09-5.13), p=0.029	3.95 (1.06-14.75), p=0.041
<i>ARV7</i>	1.56 (0.66-3.70)	1.98 (0.60-6.50)
<i>FOLH1</i>	1.17 (0.55-2.50)	1.99 (0.62-6.37)
<i>AR</i>	2.00 (0.46-8.57)	60.89 (5.39-688.62), p=0.001
<i>SYP</i>	1.53 (0.46-5.09)	2.15 (0.26-17.97)
<i>ENO2</i>	N/A	N/A
<i>VEGFA</i>	1.96 (0.82-4.67), p=0.130	4.44 (1.11-17.80), p=0.035
<i>ALDH1A1</i>	0.62 (0.21-1.81)	0.85 (0.18-4.07)
<i>CD44</i>	2.12 (0.71-6.29), p=0.177	7.59 (1.68-34.38), p=0.009
<i>EZH2</i>	2.06 (0.61-6.98)	1.33 (0.16-11.07)
<i>NANOG</i>	1.74 (0.81-3.73), p=0.156	4.26 (0.95-19.16), p=0.059
<i>POU5F1</i>	1.38 (0.64-2.99)	4.08 (0.90-18.36), p=0.128
<i>SOX2</i>	1.47 (0.64-3.33)	3.22 (0.71-14.52)
Number of genes	1.13 (0.99-1.29), p=0.071	1.43 (1.13-1.79), p=0.002
Number of genes excluding <i>CD44</i> and <i>SOX2</i>	1.16 (0.98-1.38), p=0.078	1.60 (1.19-2.15), p=0.002
Number of luminal genes	1.40 (0.99-1.98), p=0.057	2.16 (1.23-3.80), p=0.008
Number of neuroendocrine genes	1.41 (0.83-2.40)	2.20 (0.93-5.19), p=0.073
Number of stem cell genes	1.12 (0.91-1.37)	1.41 (0.97-2.04), p=0.070
Any luminal gene	1.48 (0.65-3.37)	4.27 (0.54-33.90), p=0.169
Any neuroendocrine gene	1.96 (0.82-4.67), p=0.130	4.44 (1.11-17.80), p=0.035
Any stem cell gene	1.13 (0.91-1.40)	1.50 (1.00-2.25), p=0.048
Any luminal + any neuroendocrine gene	2.19 (0.80-5.97), p=0.127	6.81 (1.69-27.52), p=0.007
Any luminal + any stem cell gene	1.81 (0.83-3.98), p=0.139	9.62 (1.93-47.86), p=0.006
Any neuroendocrine + any stem cell gene	1.96 (0.82-4.67), p=0.130	4.44 (1.11-17.80), p=0.035
Overexpression of at least one gene in all three groups	2.19 (0.80-5.97), p=0.127	6.81 (1.69-27.52), p=0.007
No gene	0.48 (0.11-2.04)	N/A

Hazards ratios and 95% confidence intervals for disease progression and death in patients overexpressing the selected circulating genes. Hazards ratios and respective 95% confidence

intervals were calculated using Cox proportional hazard models. N/A: Non-Applicable. Numbers are in bold when $p < 0.05$ (p-values were added when < 0.20).

Table 2: Clinical relevance of circulating genes overexpressed in patients with advanced prostate cancer.

Characteristics		Luminal	Neuroendocrine	Stem
Age ¹		0.68 (0.26-1.78)	1.46 (0.21-10.38)	0.88 (0.20-3.80)
PSA ¹		2.54 (1.03-6.29) p=0.044	12.0 (1.10-132.1) p=0.042	4.00 (0.92-17.44) p=0.065
Bone metastases		3.71 (0.85-16.24) p=0.082	0.91 (0.07-11.54)	1.87 (0.25-13.87)
Visceral metastases		2.01 (0.61-6.62)	N/A	0.88 (0.12-6.18)
ECOG performance score ²		3.64 (1.21-10.97) p=0.022	0.49 (0.03-8.54)	2.22 (0.35-14.21)
Lines of therapy		1.48 (0.97-2.27) p=0.071	2.02 (0.85-4.83) p=0.111	1.66 (0.84-3.31) p=0.147
Progressive disease		2.99 (1.21-7.39) p=0.018	6.15 (0.53-70.90) p=0.146	3.00 (0.68-13.32) p=0.149
Initial therapy	Surgery	1.09 (0.40-2.97)	3.82 (0.54-26.84) p=0.178	5.20 (1.25-21.69) p=0.024
	Radiotherapy	0.43 (0.09-2.18)	0.83 (0.04-17.15)	1.16 (0.13-10.30)
	Non-curative therapy	1.29 (0.49-3.40)	0.27 (0.03-2.20)	0.19 (0.04-0.79) p=0.023
Past mCRPC therapy	Any AR inhibitor	1.64 (0.64-4.21)	13.3 (1.24-142.3) p=0.032	7.77 (1.81-33.38) p=0.006
	Abiraterone	1.46 (0.56-3.81)	7.88 (1.16-53.55) p=0.035	6.14 (1.44-26.10) p=0.014
	Enzalutamide	2.05 (0.46-9.12)	1.18 (0.05-27.66)	1.09 (0.10-12.43)
	Any taxane	2.7 (1.07-6.82) p=0.035	0.61 (0.08-5.03)	0.96 (0.21-4.39)
	Docetaxel	3.19 (1.27-7.99) p=0.013	0.71 (0.09-5.88)	0.69 (0.15-3.24)
	Cabazitaxel	5.25 (1.33-20.75) p=0.018	2.83 (0.19-42.33)	2.87 (0.29-28.53)
Current mCRPC therapy	None	4.09 (1.15-14.57) p=0.030	2.76 (0.19-39.90)	2.50 (0.30-20.58)
	Any AR inhibitor	0.34 (0.14-0.83) p=0.018	0.31 (0.04-2.51)	0.45 (0.10-2.00)
	Abiraterone	0.41 (0.16-1.13) p=0.086	0.61 (0.08-5.03)	0.76 (0.16-3.57)
	Enzalutamide	0.45 (0.09-2.20)	N/A	0.24 (0.02-2.77)
	Any taxane	1.46 (0.45-4.68)	3.33 (0.44-25.02)	2.66 (0.47-15.16)
	Docetaxel	0.36 (0.06-2.27)	2.83 (0.19-42.33)	1.97 (0.19-20.40)
	Cabazitaxel	3.89 (0.94-16.10) p=0.060	2.83 (0.19-42.33)	2.87 (0.29-28.54)
	PARP inhibitor	1.9 (0.24-15.28)	3.62 (0.09-33.22)	6.23 (0.30-128.61)
	Radium-223	3.58 (0.23-56.71)	N/A	N/A

Odds ratios (OD) with 95% confidence intervals (in parentheses) identifying variables associated with luminal, neuroendocrine and stem cell markers. ¹Patients were stratified below and above the median. ²Patients were stratified into two groups: ECOG 0-1 and ECOG 2-3. Multi-level, mixed effects logistic regression models were used to calculate odds ratios and their respective 95% confidence intervals, in order to adjust for repeated measurements within individuals. Bold values are significant ($p < 0.05$), and p-values < 0.2 are indicated. Abbreviations are as defined in Table S2. N/A: Non-Applicable.

SUPPLEMENTAL MATERIALS

FIGURE S1

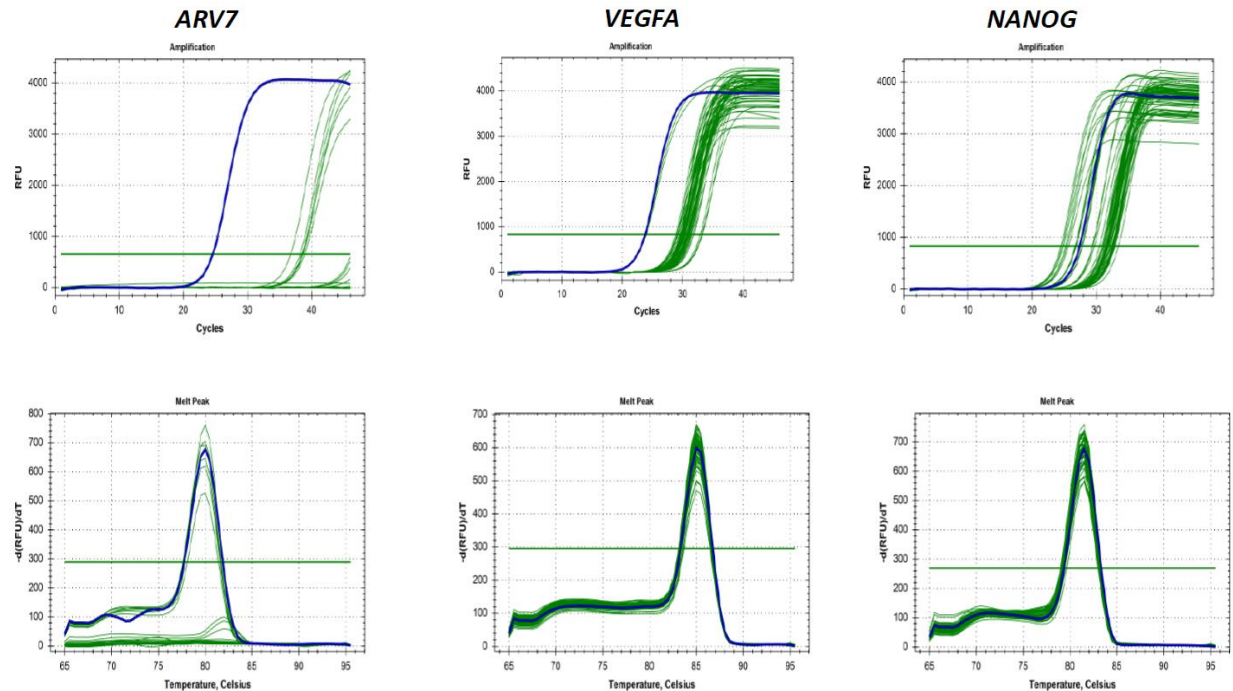


Figure S1. Examples of qPCR amplification and associated melting curves for one gene of each subtype. Amplification by RT-qPCR of ARV7 (Luminal; left panel), VEGFA (Neuroendocrine; middle panel) and NANOG (Stem cell; right panel) for patients (green curves) are represented on top panels, with associated melting curves in lower panels. Blue curves correspond to signal obtained from the positive control (RNA from a mix of human PCa cell lines). For ARV7, signal appears early in positive cell lines, showing a strong amplification, and later in patients. Low signals observed after 40 cycles in the upper left panel and small peaks in associated melting curves in the lower panel are non-specific and were excluded. Specificity can be appreciated by a single peak in melting curves (lower panels) for patients and the positive control. Different levels of amplification are observed for patients in upper panels.

FIGURE S2

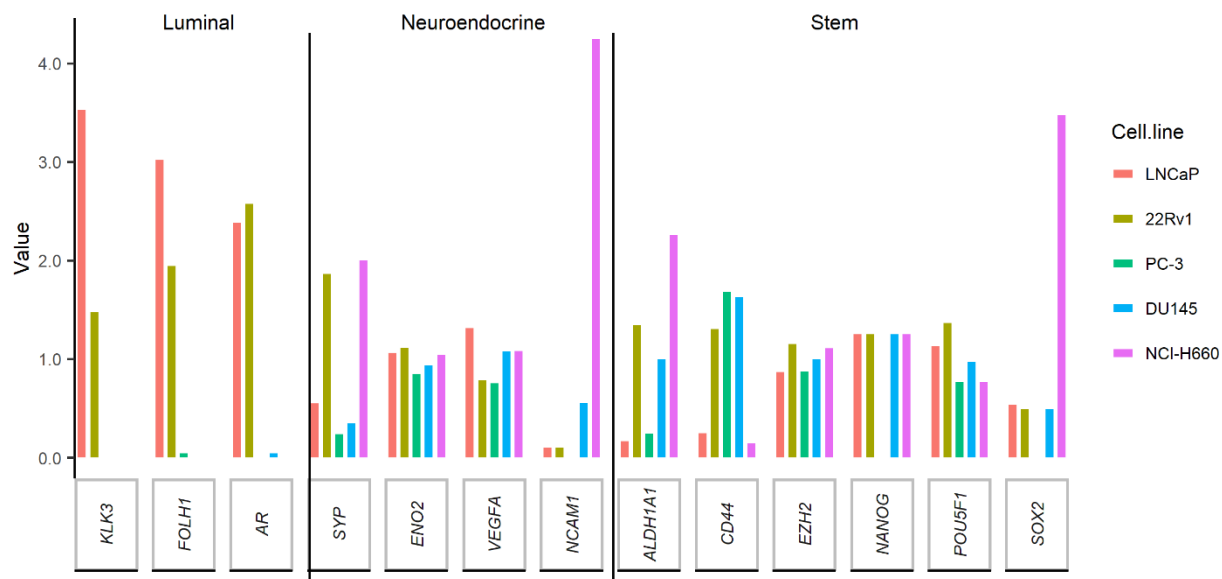


Figure S2: Expression of the cell subtype genes in CCLE Expression Atlas.

RNA sequencing data on the 14 genes of interest was obtained from the CCLE Expression Atlas as transcripts per million (for luminal ARV 7 not available), neuroendocrine and stem cell genes for the same five cell lines (color coded right) Expression is presented as Log 2 (TPM+1).

FIGURE S3

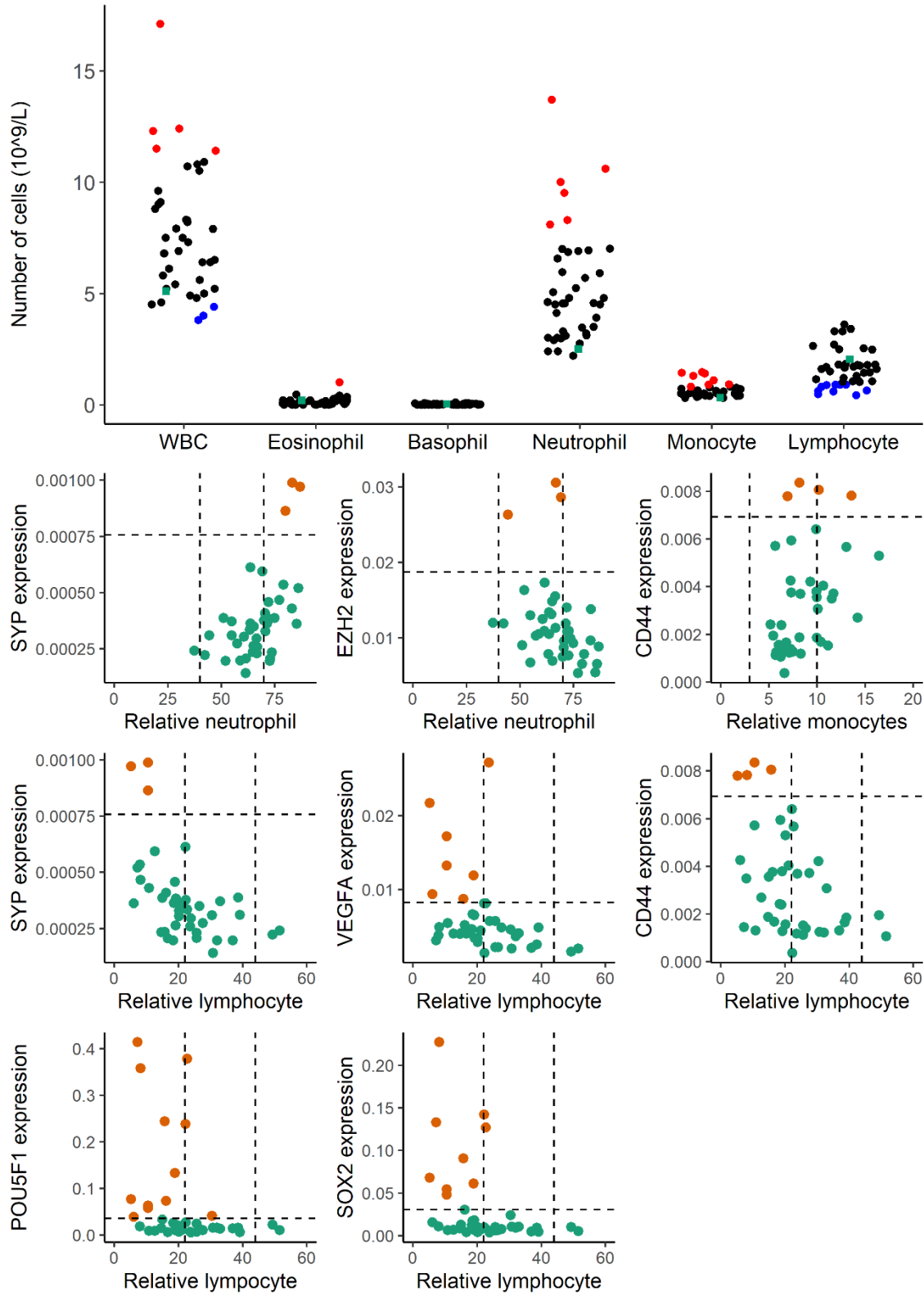
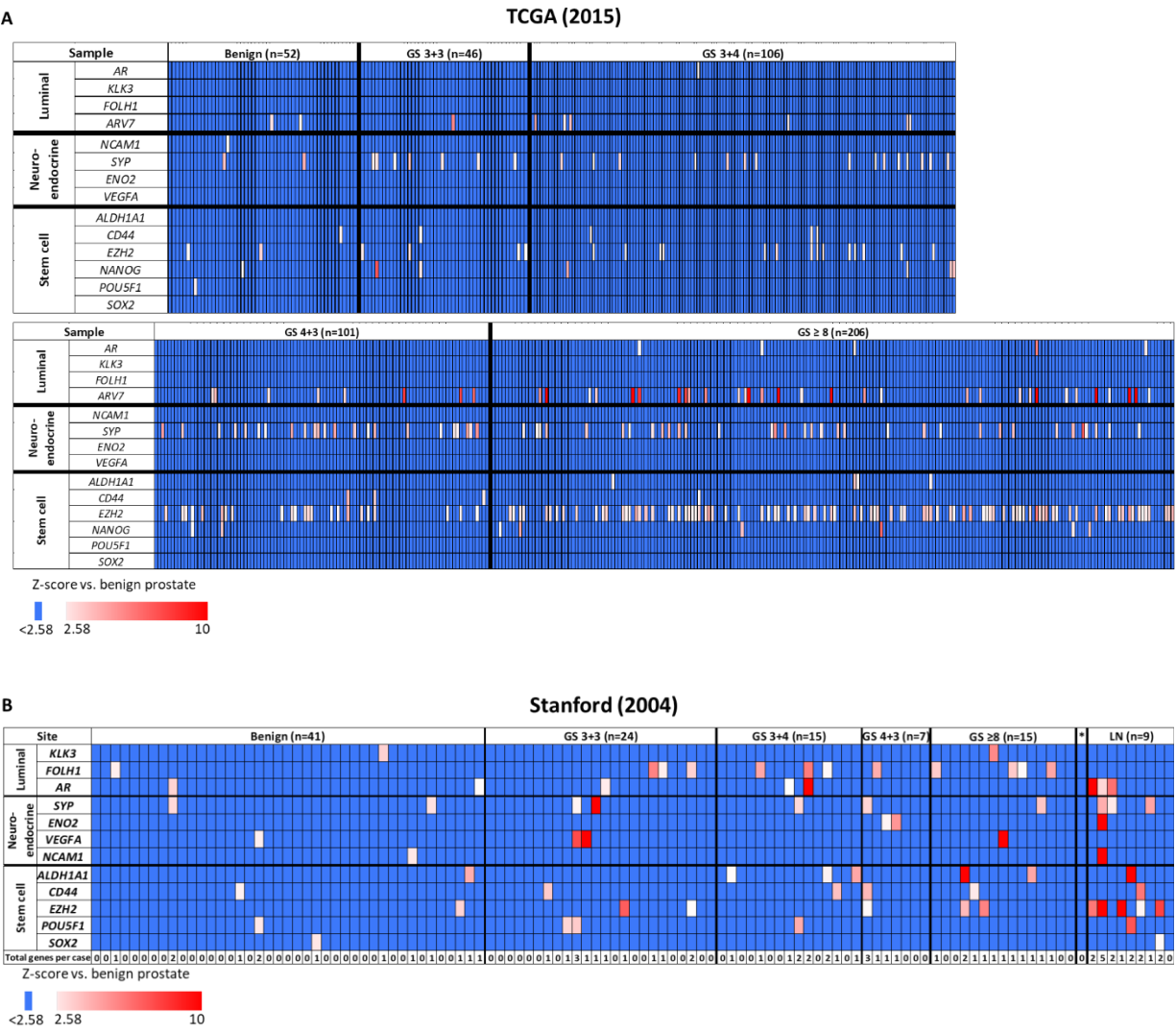


Figure S3: Overexpression of cell subtype genes in WBCs of patients. (A) WBC counts in the 40 mCRPC patients at time of blood draw. Normal counts are represented in black, high in red, and low in blue. WBC data from a healthy volunteer whose circulating genes were tested as a control are included (green square). Overall, 75 of patients' WBC and lineage counts were within the normal range of the clinical Hematology labs (MUHC and JGH). (B) Scatter plots of gene expression vs different WBC proportions. Selected genes were based on associations (negative or positive) found in Figure 2 B. Vertical lines represent the minimum and maximum for the normal range for relative cell counts. Horizontal lines represent the threshold for each gene overexpression. Orange circles represent cases overexpressing the respective gene, and green circles represent basal level of expression.

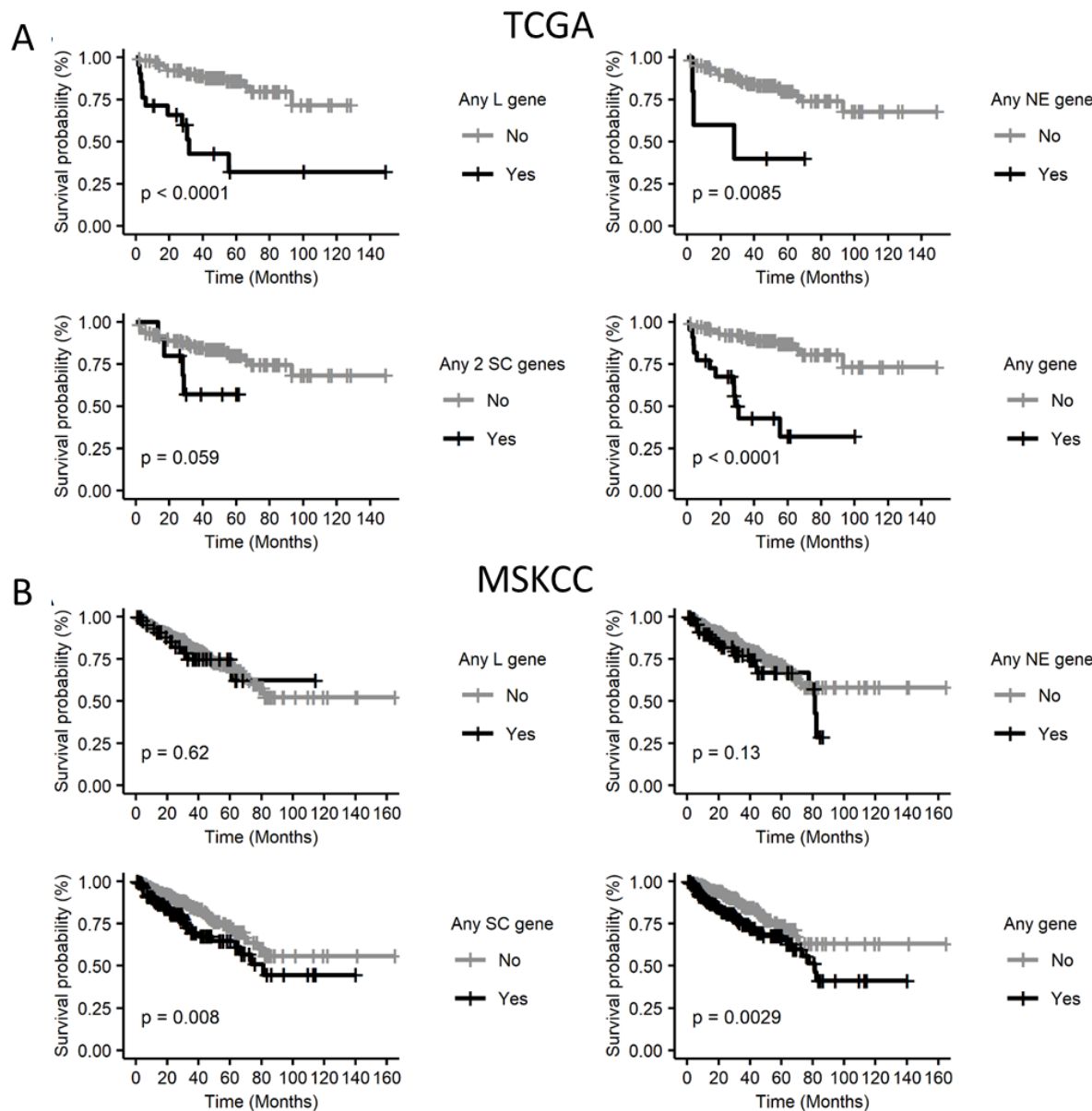
FIGURE S4



C



FIGURE S5



C

Dataset	Overexpressed genes	Hazard ratio (confidence interval)	
		Univariate	Multivariate
TCGA	Any L gene	1.2 (0.6-2.3)	0.86 (0.4-1.7)
	Any NE gene	1.5 (0.9-2.4)	1.5 (0.9-2.4)
	Any SC gene	1.8 (1.2-2.7)	1.1 (0.7-1.8)
	Any gene	1.9 (1.2-2.8)	1.3 (0.8-2.0)
MSKCC	Any L gene	5.5 (2.5-12)	2.3 (0.95-2.8)
	Any NE gene	4.4 (1.3-15)	4.1 (0.98-17.3)
	Any 2 SC genes	2.7 (0.9-8)	0.94 (0.3-3.0)
	Any gene	6.4 (2.9-14)	2.2 (0.9-5.6)

Figure S5. Clinical significance of cell subtype genes on disease-free survival in PCa transcriptomic datasets. (A, B) Kaplan-Meier analyses for the number of markers overexpressed by cell subtype, or overall, in (A) TCGA and (B) MSKCC cohorts, and by (C) Uni- and multivariable Cox analyses for the number of overexpressed genes. Covariates are Gleason score and T stage for TCGA; pre-operative PSA, Gleason score, and T stage for MSKCC.

TABLE S1: Primer list with NCBI reference sequence, exon location, amplicon size (bp/base pairs), calibration curves with efficiency, r² with slope and y intercept.

Gene	Primers	NCBI reference sequence	Location	Size (bp)	Efficiency	r ²	Equation/Slope
AR	ATGGTGAGCAGAGTGCCCTATC ATGGTCCCCTGGCAGTCTCCAAA	NM_000044.4	Exon 1 Exon 2	117	112.30%	1	y= - 3.058+27.442
KLK3	CGCAAGTTCACCCTCAGAAGGT GACGTGATACCTTGAAGCACAC C	NM_001030047.1	Exon 4 Exon 5	130	105.60%	0.99	y= - 3.196+31.438
FOLH1	GGAGAGGAAGTCTCAAAGTGC C TGGTTCCTACTGCTCCTCTGAGA	NM_001014986.2	Exon 8 Exon 9	149	106.90%	1	y= - 3.167+27.477
ARV7	CAGGGATGACTCTGGGAGAA GCCCTCTAGAGCCCTCATTT	NM_001348061.1	Exon 3 Exon 4	82	107.90%	1	y=- 3.147+30.468
SYP	TCGGCTTTGTGAAGGTGCTGCA TCACTCTCGGTCTTGTGGCAC	NM_003179.2	Exon 2 Exon 3	115	87.90%	0.98	y=- 3.650+33.498
ENO2	CTGTATCGCCACATTGCTCAGC AGCTTGTTGCCAGCATGAGAGC	NM_001975.3	Exon 6 Exon 7	101	98.90%	1	y=- 3.348+29.941
VEGFA	TTGCCCTTGCTGCTCTACCTCCA GATGGCAGTAGCTGCGCTGAT A	NM_001025366.2	Exon 1 Exon 3	126	103.20%	1	y=- 3.248+28.972
NCAM1	CATCACCTGGAGGACTTCTACC CAGTGACTGGATGCTCTTCAG G	NM_000615.6	Exon 8 Exon 9	122	107.60%	1	y=- 3.153+30.644
ALDH1A1	CGGGAAAAGCAATCTGAAGAG GG GATGCGGCTATACAACACTGGC	NM_000689.5	Exon 8 Exon 9	147	106.10%	1	y=- 3.184+28.708
CD44	CCAGAAGGAACAGTGGTTTGG C ACTGTCCTCTGGGCTTGGTGTT	NM_000610.3	Exon 10 Exon 11	151	123.30%	0.97	y=- 2.866+33.873
EZH2	GACCTCTGTCTTACTTGTGGAG C CGTCAGATGGTGCCAGCAATA G	NM_001203247.1	Exon 15 Exon 16	115	117.60%	1	y=- 2.962+27.577
NANOG	CTCCAACATCCTGAACCTCAGC CGTCACACCATTGCTATTCTTC G	NM_001297698.1	Exon 2 Exon 3	115	1115.20%	1	y=- 3.004+32.460
POU5F1	CCTGAAGCAGAAGAGGATCAC C AAAGCGGCAGATGGTCGTTTG G	NM_001173531.2	Exon 2 Exon 3	106	105.00%	1	y=- 3.209+30.086
SOX2	GCTACAGCATGATGCAGGACC A TCTGCGAGCTGGTCATGGAGTT	NM_003106.4	Exon 1 Exon 1	135	107.00%	1	y=- 3.165+29.840
PKG1	CCGCTTTCATGTGGAGGAAGAA G CTCTGTGAGCAGTGCCAAAAGC	NM_000291.4	Exon 4 Exon 5	149	108.00%	0.99	y=- 3.145+24.892
PP1B	AACGCAGGCAAAGACCAAC G TCTGTCTTGGTGCTCTCCACCT	NM_000942.4	Exon 4 Exon 5	140	108.00%	1	y=- 3.145+26.756
RPLP0	TGGTCATCCAGCAGGTGTTCTGA ACAGACACTGGCAACATTGCG G	NM_001002.4	Exon 6 Exon 7	119	109.20%	1	y=- 3.119+22.121

TABLE S2. Patients' characteristics.

Parameters			CRPC Patients (n=40)
Age, median (range), years			70 (49-89)
ECOG score	0-1		33 (82.5%)
	2-3		7 (17.5%)
PSA, median IQR, ng/mL			6.1 (1.2-41.3)
Gleason score at biopsy	6		2 (5.0%)
	7		4 (10.0%)
	8		14 (35.0%)
	9		12 (30.0%)
	Not reported		8 (20.0%)
Treatment of initially localized disease	Surgery		14 (35.0%)
	Radiotherapy		5 (12.5%)
Treatment of initially metastatic disease	ADT		10 (25.0%)
	ADT + pelvic radiotherapy		4 (10.0%)
	ADT + docetaxel		6 (15.0%)
	Zibotentan		1 (2.5%)
Time, median (IQR) in months	Between diagnosis to CRPC		59 (20-120)
	Between CRPC and inclusion		19 (8-35)
Metastatic site at the time of blood draw	None		1 (2.5%)
	Bones		33 (82.5%)
	Visceral		7 (17.5%)
	Lymph nodes (pelvic)		17 (42.5%)
	Lymph nodes (extra-pelvic)		14 (35.0%)
Line of mCRPC therapy at the time of blood draw	None yet		4 (10.0%)
	1 st line		20 (50.0%)
	2 nd line		10 (25.0%)
	≥ 3 rd line		6 (15.0%)
Treatment at the time of blood draw	ADT only		4 (10.0%)
	AR inhibitors	Abiraterone	15 (37.5%)
		Enzalutamide	5 (12.5%)
	Taxanes	Docetaxel	4 (10.0%)
		Cabazitaxel	4 (10.0%)
	Others	Olaparib (PARP inhibitor)	2 (5.0%)
		Radium-223	1 (2.5%)
End-of-life palliative care		5 (12.5%)	
Progressive disease at the time of blood draw			21 (52.5%)

*CRPC: Castrate-Resistant Prostate Cancer; ECOG: Eastern Cooperative Oncology Group; PSA: Prostate Specific Antigen; IQR: Inter Quartile Range (Q25-Q75); ADT: Androgen-Deprivation Therapy; AR: Androgen Receptor. Zibotentan is an endothelin receptor antagonist; Olaparib is a PARP (Poly(ADP-Ribose) Polymerase 1) inhibitor.

TABLE S3: Threshold determination for overexpression of prostate cell subtype genes in patients' liquid biopsies.

Gene	Controls (n=15)			Patients (n=40)
	Mean	SD	Threshold	n > threshold
<i>KLK3</i>	0.000	0.000	0.000	16
<i>ARV7</i>	0.000	0.000	0.000	8
<i>FOLH1</i>	0.000	0.000	0.000	15
<i>AR</i>	0.002	0.001	0.005	2
<i>SYP</i>	0.094	0.039	0.195	3
<i>ENO2</i>	0.598	0.122	0.913	2
<i>VEGFA</i>	0.091	0.040	0.195	6
<i>NCAM1</i>	0.215	0.146	0.593	0
<i>ALDH1A1</i>	0.898	0.417	1.975	5
<i>CD44</i>	2.509	1.613	6.671	4
<i>EZH2</i>	0.121	0.071	0.193	3
<i>NANOG</i>	0.071	0.040	0.175	10
<i>POU5F1</i>	0.283	0.092	0.521	12
<i>SOX2</i>	0.578	0.263	1.255	9

Gene overexpression in patients (right column) was defined as expression greater than the threshold set for each gene (third column) at 2.58 standard deviations (SD) above the mean expression value in blood RNA from 15 healthy controls (two left columns) at a 99.5 % confidence interval (Mean + 2.58xSD).

MANUSCRIPT 3: Stratifying prostate cancer patients through circulating genes related to epithelial cell subtypes, drug targets, and therapeutic resistance

Seta Derderian, Edouard Jarry, Arynne Santos, Mohananchary Amaravadi, Quentin Vesval, Lucie Hamel, Raphael Sanchez-Salas, Alexis Rompré-Brodeur, Wassim Kassouf, Raghu Rajan, Marie Duclos, Armen Aprikian, Simone Chevalier

In preparation for submission to Molecular Oncology.

PREFACE

In Manuscript 2, we established the traceability and clinical relevance of 14 cell subtype specific genes in the blood of mCRPC patients. However, even with this additional knowledge of patients' disease, we remain limited by the available treatments. Therapies at the mCRPC stage are still mainly limited to AR inhibitors, taxanes, PARP inhibitors in select cases, and the incoming use of PSMA theranostics. Nonetheless, drugs for several other targets have been tested in clinical trials for mCRPC patients. While they have not made their way into the clinic, some have shown promising results in subsets of unselected patients. For example, in a phase 2 trial of the AURKA inhibitor alisertib, 4/60 patients showed exceptional response; these patients were confirmed to display overactivity of NMYC/AURKA.¹¹¹ We propose that such patients may be selected through non-invasive testing of the relevant genes in their blood. Such an approach would be easily incorporated in future clinical trials, and eventually to clinical use, for patient selection.

In Manuscript 3, we build on a similar approach to study an expanded gene panel in whole blood RNA of patients compared to controls. This new panel includes additional genes representative of each cell subtype, genes known to be involved in resistance to AR inhibitors and taxanes, and alternative drug targets of interest based on results of clinical trials in PCa. We present an improved

methodology, provide more strict criteria for gene selection, and include additional steps of technical and biological validation. We also incorporate additional categories of patients, from diagnosis of localized disease to mCRPC.

CONTRIBUTION OF AUTHORS

Seta Derderian: Selected PCa-relevant genes for testing in blood RNA, including genes of cell subtypes, treatment resistance, and alternate drug targets based on literature review and results of clinical trials. Performed bioinformatic analyses of selected genes in transcriptomic datasets of PCa and blood. Banked blood and extracted RNA from patients and controls. Provided cultured human PCa cell lines. Optimized RT-qPCR assays in cell line RNA. Performed technical and biological validation of assays, including longitudinal testing in blood of healthy controls. Tested all genes in the majority of blood samples collected from patients and controls. Performed statistical analyses of patients' overexpressed genes in relation to age, white blood cells, clinical parameters, treatments, and outcomes. Wrote and edited the manuscript.

Edouard Jarry (Master 2, French system): Banked blood and extracted RNA from patients and controls. Tested the initial set of 14 genes in a first set of mCRPC patients and controls. Analyzed results in relation to therapies. Performed experiments on biological validation, including longitudinal testing in blood of healthy controls.

Aryenne Santos (McGill MSc student): Banked blood and extracted RNA from patients and controls. Selected neuroendocrine genes for testing in blood RNA based on literature review.

Optimized RT-qPCR assays for neuroendocrine genes. Tested neuroendocrine genes in a first set of mCRPC patients and controls. Analyzed results in relation to patients' characteristics.

Mohanachary Amaravadi (McGill post-doctoral fellow): Optimized of RT-qPCR assays for the initial set of 14 genes (TaqMan vs. SYBRGreen). Tested *ARV7* in a first set of mCRPC patients and controls. Supervised students involved in the project.

Quentin Vesval (Master 2 student, French system): Participated in the Ethics renewal of the project for continuing the enrolment of PCa patients and controls. Banked blood samples from patients and controls.

Lucie Hamel: Provided cultured human PCa cell lines. Recruited patients, banked blood, and extracted RNA. Periodically updated patients' clinical data.

Raphael Sanchez-Salas: Urologist following MUHC patients scheduled for radical prostatectomy and enrolled in the study. Analyzed clinical data.

Alexis Rompré-Brodeur: Urologist following MUHC patients scheduled for radical prostatectomy and enrolled in the study. Analyzed clinical data.

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Raghu Rajan: Oncologist seeing PCa patients at advanced stage at the MUHC. Analyzed clinical data.

Marie Duclos: Radiation oncologist seeing MUHC PCa patients for curative therapy by RT and cases that need adjuvant RT during the course of their trajectory and enrolled in the study. Analyzed clinical data.

Armen Aprikian: Urologist following several MUHC patients enrolled in the study. Participation in the project as co-investigator and involved in analysis of clinical data. Edited the manuscript.

Simone Chevalier: Senior Scientist at the Research Institute of the MUHC: Planned and directed the research project; supervised of Seta Derderian, Dr E. Jarry, A. Santos, Dr M. Amaravadi, and Q. Vesval. Participated in all steps of the project, from setting up methods to analyzing results, and determining their meaning and value in the field of PCa. Edited the manuscript.

TITLE PAGE

STRATIFYING PROSTATE CANCER PATIENTS THROUGH CIRCULATING GENES RELATED TO EPITHELIAL CELL SUBTYPES, DRUG TARGETS, AND THERAPEUTIC RESISTANCE

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Running title: Circulating transcripts for PCa stratification

ABSTRACT

Patient stratification remains an obstacle for the optimal treatment of prostate cancer (PCa). Progression involves different prostate epithelial cell subtypes which are not all targeted by current treatments. We hypothesize that genes representative of epithelial cell subtypes, resistance to current treatments, or encoding drug targets are detectable in whole blood RNA as non-invasive biomarkers for patient stratification.

We built a panel of 64 genes overexpressed in advanced PCa but showing low or no expression in control blood. The panel was tested by RT-qPCR in blood of 26 controls and 89 samples from 65 patients. Patients with aggressive pathological features at diagnosis showed more genes overexpressed, including subtype-specific genes. In metastatic patients, signatures of subtypes or resistance were associated with treatments and outcomes. Drug target genes were overexpressed in subsets of patients, suggesting that they may benefit from more relevant treatments.

Overall, circulating transcripts show predictive value throughout the disease. These may be clinically meaningful to stratify patients and predict responses. Genes encoding drug targets may justify clinical trials of personalized treatments to impact on PCa mortality.

INTRODUCTION

Prostate cancer (PCa) is the most common cancer in North American men and a major cause of cancer mortality. Among patients diagnosed with presumably organ-confined disease, 25-35% experience a recurrence after curative therapies, including some patients initially considered at low risk. This clinical heterogeneity demonstrates our inability to predict cancer aggressiveness with certainty at diagnosis, along with the failure of blood PSA (prostate specific antigen) and imaging to detect cancer spread beyond the prostate until the disease is sufficiently advanced.¹ Recurrent disease is treated by androgen deprivation therapy (ADT), which eventually fails and leads to metastatic castration resistant PCa (mCRPC).

Intra-tumoral heterogeneity contributes to clinical heterogeneity; PCa is composed of a majority of luminal-like AR-positive cells interspersed with rare AR-negative neuroendocrine (NE) and stem-like cells.^{2, 3} ADT and AR inhibitors (ARIs) target only AR-positive cells than depend on androgens for survival. Resistance is attributed to AR-dependent and -independent mechanisms, including selection for (and differentiation towards) AR-negative NE and stem-like phenotypes.³⁻

⁵ Taxanes are also used, but none of these treatments are curative.

Drugs for alternate promising targets have been tested in clinical trials for mCRPC. Few are implemented in practice, despite some demonstrating clinical benefit in subsets of unselected patients. This emphasizes the importance of patient stratification to offer personalized therapies. Unfortunately, stratification based on markers in primary tumours may no longer be relevant after years of treatments. Biopsies of metastases would be ideal for identification of reliable prognostic markers, but this invasive procedure is not commonly part of clinical practice. Liquid biopsies, particularly blood, are attractive non-invasive alternatives which can be collected longitudinally to provide a global view of molecular changes in each patient.

Blood-based assays can be based on proteins, RNAs, cell-free (cf) nucleic acids, extracellular vesicles (EVs), and circulating tumour cells (CTCs).⁶ Whole blood RNA is useful as it encompasses transcripts released from tumours in CTCs and EVs. Most whole blood RNA studies focus on early diagnosis, differentiating between cancer and non-malignant diseases of the corresponding tissue, and early detection of recurrence or metastases. Our recent study on 14 cell-subtype genes in whole blood RNA of mCRPC patients revealed phenotypic diversity in luminal, NE, and stemness genes.⁷ We showed significant correlation between gene overexpression, treatments received, and further progression, thereby confirming their clinical relevance.

Few groups have addressed circulating RNAs as predictors of therapeutic response in PCa. Blood RNA-sequencing studies identified immune signatures relevant to immunotherapy, targeted therapy, and chemotherapy response.⁸⁻¹¹ These studies did not identify cancer- or prostate-specific genes, which were not detected due to their low expression. In RT-qPCR studies, progression on taxanes was related to markers of luminal-like cells, while progression on ARIs involved AR, constitutively active AR variants (mainly ARV7), AR target genes, and NE or stemness genes.^{7, 12-16} Altogether, the potential of blood RNA to predict treatment response has been minimally explored, especially in the context of novel therapies.

The present investigation stems from our previous work on cell-subtypes in blood of mCRPC patients. We aimed to determine the clinical relevance of an expanded gene panel in whole blood RNA of patients at different stages of disease. These include not only additional cell-subtype specific genes, but also genes reflecting ARI/taxane resistance or encoding alternate therapeutic targets tested in PCa clinical trials. We demonstrate the predictive value of several circulating genes and suggest that this approach may be useful for identifying subsets of patients for whom more relevant therapies should be considered.

MATERIALS AND METHODS

Patients and controls

This study was approved by the Ethics Review Board of the McGill University Health Center (MP-37-2017-3158 and MP-37-2017-3189 for project and bank, respectively). Participants signed informed consent forms to donate blood for research. Eighty-nine blood samples were drawn from 65 patients: 16 before radical prostatectomy (RP), 26 post-curative therapies but not metastatic (including five that were also collected before RP), and 28 metastatic. Blood was also collected from 26 controls with no history of cancer or prostatic diseases. A pseudonymized database was generated from patients' medical records.

Blood processing and RNA extraction

Peripheral blood was collected in PAXgene tubes (Qiagen; Germantown, MD, USA), incubated at room temperature for 2 hours for cell lysis, frozen at -20°C, and stored long-term at -80°C. RNA was extracted using PAXGene RNA Extraction kits following Qiagen protocols. RNA integrity (RIN) and concentration were determined by BioAnalyzer 2100 (Agilent, Millcreek, ON, Canada) and Nanodrop ND-1000 (Thermo Scientific, Waltham, MA, USA), respectively. RNA was stored at -80°C.

RT-qPCR assays

Total RNA (500 ng) was reverse transcribed using the Maxima H Minus First Strand cDNA Synthesis Kit with dsDNase (Thermo Scientific, Waltham, MA, USA). A negative control with no template was run in parallel. TaqMan assays were obtained from Thermo Scientific or Integrated

DNA Technologies, either as pre-designed or custom assays. Probe and primer information are available in Table S1. All qPCR reactions were carried out in triplicate using the TaqMan Fast Advanced Master Mix (Thermo Scientific) on the CFX384 Touch™ (Bio-Rad, Mississauga, ON, Canada) with the following program: UNG incubation 50°C 2 minutes, polymerase activation 95°C 20 seconds, 45 cycles of denaturation (3s 95°C) and annealing/extension (30s 60°C).

Assays for each gene were first tested in RNA from five human PCa cell lines (PC3, LNCaP, 22Rv1, DU145, NCI-H660) and in serial dilutions of a RNA mix from these cells to verify efficiency (Table S1). RNA from the NCI-H660 cell line alone was used to quantify genes specific to this cell line.

For experiments in blood RNA, each plate included a negative control (no template in cDNA synthesis) and serial dilutions (RNA mix from cell lines) for each gene and at least one reference gene. Relative normalized gene expression was determined by the $2^{-\Delta C_t}$ method. Reference genes (*PGK1*, *PPIB*, *RPLP0*) were chosen as previously described.⁷

Statistical analyses

Gene overexpression in blood was defined as 2.58 standard deviations above the mean expression in blood of controls *OR* the maximum expression detected in controls (whichever was highest). Female controls were excluded from cutoff determination if they were different from male controls ($p < 0.1$).

Spearman correlation was used to analyze correlations between gene expression with patient's age and blood counts. To analyze the association of individual genes vs. patient's characteristics or treatments, differences were evaluated using Chi-square tests. To identify characteristics/treatments associated with overexpression of gene sets, we adjusted for repeated

measures within individuals using multi-level mixed effects logistic regression, while also adjusting for multiple blood draws for some patients. Kaplan Meier and Cox proportional hazards models were used in survival analyses for disease progression and overall survival. Progression was either clinical (worsening of symptoms), biological (PSA rising in two subsequent measurements), or radiological (new lesion or increased size of existing lesions). Results with $p < 0.05$ were considered significant. All statistical analyses were performed using Stata v16.1 and R.

Bioinformatic analyses

Cell line RT-qPCR results were compared to RNA-sequencing data from the Cancer Cell Line Encyclopedia Expression Atlas.¹⁷ Gene expression data of individual white blood cell (WBC) populations was obtained from the consensus dataset from the Human Protein Atlas (proteinatlas.org).¹⁸ Gene expression microarray data were accessed through Gene Expression Omnibus for the Stanford (GSE3933), Cambridge (GSE70770), and MSKCC (GSE21032) datasets. For the Stanford dataset, data were normalized as initially reported. For MSKCC and Cambridge datasets, data were normalized using the LIMMA Bioconductor package in R. Publicly available RNA-sequencing datasets were accessed through Gene Expression Omnibus for red blood cells (GSE108378), platelets (GSE89843), and whole blood (GSE181228). RNA-sequencing data under controlled access were obtained through dbGaP for SU2C (phs000915.v2.p2) and GDC for TCGA (phs000178.v11.p8). Raw data was processed using the Canadian Centre for Computational Genomics GenPipes RNA-seq pipeline, with count normalization using the DESeq2 Bioconductor packages in R. Gene overexpression in tumours was defined as 2.58 standard deviations over the expression in benign samples from the same

dataset; for SU2C, metastases were compared to TCGA benign samples after batch correction using the LIMMA Bioconductor packages in R.

RESULTS

Selected genes reflect the aggressiveness of PCa

Our initial panel included 14 genes representative of prostate luminal, neuroendocrine, and stem-like cells.⁷ We first aimed to better represent intra-tumoral heterogeneity not only with additional genes of cell subtypes, but also account for mechanisms of resistance to ARIs and taxanes and include genes encoding targets for existing drugs tested in PCa clinical trials (Figure 1A). Inclusion and exclusion criteria for expanding this panel are detailed in Table 1, and the final list of 64 genes is presented in Figure 1. The chosen genes were validated in PCa transcriptomic datasets representing different categories of patients (Figure 1B). Each gene was overexpressed in at least one category of advanced disease, predominantly in various metastases.

As described in Table 1, genes with high expression in blood or specific blood cells were excluded. Indeed, 52/64 genes showed negative or minimal expression in blood components (Figure 1C). For certain genes showing expression in blood, we opted for splice variants more cancer-specific and less expressed in blood (e.g. *NCAM180*, *CD44E*, and *FYN-B*). Other genes showing expression in blood were kept from our previous study, as we showed they do not correlate with patients WBCs or platelets (*ENO2* in platelets, *ALDH1A1* in monocytes).⁷ All 64 genes were kept for further validation. Altogether, these findings support the suitability of the selected genes to monitor the PCa in blood.

Selected assays allow for specific and reproducible testing of circulating genes

We next carried out technical and biological validation of the chosen assays. Assays for each gene were first tested in five PCa cell lines, showing expression patterns in line with RNA-sequencing data available from the CCLE (Figure S1A). Testing in serial dilutions showed that all were quantifiable in a broad range of concentrations (assay efficiencies noted in Table S1).

To determine cutoffs for gene overexpression in patients, we tested all assays in the blood of 26 controls (Figure 2A). 12/64 genes were negative in all controls, while the rest showed low levels of expression. No genes correlated with age, despite the increased incidence of prostatic diseases in aging men. However, some genes (*EPCAM*, *ENO2*, *INSM1*, *POU3F2*) differed between male and female controls. To account for these sex-based differences, and considering that PCa patients are male, female controls were excluded for genes with a $p < 0.1$ to avoid any over- or underestimation of cutoffs. Cutoffs for overexpression were set at the 99.5% CI of expression in controls, or highest level detected in controls.

We next demonstrated that these assays yield reproducible results. A control sample tested in two separate RNA extractions and two separate cDNA synthesis reactions yielded consistent results remaining below thresholds (Figure S1B). Furthermore, longitudinally collected control samples (15-18 months) showed that gene expression stayed below set thresholds and was relatively constant (Fig. S2). Testing in 10 patients showed reproducible results of gene overexpression for 58/64 genes throughout batches of experiments (Figure S3). The remaining six genes (*CALCR*, *INSM1*, *NTSR1*, *SCG2*, *MTOR*, and *WNT5A*) were excluded from further analyses.

Next, we verified that there is minimal correlation between genes and patients' age and blood counts (Figure 2B). The few positive correlations were further analyzed and found to be weak and

non-linear (Figure S4). Collectively, these findings confirmed the specificity and reproducibility of assays to test circulating genes in whole blood RNA.

Circulating genes are overexpressed at all stages of PCa

The 58 gene panel was next tested in a total of 89 blood samples from patients, including 16 cases prior to RP, 30 samples from 26 non-metastatic hormone-sensitive cases, and 43 samples from 28 metastatic patients. Gene overexpression results are presented in a heatmap (Figure 3A). A total of 44/58 genes were overexpressed in at least one sample, 5/58 showed baseline expression but no overexpression, and 9/58 were completely negative in all blood samples tested.

Given that these genes were mostly relevant in advanced and/or metastatic disease (Figure 1), we compared their overexpression in blood of metastatic vs. non-metastatic patients. Chi squared analyses showed that six genes were overexpressed more often in metastatic cases (Figure 3B), including some seen strictly in mCRPC (*KLK3*, *KIF2C*). Interestingly, mixed effects logistic regression models revealed that metastatic cases were more likely to overexpress luminal genes and genes related to treatment resistance compared to non-metastatic patients (Table 2). Overall, circulating gene patterns are heterogeneous between and within categories of patients.

Circulating genes at diagnosis are related to aggressive pathological features

Fewer circulating genes were detected in patients prior to RP (Figure 3A). Yet, 30/58 genes were overexpressed in blood of at least one patient, of which four were found in at least 25% of cases (*FOLH1*, *SSTR2*, *L1CAM*, *NCAM180*) (Figure 3A). Chi squared results (Figure 4A) revealed correlations between patients' clinical and pathological parameters with the number of genes

overexpressed, cell subtype specific genes, and the number of cell subtypes represented. Patients with higher blood PSA at diagnosis were more likely to have four or more genes overexpressed (Figure 4B). Patients with intraductal carcinoma (IDC) were more likely to have four or more genes overexpressed overall or at least one NE (Figure 4C). These patients were also the only ones overexpressing stemness genes and genes of more than one cell subtype (Figure 4C, D). Finally, patients with a higher CAPRA-S score had more NE genes (Figure 4E).

In non-metastatic hormone-sensitive patients, analyses were more restrictive as no patients were actively progressing and the cohort was heterogeneous. Nevertheless, we noticed that patients with the most genes overexpressed were not actively being treated (not on ADT; Figure 4F). Furthermore, we found the NE gene *IGFBP2* and the stemness gene *EZH2* were more commonly overexpressed in patients who underwent radiotherapy, and *EZH2* was also more common in patients on ADT (Figure 4G).

Overall, genes overexpressed in blood at diagnosis are related to worse clinical features and may be relevant to monitoring disease progression, as phenotypic changes are detected after curative therapies.

Circulating genes in metastatic cases correlate with clinical characteristics, treatments, and outcomes

The most meaningful findings were obtained in metastatic patients, where 41/58 genes were overexpressed in at least one sample. Various genes were related to clinical features and treatments (Figure 5A), with examples shown in Figure 5B. Interestingly, no genes were significantly over-represented in CRPC cases compared to HSPC. *AURKA* was over-represented in patients on current taxane treatment, especially in those progressing at inclusion, and in patients who have

progressed on various treatments. The stemness gene *KIF2C* came up in cases treated with chemotherapy at diagnosis, those who progressed on ARIs, and in cases currently on taxanes. The NE gene *SSTR2* was exclusively overexpressed in patients who received pelvic radiotherapy. Overexpression of luminal genes *GRHL2* and *STEAP1*, and the NE gene *AURKA* was associated with an increased risk of death, while the NE genes *AURKA* and *IGFBP2* were associated with progression (Table S2).

While studying individual genes yields interesting results, considering them as signatures of cell subtypes or resistance to treatments can better represent the heterogeneity of cancer cells. Classification of patients by cell subtype or combinations thereof revealed that testing additional luminal, NE, and stemness genes showed significantly more patients overexpressing two or more subtypes compared to our initial panel of 14 genes (Figure 6A; $p = 0.039$).

Survival analyses by cell-subtype genes showed relevance of PCa-specific luminal genes for overall survival, while NE and EMT genes were more relevant to progression (Table 3). Patients with genes from all subtypes had shorter time to progression and shorter overall survival. To simplify the cell subtype models, we considered the best 3-gene signatures for each phenotype (luminal: *ARV7*, *KLK3*, *GRHL2*; NE: *AURKA*, *SYP*, *NCAM180*; Stem: *EZH2*, *PROM1*, *KIF2C*). This showed a stronger effect on both overall survival and disease progression (Table 3; Figure 6B, C). Looking at broader gene panels, we found that gene signatures of ARI resistance and the three best genes related to ARI/taxane resistance were relevant to both disease progression and overall survival in Cox analyses (Table 3). Thus, both phenotypic and functional genes are relevant to mCRPC patients' outcomes.

Further analyses by gene signatures showed that PCa-specific luminal genes and stemness genes were significantly associated with higher PSA, while NE genes were more common in patients

with progressive disease (Table 4). Resistance signatures were associated with higher PSA, more lines of treatment, progressive disease, and current taxane treatment, thus being related to more advanced disease overall. Interestingly, the choice of initial treatment also affects gene patterns: NE, EMT, and ARI resistance genes were less common in cases initially treated by RP as opposed to radiotherapy or non-curative treatments, suggesting that these genes were more common in cases who initially presented with more aggressive disease. Altogether, several PCa-relevant genes, including genes representative of each PCa epithelial cell subtype, were overexpressed in blood of mCRPC patients, with gene patterns associated to clinical features and outcomes.

As 23/58 genes were targets of drugs tested in clinical trials for PCa, 11 of which were also cell-subtype specific, we questioned whether they were commonly overexpressed (Figure 6D). Drug target genes were detected in 70% of samples overall. 39-60% of samples overexpressed drug target genes specific to a subtype, and 38% showed targets not specific to a subtype, including 20% of cases with no cell-subtype genes represented. The most common genes by subtype were *FOLH1*, *VEGFA*, and *TACSTD2* (Figure 6E). Overall, genes encoding drug targets are commonly overexpressed and may be relevant for choosing optimal personalized treatments.

DISCUSSION

This investigation demonstrates that a panel of circulating genes representing PCa cell subtypes, treatment resistance, and alternative therapeutic targets shows clinical relevance, thereby opening the way to patient stratification from diagnosis to the end stage of disease.

Liquid biopsies have grown increasingly popular for longitudinally assessing molecular changes in tumours. Commonly studied analytes include CTCs, EVs, and cfDNA; their usefulness, opportunities, and limitations have been described at length in previous reviews. Most CTC

isolation methods based on expression of specific markers or size exclude subsets of cancer cells.¹⁹⁻

²¹ EV isolation yields and purity depend greatly on methodology, and in some applications, rely on marker-based isolation to identify cancer-EVs.²² cfDNA is found in low amounts and identification of actionable alterations requires either previous knowledge about the patient (if utilizing targeted approaches) or NGS which has its own limitations due to cfDNA fragmentation and damage.²³

We elected to study transcripts overexpressed in whole blood to encompass all blood components and overcome the abovementioned technological limitations. However, this approach remains purposefully agnostic to the origin of the transcripts, relying heavily on the choice of genes and methodology. Studies of cancer-specific transcripts in whole blood as non-invasive markers of disease by RT-PCR date back to the 1990s, including *KLK3* in PCa.²⁴ Gene expression microarray and RNA-sequencing have shown interesting results in various cancers, providing a global view of changes in leucocytes. Searching for cancer-specific genes by these approaches has yielded minimal results due to very low expression, as opposed to qPCR which provides a much wider dynamic range of quantitative detection. We opted for TaqMan based RT-qPCR assays, providing higher sensitivity and specificity than SYBR Green-based assays. A smaller amount of sample can be used, allowing the testing of more genes. We carried out extensive technical and biological validation, showing that our results are consistently quantifiable and reproducible, with minimal expression in longitudinally tested controls.

Our strategy for building the gene panel was crucial in order to specify genes relevant to the disease and not influenced by normal interindividual differences, fluctuations in WBC populations, or changes with aging or lifestyle. Our panel was carefully curated to mirror various aspects of advanced disease and genes encoding drug targets. While choosing top upregulated genes in global

expression data is the commonly used approach to select genes of interest, this strategy would easily miss genes representative of NE and stem-like cells due to their rarity, especially in hormone-naïve samples. Thus, we opted to choose genes first based on previous literature and next validating them in transcriptomic datasets allowed us to ensure that these genes are representative of each cell phenotype. We showed the importance of each gene in different categories of patients based on the proportion of cases overexpressing the gene compared to benign prostate rather than in terms of overall differential expression results.

Our exclusion criteria meant that many genes of interest could not be considered, such as the stemness gene *ITGB1* (expressed in WBCs and platelets) and the prostate-specific marker *ACP3* (encoding prostatic acid phosphatase) involved in PCa bone metastasis (expressed in eosinophils).^{25, 26} *VIM* is an important EMT marker but is highly expressed in most WBCs; it is more relevant to study in CTCs than in whole blood RNA.²⁷ We also considered splice variants that are specific to cancer and not expressed in blood. Consequently, the design and choice of primers and probes was of critical importance for higher specificity and lower background expression of *AR*, *NCAM1*, and *CD44*. Overall, our thorough selection criteria proved to be useful, as our results did not correlate to WBCs or age, while several genes and gene signatures correlated with clinical and pathological characteristics and treatments.

Circulating cancer-specific transcripts likely originate from CTCs and EVs released from tumours. For example, overexpression of *AURKA* and *KIF2C* in blood of metastatic patients exclusively may be due to their overexpression specifically in advanced disease. *KLK3* and *FOLH1* are expressed in tumours even in early disease and may be representative of disease burden. Interestingly, while *FOLH1* is detected even at diagnosis, *KLK3* is exclusively detected in mCRPC cases. This may be due to both the lower levels of CTCs/cancer-EVs released in blood at these

early stages or differences in sensitivity between assays. An issue of sensitivity is also seen for genes such as *EGFR* which was not detected in any blood samples but is a common marker of CTCs in PCa.^{28, 29} This can be confirmed by using more sensitive assays, such as ddPCR or targeted sequencing.

Our results are in line with the concept of luminal features reflecting tumour burden, with circulating luminal genes being significantly more common in metastatic patients. While NE and stemness features are known to increase with stage and upon treatments, the predominating cells in most tumours remain luminal-like. This is further evidenced by blood PSA, a marker of inherently luminal-like AR+ PCa cells, reflecting disease progression even at late stages for most patients. The finding that ARI resistance genes are most common in mCRPC cases is in line with the ADT sensitivity at the HSPC stage.

An important concern is whether circulating genes may help to stratify PCa patients at diagnosis and upon recurrence. While this panel was specifically curated to study advanced disease, cell-subtype genes were still relevant for patient stratification at diagnosis. Patients with higher PSA at diagnosis overexpressed more genes, and only intermediate- and high-risk patients overexpressed NE genes. The main findings here showed that cases with intraductal carcinoma, which is associated with poor outcomes,³⁰ overexpressed more genes, especially NE and stemness genes. This shows potential as a non-invasive predictor of intraductal carcinoma, alongside recent findings in cfDNA and multiparametric MRI.^{31, 32} Further follow-up is necessary to determine the relevance of these genes to outcomes after RP.

Testing specific circulating genes in metastatic patients can be promising to offer tailored therapies. We found that *AURKA* was highly predictive of progressive disease at inclusion and was the only gene to predict outcomes in Cox analyses. It came up mainly in cases who progressed on

ARIs and taxanes, and who were on taxanes at the time of blood draw. AURKA is a potential target for metastatic NEPC where it is amplified, being involved in genomic instability via its role in mitosis. Its role in microtubule formation at the spindle pole may be directly related to taxane resistance. A phase II trial of alisertib showed promising results, where 4/60 unselected patients had exceptional response and were confirmed to have NMYC/AURKA overactivity.³³ Evaluating disruptions in NMYC/AURKA in this manner could be useful in the context of clinical trials to either stratify patients without the need for tumour biopsies and whether this can be monitored longitudinally to assess response to these treatments.

Another remarkable NE gene in mCRPC cases was *SSTR2*, overexpressed exclusively in patients who received pelvic radiotherapy. Radiation is known to induce PCa NE differentiation.³⁴ This is in line with the increase in *SSTR2* upon radiation in other cancers, making *SSTRs* a good target for theranostics of NE tumours.³⁵⁻³⁷ Further studies assessing *FOLH1* vs. *SSTR2* overexpression in blood of PCa patients are of interest as measures of tumour heterogeneity and as predictors of response to their respective radionuclide therapies.³⁸

We considered *KIF2C* as a stemness-related gene based on studies in breast, kidney, and bladder cancer.³⁹⁻⁴² It is also a potential therapeutic target overexpressed in both castration-resistant and docetaxel-resistant PCa.⁴³ It was found overexpressed upon paclitaxel resistance, including in triple-negative breast cancer where its inhibition sensitized cells to paclitaxel.⁴⁴ On the other hand, it may be a target of cabazitaxel according to *in vitro* and *in vivo* findings in PCa.^{45, 46} In line with these findings, we find *KIF2C* overexpressed in the blood of patients treated with taxanes at diagnosis or who were currently on taxanes. We could not assess the difference between docetaxel and cabazitaxel response in our limited cohort, but this should be further explored.

In survival analyses, a subset of cell-subtype genes, chosen as the most relevant to survival for each subtype, were highly predictive of time to progression and overall survival. These included genes that were significantly more common in metastatic cases. Choosing this limited set of nine genes may not be sufficient for classification of patients by cell subtype represented in their blood, as it does not represent the heterogeneity covered by our full panel. These genes represent the most relevant in terms of outcomes, while our extended panel may be more important for guiding treatment decisions.

Genes related to ARI and taxane resistance also predicted progression and survival, suggesting that investigating specific mechanisms of resistance may be helpful to better treat patients, especially as some of these genes are targetable. Mixed effects models showed the relevance of these gene sets, where all were overrepresented in cases with higher PSA and progressive disease. ARI resistance genes came up mostly in heavily treated patients currently on taxanes, which is in line with their previous progression on ARIs. Interestingly, NE, EMT/stem, and ARI resistance signatures were significantly under-represented in patients initially treated by surgery as opposed to radiation or non-curative therapies.

A limitation of this study is the inconsistent timing of blood draws, particularly in metastatic cases. It would be ideal to test this panel before treatment initiation, with disease progression, and with change in therapy. Such well-defined cohorts are necessary to refine our signatures. Furthermore, the fact that various targets come up in mCRPC patients and show clinical relevance alone or as part of signatures suggests that this approach may be applied to clinical trials to determine which patients would best benefit from the corresponding drugs.

While the patients we tested at diagnosis all underwent RP, this approach can also be applied to presumably low-risk cases offered active surveillance, focal therapy, or radiotherapy as a curative

treatment. Our gene panel is limited to prostate- and cancer-specific genes. It can be expanded to include genes reflecting systemic immune signatures related to treatment response/resistance or coupled to longitudinal testing of cfDNA, miRNAs, EVs, or CTCs.

CONCLUSION

This study demonstrates the relevance of circulating PCa-relevant genes in whole blood RNA of patients for following phenotypic and functional changes non-invasively and longitudinally throughout the disease trajectory. Our findings revealed associations of cell subtype-specific genes in patients' blood at diagnosis of localized disease with worse pathological features, suggesting they may benefit from more aggressive treatment. At the metastatic stage, individual genes and gene sets relevant to cell subtypes, resistance to current therapeutic modalities, and genes encoding alternate drug targets correlated with clinical parameters and treatments and were predictive of outcomes. Altogether, a better stratification of patients will allow for new clinical trials bringing us closer to personalized therapies for a heterogeneous, unpredictable, and lethal disease.

AUTHOR CONTRIBUTIONS

SC and SD designed the project and were involved in all steps. RSS, ARB, WK, RR, and MD provided clinical information and discussed clinical relevance of results. LH periodically updated patients' clinical information. SD, EJ, AS, QV, and LH processed and banked blood samples. SD, EJ, AS, and MA optimized assays and performed RT-qPCR experiments. SD performed bioinformatic and statistical analyses. S.D., S.C., and A.A. wrote and edit the manuscript, also reviewed by all co-authors.

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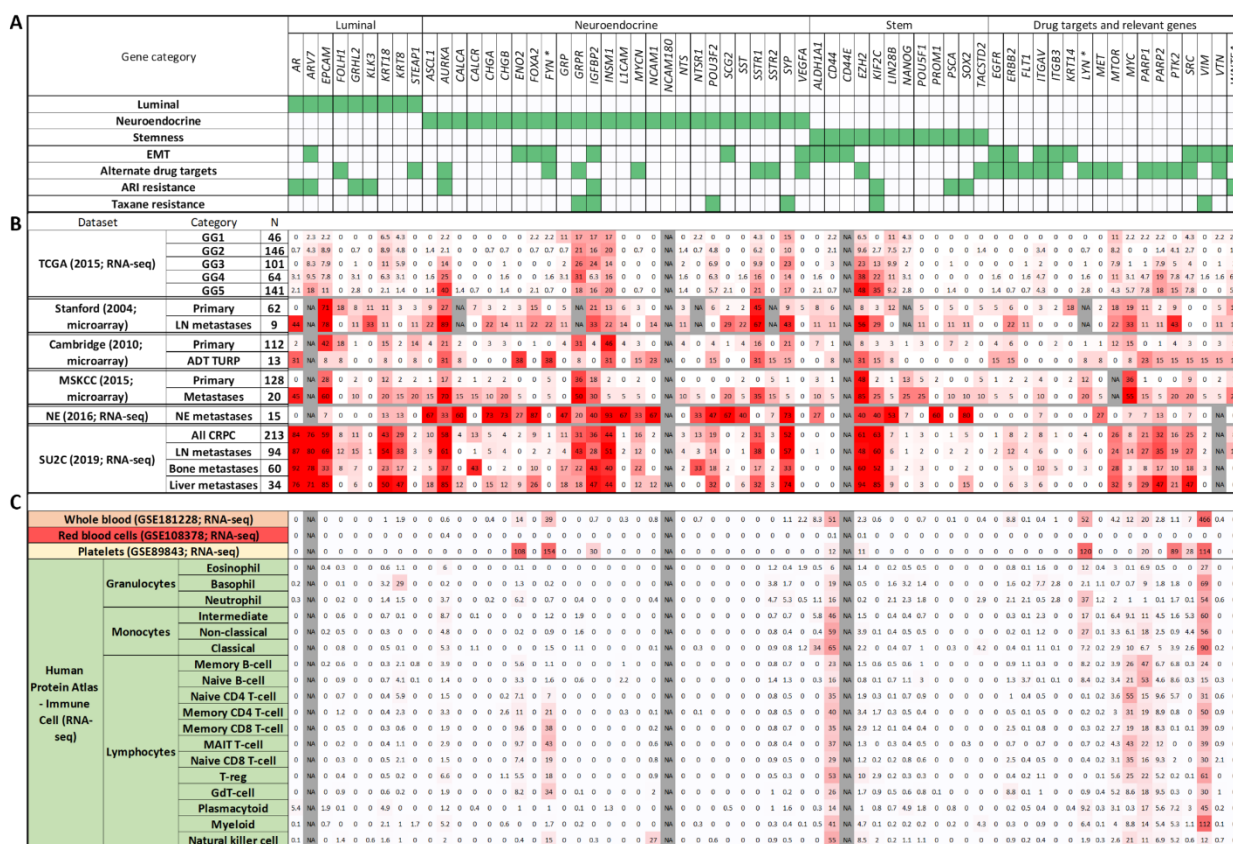
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FIGURES

Figure 1



in the same dataset. For the neuroendocrine (NE) dataset, NEPC cases were compared to non-NE CRPC cases, with a cutoff of 2.58SD. In the SU2C dataset, mCRPC metastases were compared to TCGA benign samples after normalization, as described in Methods. The gradient from white to red represents increasing proportion of cases with overexpression. (C) Expression levels of the same genes in blood datasets. For whole blood, red blood cells, and platelets, RNA-sequencing results are presented as transcripts per million (TPM). Results for white blood cell (WBC) populations are presented in normalized TPM, as described on the Human Protein Atlas. The gradient from white to red represents increasing expression.

Figure 2

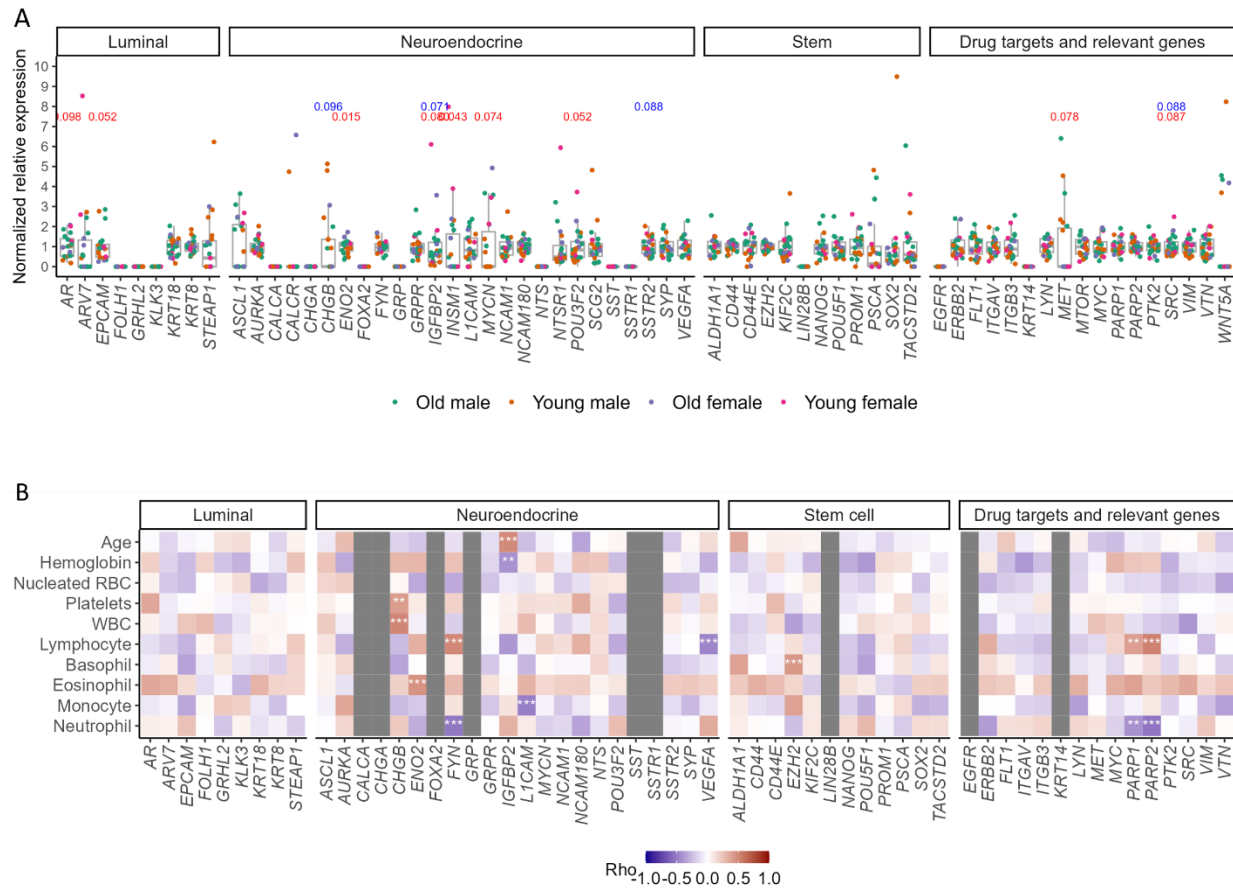


Figure 2: Biological validation of gene panel. (A) Genes expression is presented for healthy controls, grouped as younger vs. older (< vs. > 55 years) and male vs. female, with comparisons by Wilcoxon rank-sum test; p values < 0.1 are shown in blue and red, for differences by age and biological sex, respectively. (B) Heatmap of Spearman correlation results between gene expression in patients' blood vs. age, hemoglobin, platelets, and WBC counts. Only genes showing consistent results are presented, as described in Figure S3. Colors indicate by Rho values, where red and blue represent positive and negative correlations, respectively. Significant p-values are denoted by * for p<0.05; ** for p<0.01; *** for p<0.001.

Figure 3

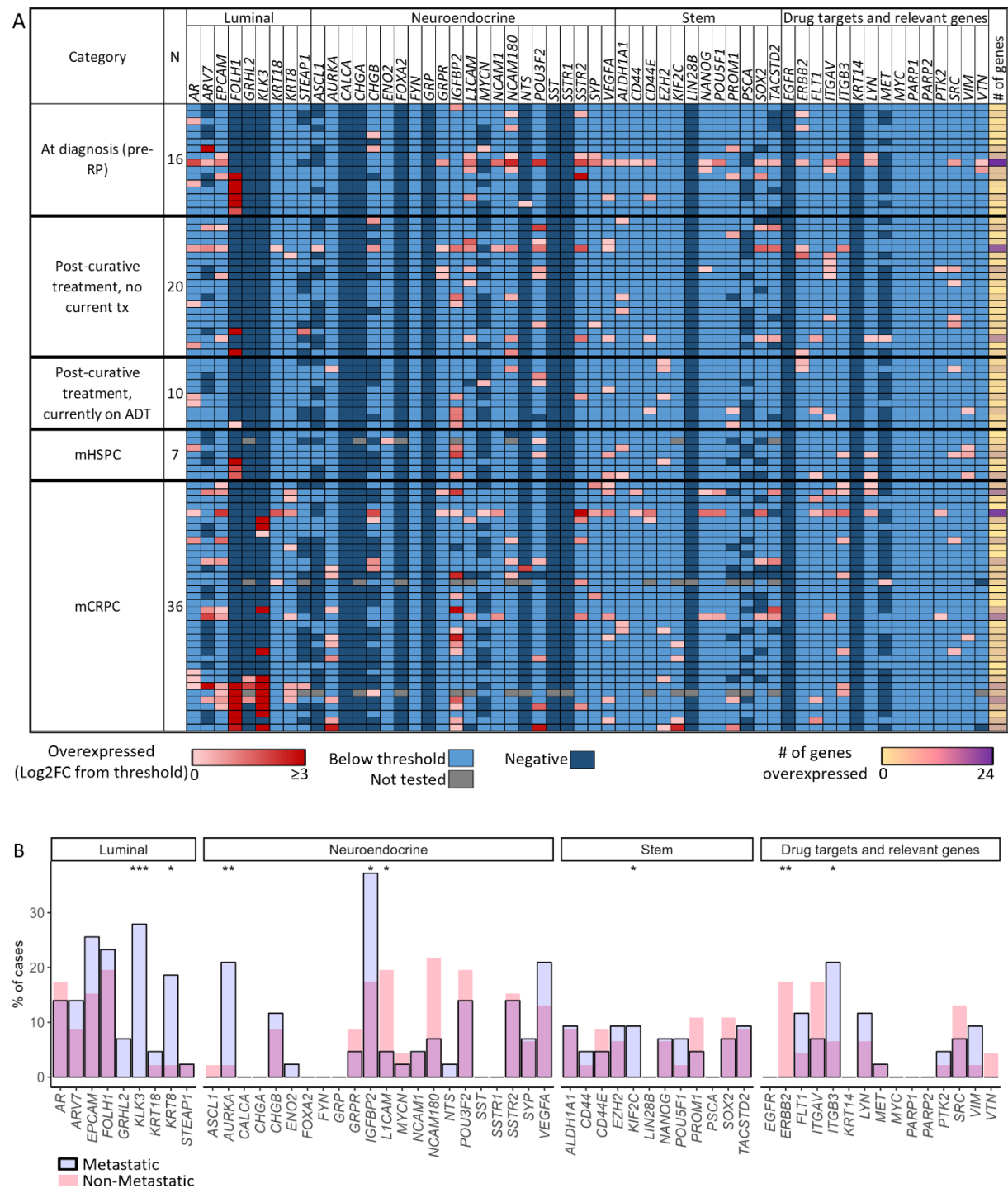


Figure 3: Gene expression patterns in PCa patients at different stages of disease. (A) Heatmap presenting gene expression results in all categories of patients. Gene overexpression is presented as a log2 fold change from the threshold (gradient of pink to red). For genes with no expression in controls (e.g. *KLK3* and *FOLH1*), the gradient represents the fold change from the lowest positive signal detected in patients. Light blue represents expression below overexpression threshold, and dark blue represents no expression. Grey represents genes not tested. The number of overexpressed genes per patient is presented on the right (gradient of yellow to purple). Only genes showing consistent results are presented, as described in Figure S3. **(B)** Bar graph representing the proportion of cases overexpressing each gene for metastatic (blue bars) and non-metastatic cases (pink bars). Significant Chi2 p-values are denoted by * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$.

Figure 4

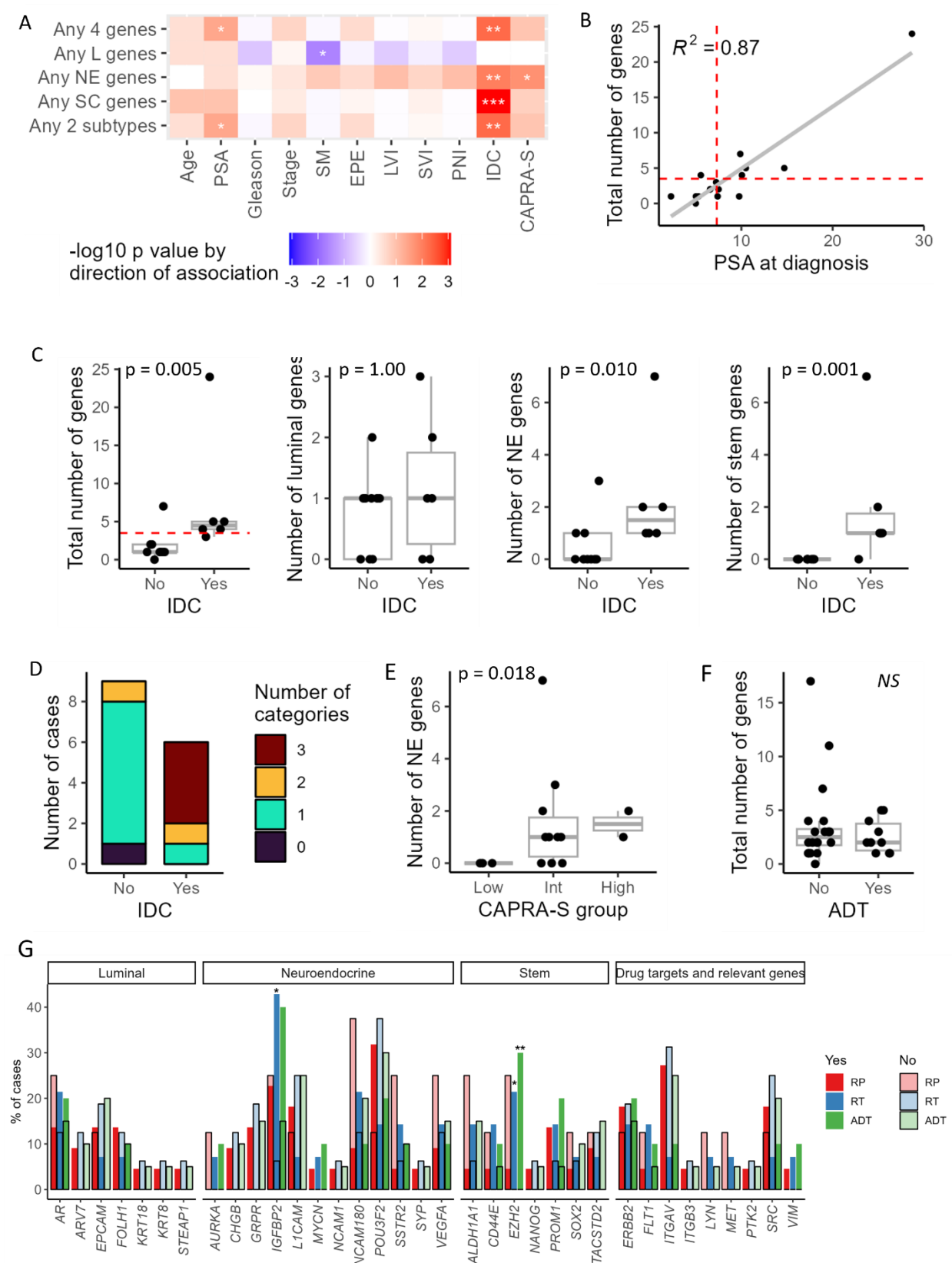


Figure 4: Clinical relevance of circulating genes overexpressed in patients prior to RP and upon recurrence.

Analyses were carried out in blood of patients prior to RP (**A-E**) or post-curative therapies (**F, G**)

(**A**) Heatmap of chi2 results for associations between clinical characteristics or treatments and overexpression of genes in patients pre-RP. Red and blue represent positive and negative correlations, respectively. P-values are entered for significant associations, denoted by * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$. Abbreviations: SM: surgical margin; EPE: extraprostatic extension; LVI: lymphovascular invasion; SVI: seminal vesicle invasion; PNI: perineural invasion; IDC: intraductal carcinoma; CAPRA-S: Cancer of the Prostate Risk Assessment Score (surgical). (**B**) Scatter plot representing the total number of genes overexpressed vs. blood PSA at diagnosis. The vertical dashed line represents the median PSA in these cases. (**C**) Box plots representing the number of genes overexpressed overall or by cell subtype as a function of intraductal carcinoma (IDC). (**D**) Stacked bar graphs representing the number of cell subtypes represented in each case by presence or absence of IDC. (**E**) Box plots representing the number of neuroendocrine (NE) genes overexpressed vs. CAPRA-S risk groups. (**F**) Box plots representing the total number of genes overexpressed vs. current ADT treatment in patients post curative therapies. (**G**) Bar graph representing the proportion of cases overexpressing each gene vs. whether they have undergone specific treatments (RP, RT, and ADT; in solid dark bars), or not (light bars with black border). Significant Chi2 p-values are denoted by * for $p < 0.05$; ** for $p < 0.01$.

Figure 5

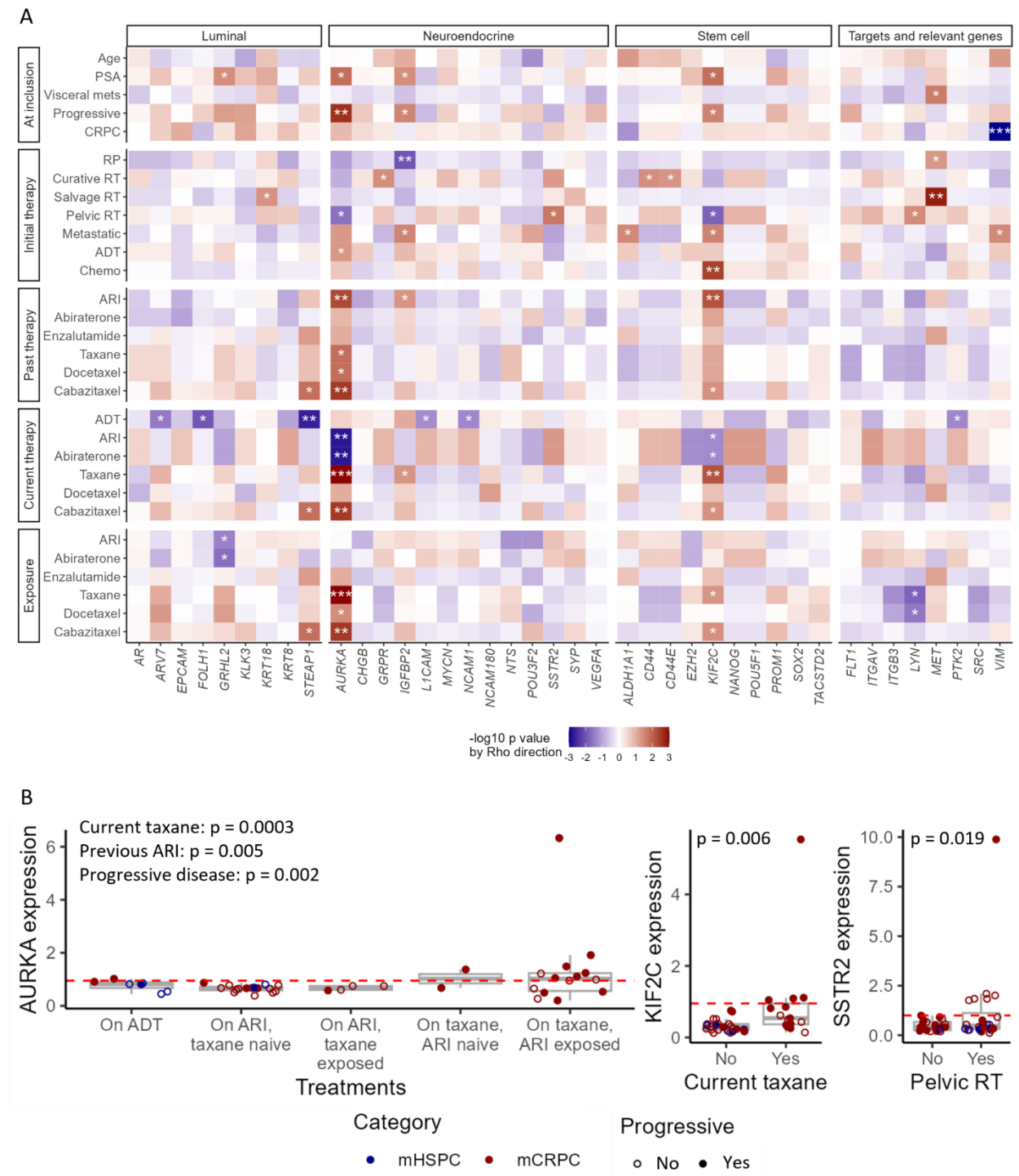


Figure 5: Circulating genes correlate with clinical parameters and treatments in metastatic patients. (A) Heatmap of chi2 results for associations between clinical characteristics or treatments and overexpression of genes in metastatic patients. Red and blue represent positive and negative correlations, respectively. P-values are entered for significant associations, denoted by * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$. (B) Box plots representing expression of *AURKA*, *KIF2C*, and *SSTR2* vs. clinical parameters. Cases with mHSPC and mCRPC are presented in blue and red, respectively; cases with progressive disease are represented as filled red/blue circles, while patients not actively progressing are shown as empty circles. P values for chi2 results are noted.

Figure 6

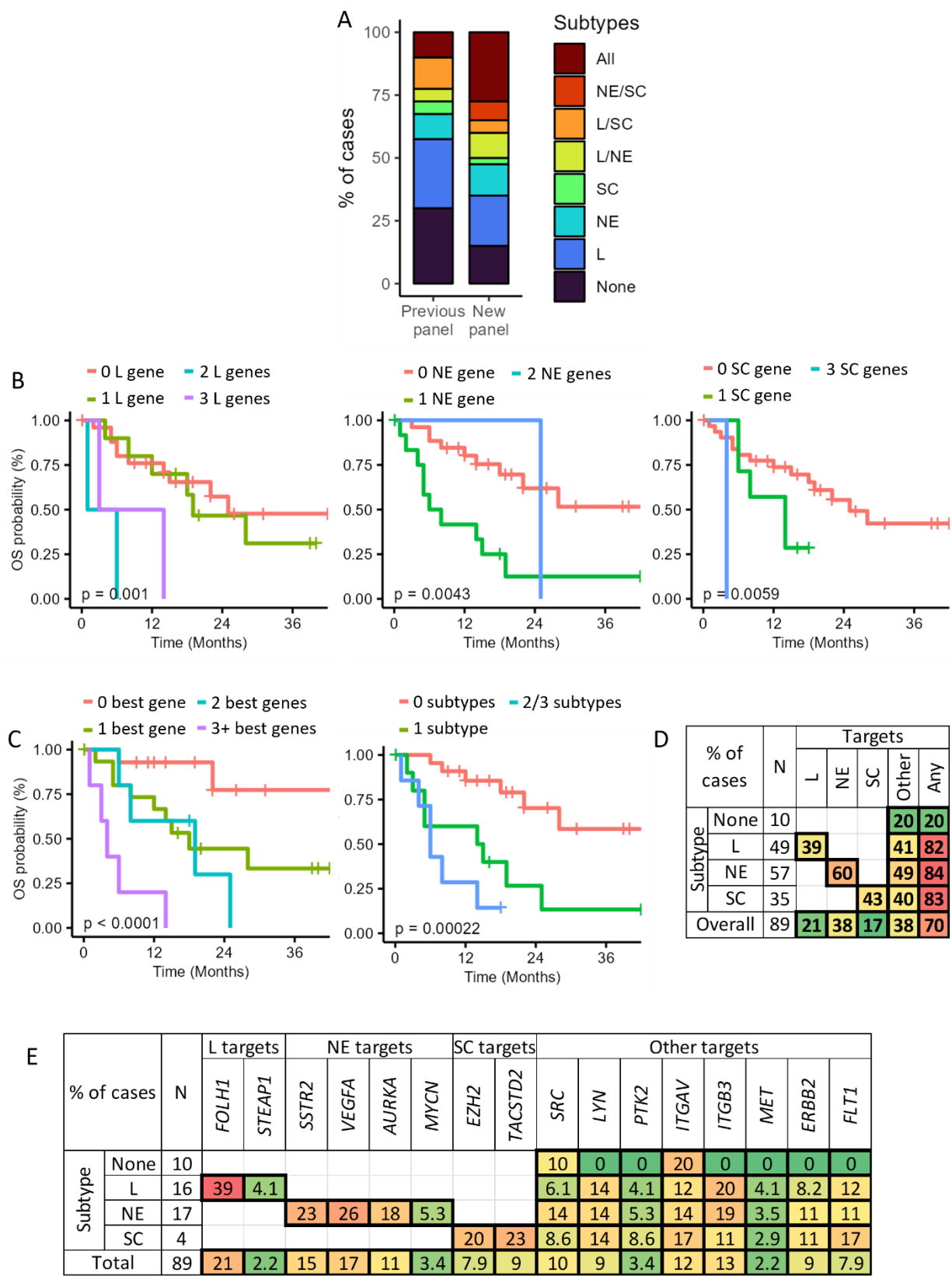


Figure 6: Cell subtype signatures are predictive of outcomes in metastatic patients and drug target genes are commonly overexpressed.

(A) Stacked bar graph comparing results using the initial 14 cell-subtype gene signature vs. all cell-subtype genes included in the 64-gene panel. Results are presented as the proportion of cases classified by subtypes or combinations thereof. (B-C) Kaplan-Meier curves for overall survival stratified by (B) the best luminal, NE, and stemness signatures, (C) the number of genes overexpressed among these three signatures, and the number of cell subtypes represented. (D, E) Heatmaps demonstrating the proportion of cases (classified by cell subtype) for which targetable genes are found overexpressed. The heatmap in (D) summarizes drug targets by cell subtype vs. non-subtype-specific (Other) or any target (Any), whereas results for individual genes are presented in (E).

TABLES

Table 1: Criteria for gene panel selection.

Inclusion criteria	Source
Epithelial cell-subtype specific	Systematic literature review on luminal, neuroendocrine, and stemness genes in PCa Upregulated in NEPC vs. non-NE CRPC (NEPC dataset)
EMT in PCa	Systematic literature review of EMT signatures in PCa
Targets of most interesting drugs tested in clinical trials for PCa	Systematic review of clinical trial results and literature on drugs/targets
Resistance to ARIs	Systematic literature review on AR inhibitor resistance, including clinical studies, <i>in vitro</i> testing, and CTCs in ARI-treated mCRPC cases RNA-seq of diverse mCRPC metastases (ARI-treated vs. ARI-naïve)
Resistance to taxanes	Systematic literature review on taxane resistance, including clinical and <i>in vitro</i> studies RNA-seq of diverse mCRPC metastases (taxane-treated vs. taxane-naïve; SU2C dataset)
Exclusion criteria	Source
High expression in whole blood RNA	GSE181228 (RNA-seq)
High expression in specific WBC population(s)	Human Protein Atlas (RNA-seq)
High expression in red blood cells	GSE108378 (RNA-seq)
High expression in platelets	GSE89843 (RNA-seq)
Difference by age in whole blood of healthy individuals	GSE33828 (microarray)
Difference by smoking status in whole blood of healthy individuals	GSE20189 (microarray)
Difference by diet in whole blood of healthy individuals	GSE18897 (microarray)

ARI: androgen receptor inhibitors; CRPC: Castration-resistant prostate cancer; CTC: circulating tumour cell; EMT: epithelial to mesenchymal transition; NE: neuroendocrine; WBC: white blood cell.

Table 2: Comparison of circulating genes in metastatic vs. non-metastatic patients.

Metastatic vs. non-metastatic	HR	95% CI
Luminal	2.79	1.30-5.98
Luminal (Prostate specific)	2.50	1.09-5.71
Luminal/Epithelial	3.59	1.11-11.55
Neuroendocrine	1.01	0.53-1.94
Stem cell	0.91	0.36-2.28
EMT	1.30	0.61-2.77
SC+EMT	1.08	0.35-3.35
ARI resistance	3.29	1.57-6.93
Taxane resistance	2.00	0.85-4.72
ARI and Taxane resistance	5.44	1.56-6.93

Mixed effects logistic regression for gene signatures in metastatic vs. non-metastatic patients.

Significant results are presented in bold. ARI: androgen receptor inhibitor; CI: confidence interval;

EMT: epithelial to mesenchymal transition; HR: hazard ratio; SC: stem cell.

Table 3: Survival analyses by gene signature in metastatic patients.

Category	PFS		OS	
	HR	95% CI	HR	95% CI
Number of genes	1.07	1.00-1.14	1.00	0.93-1.09
Number of luminal genes	1.13	0.93-1.38	1.17	0.90-1.51
Number of PCa-specific luminal genes	1.20	0.91-1.58	1.49	1.05-2.09
Number of general/epithelial luminal genes	1.00	0.68-1.48	0.84	0.53-1.35
Number of NE genes	1.26	1.03-1.54	1.06	0.84-1.33
Number of stem genes	1.23	0.92-1.64	0.94	0.65-1.35
Number of EMT genes	1.28	1.03-1.59	1.05	0.81-1.37
Number of stemness and EMT genes	1.15	1.01-1.32	1.02	0.87-1.20
Any luminal gene	0.83	0.41-1.64	0.92	0.38-2.26
Any PCa-specific luminal genes	1.14	0.57-2.28	1.28	0.52-3.13
Any general/epithelial luminal genes	0.80	0.40-1.58	0.71	0.29-1.72
Any NE gene	2.68	1.25-5.72	2.18	0.84-5.69
Any stemness gene	1.61	0.81-3.22	1.24	0.51-2.98
Any EMT gene	2.48	1.16-5.27	2.19	0.83-5.71
Any stemness or EMT gene	2.47	1.12-5.48	3.05	1.01-9.15
Any 2 luminal (PCa), 2 NE, and 1 stem gene	2.36	0.69-8.10	6.42	1.77-23.30
Number of subtypes - 1	2.82	1.07-7.40	2.81	0.84-9.39
Number of subtypes - 2	2.60	1.03-6.56	1.44	0.39-5.38
Number of subtypes - 3	4.97	1.20-20.57	10.80	2.28-51.08
3 best luminal genes	2.48	0.82-7.48	6.71	2.08-21.68
3 best NE genes	3.54	1.46-8.55	3.72	1.53-9.04
3 best SC genes	1.56	0.66-3.65	2.77	1.00-7.73
Any 2 of the best genes	3.40	1.39-8.31	4.09	1.65-10.17
Number of best genes	1.43	1.11-1.85	1.72	1.31-2.27
Number of subtypes - 1	2.24	0.94-5.36	4.44	1.53-12.89
Number of subtypes - 2/3	4.10	1.44-11.63	8.34	2.48-28.05
Number of ARI resistance genes	1.51	1.18-1.94	1.43	1.07-1.90
Number of taxane resistance genes	1.68	1.11-2.55	1.24	0.78-1.95
Number of any resistance genes	1.78	1.21-2.59	1.72	1.12-2.62
Any ARI resistance gene	3.89	1.66-9.12	3.51	1.03-12.00
Any taxane resistance gene	2.59	1.22-5.48	2.06	0.85-4.99
Any 2 resistance genes	2.93	1.19-7.24	3.76	1.47-9.62

Cox analyses were carried out by gene signature for progression-free survival (PFS) or overall survival (OS). Significant results are presented in bold ($p < 0.05$). ARI: androgen receptor inhibitor; CI: confidence interval; EMT: epithelial to mesenchymal transition; HR: hazard ratio; NE: neuroendocrine; SC: stem cell.

Table 4

HR (95% CI)		Luminal	Neuroendocrine	Stemness	Stem/EMT	ARI resistance	Taxane resistance
Age ¹		0.66 (0.22-2.01)	1.28 (0.51-3.24)	1.82 (0.48-6.81)	1.77 (0.58-5.36)	0.91 (0.37-2.19)	1.86 (0.59-5.90)
PSA ¹		3.11 (1.12-8.64)	1.85 (0.83-4.12)	4.00 (1.04-15.28)	4.54 (1.48-13.92)	4.00 (1.98-8.09)	3.93 (1.25-12.35)
Initial therapy	Surgery	0.43 (0.11-1.75)	0.18 (0.05-0.70)	0.11 (0.01-1.13)	0.13 (0.03-0.64)	0.18 (0.05-0.66)	0.14 (0.02-1.12)
	Curative RT	1.13 (0.33-3.80)	1.15 (0.42-3.14)	1.22 (0.31-4.79)	1.21 (0.36-4.07)	0.63 (0.24-1.65)	0.85 (0.25-2.95)
	Non-curative	1.34 (0.44-4.10)	1.54 (0.60-3.91)	2.01 (0.54-7.51)	1.96 (0.65-5.92)	2.50 (1.10-5.70)	2.00 (0.68-5.88)
	Any pelvic RT	1.31 (0.42-4.10)	1.30 (0.50-3.36)	1.36 (0.38-4.92)	1.55 (0.51-4.71)	0.62 (0.26-1.45)	0.72 (0.24-2.20)
Past mCRPC therapy	ARI	0.41 (0.12-1.43)	1.06 (0.40-2.81)	0.74 (0.19-2.83)	0.95 (0.30-3.01)	1.67 (0.70-3.99)	2.55 (0.88-7.34)
	Taxane	0.80 (0.29-2.22)	1.09 (0.50-2.36)	1.06 (0.37-3.06)	0.70 (0.31-1.60)	1.62 (0.78-3.39)	1.10 (0.38-3.19)
Current therapy	ARI	1.56 (0.56-4.30)	0.62 (0.26-1.46)	0.82 (0.25-2.68)	0.89 (0.39-2.07)	0.56 (0.26-1.21)	0.66 (0.23-1.95)
	Taxane	1.20 (0.43-3.40)	1.74 (0.76-4.00)	2.51 (0.61-10.41)	1.65 (0.65-4.20)	2.72 (1.31-5.66)	2.63 (0.91-7.60)
Line of therapy		0.89 (0.50-1.61)	1.24 (0.77-1.99)	1.16 (0.63-2.16)	0.94 (0.58-1.53)	1.57 (1.04-2.37)	1.54 (0.88-2.69)
Progressive disease		2.01 (0.80-5.05)	2.51 (1.18-5.32)	1.47 (0.53-4.02)	2.44 (1.13-5.26)	4.31 (2.19-8.49)	3.81 (1.36-10.71)

Mixed effects logistic regression for gene signatures vs. clinical parameters and treatments in metastatic patients. Significant results are presented in bold. ¹Patients were stratified below and above the median. ARI: androgen receptor inhibitor; EMT: epithelial to mesenchymal transition; PSA: prostate specific antigen; RT: radiation therapy.

SUPPLEMENTAL MATERIALS

Table S1: Assay details and efficiencies

Gene	Assay	Manufacturer	Amplicon length	RefSeq sequence	Exon boundary	Assay location	Assay efficiency (%)
<i>ALDH1A1</i>	Hs00946916_m1	Life Tech	61	NM_000689.4	8_9	1173	105.27
<i>AR (ARV7 specific)</i>	custom	Life Tech	91	NM_001348061.1	4	2465-2555	107.24
<i>AR (full length AR and AR45 specific)</i>	Hs00171172_m1	Life Tech	72	NM_000044.3	4_5	3289	107.24
<i>ASCL1</i>	Hs00269932_m1	Life Tech	60	NM_004316.3	1_2	1332	94.84
<i>AURKA</i>	Hs01582073_m1	Life Tech	117	NM_001323303.1	7_8	1211	97.41
<i>CALCA</i>	Hs01100741_m1	Life Tech	84	NM_001033952.2	2_3	160	102.18
<i>CALCR</i>	Hs00156229_m1	Life Tech	89	NM_001164737.1	10_11	1055	94.51
<i>CD44</i>	Hs01075861_m1	Life Tech	70	NM_000610.3	17-18	2462	107.75
<i>CD44 (CD44E specific)</i>	Hs01081475_m1	Life Tech	82	NM_001001390.1	5_6	1099	106.61
<i>CHGA</i>	custom	IDT	111	NM_001275.3	4_5	470-581	105.96
<i>CHGB</i>	Hs01084631_m1	Life Tech	112	NM_001819.2	3_4	498	106.43
<i>EGFR</i>	Hs01076088_m1	Life Tech	71	NM_005228.3	3_4	671	97.84
<i>ENO2</i>	Hs00157360_m1	Life Tech	77	NM_001975.2	7_8	889	100.43
<i>EPCAM</i>	HS00901888_g1	Life Tech	93	NM_002354.2	6_7	1021	96.01
<i>ERBB2</i>	Hs01001580_m1	Life Tech	60	NM_001005862.2	17_18	2223	95.16
<i>EZH2</i>	Hs01016789_m1	Life Tech	128	NM_001203247.1	16_17	2121	101.37
<i>FLT1</i>	HS01052961_m1	Life Tech	72	NM_001159920.1	8_9	1387	91.23
<i>FOLH1</i>	HS00379515_m1	Life Tech	110	NM_001014986.1	4_5	769	101.41
<i>FOXA2</i>	Hs00232764_m1	Life Tech	66	NM_021784.4	1_2	278	104.96
<i>FYN (isoform A specific)</i>	Hs00941606_m1	Life Tech	91	NM_002037.5	9_10	1469	104.76
<i>GRHL2</i>	Hs00227745_m1	Life Tech	82	NM_024915.3	12_13	1846	104.85
<i>GRP</i>	Hs01107047_m1	Life Tech	80	NM_001012512.2	1_2	239	98.99
<i>GRPR</i>	Hs01055872_m1	Life Tech	68	NM_005314.2	2_3	1422	107.87
<i>IGFBP2</i>	Hs01040719_m1	Life Tech	54	NM_000597.2	3_4	941	104.70

<i>INSM1</i>	Hs00357871_s1	Life Tech	72	NM_002196.2	1	845	109.05
<i>ITGAV</i>	Hs01041361_m1	Life Tech	90	NM_001144999.2	25_26	2580	107.32
<i>ITGB3</i>	Hs00173978_m1	Life Tech	89	NM_000212.2	10_11	1714	107.74
<i>KIF2C (variant 1/2 specific)</i>	Hs00901716_m1	Life Tech	72	NM_006845.3	2_3	279	106.25
<i>KLK3</i>	HS02576345_m1	Life Tech	83	NM_001030047.1	1_2	83	96.96
<i>KRT14</i>	Hs00265033_m1	Life Tech	64	NM_000526.4	7_8	1385	102.20
<i>KRT18</i>	Hs02827483_g1	Life Tech	64	NM_000224.2	1_2	531	95.77
<i>KRT8</i>	Hs02339473_g1	Life Tech	118	NM_001256282.1	6_7	1089	95.70
<i>L1CAM</i>	Hs00240928_m1	Life Tech	102	NM_000425.4	4_5	615	93.59
<i>LIN28B</i>	Hs01013729_m1	Life Tech	130	NM_001004317.3	2_3	401	110.45
<i>LYN (LYN56 specific)</i>	Hs01015815_m1	Life Tech	133	NM_002350.3	2_3	411	106.07
<i>MET</i>	Hs01565584_m1	Life Tech	73	NM_000245.3	3_4	1594	101.35
<i>MTOR</i>	Hs00234508_m1	Life Tech	103	NM_004958.3	20_21	3240	107.24
<i>MYC</i>	Hs00153408_m1	Life Tech	107	NM_002467.4	2_3	1325	94.31
<i>MYCN</i>	Hs00232074_m1	Life Tech	81	NM_001293228.1	2_3	1525	106.95
<i>NANOG</i>	Hs02387400_g1	Life Tech	109	NM_001297698.1	1_2	366	112.24
<i>NCAM1</i>	Hs00941830_m1	Life Tech	58	NM_000615.6	3_4	711	105.88
<i>NCAM1 (NCAM180 specific)</i>	Hs00941837_m1	Life Tech	76	NM_181351.4	8_9	1430	104.61
<i>NTS</i>	Hs00175048_m1	Life Tech	75	NM_006183.4	3_4	467	94.90
<i>NTSR1</i>	Hs00173592_m1	Life Tech	113	NM_002531.2	2_3	1290	98.34
<i>PARP1</i>	Hs00242302_m1	Life Tech	111	NM_001618.3	7_8	1187	105.55
<i>PARP2</i>	Hs00193931_m1	Life Tech	116	NM_001042618.1	4_5	358	101.07
<i>PGK1 (reference gene)</i>	Hs00943178_g1	Life Tech	73	NM_000291.3	5_6	73	102.86
<i>POU3F2</i>	Hs00271595_s1	Life Tech	100	NM_005604.3	1	1724	93.65
<i>POU5F1</i>	Hs00999632_g1	Life Tech	77	NM_001173531.2	2_3	753	103.87
<i>PPIB (reference gene)</i>	Hs00168719_m1	Life Tech	67	NM_000942.4	3_4	511	96.02
<i>PROM1</i>	Hs01009259_m1	Life Tech	66	NM_001145847.1	6_7	1186	107.87
<i>PSCA</i>	Hs04177224_g1	Life Tech	107	NM_005672.4	2_3	216	104.00
<i>PTK2</i>	Hs01056441_m1	Life Tech	73	NM_001199649.1	16_17	1640	101.12

<i>RPLP0 (reference gene)</i>	Hs00420895_Gh	Life Tech	76	NM_001002.3	3_4	406	100.17
<i>SCG2</i>	Hs01920882_s1	Life Tech	116	NM_003469.4	2	2205	96.56
<i>SOX2</i>	Hs01053049_s1	Life Tech	91	NM_003106.3	1	1539	108.54
<i>SRC</i>	Hs01082246_m1	Life Tech	67	NM_005417.4	11_12	1567	101.51
<i>SST</i>	Hs00356144_m1	Life Tech	86	NM_001048.3	1_2	263	91.63
<i>SSTR1</i>	Hs.PT.58.3617180.g	IDT	124	NM_001049.3	2_3	289-412	107.25
<i>SSTR2</i>	Hs00265624_s1	Life Tech	70	NM_001050.2	2	917	104.81
<i>STEAP1</i>	Hs00185180_m1	Life Tech	78	NM_012449.2	4_5	965	99.29
<i>SYP</i>	Hs00300531_m1	Life Tech	63	NM_003179.2	3_4	244	103.42
<i>TACSTD2</i>	Hs00242741_s1	Life Tech	64	NM_002353.2	1	80	103.33
<i>VEGFA</i>	Hs00900055_m1	Life Tech	59	NM_001025366.2	3_4	1352	100.27
<i>VIM</i>	Hs00185584_m1	Life Tech	73	NM_003380.3	2_3	978	102.62
<i>VTN</i>	Hs00940757_g1	Life Tech	69	NM_000638.3	1_2	219	104.58
<i>WNT5A</i>	Hs00180103_m1	Life Tech	101	NM_001256105.1	2_3	205	94.66

Gene	Forward primer sequence	Reverse primer sequence	Probe sequence
<i>ARV7</i>	GGCTGACTTGCCTCATTCAAAT	AACCTAAGCTCCTAAGCCTCAGA	CCAGGTTTCTCCAGACTAT
<i>CHGA</i>	GAGACATCAGAATTTACTGAAGGAG	ACCTCTGAGAGTTCATCTTCAA	CAAGGAGAGGGCACATCAGCAGAA
<i>SSTR1</i>	AGATCGTCAACCTGCTAACAG	CTGGACTGGTAAATGATTAATAGGC	ACTGAGACGTAGATGGTCGGTGCA

Assays bought from Life Technologies do not provide primer and probe sequences, but do provide assay location, amplicon length, exon boundaries, and variant specificity information.

Table S2: Cox analyses by gene for overall survival and progression-free survival

Category	Genes	Progression-free survival		Overall survival	
		HR	95% CI	HR	95% CI
Luminal	<i>AR</i>	1.23	0.47-3.19	1.03	0.30-3.50
	<i>ARV7</i>	1.94	0.79-4.80	1.75	0.64-4.76
	<i>EPCAM</i>	1.08	0.51-2.31	1.05	0.41-2.72
	<i>FOLH1</i>	0.77	0.34-1.77	0.83	0.28-2.48
	<i>GRHL2</i>	2.36	0.69-8.10	4.67	1.29-16.90
	<i>KLK3</i>	1.13	0.54-2.38	1.81	0.76-4.32
	<i>KRT18</i>	1.45	0.34-6.14	0.59	0.08-4.44
	<i>KRT8</i>	0.72	0.31-1.69	0.40	0.11-1.38
	<i>STEAP1</i>	2.35	0.31-17.68	12.49	1.30-120.11
Neuroendocrine	<i>AURKA</i>	3.45	1.41-8.44	3.58	1.46-8.78
	<i>CHGB</i>	1.25	0.43-3.60	0.65	0.15-2.81
	<i>GRPR</i>	1.37	0.32-5.76	0.48	0.06-3.67
	<i>IGFBP2</i>	2.53	1.12-5.27	1.80	0.75-4.34
	<i>L1CAM</i>	0.72	0.17-3.07	NA	NA
	<i>MYCN</i>	2.47	0.33-18.56	NA	NA
	<i>NCAM1</i>	1.93	0.45-8.24	NA	NA
	<i>NCAM180</i>	1.86	0.55-6.26	2.03	0.59-7.00
	<i>NTS</i>	2.35	0.31-17.68	1.97	0.26-14.99
	<i>POU3F2</i>	1.53	0.58-4.00	2.35	0.77-7.14
	<i>SSTR2</i>	1.01	0.42-2.46	0.46	0.11-1.97
	<i>SYP</i>	1.67	0.38-7.25	0.86	0.12-6.48
	<i>VEGFA</i>	2.02	0.88-4.64	0.87	0.29-2.60

Stemness	<i>ALDH1A1</i>	1.61	0.55-4.69	0.82	0.19-3.56
	<i>CD44</i>	1.37	0.32-5.76	0.48	0.06-3.67
	<i>CD44E</i>	0.81	0.19-3.47	NA	NA
	<i>EZH2</i>	1.04	0.32-3.42	2.57	0.73-9.05
	<i>KIF2C</i>	2.57	0.85-7.81	2.78	0.78-9.87
	<i>NANOG</i>	1.44	0.44-4.77	0.30	0.04-2.28
	<i>POU5F1</i>	1.44	0.44-4.77	0.30	0.04-2.28
	<i>PROM1</i>	2.29	0.53-9.92	3.68	0.83-16.41
	<i>SOX2</i>	2.15	0.64-7.26	0.35	0.05-2.63
	<i>TACSTD2</i>	1.55	0.54-4.47	1.19	0.34-4.15
Drug targets and relevant genes	<i>FLT1</i>	1.36	0.52-3.55	0.61	0.14-2.62
	<i>ITGAV</i>	1.44	0.44-4.77	0.30	0.04-2.28
	<i>ITGB3</i>	1.10	0.48-2.54	1.08	0.39-2.96
	<i>LYN</i>	1.54	0.53-4.45	0.34	0.04-2.51
	<i>MET</i>	1.01	0.14-7.51	1.55	0.21-11.65
	<i>PTK2</i>	1.93	0.45-8.24	NA	NA
	<i>SRC</i>	2.15	0.64-7.26	2.54	0.56-11.51
	<i>VIM</i>	2.24	0.76-6.59	0.61	0.08-4.56

Significant results are presented in bold. HR: hazard ratio; CI: confidence interval.

Figure S1

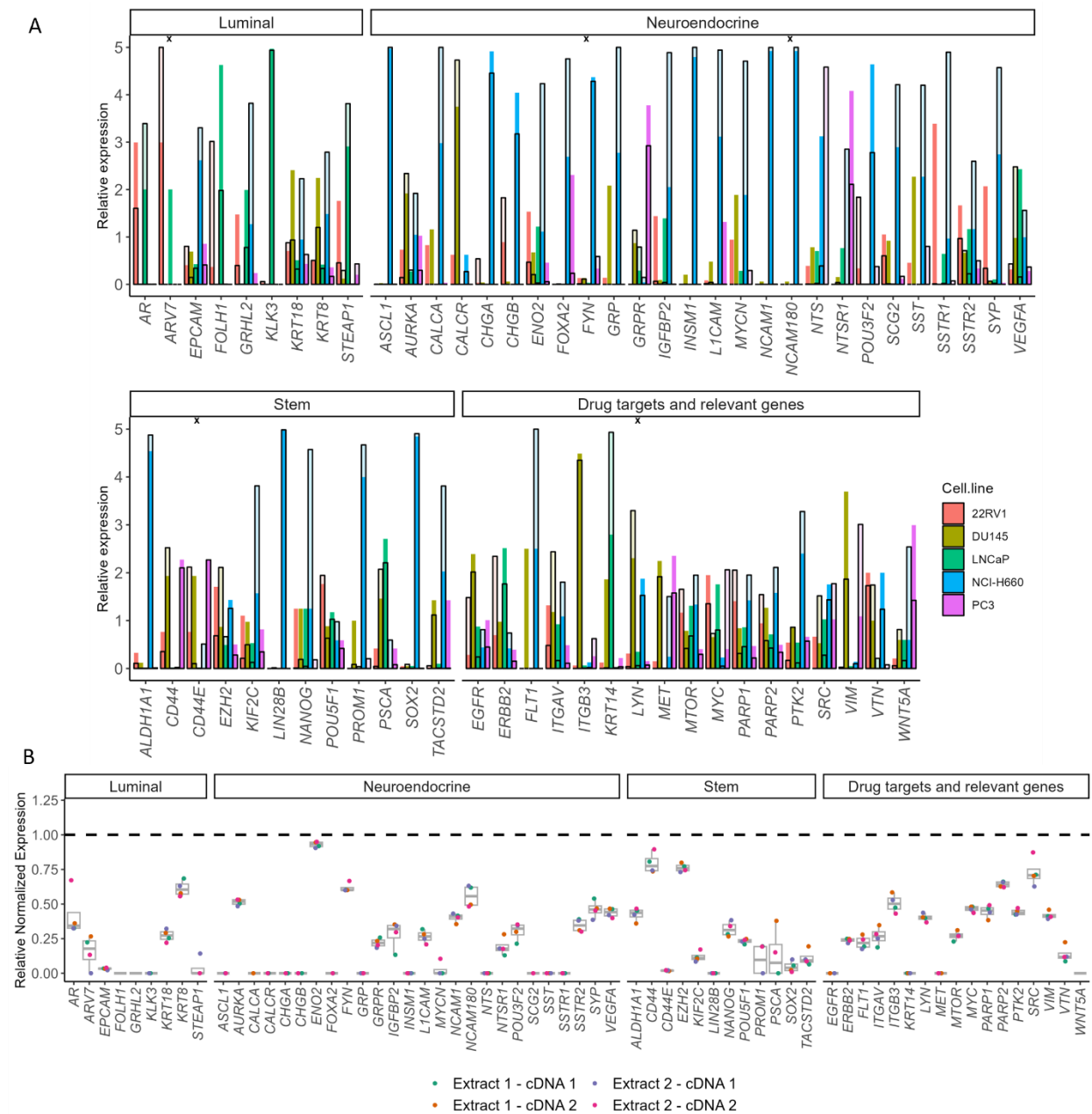


Figure S1: Gene expression is in line with results in cell lines and shows consistent results throughout batches of RNA extraction and cDNA synthesis.

(A) Genes were tested by RT-qPCR in RNA of five PCa cell lines and compared to expected expression based on RNA-seq results from the CCLE dataset. Results are presented as relative expression among the five cell lines, based on $2^{-\Delta\text{Ct}}$ for qPCR results and TPM for RNA-seq

results. Contoured bars represent qPCR data and filled bars represent RNA-seq data. Genes marked with an x were tested as specific splice variants and are not expected to correlate with RNA-seq data. (B) Each gene was tested in a single control blood collection from which two batches of RNA extraction and two batches of cDNA synthesis were performed. Extractions and cDNA synthesis were performed on separate days and using separate kits. Expression is presented as the fold change from overexpression threshold (dashed line).

Figure S2

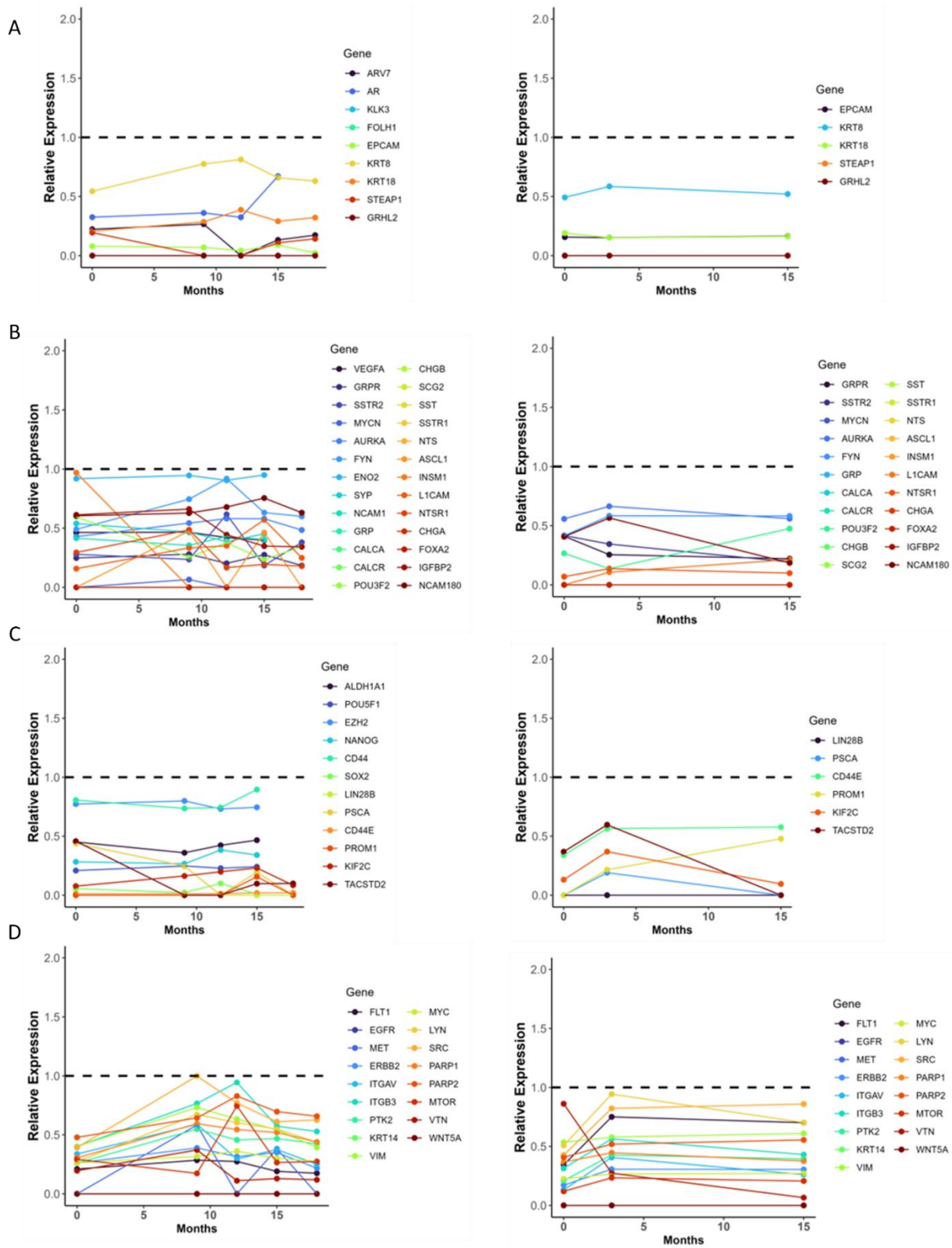


Figure S2: Longitudinal testing in control blood. All genes were tested in longitudinally collected blood samples from two healthy controls (left and right panels). Results are presented as fold change from the overexpression threshold for (A) luminal, (B) neuroendocrine, (C) stemness, and (D) drug targets and relevant genes.

Figure S3

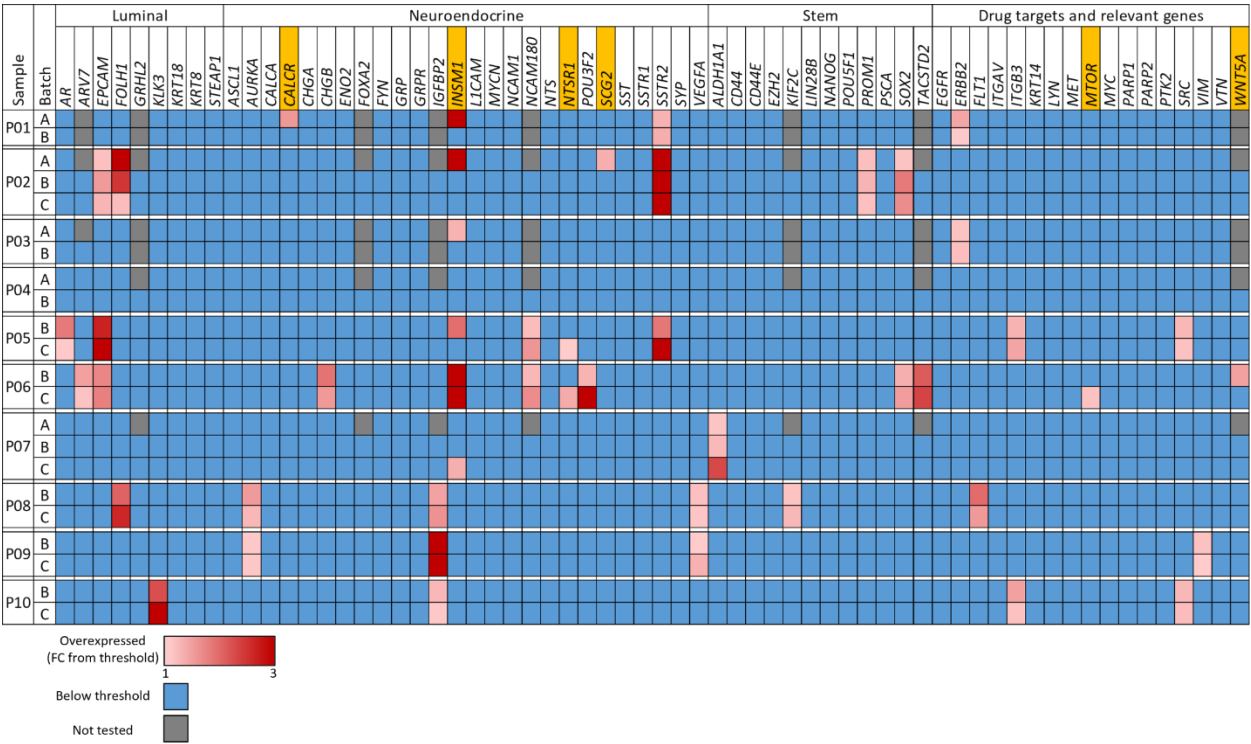
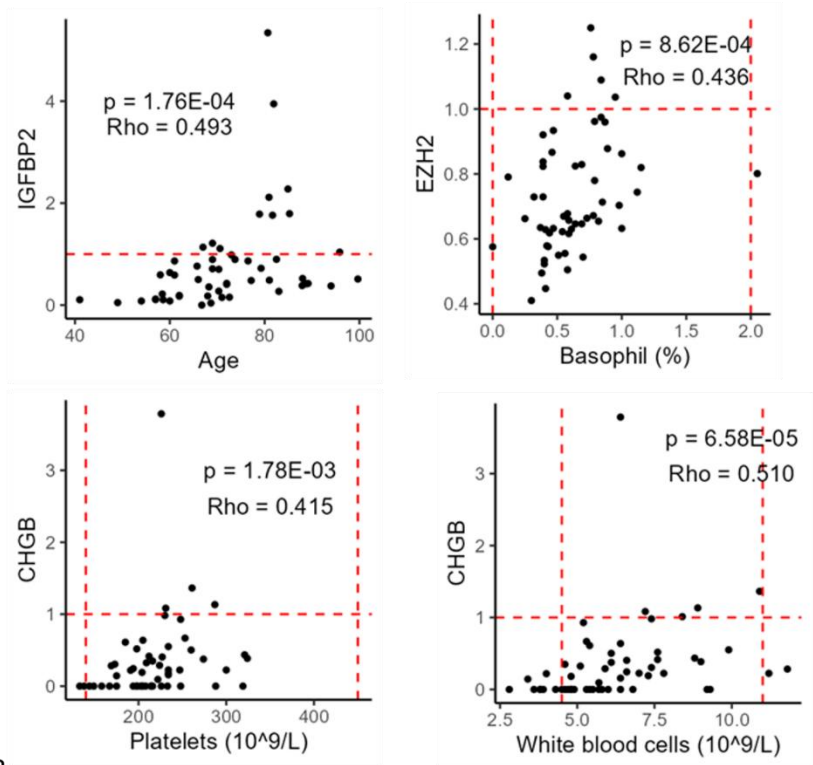


Figure S3: Reproducibility of gene expression in patients' blood RNA.

Results are presented as a heatmap, where gene overexpression is presented as a fold change from the threshold in a gradient of pink to red. For genes with no expression in controls (e.g. *KLK3* and *FOLH1*), the gradient represents the fold change from the lowest positive signal in patients. Blue represents expression below overexpression threshold. Grey represents genes not tested in the batch. The six genes in orange showed inconsistent results and were excluded from further analyses.

Figure S4

A



B

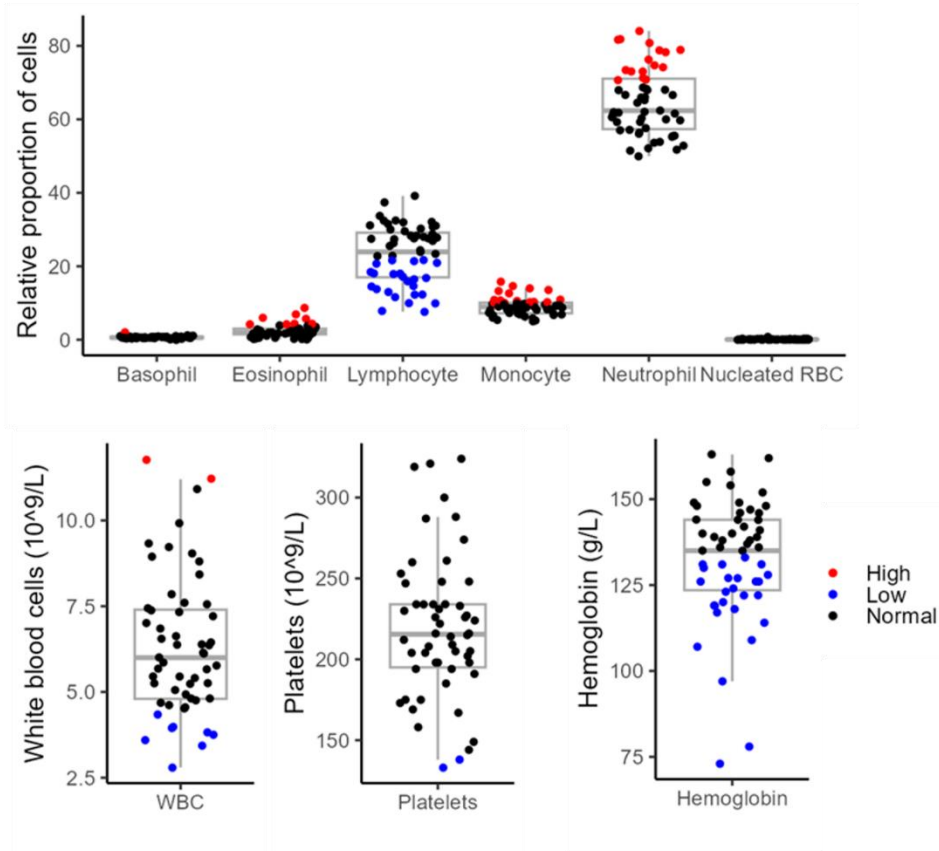


Figure S4: Comparison of patients' blood counts vs. gene expression. (A) Scatter plots for genes vs. parameters that were significantly positively correlated (see Figure 2B). The horizontal red dashed line represents the overexpression threshold; vertical red dashed lines represent normal limits for blood components. (B) Relative proportions of WBC populations, total WBCs, platelets, and hemoglobin in patients' blood at the time of blood draw. Black represents normal results, red higher than the normal range, and blue lower than the normal range.

MANUSCRIPT 4: Transcriptomics of canine prostate cancers reveals similarities to aggressive features of human metastatic castration resistant disease

Seta Derderian, Eleonora Scarlata, Lucie Hamel, Murillo Luz, Armen Aprikian, Maurice Anidjar, Simone Chevalier

In preparation for submission to Cancer Cell International.

PREFACE

While PCa is all too common in humans, it is almost completely absent in all other mammals with the exception of dogs. The prostate of men and dogs are anatomically and histologically similar, especially when compared to that of rodents which are commonly used as experimental models of PCa.²⁹⁷ Canine PCa is rare (~1%) and is diagnosed at a mean age of 10 years; it is most common in medium and large breeds. Approximately 80% develop metastases, the main sites being LNs, bone, and lungs.^{298, 299} It can be classified as prostatic adenocarcinoma, urothelial carcinoma, or mixed, where urothelial carcinoma can usually be distinguished by CK7 and uroplakin III staining.³⁰⁰ Morphology for prostatic adenocarcinoma is heterogeneous, including tubular, solid, papillary, cribriform, micropapillary, and sarcomatoid patterns.^{300, 301} A modified Gleason score derived for canine disease classified 46.7% of cases as Gleason score 10.³⁰² Overall, canine disease is aggressive compared to human primary PCa.

Canine PCa is androgen independent, irrespective of castration status. It remains unclear whether castration has any effect on canine PCa incidence, although it does seem to increase survival time.³⁰³ The known molecular features of canine disease include loss of AR, deletion or mutation of *PTEN*, increased PI3K/AKT/mTOR signaling, and features of stemness and EMT; these are in line with many features seen in AR-independent human mCRPC.^{304, 305} Interestingly, there is

disagreement on the presence of NE cells in the normal canine prostate and PCa.³⁰⁴⁻³⁰⁷ Overall, canine PCa is of interest as a spontaneous model to better understand human castration-resistance.

The host lab have previously reported on the canine DPC-1 cell line, derived from a poorly differentiated prostate adenocarcinoma.³⁰⁸ After intra-prostatic implantation in large dogs, it forms LN, bone, and lung metastases with micropapillar and cribriform growth patterns.³⁰⁹ This model was used to test an PSMA-based SPECT/CT imaging approach to detect lesions, further establishing the relevance of canine models in terms of technology transfer to the clinic.³¹⁰

Having access to DPC-1 tumours from previous studies in large dogs and to transcriptomics of canine and human PCa, we conducted a comparative oncology study to further dissect canine disease with an emphasis on the DPC-1 model. We tested whether our findings on human prostate epithelial cell subtypes were applicable to canine PCa models and naturally occurring canine PCa. We further explored the canine disease to identify the most promising markers and potential targets to manage the disease in both dogs and humans. Finally, we monitored PCa-relevant genes in longitudinally collected blood of dogs bearing DPC-1 tumours. Overall, we achieved a better understanding of the DPC-1 model and naturally occurring PCa in dogs at the molecular level and characterized its relevance to advanced human disease.

CONTRIBUTION OF AUTHORS

Seta Derderian: Performed all bioinformatic analyses of transcriptomic data from canine and human PCa. Extracted RNA from banked blood samples. Identified genes to test in blood and optimized assays. Performed RT-qPCR in blood RNA and analyzed results. Wrote the manuscript.

Eleonora Scarlata (veterinary pathologist): Performed necropsy of dogs bearing DPC-1 tumours, including collection of primary tumours and metastases. Analyzed all FFPE sections of prostate tissues and metastases. Dissected fresh-frozen tumours for RNA extraction. Edited the manuscript.

Lucie Hamel: Obtained ethical approval from the Animal Committee of the RI-MUHC. Provided cultured DPC-1 cells for implantation. Processed harvested tissues and blood samples for banking. Extracted RNA from DPC-1 tumours.

Murillo Luz: Surgical Oncologist involved in the PSMA imaging project in dogs bearing DPC-1 tumours and metastases. Performed subcutaneous injection of DPC-1 cells to follow tumour growth. Performed ultrasound imaging of the dog prostate after implantation of DPC-1 cells.

Armen Aprikian: McGill Full Professor and urologist at the MUHC participating in the project on PSMA imaging of metastases in dogs bearing DPC-1 cells.

Maurice Anidjar: McGill Associate Professor and urologist at the MUHC and Jewish General Hospital directly involved as a co-investigator in projects on dogs. Performed surgery for implanting DPC-1 cells within the prostate.

Simone Chevalier: McGill Full Professor and Senior Scientist at the RI-MUHC: Planned and directed all research projects on dogs; supervised Seta Derderian. Participated in all steps of the project, from setting up methods to analyzing results, and determining their meaning and value in the field of PCa. Edited the manuscript.

TITLE PAGE

TRANSCRIPTOMICS OF CANINE PROSTATE CANCERS REVEALS SIMILARITIES TO AGGRESSIVE FEATURES OF HUMAN METASTATIC CASTRATION RESISTANT DISEASE

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ABSTRACT

Dogs are among the rare mammals that spontaneously develop prostate cancer (PCa) with advancing age, although at a low prevalence. The disease is usually diagnosed at an advanced stage and does not respond to hormonal therapy, as seen with advanced PCa in men. We previously reported that the dog prostate cancer (DPC)-1 cell line derived from a poorly differentiated adenocarcinoma recapitulates in large dogs the key features of advanced human PCa. To further assess the relevance of this model to human disease, we carried out transcriptomic characterization of DPC-1 tumours compared to naturally occurring canine PCa, normal prostate, and human PCa. First, we compared canine PCa cell lines to their paired tumours, identifying specific consistently altered genes and features. Next, DPC-1 subcutaneous and orthotopic models were compared, showing that orthotopic tumours and metastases derived therefrom are more similar to naturally occurring canine PCa. DPC-1 tumours recapitulated significant heterogeneity similar to what is seen for naturally occurring canine tumours, varying in features of cell plasticity, epithelial to mesenchymal transition, and PI3K/AKT signaling. The top upstream regulators and drug targets showed many targetable pathways such as growth factor receptors and estrogen signaling through estrogen receptor alpha (ER α) which are relevant to advanced human PCa. Furthermore, by comparing canine to human tumours, we found that they most closely resemble a subset of castration-resistant PCa with mesenchymal and stemness features, along with a distinct neuroendocrine signature. Lastly, PCa-specific transcripts were detectable in liquid biopsies of dogs bearing DPC-1 tumours, allowing for the potential earlier diagnosis. Altogether, by exploring the molecular similarities between the DPC-1 model and human PCa, we identified key for both species. These findings substantiate the suitability of the canine model as a reliable tool to better fill the gap between rodents and humans.

INTRODUCTION

While prostate cancer (PCa) is the second most commonly diagnosed cancer in men worldwide, it is otherwise almost completely absent in other species.^{1, 2} Dogs are the exception, with an estimated 0.35% developing spontaneous PCa.³ Canine PCa is often diagnosed at late stages, with 40% presenting metastases.⁴ In both species, the normal prostate epithelium is composed of androgen receptor (AR)+ luminal cells, AR+/- basal cells, and rare subsets of AR- neuroendocrine and stem cells.⁵ Pioneer studies by Huggins et al. showed the androgen-dependence of the canine prostate, paving the way to androgen deprivation therapy (ADT) as a mainstay of treatment for men with PCa.^{6, 7} In human PCa, while ADT kills the majority of cancer cells which are luminal-like and AR+, subpopulations of surviving cells develop resistance while remaining AR+.^{8, 9} Furthermore, there can be a concomitant differentiation towards or selection for AR-negative neuroendocrine and stem-like populations.^{8, 10} Thus, men on ADT eventually develop castration-resistant PCa (CRPC). In contrast, despite AR being integral for the development and homeostasis of the dog prostate, there is no or minimal AR expression in canine PCa, making it androgen/AR-independent.¹¹ This is more similar to the situation in human metastatic (m)CRPC, although in the later case AR often remains relevant. This makes the canine disease an interesting comparative model for advanced human PCa, especially in terms of androgen insensitivity, whereas most animal studies focus on rodents which do not naturally develop PCa.

Several canine PCa cell lines have been established, including the DPC-1 cell line derived from a primary poorly differentiated prostatic carcinoma.¹² Its orthotopic implantation in the prostate of large dogs has led to a model which recapitulates the features of advanced human PCa, with numerous metastases to lymph nodes, lungs, and bone.¹³ This canine model is highly relevant,

allowing for PSMA imaging which is currently an important target for imaging and treatment in human PCa.¹⁴

In this study, we aimed to better define the molecular characteristics of the DPC-1 model and compare it to canine and human PCa through transcriptomic analyses. We performed RNA-sequencing of the DPC-1 cell line, tumours from DPC-1 orthotopic and subcutaneous models including LN and lung metastases, and normal canine prostate. These results were compared to RNA-sequencing of normal canine prostate, naturally occurring canine PCa and cell lines derived therefrom, and human PCa to identify their unique and shared features. Top regulators and potential targets were identified for further study. Finally, we tested commonly overexpressed genes in longitudinally banked blood from dogs bearing DPC-1 tumours to determine their relevance for cancer detection.

MATERIALS AND METHODS

DPC-1 cell culture

The DPC-1 cell line (patent US2003013191: Cussenot and Villette, France) was maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% antibiotic (penicillin-streptomycin; all from Wisent Inc., Saint-Jean-Baptiste, QC, Canada).

DPC-1 tumour samples

The experimental protocol was approved by the Animal Ethic Committee of the McGill University Health Centre Research Institute and the McGill University Animal Ethic Committee, in accordance with Canadian regulations for experimentation in animals. Methods for DPC-1

intraprostatic implantation, follow-up, euthanasia, and histopathology were defined in previous studies.^{13, 14} The same approach was used for the subcutaneous implantation of 2×10^6 cells in the back of the neck. Fresh-frozen tumour samples were banked at necropsy. Blood was also periodically banked from a subset of dogs prior to cyclosporine, at time of implantation, when performing prostate biopsies, and prior to reaching experimental endpoint.

15 tumour samples from four dogs (referred to as E, K, S, and T) were chosen for further analysis. These include one primary tumour, seven LN metastases (iliac and sacral), three lung metastases, and four subcutaneous masses. DPC-1 tumour samples are referred to as “site_animal” when mentioned in Figures (e.g. LNiliac_S). One normal prostate sample from a dog with no prostatic disease (termed “Normal”) and two samples of the DPC-1 cell line were also included. RNA was extracted using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Toronto, ON, Canada), quantified by NanoDrop (Thermo Scientific, Waltham, MA, USA), and quality checked by BioAnalyzer 2100 (Agilent, Millcreek, ON, Canada). Only samples with RNA integrity number (RIN) > 7 were sent for sequencing.

RNA sequencing and bioinformatic analysis

Library preparation (Illumina Stranded mRNA Prep; Illumina, San Diego, CA, USA) and RNA sequencing (NovaSeq 6000 System, Illumina) at 50M reads were performed at Genome Québec (Montreal, Canada).

Raw RNA-sequencing FASTQ files were also obtained for other canine samples, including naturally occurring PCa (n=14; referred to as P1-P13 and LN4), normal prostate (n=14; Bn1-14), and cell lines (n=6) from GSE190374, GSE162832, and GSE122916 through Gene Expression

Omnibus.¹⁵⁻¹⁷ FASTQ files for human PCa datasets were obtained by authorized accessed through dbGaP for SU2C (phs000915.v2.p2) and GDC for TCGA (phs000178.v11.p8).

All raw data were processed using the GenPipes RNA Sequencing pipeline with the assembly method set to “stringtie” to obtain HTSeq raw counts.¹⁸ All subsequent analyses were conducted in R version 4.2.2. Details of analyses are reported in Supplemental Methods. Gene sets used in analyses are presented in Table S1.

Blood RNA extraction and RT-qPCR

Blood RNA was extracted from 20 banked buffy coats from eight dogs using the RNeasy Mini kit (Qiagen, Toronto, ON, Canada). RNA quantity and quantity were determined as described above.

500ng of RNA was reverse transcribed for a final volume of 20µl cDNA per sample (Maxima H Minus First Strand cDNA Synthesis Kit with dsDNase; Thermo Scientific, Waltham, MA, USA). Each RT-qPCR assay was carried out in triplicate, using 1µl of cDNA in 10µl reactions. TaqMan Fast Advanced Master Mix was used (Thermo Scientific) on the CFX96 Touch™ (Bio-Rad, Mississauga, ON, Canada) with the following program: UNG incubation 50°C 2 minutes, polymerase activation 95°C 20 seconds, 45 cycles of denaturation (3s 95°C) and annealing/extension (30s 60°C). TaqMan assays were designed for *FOLH1*, *KLK2*, *L1CAM*, and *PSCA* as genes of interest, and *GAPDH*, *GUSB*, and *ACTB* as reference genes. Assays were first tested in serial dilutions of DPC-1 cell line or normal prostate RNA to confirm efficiency. Assay details are presented in Table S2. All plates included positive (cell line or normal prostate cDNA) and negative (no template in cDNA synthesis) controls. Expression was analyzed as $2^{-\Delta\text{Ct}}$.

RESULTS

DPC-1 orthotopic tumours and metastases recapitulate feature of naturally occurring cancers not represented by cell lines and subcutaneous masses

To characterize the molecular features of the DPC-1 model, we performed RNA-sequencing of the cell line and its derived tumours (intraprostatic, metastasis, or subcutaneous). We combined our data with publicly available canine data of normal prostates, naturally occurring PCa, and cell lines derived therefrom.¹⁵⁻¹⁷ Principal component analysis showed clear clustering of cell lines, tumours, and normal prostates, irrespective of the dataset (Figure 1A). DPC-1 tumours clustered with naturally occurring canine tumours. Interestingly, the DPC-1 primary tumour clusters more closely with naturally occurring cancers than to other DPC-1 tumours.

While all cell lines cluster together, we can identify features specific to DPC-1: 856 differentially expressed genes (DEGs) were identified in DPC-1 at a five-fold change in either direction (Figure 1B). Among genes overexpressed in DPC-1, we see: *LY6D* and *CTSE* which are bi-lineage genes in PCa;^{19, 20} *CD74* related to invasion in androgen independent models;²¹ and *ITGBL1* involved in epithelial to mesenchymal transition (EMT), invasion, and migration.²²

While the DPC-1 tumours are derived from the cell line, the studies we are comparing to had isolated tumours and derived cell lines therefrom. Thus, we questioned whether DPC-1 orthotopic tumours and metastases differ from naturally occurring tumours due to their previous *in vitro* expansion. By comparing the DPC-1 orthotopic model and naturally occurring tumours to their respective cell lines, we found a high number of DEGs, most of which were common between the natural and DPC-1 models (Figure S1A). Several cancer hallmarks and signaling pathways were enriched in tumours compared to cell lines, including immune signatures, EMT, PI3K, and KRAS signaling (Figure 1C, S1B). These profiles were similar between DPC-1 and natural tumours.

Next, to determine whether subcutaneous DPC-1 masses resemble orthotopic tumours and metastases, we compared these models to naturally occurring PCa. Most genes were found differentially expressed in the same direction for all tumours compared to normal prostate (Figure S2A). Over-representation analysis (ORA) showed great overlap between naturally occurring PCa and the orthotopic model relating to immunity, proliferation, and signaling pathways (Figure 1D, S2B). The subcutaneous model showed a similar proliferation profile but differed in signaling and immune pathways. For example, VEGF signaling and various immune pathways were over-represented only in the orthotopic model and natural PCa (Figure S2B). Interestingly, these differences extend to specific PCa-relevant genes, which can change considerably between paired cell lines vs. tumours (e.g. *FOLH1*, *ESR1*) and between orthotopic vs. subcutaneous models (e.g. *TP63*, *ENO2*; Figure 1E).

Altogether, the DPC-1 orthotopic model and metastases resemble natural canine PCa despite prior *in vitro* passaging of the cell line. Differences between paired cell lines vs. tumours are consistent between the DPC-1 model and naturally occurring PCa. While subcutaneous masses share features with the orthotopic model, differences are sufficient to restrict further analyses to DPC-1 orthotopic tumours and metastases.

The heterogeneity of DPC-1 tumours is comparable to natural canine PCa

To better discern the heterogeneity among natural and DPC-1 tumours, we performed a principal component analysis among these samples to better define their variance (Figure 2A). Remarkably, the DPC-1 samples clustered among the natural tumours and did not clearly separate by site or by animal. Furthermore, the castration status did not account for much variability among natural PCa (Figure S3).

Exploring the top genes comprising these principal components (Figure 2A), we see that the PC1(-) includes various stemness and bi-lineage markers (*LY6D*, *PSCA*, *CTSE*). ORA of the top genes for each direction in PC1 and PC2 revealed that PC1(+) is enriched in EMT, angiogenesis, and signaling through PI3K, AGE-RAGE, and Relaxin; PC2(+) is enriched in NFkB and B cell receptor signaling gene sets; and PC2(-) is enriched in PI3K and IL-17 signaling (Figure 2B, C).

Analyzing the differentially expressed genes in DPC-1 or natural tumours vs. normal prostate showed most genes changing in the same direction (Figure S4 A, B). Gene set enrichment analysis (GSEA) showed similar immune and proliferation profiles for DPC-1 tumours and natural PCa vs. normal prostate (Figures S4 C, D). While most signaling pathways also showed similar results, there were some noticeable differences, such as a high enrichment of EMT in natural PCa which was not seen in DPC-1 tumours. To better visualize the heterogeneity between samples, gene set variation analysis (GSVA) results were plotted as heatmaps by hierarchical clustering (Figure 2D, S5). Once again, DPC-1 tumours showed no clear distinctions by animal or by metastatic site, and clustered within the natural PCa. TGFβ signaling, MYC targets, G2M checkpoint, E2F targets, cholesterol homeostasis, EMT, and IFN alpha response were among the most variable gene sets among tumours (Figure 2D).

Altogether, DPC-1 cells form heterogeneous tumours recapitulating some of the diversity found in natural PCa. This variability is not explained only by site-specific or inter-individual variation, but recapitulates some intra-individual variation.

Upstream regulators for canine PCa include targetable pathways

To decipher some of the mechanisms contributing to canine PCa, we identified the top upstream regulators in DPC-1 and natural tumours through Ingenuity Pathway Analysis (Figure 3A-C). The

top activated kinases included common targetable hits for which drugs have been tested in human clinical trials, such as HER2, EGFR, and SRC family kinases (Figure 3A). Complexes of interest included the PI3K, NFkB, and PDGF. Many growth factors were highly activated, including ligands for MET, EGFR, VEGFRs, and cytokines such as IL6 along with the downstream JAK and STAT3 (Figure 3B). Among top transcription factors, we find activation of MYC and E2F1, while RB1 and PTEN are inhibited (Figure 3C).

Interestingly, ER α , ER β , and AR were all highly activated upstream regulators, along with beta-estradiol which was among the top 10 activated hits in both DPC-1 and natural tumours (Figure 3B, C). While *ESR1* was highly activated in both natural and DPC-1 tumours, *ESR2* only came up for natural PCa. Furthermore, most tumours show no or very low expression of AR and *ESR2*, while *ESR1* is present at high levels in all DPC-1 tumours and 10/14 natural tumours (Figure 1E). The top upstream regulators were in line with the literature on human PCa (Figure S6A).

To understand how AR is activated despite a >10x downregulation and loss in most DPC-1 and natural tumours, we studied the upregulated genes which suggest AR as an upstream regulator (Figure S6B). The resulting biological processes were mainly involved in cell cycle progression and mitosis, suggesting a prostate-specific proliferation signature, and not necessarily reflecting specific AR activation. Furthermore, 50% of these genes are also known to be controlled by MYC, EGFR, HER2, and ERs, suggesting overlap with these pathways.

We next identified the top therapeutic targets for canine PCa and compared these to human disease using Connectivity Map. The top hits were similar between canine and advanced human PCa (high grade or CRPC), but not with low grade PCa (Figure 3D). These included various chemotherapeutics as expected, along with inhibitors of MAPK, NFkB, and PI3K pathways, and

receptor tyrosine kinases such as VEGFRs and EGFR. Among targeted nuclear hormone receptors (NHR), ER antagonists were predicted to have anti-tumour effects, compared to ER agonists showing possible pro-tumour effects. These results are concordant with the predicted upstream regulators.

Overall, the top upstream regulators and targets in canine PCa implicate multiple pathways of interest to human disease and emphasize the role of estrogen receptors.

Canine PCa displays mesenchymal and stem-like signatures seen in human mCRPC

We questioned how canine PCa compares to human PCa at the transcriptomic level. Among cell lines from several types of human cancer, we find that canine PCa cell lines most closely resemble human prostate and breast cancer cells (Figure S7A). We next combined data from natural canine PCa, DPC-1 orthotopic and metastases, and normal prostate with human datasets. These included benign prostate, primary tumours, and mCRPC metastases. A striking overlap was observed between canine tumours and human mCRPC, reinforcing the notion that canine PCa are advanced and androgen-independent (Figure 4A). Interestingly, while human benign samples clustered closely with human primary tumours, canine normal samples were completely separate.

As human mCRPC metastases show phenotypic heterogeneity, we questioned whether canine cancers could also be categorized by specific subtypes of mCRPC, namely AR-driven (ARPC), mesenchymal and stem-like (MSPC), or neuroendocrine (NEPC). Overlaying the principal component analysis plot from Figure 4A with GSVA results representative of these mCRPC subtypes revealed that canine tumours show features of MSPC as opposed to ARPC or NEPC (Figure 4B). Interestingly, while PC1 showed clear differences between sample types, PC2 showed

striking gradients of ARPC and MSPC scores. Moreover, canine tumours showed increased signatures of bi-lineage cells and early markers of CRPC differentiation, similarly to most CRPC samples, as well as high signatures of aberrant PTEN, RB1, and P53 and (Figure S7 C, D). There was no clear distinction between intact and castrated cases for any results shown.

We further visualized the mCRPC subtype signatures gene by gene for canine samples in heatmaps. As expected, MSPC genes were higher in tumours compared to normal prostate and ARPC genes were decreased, but NEPC genes showed mixed trends (Figure S8A). Results for luminal, basal, and bi-lineage genes reinforced the upregulation of most bi-lineage markers (Figure S8B). Among luminal genes, *AR* and *KLKs* were lost, but *FOLH1* was upregulated.

To better understand the mixed results for neuroendocrine genes in canine samples, we plotted a larger list of neuroendocrine genes as a heatmap (Figure 4C). While some common neuroendocrine markers showed no trend, others were higher in tumours (*ENO2*, *FOXA2*, *SSTR2*), and some were expressed mainly in normal samples (e.g. *CHGA*, *SYP*).

Altogether, canine PCa closely resembles human mesenchymal and stem-like mCRPC tumours. They show bi-lineage and CRPC features known to exist even in a subset of aggressive cells present in early human disease. While neuroendocrine signatures show mixed results, some genes are highly upregulated and support the presence of this cell type in canine tumours.

Canine PCa-relevant transcripts are detectable in blood RNA

Liquid biopsies have recently generated great promise in the field of cancer to test prognostic markers. We reported that prostate cell-subtype-specific genes are traceable in whole blood RNA from mCRPC patients.²³ We questioned whether such non-invasive tests would detect DPC-1

transcripts in the blood of dogs bearing DPC-1 tumours (Figure 5). We chose cell-subtype genes overexpressed in canine tumours based on our analyses, but which are not known to be expressed in normal blood: *FOLH1* as a luminal marker, *L1CAM* for neuroendocrine, and *PSCA* for stemness. The luminal AR target gene *CPSE*, equivalent to human *KLK3*, was also included. These four genes were tested in 20 blood RNA samples available from eight dogs. *FOLH1* and *L1CAM* were detected in 0/5 samples pre-implantation but 14/15 and 8/15 samples post-implantation, respectively. While *FOLH1* tended to increase with further progression, *L1CAM* was less consistent. *PSCA* showed unexpected baseline expression in all blood samples and did not significantly increase post-implantation. *CPSE* was not expressed in any blood samples, as expected. Overall, two PCa-specific circulating genes were detected in dogs bearing DPC-1 tumours, thereby suggesting such an approach for following dogs with spontaneous PCa.

DISCUSSION

While rodent models provide the flexibility of genetic engineering and xenografts, dogs remain relevant because of their shared embryological origin of the prostate and spontaneous development of PCa with age.²⁴ These models are complementary for understanding the disease, with dogs being especially relevant for testing treatments before moving to human trials. The DPC-1 canine model and other cell lines also offer the potential for genetic manipulation even in the canine context as a first step towards testing treatments in spontaneous disease.

Several animal models are based on subcutaneous injections of cancer cell lines. In the DPC-1 model, we show that subcutaneous masses display distinct features compared to orthotopic and naturally occurring tumours. Of note, these findings were obtained from DPC-1 injected in the same dogs, accounting for differences in age, breed, and normal inter-individual variation.

Unfortunately, little research has been carried out directly comparing transcriptomes of subcutaneous vs. orthotopic tumours derived from a same cell line. However, while patient-derived xenografts do show some similarities between the two models, this varies by cancer type, and major target pathways and biomarkers can be affected.^{25, 26} For example, this affects tumour vasculature which can in turn affect treatment response, including in PCa xenograft models.^{27, 28} Similarly, comparing cell lines to the tumours from which they are derived, we found that while some traits are preserved, other features differ consistently *in vitro*. Interestingly, many of these changes were reverted upon orthotopic implantation in the DPC-1 model. Even genes of high importance in our study were consistently differentially expressed *in vitro*, such as *ESR1*, *ACPP*, and *TP63*. This may be partly due to the interplay between the tumour microenvironment and the cancer cells. Overall, differences between *in vitro*, subcutaneous, and orthotopic models should be accounted for whenever developing new models in any species, especially in relation to the markers/targets/pathways of interest.

The DPC-1 orthotopic model and resulting metastases showed marked heterogeneity in line with naturally occurring tumours. This variability in DPC-1 tumours were not solely due to inter-individual or site-specific differences, and castration status did not contribute to the variability of naturally occurring tumours. Morphologically, we found similar micropapillary and cribriform patterns in all DPC-1 tumours.¹³ Overall, the most variable features were EMT, PI3K signaling, NFkB signaling, and genes related to stemness and bi-lineage cell populations, although variation in other signaling and metabolic pathways were also observed. Interestingly, bi-lineage genes seen in these analyses, including LY6D and CTSE, were among the top overexpressed genes in DPC-1 compared to other cell lines. Differences in immune pathways were seen but were not studied in depth due to immunosuppression in the DPC-1 model. Thus, spatial profiling within the prostatic

and metastatic microenvironments may allow a better understanding of DPC-1 tumour heterogeneity, which is relevant to naturally occurring disease.

Our results of pathways, upstream regulators, and top targets of interest were in line with the literature on human PCa and corroborated previous findings on canine PCa.^{11, 29} These include activation of the PI3K/AKT/MTOR pathway; signaling through tyrosine kinases such as EGFR, HER2, c-KIT; transcription factors like MYC, STAT3; WNT signaling, EMT, and stemness. We also identified aberrant PTEN and P53 signaling which have previously been reported in canine PCa, and RB1 which has not previously been identified.³⁰⁻³² Alterations of these three tumour suppressors are common in human PCa, with concurrent loss of P53 and PTEN being associated with AR-independent disease, and loss of RB1 is especially common in human NEPC.³³⁻³⁵

The implication of steroid hormones other than androgens in canine PCa deserves consideration given that *ESR1* and estrogens were among the top activated upstream regulators and targets of interest. *ESR1* expression was high in most tumours despite being lower than in the normal prostate. A recent study showed a loss of ER α in canine PCa, suggesting both androgen- and estrogen-independence.³⁰ This deserves further investigation, since previous studies found ER α remained highly expressed in a subset of cells intermingled with the remaining negative cells.^{36, 37} Interestingly, the reported ER α expression patterns resemble AR expression patterns in human PCa upon ADT.^{8, 36, 37} We have previously shown estrogen supplementation in both intact and castrated dogs induces COX-2 expression in the prostate and leads to squamous metaplasia.³⁸ Indeed, there seems to be a positive feedback loop between COX-2 and estrogen signaling.³⁹ It is known that in human cancers, COX-2 is involved in proliferation, migration, EMT, immune evasion and stemness.⁴⁰ Our results showing consistent overexpression of COX-2 in both spontaneous and DPC-1 tumours compared to the normal prostate further support the findings of

increased ER α activity. This is in line with reports in human CRPC, where ER α emerges as an alternative to AR signaling, whereas ER β is lost.⁴¹ Overall, this suggests that ER inhibitors may be of use for the treatment of canine PCa, in combination with additional targeted therapies. Single-cell and spatial analyses are necessary for elucidating the role of ER α in canine PCa and other cell types contributing to this disease.

The expression of specific proteins and corresponding pathways of interest studied in canine PCa have lead to the general consensus that it resembles advanced human CRPC, especially considering its androgen independence.^{4, 11, 29} However, CRPC is also heterogeneous, and it is not yet clear which phenotypes are seen in canine disease. Three main categories of CRPC have been established: ARPC, NEPC, and DNPC (double-negative, i.e. AR-/NE-).⁴² A recent study further examined the DNPC variant, reclassifying it as MSPC.⁴³ Due to its lineage plasticity, features of MSPC were also found in some ARPC or NEPC cases. The ARPC/MSPC signatures account for the variability among human CRPC cases, and canine tumours were mostly characterized by a MSPC phenotype. Accordingly, canine PCa is a relevant model of human DNPC/MSPC even in a treatment-independent context. Thus, it may be suitable for identification of novel therapeutic targets for this currently incurable disease.

Despite the mainly mesenchymal and stemness features of canine tumours, transcripts representing luminal, basal, bi-lineage, and NE cells were also overexpressed. The presence of features specific to bi-lineage/intermediate cells with co-expression of luminal and basal cell genes is remarkable, as these cells are inherently castration resistant and correlate with advanced disease.^{19, 20} The presence of such cells is determined by hormone levels, where the presence of androgens allows their terminal luminal differentiation, as may be necessary to renewal of the normal luminal layer upon castration.²⁰ Thus, it remains unclear why these bi-lineage signatures are not specific to

castrated animals, being seen in intact animals with naturally occurring disease or bearing DPC-1 tumours. This may be a limitation due to the low number of samples, as castration did not seem to affect the transcriptome of natural canine PCas consistently. To date there is no clear indication that castration affects incidence and outcomes of canine PCa.³ These results support that transformed stem cells are at the origin of spontaneous PCa development in neutered dogs.

NE cells have been minimally studied in the canine prostate. We have previously demonstrated NE differentiation upon androgen ablation in the normal canine prostate, but some authors have disregarded this phenomenon in canine PCa due to the absence of conventional NE markers and lack of treatment pressures.^{5, 29, 44} However, given the an androgen-independent phenotype, some NE differentiation may take place despite the lack of further treatment. In line with this notion, a recent study showed expression of neuron specific enolase (encoded by *ENO2*) in a subset of canine PCas.⁴⁵ We confirm here that *ENO2* and other NE genes such as *FOXA2*, *SSTR2*, and *NTSR1* are overexpressed in cancers, while the more common NE markers (*CHGA*, *SYP*) are found mainly in the normal prostate. We have shown CHGA staining in NE cells of the normal dog prostate (unpublished). Thus, a unique canine NE-like signature in tumours can be validated in further studies to characterize these cells and understand their evolution and contribution to canine disease.

The lack of therapies for dogs with PCa has been attributed to diagnosis at an advanced metastatic stage. CPSE protein in plasma, the canine equivalent of PSA, is useful for detection of canine benign prostatic hyperplasia, but not useful for detecting PCa due to its AR-dependent expression. We have previously shown that specific PCa-relevant genes in blood RNA are predictive of outcomes in mCRPC patients.²³ Such genes also correlate with clinical and pathological features at diagnosis. Here, we showed that *FOLH1* is detectable in blood RNA of dogs bearing DPC-1

tumour, with high sensitivity and specificity. While we were limited to few genes based on the available amount of blood RNA, our study has also highlighted other genes worth considering as biomarkers in the blood of dogs for diagnosing or monitoring canine PCa: *LY6D* for bi-lineage cells and mesenchymal/stem-like features, or *ENO2*, *FOXA2*, or *POU3F2* for NE-like cells. This approach, with the addition of other genes to account for interpatient heterogeneity, may be useful for early detection of canine cancers or to monitor response to therapies and further progression.

CONCLUSION

This molecular characterization of the DPC-1 cell line and its derived tumours elucidated the key features mirroring naturally occurring PCa in dogs. Caution is warranted when studying cell lines and subcutaneous masses, as we observed clear differences when comparing orthotopic tumours, metastases, and naturally occurring disease. The evaluation of molecules or pathways of interest should be well studied before moving forward with specific hypotheses. In addition, DPC-1 tumours recapitulate some of the heterogeneity of naturally occurring canine PCa. Overall, canine disease resembles human mesenchymal and stem-like mCRPC and shows distinct neuroendocrine features compared to human PCa. Estrogens and *ESR1* are among the most important targets and should be further considered, along with various other targets also relevant to human PCa. Lastly, canine disease is traceable in blood RNA, allowing for the potential monitoring of a disease that is often caught late. Altogether, canine PCa and the DPC-1 model are suitable for better understanding and developing treatments for advanced human PCa.

DECLARATIONS

Ethics approval and consent to participate: The experimental protocol was approved by the Animal Ethic Committee of the McGill University Health Centre Research Institute and the McGill University Animal Ethic Committee, in accordance with Canadian regulations for experimentation in animals.

Consent for publication: Not applicable

Availability of data and materials: The DPC-1 dataset generated during the current study are available from the corresponding author on reasonable request. Additional canine datasets analyzed during the current study are available through Gene Expression Omnibus: [GSE122916](#), [GSE162832](#), [GSE190374](#). Raw data for human datasets were obtained by authorized accessed through dbGaP for SU2C ([phs000915.v2.p2](#)) and GDC for TCGA ([phs000178.v11.p8](#)).

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Authors' contributions: SD performed bioinformatic analyses, tested genes in blood RNA, and wrote the manuscript. ES performed necropsy on DPC-1 dogs, collected tumour samples, and dissected fresh-frozen tumours for RNA extraction. LH cultured DPC-1 cells for implantation, processed harvested tissue and blood samples at necropsy, and extracted tumour RNA for sequencing. ML performed subcutaneous injections and monitored intraprostatic tumours by ultrasound. MA performed surgery for the intraprostatic implantation of DPC-1 cells. SC conceived and directed the project.

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FIGURES

Figure 1

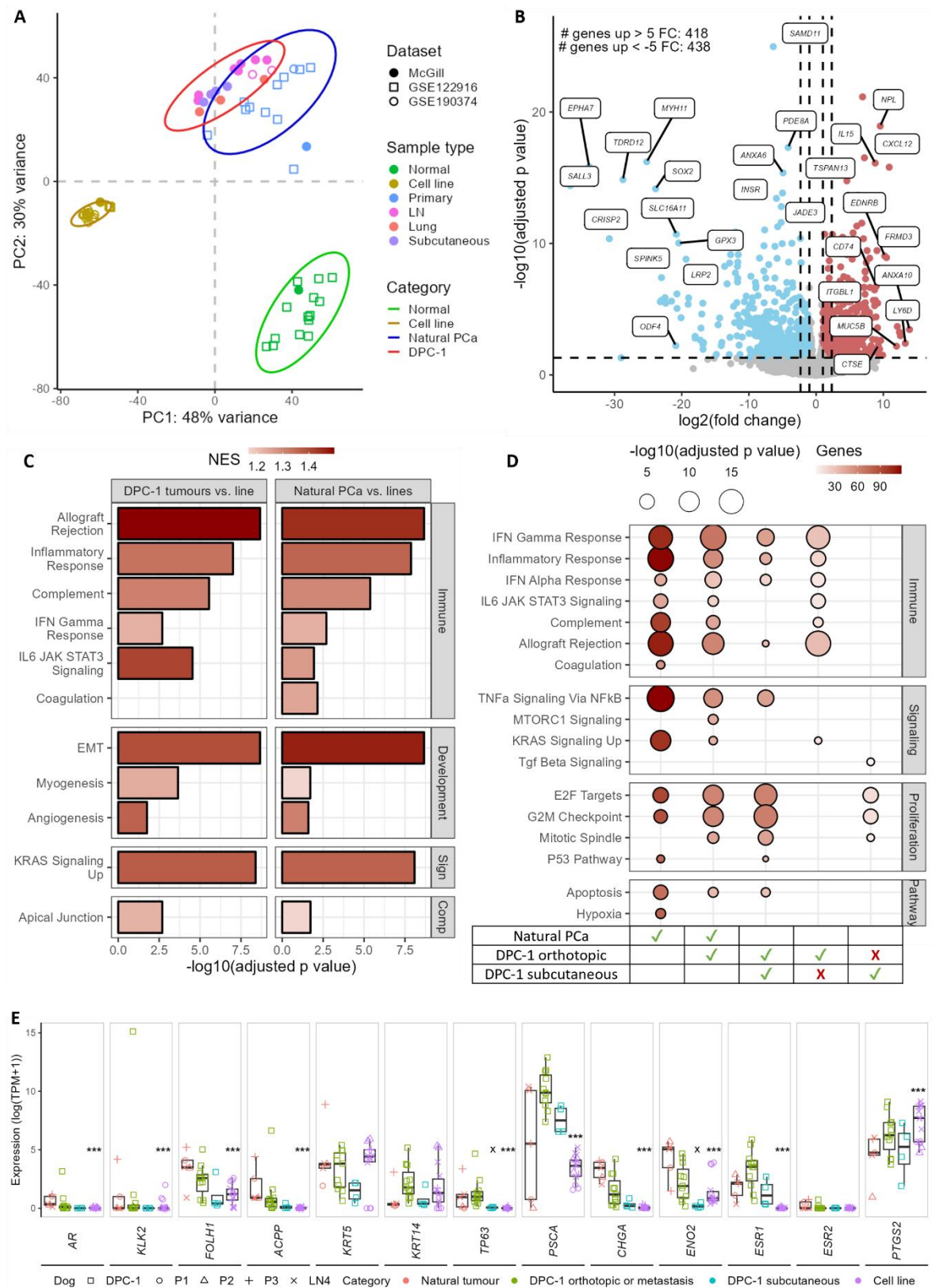


Figure 1: The DPC-1 cell line and subcutaneous masses recapitulate some, but not all, features of DPC-1 orthotopic tumours and metastases.

Transcriptomic data from the DPC-1 cell line and derived tumours were compared to canine PCa, derived cell lines, and normal prostate. (A) Principal component analysis plot showing the variability among all canine samples. For each sample, the shape represents the dataset, while the color represents the sample type. Ellipses represent the 95% confidence interval of sample categories. (B) Volcano plot of differential expression analysis results for the comparison between the DPC-1 cell line vs. other canine PCa cell lines. Red and blue represent significantly over- and under-expressed genes (adjusted $p < 0.05$ and fold change >2 or <-2), respectively. Grey represents genes not differentially expressed. Selected top genes are annotated with gene symbols. (C) Bar graphs showing gene set enrichment analysis results comparing DPC-1 orthotopic tumours and metastases tumours or natural PCa vs. their paired cell lines for the Hallmarks of Cancer gene sets. The length of the bars represents the $-\log_{10}(\text{adjusted } p \text{ value})$ and colors represent normalized enrichment scores (NES). (D) Bubble plot of over-representation analysis results for Hallmarks of Cancer gene sets for the lists of genes uniquely or commonly differentially expressed in tumours (natural PCa, DPC-1 orthotopic tumours/metastases, or DPC-1 subcutaneous masses) vs. normal prostate. Gene lists are defined at the bottom, where ✓ and X represent the inclusion and exclusion of genes upregulated in these samples vs. normal prostate, respectively. The size of the bubbles represents the $-\log_{10}(\text{adjusted } p \text{ value})$ and the color represents the number upregulated genes corresponding to the gene set. (E) Box plots showing expression of specific genes in tumours and cell lines. Gene expression is presented as $\log(\text{TPM}+1)$. Colors represent the sample categories (natural PCa, DPC-1 orthotopic tumour/metastases, DPC-1 subcutaneous masses, and cell lines). Shapes represent individual dogs with natural PCa for ease of comparison between tumours and derived cell lines; samples from which no cell line was derived were excluded. Adjusted p values from differential expression analyses are shown at the top for tumours vs. paired cell lines (*) and DPC-1 orthotopic/metastases vs. subcutaneous masses (x). Significance levels are presented as: */x < 0.05 ; **/xx < 0.01 ; ***/xxx < 0.001 . Abbreviations: EMT: epithelial to mesenchymal transition; Sign: Signaling; Comp: Cell component; TPM: Transcripts per million.

Figure 2

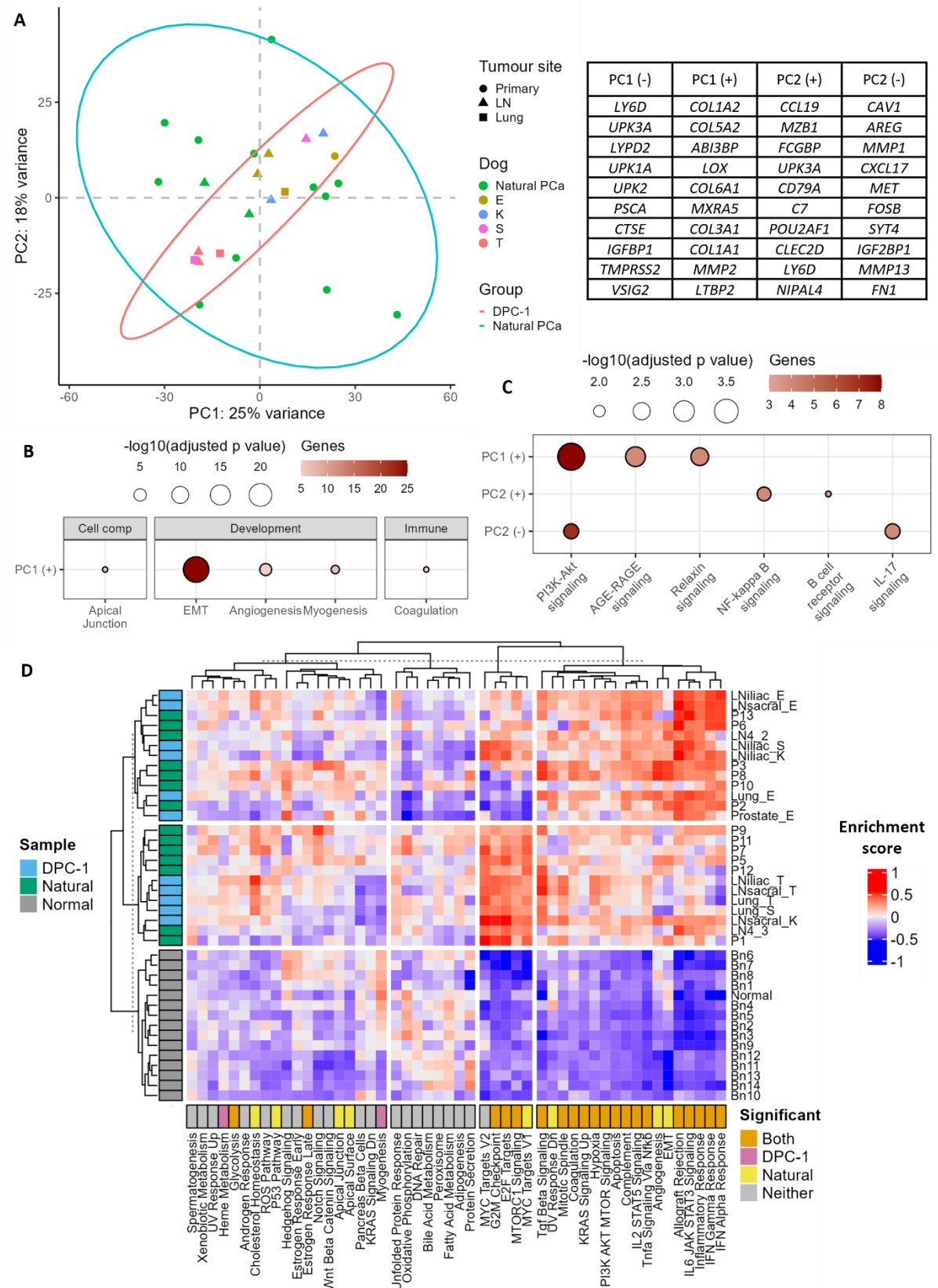


Figure 2: DPC-1 tumours are heterogeneous and comparable to naturally occurring primary tumours in dogs.

Comparisons between the DPC-1 orthotopic model and naturally occurring canine PCa. (A) Principal component analysis plot showing the variability among tumour samples. For each sample, the shape represents the tumour site, while the color represents the animal (natural PCa vs. DPC-1 dogs E, K, S, and T). Ellipses represent the 95% confidence interval of sample categories. The table on the right shows the top 10 genes contributing to each direction of principal component (PC)1 and PC2, in order of importance. (B-C) Bubble plots of over-representation analysis results for the top 50 genes contributing to each direction of PC1/PC2. Results are presented for Hallmarks of Cancer (B) and selected pathways from the KEGG gene set collection (C). The size of the bubbles represents the $-\log_{10}(\text{adjusted } p \text{ value})$, while the color represents the number of genes corresponding to each gene set. (D) Heatmap of gene set variation analysis results of the Hallmarks of Cancer gene sets, where the bottom annotation represents significance by gene set enrichment analysis (significant gene set in DPC-1 vs. normal prostate, natural PCa vs. normal prostate, both, or neither, as seen in Figure S4C, D) and the left annotation represents sample category (DPC-1, natural PCa, normal prostate).

Figure 3

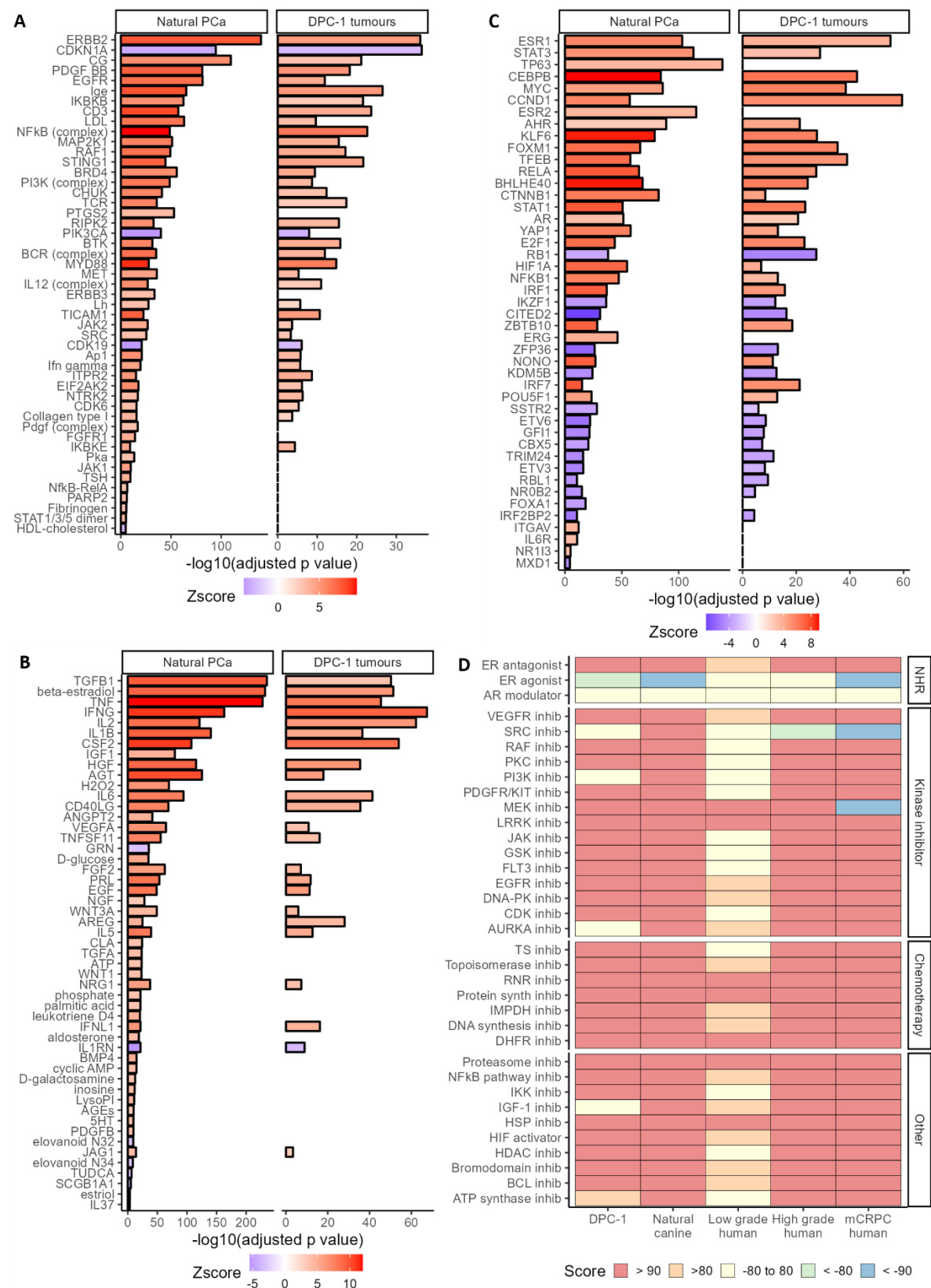


Figure 3: Top upstream regulators and targets for canine PCa are relevant to advanced human disease.

(A-C) Bar graphs depicting upstream regulators identified in Ingenuity Pathway Analysis for both DPC-1 tumours and naturally occurring PCa. The top hits are presented for (A) kinases, ion channels, enzymes, complexes, and other proteins (B) endogenous chemicals, cytokines, and growth factors; and (C) transcription factors, nuclear hormone receptors (NHR), transmembrane proteins, and G-protein coupled receptors. Activation Zscores are represented by color, while bar length shows $-\log_{10}(\text{adjusted p value})$. (D) Heatmap representing the top drugs and targets of interest based on Connectivity Map results for DPC-1 tumours, naturally occurring PCa, low vs. high grade human primary tumours, and human CRPC metastases. Scores >90 are top hits warranting further consideration.

Figure 4

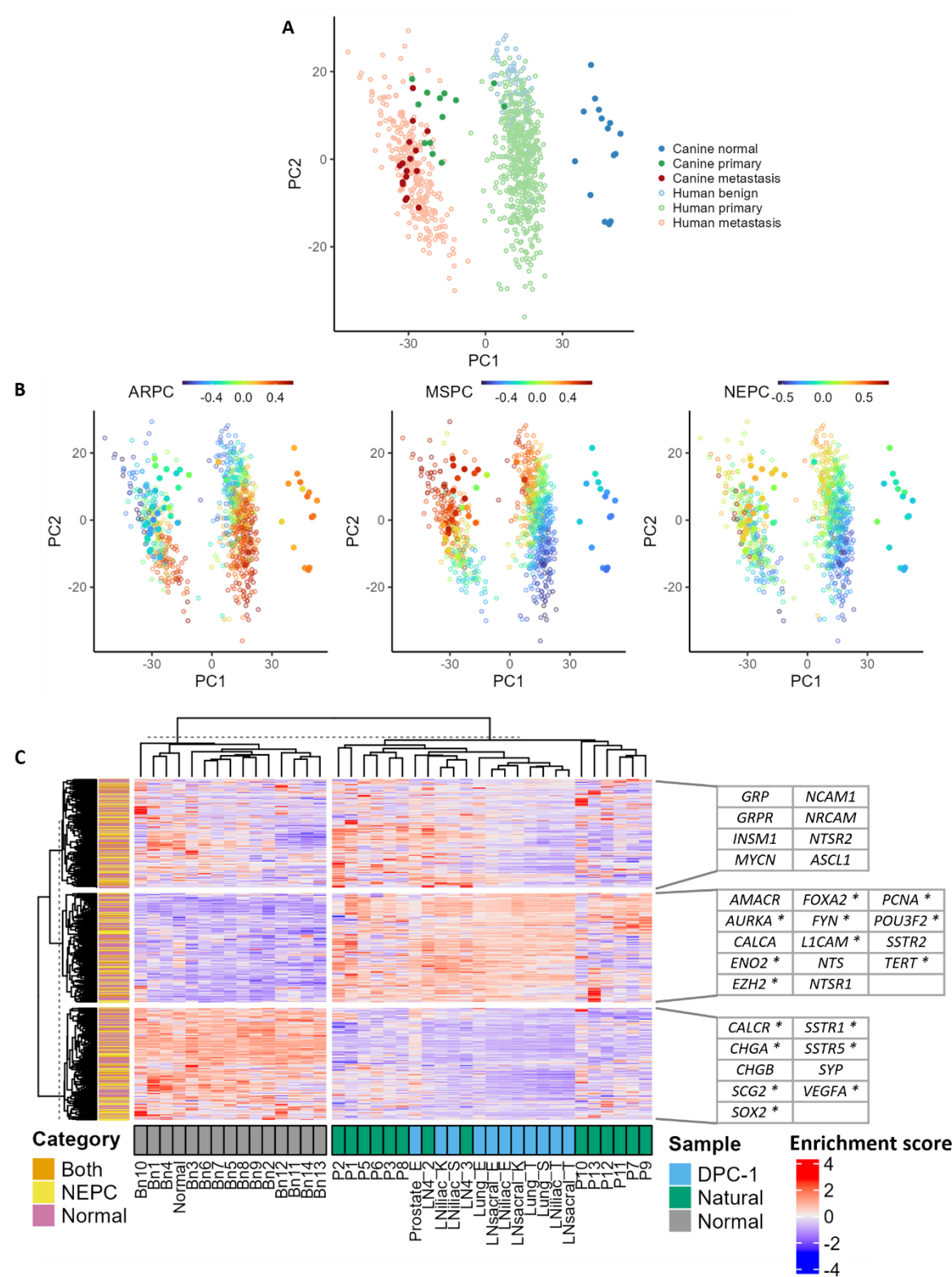


Figure 4: Canine PCa resembles mesenchymal and stem-like human mCRPC.

Analysis of transcriptomic datasets of human PCa vs. canine disease. (A) Principal component analysis plot showing variability among human and canine prostate and PCa samples. Species and sample categories (normal/benign, primary tumour, metastasis) are represented by different shapes and colors. (B) The same plot from (A) was overlaid with gene set variation analysis results for mCRPC subtypes: ARPC (AR-driven PCa), MSPC (mesenchymal and stem-like PCa), and NEPC (neuroendocrine PCa). Scores are denoted by color. Canine and human samples are shown as filled and empty circles, respectively. (C) Heatmap for expression of neuroendocrine (NE) genes in canine PCa and normal dog prostate samples. The annotation on the left designates whether genes are derived from a gene set of normal NE cells, NEPC, or both (see Methods for details of gene sets). The bottom annotation designates the sample category. Tables on the right show examples of genes in different clusters (upregulated in cancer, downregulated in cancer, or mixed), where a * denotes significant differential expression between tumour samples and normal prostate.

Figure 5

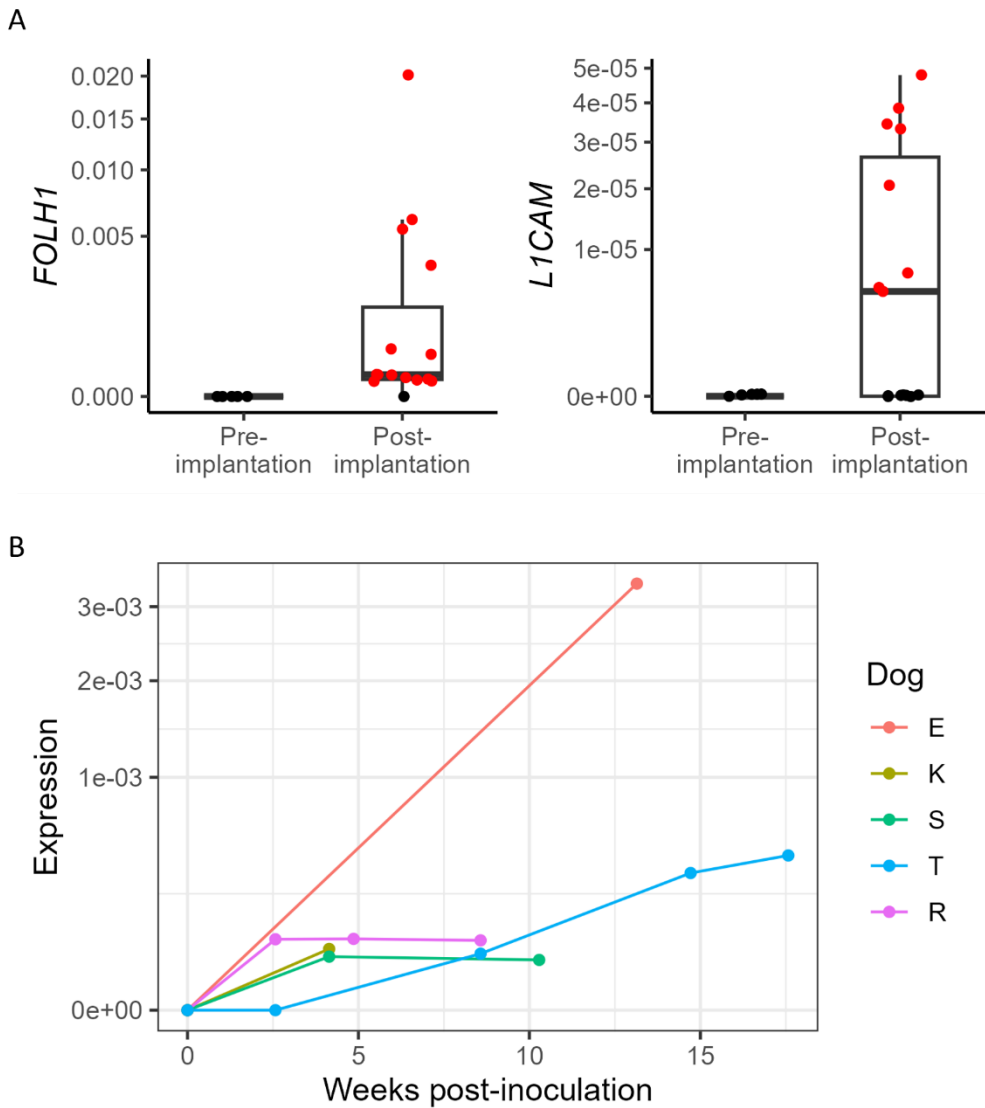


Figure 5: Circulating PCa-specific transcripts are detectable in dogs bearing DPC-1 tumours.

(A) Expression of *FOLH1* and *L1CAM* in blood RNA are presented as $2^{-\Delta\text{Ct}}$ for samples pre- and post-implantation of DPC-1 cells. (B) Expression of *FOLH1* in longitudinally collected blood samples. Colors represent individual dogs.

SUPPLEMENTAL MATERIALS

Supplemental Methods: Details of bioinformatic analysis.

RNA-sequencing raw counts obtained from HTSeq were normalized using DESEQ2 followed by correction using LIMMA to remove batch effects between datasets.^{1, 2} Sample type (normal prostate, PCa, cell line) was accounted for in the LIMMA model matrix. For analyses combining canine and human data, only genes with one-to-one orthologs were considered, and species was accounted for in the model matrix. For plotting individual genes (as in Figure 1E), raw counts were batch corrected using the R package ComBat-seq and normalized as Transcripts Per Million (TPM) for ease of visualization.³

Differential expression results and principal component analysis were obtained from DESEQ2.¹ Principal component analysis plots obtained from the plotPCA function of DESEQ2 used the top 500 genes selected by highest variance across samples. Ellipses overlaid on principal component analysis plots represent the 95% confidence interval of sample categories, obtained by the stat_ellipse function in ggplot2. Gene set enrichment analysis (GSEA) and over-representation analysis (ORA) were carried out using the R package clusterProfiler to identify gene sets of interest in comparisons between sample categories.⁴ Gene set variation analysis (GSVA) was carried out using the GSVA package to identify gene sets of interest in individual samples.⁵ MSigDB gene sets were used for these analyses (Hallmarks of Cancer and KEGG),⁶⁻⁸ as well as various curated gene sets summarized in Table S1.⁹⁻¹⁸

Heatmaps and venn diagrams were plotted using ComplexHeatmap and ggVennDiagram R packages.^{19, 20} All Figures were plotted using ggplot2 R package.²¹

DESEQ2 results for DPC-1 tumours vs. normal prostate and natural PCa vs. normal prostate were analyzed with the use of QIAGEN IPA (Ingenuity Pathway Analysis, QIAGEN Inc., <https://digitalinsights.qiagen.com/IPA>) to evaluate upstream regulators.²² Only hits with adjusted p value < 0.05 and Zscore >2 or <-2 were considered.

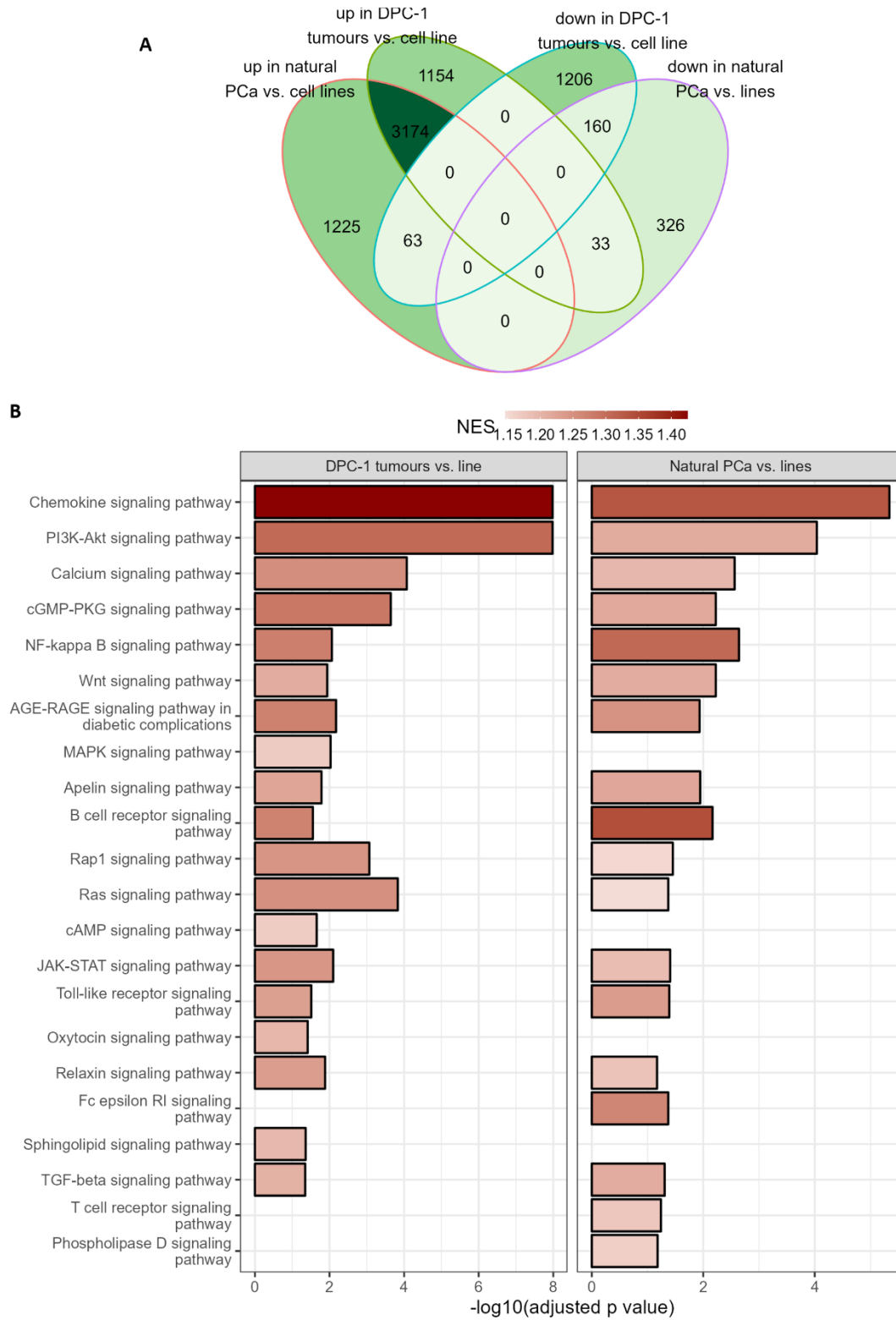
Top targets of interest were determined by Connectivity Map (CMap).²³ The top 150 upregulated genes among one-to-one orthologs were used based on adjusted p value.

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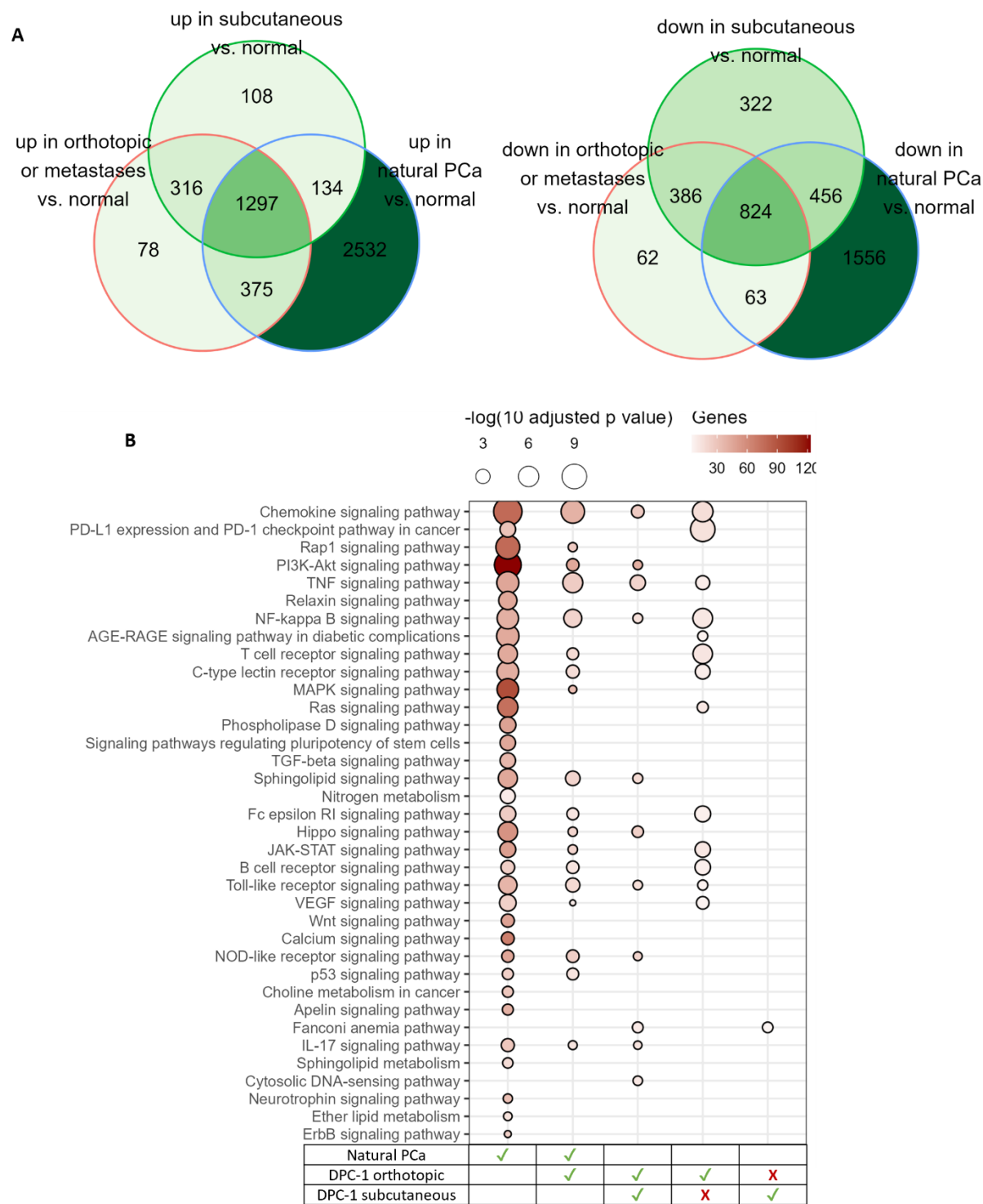
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Figure S1



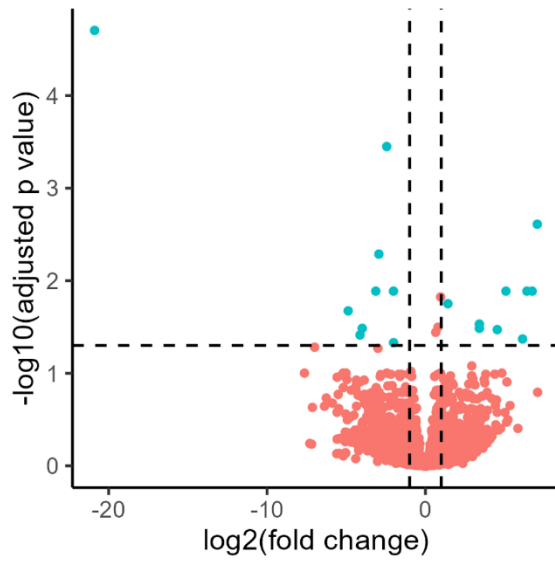
Supplemental Figure 1: Differences between the DPC-1 cell line and its derived tumours are also seen in natural PCa and their derived cell lines. (A) Venn diagram of differentially expressed genes for natural PCa or DPC-1 tumours vs. their paired cell lines (cutoffs at adjusted $p < 0.05$, fold change > 2 or < -2). (B) Bar graphs showing gene set enrichment analysis results comparing DPC-1 orthotopic and metastatic tumours or natural PCa vs. their paired cell lines for selected pathways from KEGG gene sets. The length of the bars represents the $-\log_{10}(\text{adjusted } p \text{ value})$ and colors represent normalized enrichment scores (NES).

Figure S2



Supplemental Figure 2: DPC-1 subcutaneous masses differ from orthotopic tumours and metastases. (A) Venn diagram of differentially expressed genes between DPC-1 orthotopic tumours and derived metastases or subcutaneous masses (cutoffs at adjusted $p < 0.05$, fold change > 2 or < -2). (B) Bubble plot of over-representation analysis results for selected pathways in KEGG gene sets for the lists of genes uniquely or commonly differentially expressed in tumours (natural PCa, DPC-1 orthotopic tumours/metastases, or DPC-1 subcutaneous masses) vs. normal prostate. Gene lists are defined at the bottom, where ✓ and X represent the inclusion and exclusion of genes upregulated in these samples vs. normal prostate, respectively. The size of the bubbles represents the $-\log_{10}(\text{adjusted } p \text{ value})$ and the color represents the number upregulated genes corresponding to the gene set.

Figure S3



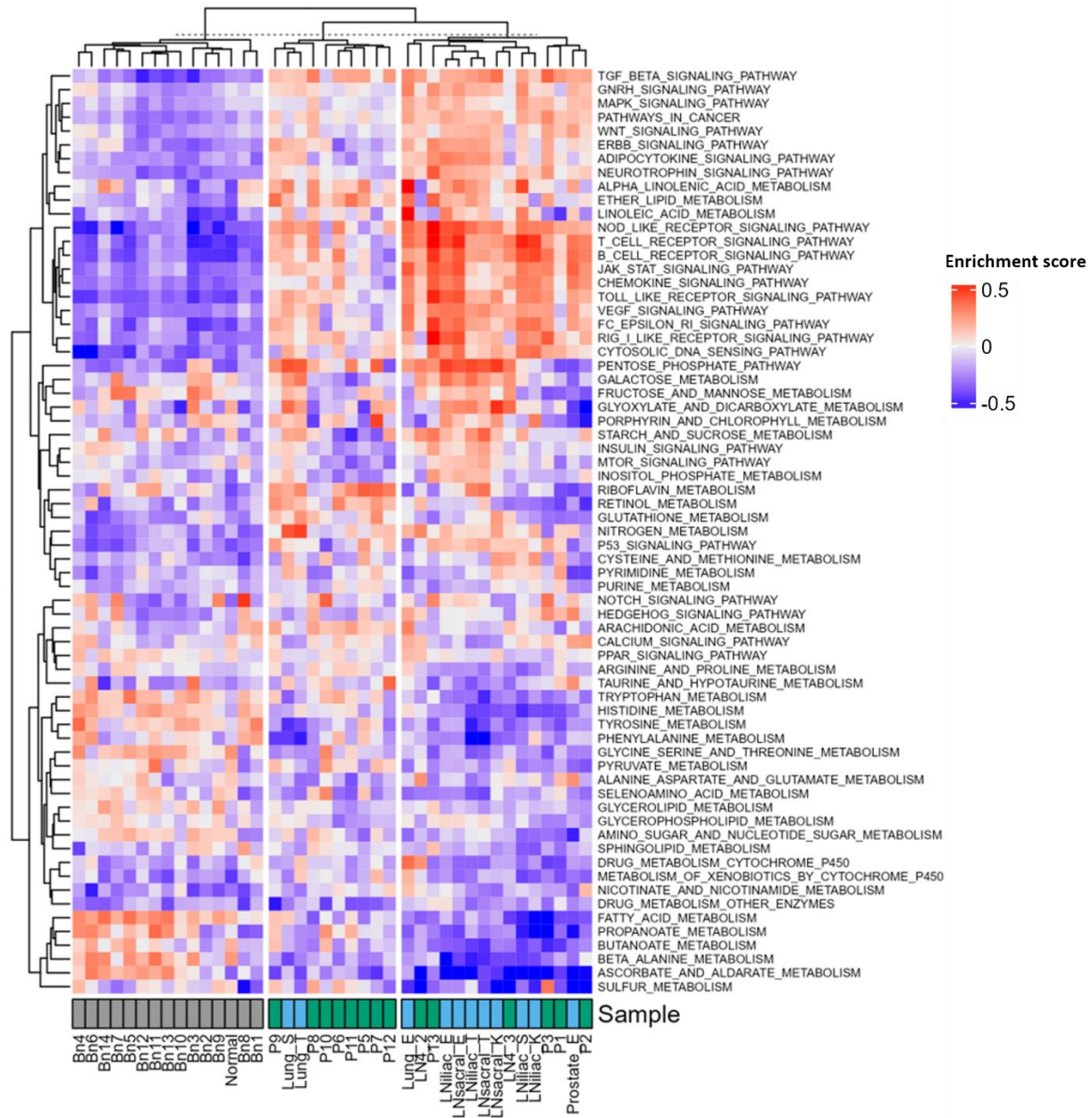
	Gene	Log2 Fold Change	Adjusted p value
Upregulated in Neutered	ENSCAFG00000029568	-20.89	1.98E-05
	<i>RPL4</i>	-4.88	2.12E-02
	<i>SLAMF1</i>	-4.13	3.86E-02
	<i>CCL17</i>	-3.99	3.27E-02
	<i>CX3CL1</i>	-3.13	1.30E-02
	<i>HIST1H1C</i>	-2.94	5.16E-03
	<i>VNN1</i>	-2.44	3.55E-04
	<i>PFN2</i>	-2.02	1.30E-02
	<i>FOXN2</i>	-2.01	4.67E-02
Upregulated in Intact	<i>C21orf2</i>	1.42	1.77E-02
	ENSCAFG00000003238	3.40	2.94E-02
	ENSCAFG00000011827	3.41	3.27E-02
	<i>GPR156</i>	4.53	3.38E-02
	<i>HRASLS</i>	5.08	1.30E-02
	<i>RTN1</i>	6.13	4.26E-02
	<i>VIL1</i>	6.41	1.30E-02
	<i>PKIA</i>	6.74	1.30E-02
	<i>NELL2</i>	7.06	2.45E-03

Supplemental Figure 3: Castration has a limited effect on the transcriptome of natural PCa.

Volcano plot of differential expression analysis results for the comparison between natural PCa in intact vs. neutered dogs. Red represents genes not differential expressed (adjusted p value < 0.05 and/or fold change < 2 or > -2), while blue represents significantly differentially expressed genes (adjusted p < 0.05 , fold change > 2 or < -2). The table below shows all differentially expressed genes for this comparison.

Supplemental Figure 4: DPC-1 tumours resemble natural canine PCa. (A-B) Volcano plots of differential expression analysis results for (A) DPC-1 tumours vs. normal prostate or (B) natural PCa vs. normal prostate. For results in the DPC-1 tumours, colors indicate whether the same gene was differentially expressed in natural tumours vs. normal prostate, and vice versa. (C-D) Bar graphs showing gene set enrichment analysis results comparing DPC-1 tumours or natural PCa vs. normal prostate for Hallmarks of Cancer (C) or selected KEGG pathways (D). The length of the bars represents $-\log_{10}(\text{adjusted p value})$ and colors represent normalized enrichment scores (NES).

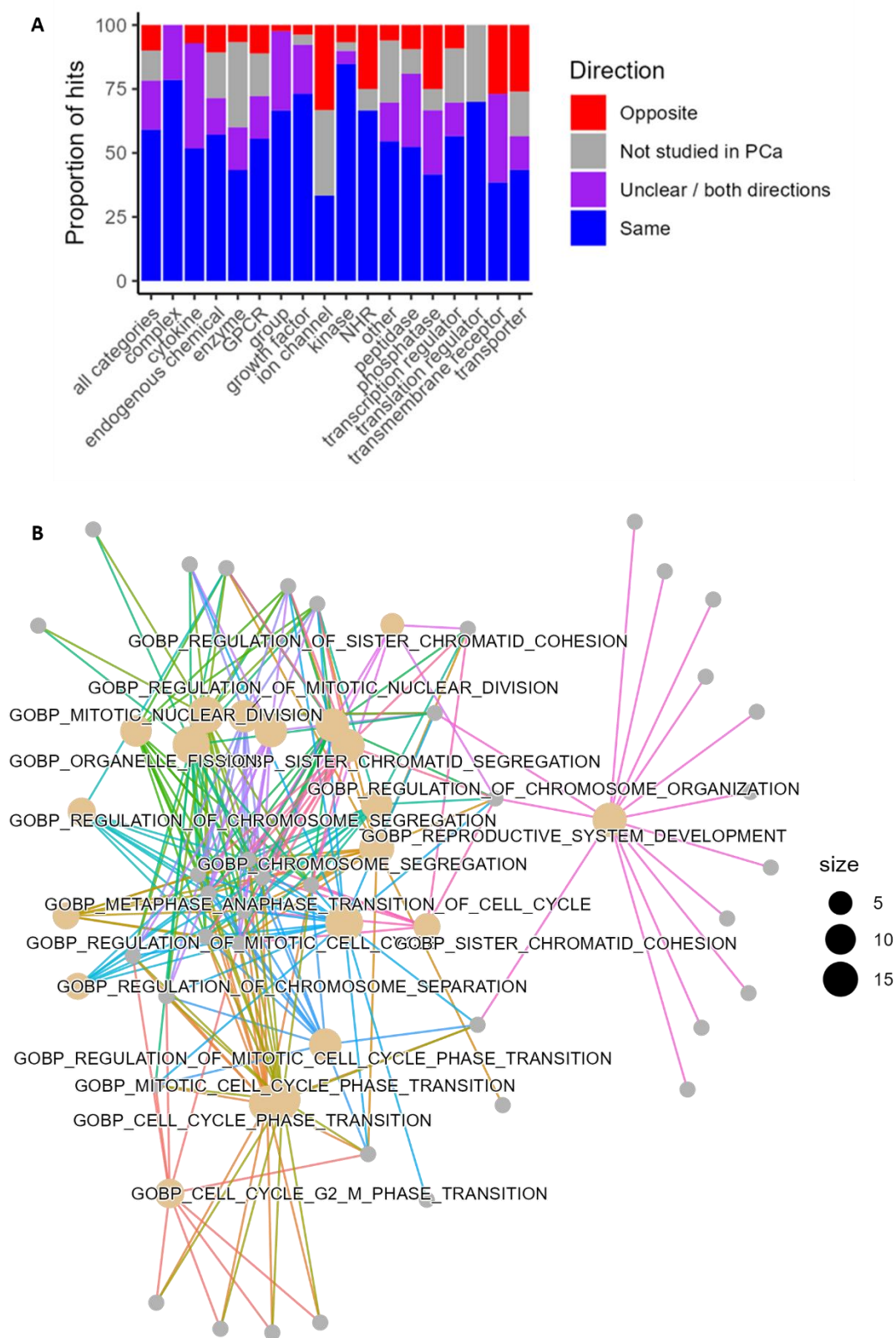
Figure S5



Supplemental Figure 5: DPC-1 tumours recapitulate the heterogeneity seen in natural PCAs.

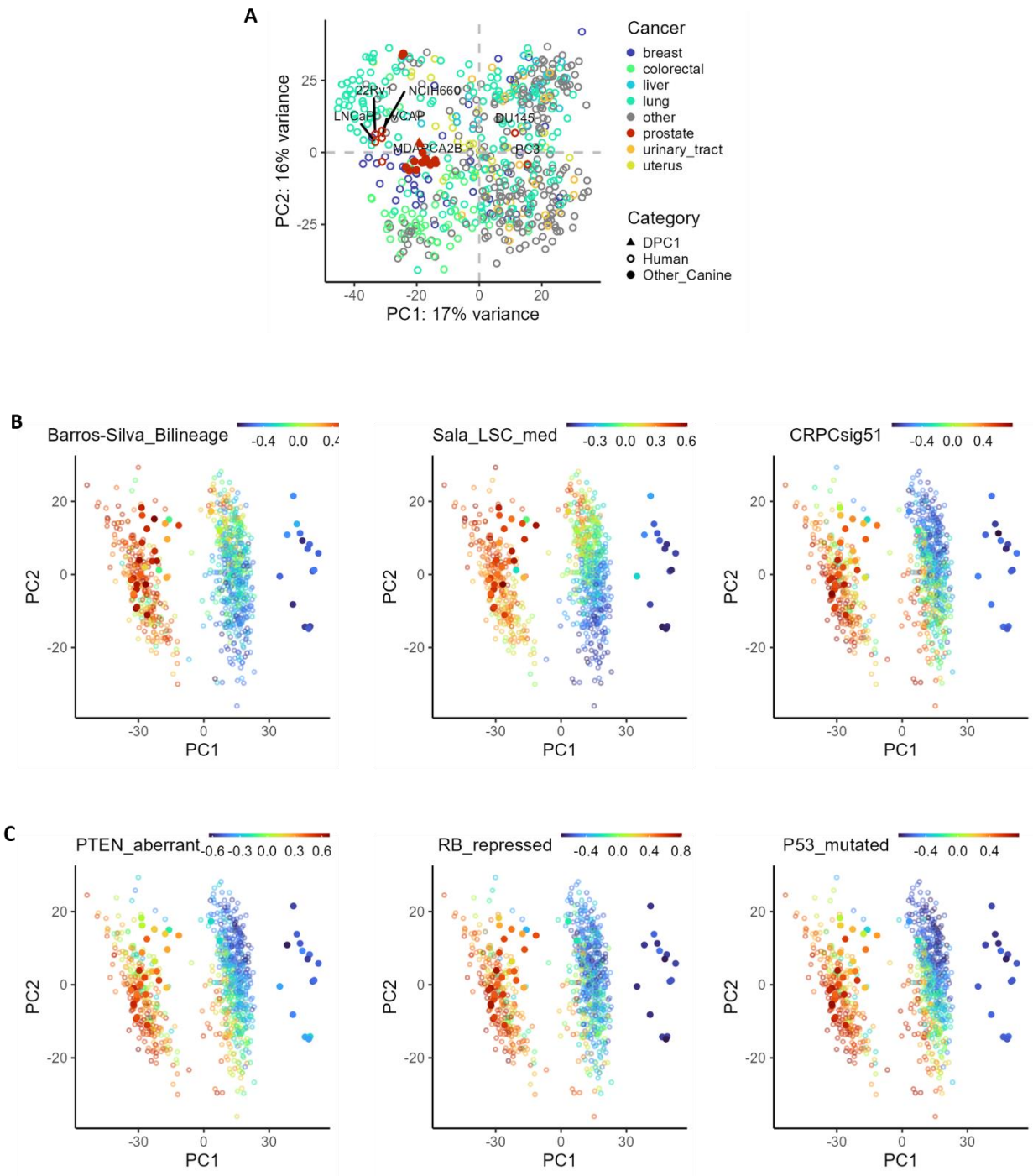
Heatmap of gene set variation analysis results of selected pathways from KEGG gene sets, where the bottom annotation denotes significance by gene set enrichment analysis (significant gene set in DPC-1 vs. normal prostate, natural PCAs vs. normal prostate, both, or neither, as seen in Figure S3C) and the left annotation denotes sample category (DPC-1, natural PCa, normal prostate).

Figure S6



Supplemental Figure 6: (A) Stacked bar graph showing the relevance of Ingenuity Pathways Analysis top upstream regulator hits vs. the literature on human PCa. Genes whose activation/inhibition is in line with the literature in human PCa are denoted as showing the “Same” direction, while those with opposite results are denoted as “Opposite”. Genes with ambiguous results in the literature and those that seem to have complex roles that are not yet fully understood are denoted as “Unclear / both directions”. (B) Cnetplot showing over-representation analysis of gene ontology gene sets for the list of upregulated genes whose upregulation suggests AR as an activated upstream regulator.

Figure S7

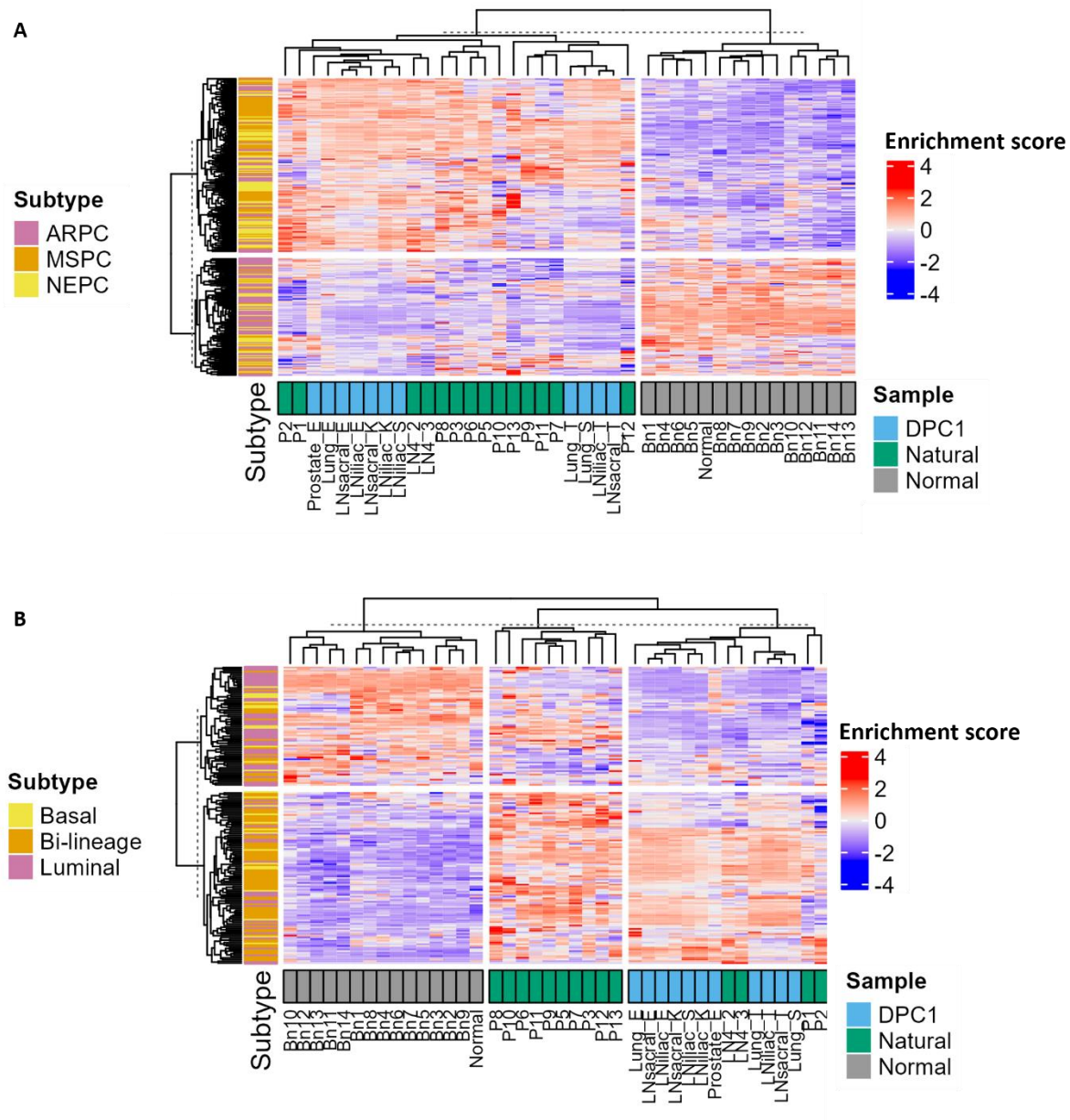


Supplemental Figure 7: Canine PCa resemble mesenchymal and stem-like human mCRPC.

(A) Principal component analysis plot for canine and human cell lines. The shape represents the species, while the color represents the cancer site. Human PCa cell lines are annotated. (B, C) The

same principal component analysis plot as in Figure 4A was overlaid with gene set variation analysis results for gene sets representing (B) an early CRPC-like cell signature (CRPC51) and two bi-lineage cell signatures, or (C) aberrant activity of common tumour suppressors (PTEN, RB1, and P53).

Figure S8



Supplemental Figure 8: Canine PCa resemble mesenchymal and stem-like human mCRPC.

Heatmaps for expression of genes from mCRPC subtype gene sets (A) and luminal, basal, and bi-lineage cell gene sets (B) in canine PCa and normal prostate samples. The annotation on the left indicates the mCRPC or cell subtype for each gene (see Methods for details of gene sets). The bottom annotation indicates the sample category.

Table S1: Assay details and efficiencies.

Gene	Assay	Manufacturer	Amplicon length	RefSeq sequence	Exon boundary	Assay location	Assay efficiency (%)
<i>ACTB</i>	Cf04931159_m1	Life Tech	52	NM_001195845.2	1-2	77	106.90
<i>FOLH1</i>	Cf02632661_g1	Life Tech	132	NM_001271778.1	16-17	1900	102.73
<i>GUSB</i>	Cf02622808_m1	Life Tech	75	NM_001003191.1	11-12	1849	94.89
<i>KLK2</i>	Cf02628870_m1	Life Tech	86	NM_001003284.1	2-3	236	98.90
<i>L1CAM</i>	Cf02725306_m1	Life Tech	115	XM_022415854.1	4-5	515	93.77
<i>PSCA</i>	CUSTOM	IDT	101	XM_038685212.1	NA	302-402	101.91

PSCA Forward primer: GCTGGACCGAGCACATC

PSCA Reverse primer: CTTGCCCACGTAGTAGTTCTG

PSCA probe: CCTGACCCTCATTAGCAAGGGCTG

RT-qPCR assays bought from Life Technologies do not provide primer and probe sequences, but do provide assay location, amplicon length, exon boundaries, and variant specificity information.

Table S2: Gene sets used in analyses.

Gene set	Source	Genes
AR-driven CRPC (ARPC)	Han et al., 2022, Cell Rep. (PMID: 35385726)	ABCC4, ACACA, ACADL, ACPP, ACSL3, ACSM3, ADRB2, AFF3, ALDH6A1, ANKRD29, ANKS1B, AR, ARHGAP6, ARHGEF26, BAIAP2, BMPR1B, C19orf48, C1orf116, C1orf21, CCND1, CDYL2, CHPT1, COLEC12, CPNE4, CREB3L4, CROT, CYP39A1, DCXR, DLX1, EHF, ELL2, ERGIC1, FASN, FGFR1, FOLH1, GALNT7, GATA2, GLUD1, GLYATL1, GREB1, GRIN3A, H2AFJ, HOMER2, HPN, IGF1R, IRS1, IRS2, IRX3, IVD, KCNN2, KLF15, KLK4, LGR4, LIMCH1, LONRF2, MAOA, MBOAT2, MCCC2, MLPH, MYC, NEDD4L, NKX3-1, PDE9A, PDLIM5, PLA2G7, PLD6, PLEKHH1, PMEPA1, PPP3CA, RAMP1, RANBP3L, RDH11, RHOU, SH3RF1, SLC22A5, SLC25A27, SLC35F2, SORD, SPDEF, SPON2, STEAP1, STEAP2, SYNGR2, TBX3, TM7SF2, TMCC3, TMEM192, TMPRSS2, TRIM36, TTL12, TUB, TUBA3D, UNC13B, VIPR1, ZBTB16, ZG16B, ZNF385B, ZNF577, ZNF613, ZNF649
Mesenchymal and stem-like CRPC (MSPC)		ADAM19, AKR1C1, ANO9, ANXA1, ANXA2, ARHGAP23, ARHGAP23, ARNTL2, ATP2A3, ATP2B4, CASP10, CDH2, CHI3L1, CHST11, CHST15, CNN2, CRIP1, CRIP2, CTHRC1, CTSC, DAB2, DAPP1, DHRS9, DOCK11, DPYSL3, EFHD2, FMNL3, FXYD5, GBP1, GIMAP2, GRAMD1B, HDAC9, HLA-A, HLA-DRB5, HLA-E, IER3, IFI16, IFI44, IFI44L, ISLR2, ITGA2, ITGB4, ITPRIP, KIF26B, LAMP3, LGALS3, LTBP1, MACC1, MBNL2, MDFIC, MLKL, MSN, MUC1, MX1, MX2, MYOF, NKD1, NMI, OAS2, OASL, OPTN, PALLD, PCDH9, PDLIM1, PGM2L1, PLCE1, PLEKHA2, PLEKHG1, PLSCR1, PLTP, PRICKLE2, PRKD3, PTPRE, PYCARD, RSAD2, SAMD9, SAMD9L, SATB1, SERPINA1, SERPINB1, SH3BGRL, SLFN13, SPATS2L, SULF1, TAGLN2, TGFBI, TLR4, TM4SF1, TMEM173, TMPRSS13, TMSB4X, TPBG, TRIM38, TTC39B, UCP2, VCAN, VIM, VOPP1, WIPF1, XAF1
Neuroendocrine CRPC (NEPC)		ABLM2, ACYP1, AMPH, AP3B2, ASF1B, BTBD3, CACNA2D1, CACNA2D3, CCDC88A, CCNE2, CDC25B, CDC7, CDKAL1, CDKN2A, CDKN2C, CDKN2D, CEP55, CHGA, CHRN2, COTL1, CRMP1, CRTAC1, CXXC4, DBNDD2, DERA, DNM1, DNMT1, DONSON, E2F7, EFR3B, ENO2, FBXO5, GAD1, GADD45A, GNAO1, GNG2, GPRIN1, GPSM2, GRIN2C, H2AFV, HES6, HOXB2, HOXB5, HOXB6, HOXB7, IL13RA1, JAM3, KATNAL1, KCNH3, KCNH6, KIF18A, KIF19, KIF2A, LAPTM4B, LGI2, LPIN1, LYPD1, MAST1, MBIP, MET, MEX3B, MTSS1, NEB, NR2F1, NRCAM, NRM, NUA1, PAM, PCNA, PEG10, PGAP1, PGBD5, PHYHL, PLEKHA1, PPM1D, PROX1, RBM38, RCAN1, RGS16, RNASEH2A, RUNDC3A, S100A6, SEZ6, SGCE, SLC36A4, SLC3A1, SMYD3, SNCAIP, SSX2IP, STMN1, STX1A, SYT11, TEAD1, TNFRSF11B, TPX2, TRIM9, TUBA1A, TUBB2B, UBE2QL1, UNC13A
Basal cells	Henry et al., 2018, Cell Rep. (PMID: 30566875)	CAV1, CAV2, CLU, CRYAB, CYR61, DEFB1, DKK1, DST, EDN1, F3, FHL2, HBEGF, HSPA1A, HSPA1B, KRT14, KRT15, KRT17, KRT5, MEG3, MYC, MYL9, PALLD, PHLDA1, RGCC, S100A2, SERPINF1, STK17A, TAGLN, TIMP3, ZFP36L2
Luminal cells		ABHD2, ACPP, ADIRF, AGR2, ALDH1A3, AZGP1, B2M, BACE2, CD24, CIB1, CLDN3, CPE, DBI, DHRS7, DSC2, ELF3, ELOVL5, FDPS, FLJ20021, FXYD3, GADD45B, GOLM1, H2AFJ, HMGS1, IL6ST, INSIG1, JUN, KLK2, KLK3, KLK4, LINC00844, LMO7, MSMB, MT1E, MT1F, MT1G, MT2A, NDRG1, NEAT1, NKX3-1, NPDC1, PART1, PLA2G2A, PLPP1, PMEPA1, PPP3CA, PSCA, RAB27B, RASEF, RDH11, RNF144B, RPS27L, SAT1, SEC11C, SERF2, SLC12A2, SLC39A6, SLC44A4, SMIM14, SORD, SPINK1, STEAP2, TFF1, TMC5, TMPRSS2, TPD52, TSC22D1, TSPAN8, TXNIP, UQCRCQ, ZG16B
Bi-lineage cells	Barros-Silva et al., 2018, Cell.Rep.	ADAM10, CD14, CD38, CD74, CD9, CDCP1, ENPP1, F3, FGFR2, FGFR3, IGF1R, IGSF3, ITGA2, ITGA5, ITGAV, ITGB4, LY6A, LY6D, PLAUR, PTGFRN, SDC1, TACSTD2, TNFRSF4, TNFRSF8
Basal cells		BCAM, ICAM1, IFITM1, ITGA1, KRT14, KRT5, TP63

Luminal cells	(PMID: 30566873)	ALCAM, BSG, CD164, CD24A, CD68, CEACAM2, EPCAM, KRT18, KRT8, NGFRAP1, NKX3-1
Bi-lineage cells	Sackmann Sala et al., 2017, J Pathol. (PMID: 28603917)	ACSM3, ALDH1A3, ANXA1, APOD, AREG, ARL14, ASPA, ATP10B, B4GALNT2, BACE2, BTC, C3, CADPS, CAPN13, CAPSL, CD55, CDC42EP5, CDK6, CLCA1, CLU, CTSE, CTSH, CXCL13, CXCL17, CYP2F1, D17H6S56E-5, DUSP4, EDN1, F5, FFAR4, GFPT2, GPRC5A, GRP137B, GSDMC, GSTA3, IGSF11, IL1RN, KCNK5, KRT23, KRT4, KRT7, LENG9, LGALS3, LTF, MEIS2, MGAT3A, MGAT5, MYOF, NBEA, NGF, NT5C2, OSBPL3, PGLYRP1, PPARG, PRSS22, PSCA, REG3A, REG3G, S100A1, SAA1, SBSN, SCARA3, SCEL, SDCBP2, SLC25A24, SMIM5, SPNS2, SPTLC3, SULT1C2, SULT1E1, TCF21, TNFAIP6, TSPAN8, TSTD1, UGT2B10, USP6NL, WFDC2
Additional luminal genes		AR, FOLH1
Neuroendocrine cells	Henry et al., 2018, Cell Rep. (PMID: 30566875)	A2M-AS1, ABCC8, ABHD16A, ABHD3, ABO, AC007389.3, AC009410.1, AC010746.3, AC013463.2, AC015849.2, AC022007.5, AC034243.1, AC053503.4, AC068282.3, AC072062.1, AC090616.2, AC093642.3, AC093802.1, AC097724.3, AC144831.3, ACAD11, ACVR1C, ADAMTS9-AS1, ADARB2, ADCY10, ADCY2, AGR3, AIDA, AL589743.1, AMACR, ANK1, ANK2, AOC1, AOX1, AP001057.1, AP3B2, APLP1, ARAP1, ARHGAP24, ARHGDIG, ARHGEF33, ARHGEF9, ARRDC3, ARSA, ASCL1, ASCL5, ASGR2, ATG9B, ATP6V0E2, ATP6V1G1, ATP6V1G2, AZGP1, B3GALT2, B3GAT1, BASP1, BEST3, BEX1, BRINP3, BRWD1-AS2, BSN, C10orf10, C11orf16, C12orf56, C12orf71, C17orf98, C1orf210, C1QL1, C2CD4A, C2CD4B, C2orf70, C2orf72, C3orf14, C4orf48, C5orf46, CA7, CA8, CACNA1A, CACNA1H, CADPS, CALCA, CAMK2N1, CBLB, CBX7, CCDC110, CCDC154, CCDC170, CCDC181, CCDC187, CCDC67, CCP110, CD24, CDK5R2, CELF3, CEND1, CFC1, CFTR, CGA, CH17-340M24.3, CHGA, CHGB, CHN1, CHN2, CLDN3, CLGN, CNTNAP2, COL9A2, CPE, CRMP1, CRY2, CSDC2, CST1, CTB-11I22.1, CTB-171A8.1, CTB-40H15.4, CTC-492K19.7, CTD-2007H13.3, CTD-2089N3.2, CTD-2095E4.5, CTD-2666L21.1, CXCR5, CXXC4, DAB1, DDC, DFNB31, DHDH, DNAJC12, DNASE1L3, DOCK11, DOK6, DOPEY1, DPP4, DPYSL3, DRAIC, DTNA, DUSP26, DYNC1I1, EAF2, EFCAB12, EFR3B, ELAVL3, ELAVL4, ENO2, EPHA4, EPHB1, EPN2-AS1, ERBB4, ERC2, ERCC4, ERICH3, ESPL1, ESPN, FAM102A, FAM131B, FAM131C, FAM134B, FAM57B, FAM71E1, FAM96A, FGF14, FGL1, FIBCD1, FLJ46066, FOCAD, FOXA2, FRGCA, FXYD6, GABRR1, GALR1, GALR2, GPD5, GJA3, GOLGA7B, GPC5-AS1, GPER1, GPR173, GPR55, GPR89A, GPRIN1, GRASP, GREB1, GRIK4, GRIN3A, GRP, GS1-393G12.14, GSTA4, GUCA2B, GUF1, HCN2, HEPACAM2, HES6, HID1, HLTf, HOXB2, HOXB3, HOXB6, HOXB8, HOXB-AS3, HSF2, HSH2D, HTR3C, ICA1L, IFI6, IGFBP5, IL20RA, ILDR1, INA, INSM1, IQSEC3, ISX, ITGAM, IZUMO4, JAKMIP1, JAKMIP2, KB-1562D12.1, KCNAB1, KCNB2, KCNG3, KCNH2, KCNH3, KCNH6, KCNJ12, KCNJ6, KCNK12, KDELR3, KIAA1211L, KIAA1324, KIAA1644, KIF1A, KIF5C, KISS1R, KLHL25, KLHL32, KLK12, KRT20, KRT40, KRTAP5-AS1, KSR2, LA16c-321D4.2, LA16c-395F10.2, LCN15, LCORL, LDLRAD1, LDLRAD4, LECT1, LINC00261, LINC00299, LINC00327, LINC01018, LINC01091, LINC01146, LINC01353, LINC01419, LINC01481, LINC01484, LINC01497, LINC01535, LINGO2, LOX, LRP2BP, LRRC24, LRRN3, LY6H, LYL1, MAGED1, MAGEE1, MAL, MAOB, MAP1A, MAP1B, MAP7D2, MAP9, MAPK12, MAZ, MB, MBOAT2, MCF2L2, MDS2, MFN1, MGARP, MIAT, MICAL1, MIR7-3HG, MLLT11, MN1, MORN5, MRAP2, MROH2A, MS4A8, MYB, MYEF2, MYO18A, NAA40, NAAA, NAT8L, NDST4, NDUFA4L2, NEBL, NEBL-AS1, NELL1, NELL2, NGF, NKAIN2, NKAIN3, NLGN4X, NLRP1, NOL4, NOS1AP, NPDC1, NPPA, NPTX2, NR0B2, NRCAM, NRL, NRSN1, NRTN, NRXN1, NXPH3, OPRK1, OR51E1, OR51E2, OVOS2, P2RX6, PAXBP1-AS1, PCDH17, PCDHB15, PCDHGA3, PCLO, PCSK1, PCSK1N, PCSK2, PDE6A, PEG10, PEX5L, PGAM2, PHGR1, PIPOX, PKD2L1, PKIB, PLAC8, PLEKHB1, PLEKHH1, PLPP6, PLXNC1, PNMA1, PNMAL1, POMK, POPDC2, POPDC3, PPFIA2, PPM1H, PRELID3A, PRKACB, PROSER2-AS1, PROX1, PRR26, PRR36, PRUNE2, PSD, PTPN22, PTPRN, PTPRN2, QDPR, QPCT, QRFP, RAB26, RAB39B, RAB3B, RANBP3L, RASA3, RBFOX1, RECQL4, RET, RGL3, RGP4D-AS1, RGS11, RGS4, RGS7, RHBDD2, RIIAD1, RIMBP2, RIMKLA, RIPPLY2, RLN2, RNF182, RNF41, RP11-130C6.1, RP11-

		131N11.4, RP11-133K1.6, RP11-140I16.3, RP11-157J24.2, RP11-166O4.6, RP11-173D3.1, RP11-179B2.2, RP11-196G18.3, RP11-1C8.7, RP11-227G15.8, RP11-247A12.2, RP11-25K19.1, RP11-2E11.9, RP11-310E22.4, RP11-315I20.1, RP11-317N12.1, RP11-333B11.1, RP11-347C12.11, RP11-380L11.4, RP11-380O24.1, RP11-386M24.9, RP11-405M12.3, RP11-422P24.11, RP11-432I5.2, RP11-434B12.1, RP11-452H21.4, RP11-482M8.3, RP11-484K9.4, RP11-511P7.5, RP11-51J9.6, RP11-532M24.1, RP11-53O19.1, RP11-553A21.3, RP11-586D19.1, RP11-602N24.3, RP11-620J15.4, RP11-632L2.2, RP11-669E14.6, RP11-675F6.4, RP11-679C8.2, RP11-170O19.17, RP11-768G7.2, RP11-794G24.1, RP11-830F9.6, RP11-839D17.3, RP11-844P9.2, RP11-867O8.5, RP11-923I11.4, RP11-964E11.2, RP11-977B10.2, RP3-395M20.8, RP3-402G11.26, RP4-594I10.3, RP5-1033H22.2, RPH3AL, RSAD2, SALL2, SAPCD1-AS1, SATB2, SCG2, SCG3, SCG5, SCGB1B2P, SCGN, SCN3A, SCN9A, SEC11C, SEC14L1, SEC62-AS1, SEMA3D, SEPSECS, SERPINA11, SHANK3, SHISA8, SKAP1, SLC18A1, SLC25A34, SLC26A4, SLC29A4, SLC35D3, SLC35E2, SLC36A1, SLC38A11, SLC46A3, SLC7A14, SLC8A1, SLITRK6, SMCO4, SMIM24, SMOC2, SNAP25, SNCAIP, SORCS1, SOX2, SOX5, SPAG5, SPINK14, SPOCK1, SPX, SRPK3, SRRM4, ST18, ST8SIA5, STARD4-AS1, STMN2, SV2A, SYN1, SYN2, SYN3, SYP, SYT1, SYT11, SYT13, SYT14, SYT4, SYT5, TAAR1, TACC2, TAGLN3, TAT-AS1, TDRG1, TEK4, TFF3, TGM3, THSD7A, TIMM22, TIMP2, TIRAP, TMEM176A, TMEM176B, TMEM178A, TMEM260, TMEM55A, TMEM59L, TMEM61, TNFSF14, TNKS2-AS1, TOX, TPD52, TPH1, TPPIP3, TREM1, TRIM9, TLL10, TLL7, TUBA4B, TUBB2B, UBXN10-AS1, UCHL1, UCN, UCN3, UCP2, UNC79, USH1C, USP54, VEGFB, VPS33A, WNK4, WNT5A-AS1, XKR6, YPEL4, ZACN, ZBP1, ZBTB18, ZBTB37, ZKSCAN3, ZNF385B, ZNF446, ZNF548, ZNF571, ZNF585A, ZNF98, ZSWIM3, ZSWIM5
Neuroendocrine PCa	Tsai et al., 2017, BMC Cancer (PMID: 29132337)	ACTL6B, ACYP1, ADCYAP1, ADD2, AMDHD1, AMER3, ANKRD65, AP3B2, APLP1, ARHGEF2, ASCL1, ASPM, ASXL3, ATCAY, BAALC, BEX1, BSN, C1orf112, CA10, CACNA1A, CALCA, CAMK1D, CCDC88A, CDC25B, CDCA8, CDH2, CDKAL1, CDKN2A, CDKN2C, CDKN2D, CELF3, CELF4, CELSR2, CEP131, CEP72, CHD7, CHGA, CHGB, CHRNA3, CKS1B, CLSPN, CPLX2, CPT1C, CRABP1, CRMP1, CRTAC1, CXXC4, DAND5, DBNDD2, DCX, DERA, DLL3, DNMI1, DNMT1, DONSON, DPYSL5, DSCAML1, DUSP26, E2F7, E2F8, EID2B, ELAVL3, ELAVL4, EMC3-AS1, ENO2, ERC2, ESPL1, EZH2, FAM132B, FAM161A, FAM57B, FANCL, FGF9, FMN2, FOXA2, FRMD5, FSD1, FYN, FZD9, GNAZ, GPRIN1, GRIN2C, GRP, H19, HAUS1, HAUS8, HCN3, HES6, HMP19, HOXB3, HOXD10, IGFBPL1, INA, INSM1, ITGB3BP, JAKMIP2, KCNB2, KCNC1, KCNC3, KCNH3, KCTD16, KIF19, KIRREL3, KLHL14, KNTC1, KPNA2, LHX2, LIG1, LMNB1, LMO3, LOC643201, MARCH11, MAST1, MCM2, MCM3, MCM6, MIAT, MIR7-3HG, MLLT11, NAV2, NDRG4, NELL1, NFASC, NGB, NHS, NKX2-1, NOL4, NPPA, NPPB, NPTX1, NR0B2, NRCAM, NRM, NRTN, NRXN1, OCIA2, OVOS2, PAFAH1B3, PCSK2, PEG10, PGBD5, PHF19, PHYHIPL, PLEKHG4B, POLD1, POU3F2, POU4F1, PRC1, PRELID2, PROX1, PSCK1, PSIP1, PSRC1, PTPRH, PTTG3P, RAB3C, RBM38, REEP2, RFX2, RGS7, RHEBL1, RIMBP2, RIMS2, RNASEH2A, RNF182, RNF183, RPRM, RUNDC3A, RUNX1T1, SCG2, SCG3, SCN3A, SEZ6, SEZ6L, SFTA3, SH3GL2, SHOX2, SIX2, SLC36A4, SLCO3A1, SLITRK6, SNAP25, SNCAIP, SOX2, SRRM3, SRRM4, SSRP1, STMN1, STMN3, STX1A, SULT4A1, SV2B, SVOP, SYT13, TAGLN3, TCERG1L, TERT, TEX30, TIMELESS, TLE2, TMEM145, TMEM179, TMEM61, TNFRSF25, TOP2A, TOX, TPX2, TROAP, TUBA1C, TUBB2B, TYMS, UBE2S, UNC13A, VGF, ZNF711
Neuroendocrine PCa	Beltran et al., 2016, Nat Med. (PMID: 26855148)	ASXL3, AURKA, BRINP1, C7orf76, CAND2, DNMT1, ETV5, EZH2, GNAO1, GPX2, JAKMIP2, KCNB2, KCND2, KIAA0408, LRRC16B, MAP10, MYCN, NRSN1, PCSK1, PROX1, RGS7, SCG3, SEC11C, SEZ6, SOGA3, ST8SIA3, SVOP, SYT11, TRIM9
CRPCsig51	Cheng et al. 2022, Eur Urol	ASFI1B, ASPM, ATAD5, BARD1, BIRC5, C1QTNF9B-AS1, CADM1, CCDC14, CCDC34, CCNB1, CDK1, CDKN3, CENPF, CENPH, CENPK, CENPU, CENPW, CEP78, CKAP2, DTYMK, FAM111B, FEN1, GGH, GLYATL1, HELLS, ISYNA1, ITGB3BP, KIAA0101, LINC00869, LINC01358, LMNB1, MAD2L1, MKI67, NUSAP1,

	(PMID: 35058087)	<i>PHF19, PRC1, RBP1, RNASEH2A, RRM2, SLC4A4, SMC2, SYP, TFF3, TK1, TMPO, TOP2A, TPX2, TYMS, UBE2C, UBE2T, ZWINT</i>
PTEN	Saal et al 2007, Proc Natl Acad Sci USA (PMID: 17452630)	<i>ANP32B, ARL6IP6, ARMC8, ATAD2, AURKA, BIRC3, BRWD1, BUB1, CBX1, CCNB1, CCNB2, CCSAP, CDC7, CDCA2, CDH12, CENPE, CENPW, CFAP20, CHAF1B, CHORDC1, CKS2, CLNS1A, CPZ, CTPS1, DCAF6, DHX58, DLGAP5, DRAP1, DTX3L, ESRP1, EXO5, FAM83D, GART, GMPS, GPBP1L1, GPKOW, GZMH, HDAC2, HLTF, HNRNPU, ILF2, KATNAL1, KIF14, KIF23, KIF2A, KIF2C, KIF4A, KIFC1, KPNA2, MANEA, MARCHF7, MCM3, MCM6, MELK, METTL18, MGST3, MLF1, MRPL3, MRPL48, MRPL50, MRS2, NEK2, NELFE, NUDCD1, NUP160, PACC1, PAICS, PARP14, PATL1, PIK3CA, PLSCR1, PNPT1, PRDX1, PSMD12, PTTG1, RAB27A, RAB6A, RACGAP1, RAD51C, RBMX, RFC4, RSF1, RUBCN, SAMD9L, SENP5, SGO2, SNAPC3, SNRPD1, SRPK1, SRSF1, STAT1, STIP1, STMN1, SUMO3, TASOR2, TCF19, TGFBRAP1, THAP12, TIMM17A, TOPBP1, TP53BP2, TRA2B, TUBB2A, U2AF1, UBQLN1, UMPS, VRK2, WDHD1, ZDHHC13, ZNF672</i>
RB1	Han et al., 2022, Oncogene (PMID: 34975152)	<i>AARS, ASF1B, ATP6V0D1, BRCA1, C19orf48, CALR, CDC45, CDC6, CDK2, CFL1, CHCHD2, DERA, DNMT1, DSN1, E2F1, FAM111B, FEN1, GMPS, H1FX, H2AFX, HES4, KLF4, KNTC1, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7, MSH6, MYBL2, NASP, NUP155, PCNA, PKMYT1, PRIM1, PTGES3, RBBP7, RFC2, S100P, SDC1, SLC1A5, SLC20A1, TUBG1, UBC, UNG, WDHD1, WDR76, WEE1</i>
P53	Cidza et al., 2021, Clin Genitourin Cancer (PMID: 32896505)	<i>ACTL6A, ADAM17, AK2, ANLN, ASF1B, ASPM, AURKA, BEND3, BRIX1, BUB1, C16orf71, C17orf53, C9orf117, CCDC150, CCDC40, CCNA2, CCNB2, CDC123, CDC20, CDC25C, CDC45, CDCA4, CDCA5, CDCA8, CDKN3, CDON, CDR2L, CENPA, CENPF, CENPI, CENPL, CIT, CKAP2L, CPSF3, CSNK2A1, DCAF13, DCUN1D5, DEPDC1B, DERL1, DGAT2, DLGAP5, DNMT3B, DPH2, DSN1, DVL3, E2F2, E2F3, ECT2, ERCC6L, ESPL1, EXO1, EZH2, FAM49B, FAM72B, FANCB, FANCF, GINS1, GINS4, GNAI3, GTF3C2, GTPBP4, HJURP, INCENP, IQGAP3, ISY1, ITGB1BP1, ITGBL1, KIAA1524, KIF18B, KIF20A, KIF23, KIF2C, KIF4A, KIF4B, KIFC1, KPNA2, LSG1, MCM10, MCM4, MELK, MLF1, MRPL13, MRPL3, MSH2, MTBP, MYBL2, NAA50, NCAPG, NCAPG2, NCAPH, NCBP2, NEIL3, NUF2, NUP85, PARP2, PDCD10, PKMYT1, PLK1, POLQ, POLR2K, PRPF38A, PSMA7, PSMC2, PSMD12, PTTG1, RAB6B, RACGAP1, RAD21, RAD51, RAD54B, RAD54L, RAE1, RBM17, RBM28, RFC4, RSPH1, SEC22A, SGOL1, SMC1B, SMC4, SMYD2, SNRPB2, SPAG5, STIP1, TACC3, TARDBP, TCEB1, TK1, TMEF132A, TOPBP1, TPX2, TRAIP, TRIP13, TROAP, TTC9C, TTK, UBE2C, VRK1, VWA3A, WDHD1, WDR53, WDR62, WDR78, XPO1, YEATS2</i>

DISCUSSION

This thesis brings new knowledge in the field of human and canine PCa. We present opportunities to better stratify patients and manage the disease differently, moving closer to precision oncology. The following discussion first considers our findings on cell subtypes and testing circulating genes, ending with canine comparative oncology. This is followed by a section on future directions.

1. Cell subtypes and drug targets: Tissue, blood, and comparative approaches

a. Tissue-based assessment of AR+/AR– subtypes at diagnosis

It is well established that all prostate epithelial cell subtypes contribute to PCa progression through various mechanisms of resistance. However, AR in luminal cells remains the main therapeutic target upon recurrence for all cases. This is for good reason, as ADT has had a huge impact on PCa mortality. As shown in Manuscript 1, ~99% of benign luminal cells are AR+, and these account for the great majority of PCa cells as well. While this is in line with the literature on PSA expression in 99.8% of PCas,³¹¹ reports on AR expression have been highly inconsistent, partly due to variability in methods of quantification and data reporting.

The most important takeaway from our cell-by-cell quantification of AR expression is that it provides a simple assessment of the proportion of luminal-like PCa cells, from which we can indirectly infer the total proportion of NE, stem-like, and mesenchymal-like PCa cells without the need to account for markers of each cell type and the heterogeneity therein. We did notice differences among NE markers in primary tumours of hormone-treated patients, showing areas with more CHGA+ cells and other foci with more SYP+. This may be attributed to the state of differentiation, where cells actively differentiating towards a NE phenotype will express different markers compared to terminally differentiated NE cells.³¹² For example, while CHGA seems to mark mainly terminally differentiated NE cells, SYP comes up during NE differentiation and

remains expressed.³¹² Of course, this heterogeneity is not limited to NE cells. While such heterogeneity remains of interest for further investigation and is applicable to other phenotypes, all of these AR– cells are resistant to ADT.

Our findings on AR expression levels further increasing in ADT-treated cases despite the lower prevalence of AR+ cells were clinically significant. This is likely indicative of androgen-independent but AR-driven features of ADT resistance. Such mechanisms may involve ARVs, cross-talks with other signaling pathways including downstream of growth factor receptor and cytokine signaling, AR mutations or amplification, etc. The gradual loss of AR+ fibroblasts with increasing grade and hormone treatment was also in agreement with earlier reports. While we did not further elaborate on this important phenomenon in our subsequent work, it remains relevant to our general theme of cellular heterogeneity and likely directly contributes to the evolution of cancer cells. Indeed, fibroblasts secrete factors that contribute to PCa cell stemness, EMT, and NED.³¹³ While fibroblastic AR have been linked to tumorigenesis and growth, its loss or inactivation contribute to progression.³⁴⁸⁻³⁵¹

Although AR quantification may be useful for detecting various AR-independent and AR-driven mechanism of eventual castration-resistance indiscriminately, it is not all-encompassing. In line with the heterogeneity within cell subtypes, further markers should be assessed to better identify the specific NE, stemness, or AR-driven pathways to target in such cases, whether at diagnosis or in case of recurrence. Furthermore, AR expression alone does not identify the luminal cells with the potential to de-differentiate or trans-differentiate towards AR– phenotypes upon ADT. Indeed, we found both AR– and AR+ cells in the basal layer, which is likely dependent on their commitment to differentiate into luminal secretory cells. Such cells in cancer may be considered similar to intermediate or bi-lineage cells if they express basal markers, and would thus show some

stemness features, AR expression, but low AR signaling.^{98, 314} These would be considered “luminal” in this study, but may switch subtypes more easily upon treatment pressure due to their plasticity.

The most comprehensive methods to address these additional questions include digital spatial profiling and single-cell (sc) analyses. While scRNA-sequencing would allow a complete transcriptomic assessment of these cell types, it loses the spatial context that is integral for understanding their interactions. Indeed, we identified areas enriched in AR+ or AR– cells, while other foci were mixed. Spatial profiling with GeoMx (NanoString) or Visium (10X Genomics) would allow whole transcriptome analyses accounting for their spatial distribution. However, these technologies do not provide true single-cell resolution (minimum ~20 cells vs. 55µm square, respectively). Newer spatial technologies, such as CosMx (NanoString) and Xenium (10X Genomics), allow for single-cell resolution, but do not yet offer whole transcriptome assessment (currently limited to 6000 and 477 genes, respectively). Spatial and single-cell approaches can be used in parallel to assess the whole transcriptome and infer their spatial distribution therefrom.³¹⁵ A key consideration in such experiments is to have a clear question so that sample and marker selection and preparation is carried out accordingly, especially when studying rare cell types.

A limitation for translation of our approach is that it would be time consuming to acquire accurate cell-by-cell quantification in a clinical setting. However, this may be overcome by digital pathology and image analysis.³⁵² Most importantly, these tissue-based methods cannot absolutely determine which types of cells have already spread beyond the prostate, what phenotypes will become predominant in metastases, and what would be best targeted for each patient down the line. We attempted to address this issue in our study of genes in liquid biopsies.

b. Cell subtype and drug target genes in blood

Cell-subtype heterogeneity is even more striking in advanced cases, whether assessed in mCRPC tumours or blood RNA. Initially, with a limited panel of 14 genes, we found an increase in cases overexpressing genes of multiple subtypes with more advanced disease (based on bioinformatics of tumour transcriptomics) and an association with overall survival (in blood RNA). We identified significant associations between cell types or specific genes with clinical features, treatments, and outcomes. Once again, heterogeneity within subtypes was noted.

Before moving forward from our panel of 14 genes in Manuscript 2, we re-assessed the pros and cons of our initial methodology, keeping in mind that whole blood RNA allows for an agnostic approach to biomarker identification, not limited by the choice and implementation of methods for isolating specific cells or blood fractions. As previously mentioned, PaxGene tubes are banked with no processing needed until RNA extraction, making them amenable to clinical applications. The main issues inherent to such biomarker studies are the sensitivity and specificity of assays for markers found in low amounts. These were considered in Manuscript 2, but were further improved in Manuscript 3, where we: (1) switched from SYBR-green based detection to TaqMan probe-based assays, (2) implemented stricter inclusion and exclusion criteria for genes of interest, and (3) performed rigorous technical and biological validation to prove quantifiability and reproducibility of our results prior to testing all patient samples to assess clinical relevance.

Our principal limitation in the choice of genes was their specificity to the prostate or to cancer, and not to blood cells or derived fractions such as platelets. For some genes, specificity can be increased by testing for specific splice variants. In Manuscript 3, we had two genes (*CD44* and *NCAM1*) from Manuscript 2 for which splice variants were added to improve their detection. While *CD44* is known as a common stemness marker in many cancers, it is also expressed in various

WBCs and shows complex alternative splicing.³¹⁶ The variant *CD44E* is more specific to epithelial cells and was shown to be associated with stemness features in PCa.^{317, 318} Upon testing in blood, we found that *CD44E* expression is lower than *CD44* overall, and that it was overexpressed in a higher number of patients. *NCAM1* is a common NE marker but is also expressed specifically in a subset of natural killer cells.³¹⁹ The variant *NCAM180* is most relevant to NE lung cancers and neuroblastoma, and our preliminary bioinformatic results show this is also true for NEPC, whereas the *NCAM140* variant is ubiquitously expressed (unpublished data).³²⁰⁻³²³ Once again, we found more patients overexpressing the *NCAM180* variant in their blood compared to *NCAM1* overall.

Keeping in mind the limited therapeutic options for patients at advanced stages, we decided to add genes encoding drug targets to our panel to test in blood in Manuscript 3. These were chosen through a review of the literature and clinical trial results to identify promising drugs for which subsets of unselected patients showed responses. The NE gene *AURKA* showed clear trends in our samples, suggesting that subsets of patients who progressed on taxanes may benefit from the relevant targeted treatments. As the *AURKA* inhibitor alisertib has recently been granted orphan drug designation for small cell lung cancer, we may propose its testing as a circulating gene for patient selection in view of future NEPC trials. Indeed, not all NEPC patients are expected to benefit from this treatment, as only 40-65% show *AURKA/MYCN* amplification.^{104, 105}

Two other targetable genes of interest were *FOLH1*, encoding PSMA, and *SSTR2*. PSMA is the main imaging and theranostic target for PCa, and *SSTR* imaging is gaining interest for mCRPC, including with the ongoing 3TMPO study in Quebec. It may be relevant to verify whether imaging results correlate with gene expression results in blood.¹⁷⁶ We found that *FOLH1* was the most commonly overexpressed luminal gene in all categories of patients tested, and *SSTR2* was shown to correlate with RT. While we would expect imaging and liquid biopsy results to correlate well,

we must keep in mind that they do not represent the same features of the tumours. Imaging will detect metastases expressing a certain marker (or showing certain metabolic features) and of a certain size, while circulating transcripts represent what is released from the tumours, mainly in CTCs or EVs. It is noteworthy that a circulating gene overexpressed in the blood, such as *KLK3*, may represent the transcript level in CTCs and EVs, whereas its equivalent protein in plasma, i.e. PSA, reflects the tumour burden.

Other promising therapeutic targets found commonly overexpressed in blood were *VEGFA* for NE cases, *EZH2* and *TACSTD2* for stemness, and *ITGAV/ITGB3*. Src family kinases and receptor tyrosine kinases also came up. Altogether, this non-invasive approach measuring various features of PCa progression, including specific drug targets, is worth considering in combination with current markers to better predict progression and response to treatments. The next step would be to test such circulating genes in the context of clinical trials, to determine whether they are useful in selecting patients who would best respond to the corresponding novel treatments. The goal is to eventually suggest alternate therapies based on each patient's gene profile.

The extended gene panel was skewed towards genes relevant in late disease. However, its application at diagnosis showed correlations between the number of genes overexpressed, cell subtypes, and aggressive clinical features. Despite the small cohort size, these results were encouraging and warrant further study. It also raises questions about the use of blood RNA-based approaches in early disease: (1) how early can we detect the cancer? (2) how accurate can we be in prognostication? (3) can such results help to implement precision oncology at early stages of disease when warranted? While the first question would require population-based studies to evaluate the predictive value for diagnosis, the second can be answered using the PROCURE cohort and biobank of 2,000 RP patients, as discussed in future directions.

A major problem encountered was the enrolment of patients, which was started in September 2019, ended abruptly in March 2020 due to COVID-19, and resumed at the end of 2021 in a limited capacity. An ongoing issue is the decrease of in-person visits to clinics and blood centers, replaced by teleconsultations and blood collections at local pharmacies, CLSCs, or other clinics. This problem is relevant to several biobanks. Furthermore, over the course of a year, blood could be collected from only three untreated BPH patients, as most cases were already prescribed 5 α -reductase inhibitors. Accordingly, we opted for healthy volunteers with no known prostatic diseases as controls. Of note, testing our circulating gene panel with the blood RNA of the three BPH cases yielded minimal expression within limits set in healthy controls.

Lastly, despite our efforts to choose genes specific to the prostate and cancer, the origin of our genes of interest remains to be determined. This can be done by various approaches. Direct comparisons between blood and tumours would be ideal. While this may be feasible for a subset of patients at diagnosis using RP or biopsy specimens, biopsies of metastases are rarely part of clinical practice for advanced disease. Another option is to verify expression in various blood fractions, e.g. plasma vs. CD45⁻ vs. CD45⁺ fractions for studying RNA from EVs/cfRNAs, CTCs, and WBCs, respectively. While verifying their absence in WBCs or PBMCs is straightforward, testing in cfRNA and CTCs would require additional steps of pre-amplification due to low yields. For CTCs, these can also be tested at the protein level, in line with our preliminary results for negative selection-based CTC isolation and immunofluorescence.

c. Canine comparative oncology

Given the similarities between canine and human PCa, we characterized the DPC-1 model at the molecular level. We achieved this using banked samples already available from dogs previously enrolled in our projects on PSMA imaging. We identified common features and potential targets and demonstrated that the disease is traceable through circulating genes in blood.

As a first step in Manuscript 4, we addressed the resemblance of *in vitro*, subcutaneous, and orthotopic models to spontaneous tumours. We noted significant and consistent differences between tumours and their respective cell lines, whether for spontaneous disease or for the DPC-1 model. While variation in some pathways was expected, changes in key players for cancer progression were not anticipated. For instance, the complete loss of *ESR1* in cell lines was clearly seen, but it figured among the top targets identified in multiple analyses in tumours, including for the DPC-1 orthotopic model and metastases. The expression patterns of the encoded protein, ER α , remain to be validated in DPC-1 tumours and in the cell line, pending identification of the *ESR1* splice variants of interest in canine disease. For the DPC-1 model, samples consisted of solid masses of cancer cells with some infiltrating immune cells. Thus, while the signal is likely coming from cancer cells, further studies are needed to confirm this. If this is the case, it would be of interest to determine how 3D culture of cell lines as spheroids or in hydrogels would affect expression of such genes.

In line with differences between *in vitro* and *in vivo* models, the choice of subcutaneous vs. orthotopic implantation also came up in the DPC-1 model. Few studies have compared the two methods head-to-head for any cancers. Based on a preliminary overview of publicly available transcriptomic data, we suggest that a meta-analysis comparing *in vitro*, subcutaneous, and orthotopic models of various cancers may be feasible to identify general and site-specific

differences. This is especially interesting and clinically relevant in the context of patient derived xenograft models, where the choice of subcutaneous vs. orthotopic implantation remains an important consideration.^{324, 325}

The main finding was that canine PCas closely resemble mesenchymal and stem-like CRPC in humans, with distinct NE features and few luminal genes. Mixed features of different subtypes in such advanced disease are in line with our findings in human tumours and blood. Furthermore, the main CRPC subtype signatures evaluated here were described as often being co-expressed, especially between stem-like and AR-driven/luminal-like signatures.⁹⁷

This raises the questions of *how* and *why* these tumours show predominant features seen mainly in heavily treated mCRPC, including in cases with no active treatment or castration. In hormone-naïve human PCa, most cancer cells differentiate towards a luminal phenotype due to their inherent androgen-responsiveness because the androgen/AR pathway allows their survival and drives them to proliferate. Less luminally-differentiated cancer cells, many of which can be considered intermediate/bi-lineage cells, show CRPC-like features at diagnosis and further arise with hormone treatment.^{98, 314, 326} It seems that this push towards luminal differentiation is more limited in the canine prostate, even in intact dogs, leaving more cells in an intermediate/bi-lineage state with higher plasticity comparable to advanced disease in humans. We suggest that this may be due to either differences in this intermediate phenotype, which may not express AR until it differentiates further in comparison to human prostate epithelial cells, or hormonal differences at advanced age having a bigger impact on the androgen-dependence of the canine prostate compared to humans. This can be studied by lineage tracing as previously described in mouse models of PCa, especially using bi-lineage markers such as LY6D which was among the highest overexpressed genes in canine tumours vs. the normal dog prostate. Furthermore, we identified a novel NE signature in

canine PCa, likely stemming from the emergence of a small number of NE cells or a more prominent population of cells differentiating towards NE phenotypes without being committed to this fate.

Spatial profiling and single-cell approaches would be useful to better understand and characterize the cell subtypes present in canine PCa in comparison with human disease. Genomic studies would also be a useful complement to our transcriptomic analyses. Overall, spontaneous canine PCa and the DPC-1 model are of interest for identifying potential targets for canine PCa and human mCRPC with predominant features of stemness and mesenchymal transition.

2. Future directions in liquid biopsy

a. Applicability to other cancers and development of high-throughput approaches

Our RNA-based liquid biopsy approach can be broadly applicable to most cancers. While tracing cell phenotypes, mechanisms of resistance, and potential targets is relevant to all cancers, a few important examples are worth mentioning. For cancers similar to PCa, such as luminal A breast cancers, with a generally long latency and common recurrences >10 years after curative treatments, this approach may be beneficial for more thorough long-term follow-up to detect recurrence. This is also relevant for aiding in diagnosis of cancer vs. non-malignant diseases of the same tissue (e.g. pancreatitis vs. pancreatic cancer, chronic obstructive pulmonary disease vs. lung cancer, inflammatory bowel disease vs. colorectal cancer, and BPH vs. PCa).³²⁷⁻³³¹ This approach is also relevant for diagnosis of veterinary cancers, with genes of both luminal and NE phenotypes consistently detectable in blood RNA of DPC-1 tumour bearing dogs. The major considerations

remain similar, starting with the selection of genes of interest and the choice of controls relevant to the cancer type/site.

Variables such as age, biological sex, and smoking status must be considered, depending on the population of interest, especially if considering immune profiles in whole blood.³³²⁻³³⁶ Other variables may be more difficult to account for, such as diet, exercise, comorbidities, medications, normal interindividual variation in total RNA yields and WBC counts, normal longitudinal changes in the same individual, time of day for blood collection, and seasonal changes.^{332, 337-341}

The main limitation for expanding on our current work and generalizing to various cancers is the move towards medium- or high-throughput assays. Testing our panel of 64 genes and three reference genes in triplicate required over 5.5µg of RNA per sample and was very labor intensive and time consuming. To illustrate the scope of the work: tests for circulating genes in Manuscripts 2 and 3 included 4,080 and 23,115 individual qPCR reactions in blood samples, respectively, excluding negative controls, serial dilutions, and additional experiments assessing reproducibility. This amount of RNA can be obtained from one or two PaxGene tubes but is a major limitation if we want to further expand the panel, especially in biobanks with only a single PaxGene tube. Multiplexing would be necessary to move forward in order to lower the amount of precious patient material needed and to reduce costs.

In our experience, RNA-sequencing appears to be viable options to explore for the reliable results including very low expression transcripts, as opposed to hybridization-based assays. The technical limitation is the inclusion of globin RNAs expressed in red blood cells, which saturate microarray and RNA-sequencing platforms and masking the detection of low abundance genes. However, globin reduction significantly improves data quality of PaxGene RNA samples with low variance

in microarray and RNA-sequencing studies, increasing the number of detected genes.³⁴²⁻³⁴⁶ Thus, globin depletion combined with high depth of sequencing may be worth considering as an alternative to further RT-qPCR assays. Targeted RNA-sequencing may also be a viable option.

b. Longitudinal multianalyte tracing for complete molecular characterization of PCa

The culmination of this work can be considered as preparation for a larger project now ongoing in the PROCURE biobank, consisting of a comprehensive molecular analysis of PCa. It is expected to allow discovery of biomarkers for risk stratification, recurrence, and response to therapies, as well as identification of novel targets for personalized treatment.

The multidisciplinary team of PROCURE has developed a multicentric cohort at four Quebec University hospitals, consisting of 2,007 RP patients from whom blood and urine were longitudinally collected since 2006, up to 15 years. They donated fresh frozen and FFPE prostate tissues to the biobank, and liquid biopsies (blood and urine) were collected longitudinally. The planned project will include WGS and RNA-sequencing of primary tumours and paired benign prostate and will focus mainly on cases with lethal disease. Additional tissue-based analyses may be considered, including epigenetic and proteomic profiling. In line with the work presented in this thesis, analyses of epithelial cell subtypes can be carried out in this bulk sequencing, as well as additional spatial profiling by cell subtype.

The main advantage with such a cohort is the long-term longitudinal collection of blood. While we showed promising results in whole blood RNA, longitudinal multianalyte tracing is necessary for in depth characterization of disease. Each analyte offers a complementary perspective relevant to the evolution of the disease. Whole blood RNA can provide information on cancer- or prostate-specific transcripts actively or passively released from tumours in CTCs and EVs, as well as

profiling systemic immune changes relevant to disease. Cell-free DNA is released from apoptotic cells and can show the evolution of genomic alterations in tumours. Most studies on cfDNA use targeted PCR to detect specific alterations known to be relevant to the disease, or they use shallow WGS to estimate the fraction of ctDNA as a proxy of tumour burden.²⁰⁶⁻²⁰⁸ However, deep WGS would allow for more accurate ctDNA fraction estimation and identification of known and novel genomic alterations.²⁰⁶⁻²⁰⁸ Fragmentation patterns and methylation may also be considered for analysis in ctDNA. Coupled with blood RNA-sequencing, the untargeted detection of somatic alterations and transcripts would provide complementary information on the molecular evolution of the disease, including detection of relapse, response, and choice of optimal therapeutic strategies.

Further deconvoluting of liquid biopsy results can be achieved partly by scRNA-sequencing of CTCs, as previously described, especially if cancer cells of all phenotypes can be captured.³⁴⁷ Plasma proteomics, lipidomics/metabolomics, and analysis of macromolecules in EVs would also be informative. Integration of these multianalyte results through machine learning and artificial intelligence would allow the identification of specific markers of clinical utility.

FINAL CONCLUSION AND SUMMARY

In this thesis, our cell-by-cell quantification of AR expression illustrated changes in epithelial cell subtypes throughout PCa initiation, progression, and hormone treatment, which are representative of multiple established mechanisms of resistance. A novel AR Score was defined and shown to be predictive of progression, allowing stratification of intermediate-risk cases. The traceability of cell subtypes as genes in whole blood was demonstrated, identifying mixed phenotypes as seen in tumours and defining their association with patients' treatments and outcomes. Expanding on this approach proved that not only phenotypic, but also functional changes can be assessed non-invasively and longitudinally in patients' blood, and that this is relevant from diagnosis to mCRPC. This may be useful for patient stratification and allow for new clinical trials bringing us closer to personalized therapies. Finally, canine PCa was found to resemble human mCRPC tumours with stemness features and displaying a subset of NE markers, with ER α being a target of interest. PCa was also traceable in blood RNA of dogs bearing DPC-1 tumours, allowing for the potential earlier diagnosis of a disease that is often caught at the metastatic stages for dogs and further validating our approach in humans.

Altogether, this work has demonstrated the relevance of PCa epithelial cell subtypes throughout progression, identified a previously under-utilized non-invasive approach for monitoring disease evolution longitudinally, and further characterized a relevant comparative model of spontaneous PCa. It has helped to set the stage for a larger study with the ultimate aim of providing precision oncology to patients suffering from this unpredictable and often lethal disease.

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