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Prognostic Immune Biomarkers In Metastasic Castration Resistant Prostate Cancer

TESIS DOCTORAL

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RESUMEN

El cáncer de próstata metastásico hormono-refractario es una enfermedad heterogénea caracterizada por la falta de respuestas duraderas a las terapias estándar aprobadas para su tratamiento. La evolución clínica de esta enfermedad y su respuesta a terapias experimentales podría mejorar con la estratificación molecular tumoral y la identificación de biomarcadores predictivos de pronóstico y respuesta, pero la disponibilidad de biopsias es limitada en la investigación de la enfermedad metastásica. En los últimos años, la inmunoterapia ha supuesto una revolución en el tratamiento de una gran variedad de tumores, consiguiendo respuestas duraderas e incrementando la supervivencia global en pacientes diagnosticados de melanoma, cáncer de pulmón, cáncer vesical y cáncer renal entre otros. Sin embargo, la respuesta a estos tratamientos en el cáncer de próstata metastásico resistente a la castración hormonal es limitada en los pacientes no seleccionados molecularmente. El objetivo de este estudio es determinar la prevalencia de biomarcadores con potencial impacto en la configuración del microambiente tumoral del cáncer de próstata metastásico resistente a la castración hormonal, la correlación entre la expresión de estos biomarcadores, y determinar su asociación con la supervivencia global de estos pacientes. Para ello, las biopsias de 100 pacientes con cáncer de próstata metastásico hormono-refractario se analizaron mediante Whole Exome Sequencing (WES), Targeted Next-Generation Sequencing (NGS), RNA sequencing (RNAseq), NanoString e inmunohistoquímica, además de la presencia de rasgos histológicos sugestivos de diferenciación tumoral neuroendocrina. El coeficiente phi se utilizó para determinar las correlaciones entre los biomarcadores de interés. La supervivencia global se calculó mediante curvas de Kaplan-Meier (KM) y Hazard Ratios ajustados (aHR) mediante el modelo de regresión de Cox. La expresión proteica de Programmed Death cell Ligand (PD-L1) y SRY (sex determining region Y)-box 2 (SOX2) se detectó por inmunohistoquímica (Combined Positive Score (CPS) de 1 y > 5% células con expresión proteica, respectivamente) en 24 (33%) y 27 (27%) de las biopsias, respectivamente; 23 (26%) de las biopsias tenían un T-cell–inflamed Gene Expression Profile (TcellinfGEP) score alto (>–0.318). Los resultados de estos análisis demostraron una correlación positiva entre la expresión de PD-L1 y el

TcellinfGEP score (phi 0.63 [0.45; 0.76]). La expresión proteica de PD-L1, (aHR: 1.90 [1.05; 3.45]), un score elevado para TcellinfGEP (aHR: 1.86 [1.04; 3.31]) y la expresión proteica de SOX2 (aHR: 2.09 [1.20; 3.64]) se asociaron con peor supervivencia global.

En conclusión, PD-L1, TcellinfGEP, y SOX2 se identificaron como biomarcadores pronósticos para el cáncer de próstata metastásico hormono-refractario. Si estos biomarcadores se validasen en cohortes prospectivas, el diseño de estudios de biomarcadores predictivos de supervivencia debería tener en cuenta estos hallazgos en el futuro.

ABSTRACT

Metastatic castration-resistant prostate cancer (mCRPC) is a heterogeneous disease characterised by the lack of durable responses to standard therapies currently approved for its treatment. Clinical outcomes of this disease and its response to experimental therapies could be improved by the molecular characterisation of the tumour and the identification of biomarkers predictive of prognosis and response, but the availability of metastatic tissue samples for research in this setting are limited. In recent years, immunotherapy has gained momentum after several immune checkpoint inhibitors demonstrated durable responses and improved overall survival in different solid tumours like melanoma, lung, bladder and renal cancers among others. However, mCRPC response to these therapies is poor in molecularly unselected patients. The objective of this study is to determine the prevalence of biomarkers with a potential impact in the tumour microenvironment of mCRPC, the correlation between the expression of these biomarkers, and to determine their association with overall survival (OS). In order to do so, mCRPC biopsies from 100 patients were assayed by whole exome sequencing (WES), targeted next-generation sequencing (NGS), ribonucleic acid sequencing (RNAseq), NanoString and immunohistochemistry (IHC), as well as the presence of neuroendocrine features. The phi coefficient determined correlations between biomarkers of interest. OS was assessed using Kaplan-Meier (KM) curves and adjusted hazard ratios (aHRs) from Cox regression. Programmed death cell ligand (PD-L1) and SRY (sex determining

region Y)-box 2 (SOX2) protein expression was detected by immunohistochemistry (Combined Positive Score (CPS) 1 and >5% cells with protein expression, respectively) in 24 (33%) and 27 (27%) mCRPC biopsies, respectively; 23 (26%) mCRPC biopsies had high T-cell–inflamed Gene Expression Profile (TcellinfGEP) scores (>–0.318). The results of these analysis demonstrated that PD-L1 protein expression and TcellinfGEP scores were positively correlated (phi 0.63 [0.45; 0.76]). PD-L1 protein expression (aHR: 1.90 [1.05; 3.45]), high TcellinfGEP score (aHR: 1.86 [1.04; 3.31]), and SOX2 expression (aHR: 2.09 [1.20; 3.64]) were associated with worse OS.

In conclusion, PD-L1, TcellinfGEP score, and SOX2 were identified as prognostic biomarkers in the mCRPC setting. If validated, predictive biomarker studies incorporating survival endpoints would need to take these findings into consideration.

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ABBREVIATIONS

ADT: Androgen Deprivation Therapy

aHR: adjusted Hazard Ratio

AR: Androgen Receptor

ATM: Ataxia Telangiectasia Mutated

ATR: Ataxia Telangiectasia and Rad3-related

ATRi: ATR inhibitors

Bcl-2: B-cell lymphoma 2

BRCA2: Breast Cancer gene 2

CAFs: Cancer Associated Fibroblasts

CD4+Th: CD4+ T helper

CD4+Tfh: CD4+ T follicular helper

CD4+Treg: CD4+ regulatory T cells

cDC: conventional Dendritic Cells

CDK: Cyclin-Dependent Kinase

CDK12: Cyclin-Dependent Kinase 12

CI: Confidence Interval

CMKLR1: Chemerin chemokine-like receptor 1

CPS: Combined Positive Score

CRPC: Castration Resistant Prostate Cancer

CTLA4: Cytotoxic T-Lymphocyte Antigen 4

DC: Dendritic Cells

DDR: DNA Damage Response

DINe: DNA Integrity Number equivalent

dMMR: Deficient Mismatch Repair

DNA: Deoxyribonucleic Acid

DSBs: Double-Strand DNA breaks

ECM: Extracellular Matrix

ECOG: Eastern Cooperative Oncology Group

EMT: Epithelial-Mesenchymal Transition

EPCs: Endothelial Progenitor Cells

F1CDx: FoundationOne CDx

FDA: Food and Drug Administration

FFPE: Formalin-Fixed Paraffin-Embedded

FGF: Fibroblasts Growth Factor

FPKM: Fragments Per Kilobase of transcript per Million mapped reads

GATK: Genome Analysis Toolkit

GZMB: Granzyme B

H&E: Hematoxylin and Eosin

HIF-a: Hypoxia-Inducible Factor a

HNSCC: Head and Neck Squamous Cell Carcinoma

HRR: Homologous Recombination Repair

ICI: Immune Checkpoint Inhibitors

ICR: Institute of Cancer Research

IFN: Interferon

IHC: Immunohistochemistry

IL: Interleukin

IL20RA: Interleukin 20 Receptor subunit Alpha

Inf-DC: Inflammatory Dendritic Cells

JAK: Janus Kinase

KM: Kaplan-Meier

LAG3: Lymphocyte-activation gene 3

LBD: Ligand Binding Domain

MANAs: Mutation-Associated Neoantigens

mCRPC: metastatic Castration Resistant Prostate Cancer

mCSPC: metastatic Castration Sensitive Prostate Cancer

MFS: Metastasis Free Survival

MHC: Major Histocompatibility Complex

mRNAseq: messenger RNA sequencing

miRNA: microRNA

MMR: Mismatch Repair

MDSCs: Myeloid Derived Suppressive Cells

M-MDSCs: Monocytic MDSCs

MSI: Microsatellite Instability

MSI-H: MSI High

MSI-L: MSI Low

MSS: Microsatellite Stable

NE: Neuroendocrine

NECs: Normal Endothelial Cells

NEPC: Neuroendocrine Prostate Cancer

NF-kB: nuclear factor kB

NGS: Next Generation Sequencing

NK: Natural Killer

NKG7: Natural Killer Cell Granule Protein 7

nmCRPC: non metastatic Castration Resistant Prostate Cancer

NMD: nonsense-mediated decay

OS: Overall Survival

PALB2: Partner And Localizer of BRCA2

PARP: Poly-ADP Ribose Polymerase

PARPi: PARP inhibitors

PCR: Polymerase Chain Reaction

PCSCs: Prostate Cancer Stem Cells

pDC: Plasmacytoid Dendritic Cells

PDGFB: Platelet Derived Growth Factor

PDGFBRB: Platelet Derived Growth Factor Receptor B

PD-1: Programmed Death 1

PD-L1: Programmed Death-Ligand 1

PI3K: phosphatidylinositol 3-kinase

PMN-MDSCs: Polymorphonuclear MDSCs

PRF: perforin

PSA: Prostate Specific Antigen

PSADT: PSA Doubling Time

PSMA: Prostate-Specific Membrane Antigen

PSMB10: proteasome B-type 10

PTEN: Phosphatase and Tensin Homolog

RMH: Royal Marsden Hospital

RNA: Ribonucleic Acid

RNAseq: RNA sequencing

ROS: Reactive Oxygen Species

rPFS: radiographic progression free survival

RTK: Receptor Tyrosine Kinase

sB7-H3: soluble B7-H3

SOX: Sry-type HMG box

SOX2: SRY (sex determining region Y)-box 2

SSBs: Single Strand DNA breaks

STAT: Signal Transducer and Activator of Transcription

STING: Stimulator of Interferon Genes

TAMs: Tumour Associated Macrophages

TANs: Tumour Associated Neutrophils

TcellinfGEP: T-cell–inflamed Gene Expression Profile

TCR: T Cell Receptor

TD: Tandem Duplications

TECs: Tumour Endothelial Cells

TGF-b: Transforming Growth Factor Beta

TIGIT: T cell immunoreceptor with Ig and ITIM domains

TILs: Tumour Infiltrating Lymphocytes

TMB: Tumour Mutational Burden

TME: Tumour Microenvironment

TNF: Tumour Necrosis Factor

UK: United Kingdom

VEGF: Vascular Endothelial Growth Factor

VEGFR: Vascular Endothelial Growth Factor Receptor

WES: Whole Exome Sequencing

1. Introduction

1.1 Disease background

Prostate cancer is the second most prevalent cancer in men worldwide and is estimated to result in the death of >70 000 men in Europe in 2020 (1). Although most advanced prostate cancers initially respond to androgen deprivation therapy, second-generation androgen inhibitors, taxane-based chemotherapies, and radionuclide therapies, advanced disease remains fatal (2).

Prostate cancer is predominantly an androgen-dependent disease (2). Endocrine therapies exploit this dependency by depleting endogenous androgen secretion or directly targeting the androgen receptor (AR) (2). AR signalling is critical for normal prostate cell physiology but is upregulated in prostate cancer cells, driving uncontrolled proliferation (2). Despite major advances in therapeutics, androgen deprivation therapy (ADT) has remained as the main treatment for advanced prostate cancer during the last century (2). However, although most advanced prostate cancers regress with ADT, they invariably progress to castration-resistant prostate cancer (CRPC), which is inevitably lethal (2). Therapies shown to improve survival for men with mCRPC include taxane-based chemotherapies, second generation hormonal agents, Radium-223 and Lutetium PSMA (3). However, none of these are curative, probably due to intrinsic and acquired resistance mechanisms (2). For that reason, tumour molecular characterisation should identify biomarkers predictive of response and prognosis to further improve mCRPC outcomes through disease stratification.

1.2 Standard of care therapies in mCRPC

1.2.1 Hormonal therapies

As previously discussed, ADT is the mainstay of therapy for advanced prostate cancer (2). Although most men with advanced prostate cancer respond to ADT, all patients invariably develop lethal metastatic CRPC. This has led to the development of second-generation hormonal therapies that have further improved the outcome for patients with advanced prostate cancer, including abiraterone, enzalutamide, apalutamide and darolutamide (4-14).

Abiraterone is a structural analog of pregnenolone that inhibits CYP17, an enzyme necessary for androgen synthesis that is expressed in testicular, prostate, and adrenal tissue (4). Inhibition of CYP17 results in reduction of androgen synthesis and serum levels of testosterone and other androgens (4). So far, treatment with abiraterone has been approved for metastatic castration-sensitive prostate cancer (mCSPC), high-risk locally advanced CSPC, and metastatic castration-resistant prostate cancer (mCRPC) pre- and post-chemotherapy (4,5,6,7).

Enzalutamide is an oral, second-generation AR inhibitor that blocks multiple steps in the AR signalling pathway and prevents the translocation of the AR from the cytoplasm to the nucleus (8). Within the nucleus, enzalutamide inhibits AR binding to deoxyribonucleic acid (DNA), which prevents further transcription of AR-responsive genes (8). Treatment with enzalutamide is currently approved for mCRPC in the post- and pre -chemotherapy setting, mCSPC, and nonmetastatic castration resistant prostate cancer (nmCRPC) (8,9,10,11).

Apalutamide is a nonsteroidal second-generation AR antagonist that binds to the ligand binding domain (LBD) of AR, preventing its nuclear translocation, DNA binding, and transcription (12). Apalutamide is currently approved by the Food and Drug Administration (FDA) for treatment of nmCRPC with prostate-specific antigen doubling time (PSADT) less than 10 months and mCSPC (12).

Darolutamide is a nonsteroidal AR antagonist that consists of a 1:1 mixture of two pharmacologically active diastereomers [(S,R)-darolutamide and (S,S)-darolutamide], which interconvert through the pharmacologically active major metabolite keto-darolutamide (13). Darolutamide and its active metabolite have been shown to inhibit testosterone-induced translocation of AR to the nucleus, thus decreasing activation of AR-responsive genes required for the growth and survival of prostate cancer cells (13). Treatment with darolutamide twice daily plus ADT was approved by the FDA after the results of the phase 3 ARAMIS trial, which showed a significant metastasis free survival (MFS) and OS benefit compared to placebo plus ADT for patients with nmCRPC with a PSADT of less than 10 months (14).

Despite the proven clinical benefit of targeting the AR signalling axis both primary and secondary resistance remain common, and further therapies are needed to improve the outcome of these patients.

1.2.2 Chemotherapy

Docetaxel

Up until 2004, there was no standard front-line or second-line chemotherapy for mCRPC. Chemotherapy at that time provided palliative but no survival benefit.

Docetaxel is a taxane derivative that binds to the cell microtubules, prevents AR nuclear translocation and causes apoptosis through B-cell lymphoma 2 (Bcl-2) phosphorylation (15). In 2004, the TAX 327 randomized phase III trial was published (15). This trial involved patients with mCRPC who were randomised to treatment with mitoxantrone 12 mg/m2 every 3 weeks compared to docetaxel 75 mg/m2 every 3 weeks, or docetaxel 30 mg/m2. The results showed that the survival rate in the group of docetaxel every 3 weeks was significantly higher than the mitoxantrone group, 18.9 months versus 16.5 months, respectively ($p = 0.009$). The group of weekly docetaxel did yield an OS of 17.4 months, not significantly higher than the mitoxantrone group ($p = 0.36$). Therefore, treatment

with docetaxel 75mg/m2 every 3 weeks became the default standard of care thereafter.

Numerous subsequent combination trials have been performed in an attempt to improve upon the efficacy of docetaxel, but most of these have been largely negative trials (16).

Cabazitaxel

After failure of docetaxel as first-line chemotherapy, second-line treatment options included mitoxantrone, retreatment with docetaxel, or clinical trials. In 2010 another chemotherapeutic drug, cabazitaxel, was approved for the treatment of mCRPC (17). The TROPIC trial assessed the role of cabazitaxel plus prednisone in patients with mCRPC who progressed after docetaxel (17). Patients were randomised to receive either cabazitaxel (25 mg/m2) or mitoxantrone (12 mg/m2) on day 1 of each 21-day cycle, and all patients received prednisone 10 mg daily. The primary endpoint of the trial was median OS and was superior in the cabazitaxel arm at 5.1 (95% CI: 14.1–16.3) months compared to mitoxantrone group at 12.7 (95% CI: 11.6–13.7) months translating to a 30% reduction in relative risk of death (HR: 0.70, 95% CI: 0.59–0.83, $p < 0.0001$). Cabazitaxel therefore remains an option for patients with mCRPC who have progressed after docetaxel.

As discussed above, both docetaxel and cabazitaxel have shown to have proven efficacy in terms of OS in the mCRPC setting. However, similarly to second-generation hormonal therapies, mCRPC invariably progresses to these therapies in this scenario, and further options are needed to treat these patients.

1.2.3 Radioactive therapies

^{177}Lu-PSMA-617

Prostate-specific membrane antigen (PSMA) is a type II membrane protein expressed in all forms of prostate tissue, including carcinoma (18). The PSMA

protein has a unique 3-part structure: a 19-amino-acid internal portion, a 24-amino-acid transmembrane portion, and a 707-amino-acid external portion (18). PSMA-617 is a molecule with high affinity for the outside domain of PSMA (18). This molecule carries a payload of Lutetium-177. Once bound to PSMA, the molecule PSMA-617 undergoes endocytosis, where it radiates the bound cell and neighbouring cells while Lutetium is radiating with beta particles **(Figure 1).**

The VISION trial evaluated the advantages of Lutetium PSMA-617 over best supportive care in improving the overall survival and image-based progression-free survival in patients with mCRPC (3). This was an international, multi-centric, phase three, open-label, randomised trial that recruited mCRPC patients who progressed after receiving both androgen-receptor-pathway inhibitors (abiraterone or enzalutamide) and either one or two taxane regimens. Lutetium PSMA-617 was given every 6 weeks in 4–6 cycles. There was a significant improvement in the overall survival in the patients who received Lutetium PSMA-617 and standard care compared to standard care alone (15.3 months vs. 11.3 months; hazard ratio [HR] - 0.62; 95% confidence interval [CI] - 0.52–0.74; $P < 0.001$) (3). The second primary endpoint, image-based progression-free survival, also showed significant improvement in the patients who received Lutetium PSMA-617 (HR - 0.40; 95% CI - 0.29–0.57; $p = 0.008$) (3).

With this prospective randomised trial showing favourable results, Lutetium PSMA-617 stands as a new option for patients with progressive PSMA-positive mCRPC.

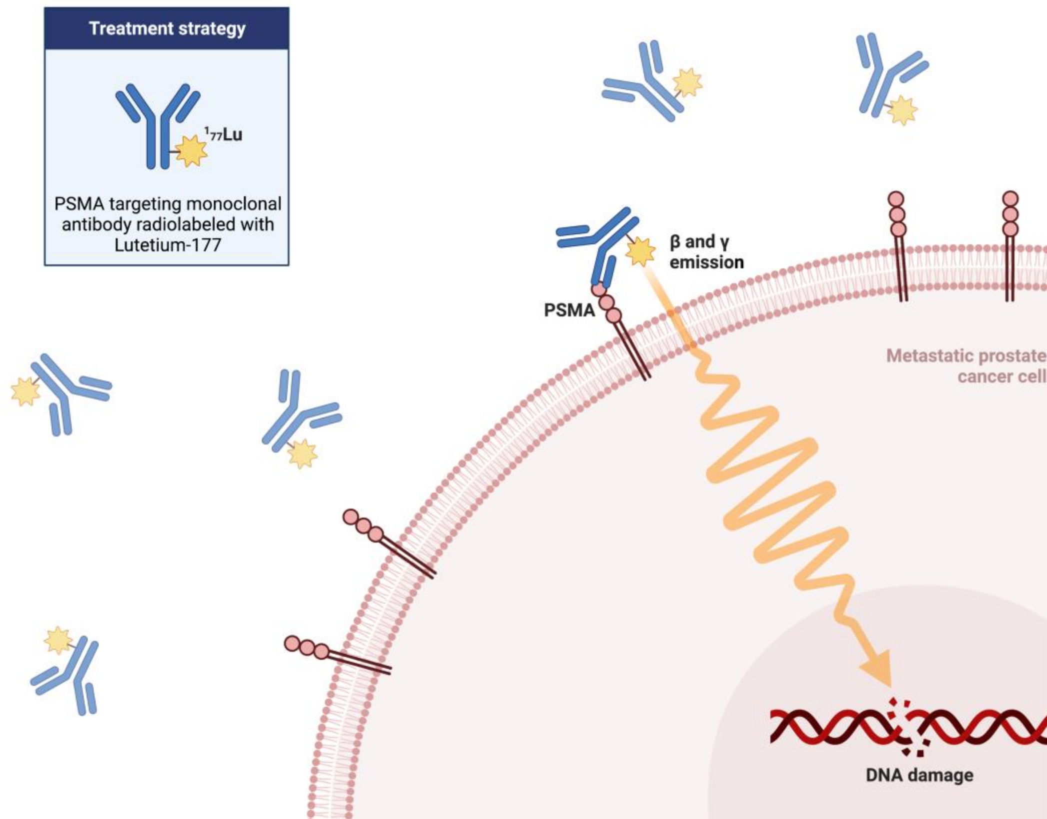


Figure 1 illustrates Lutetium PSMA-617 mechanism of action. After administration into the bloodstream, Lutetium PSMA-617 binds to prostate cancer cells that express PSMA. Once bound, emissions from the radioisotope damage tumour cells, disrupting their ability to replicate and/or triggering cell death.

Radium 223

Radium-223 selectively targets bone metastases with alpha particles (19). The ALSYMPCA clinical trial assessed the efficacy and safety of radium-223 as compared with placebo, in addition to the best standard of care, in men with castration-resistant prostate cancer and bone metastases (19). Radium-223, as compared with placebo, significantly improved overall survival (median, 14.9 months vs. 11.3 months; hazard ratio, 0.70; 95% CI, 0.58 to 0.83; p <0.001) (19). Therefore, this treatment is currently approved for patients with mCRPC and bone metastasis.

1.3 Tumour heterogeneity and new strategies

Despite the proven efficacy of the above-mentioned therapies, mCRPC is a heterogeneous disease with diverse drivers of progression and mechanisms of therapeutic resistance, and its lethality is driven by the lack of therapies capable of generating durable responses. Looking at the results in other solid tumours, immunotherapy seems and attractive approach to improve the outcomes of these patients, but the results so far have been disappointing in molecularly unselected mCRPC (20,21). In this setting, a better understanding of how genomic aberrations can correlate with the prognosis and the immune infiltrate in the tumour microenvironment (TME) of mCRPC, and how to better target these cells seems crucial to improve the response to these therapies. However, a major limitation in this approach is the scarce availability of biopsies of the metastatic disease. In fact, a large number of previous studies measuring biomarkers have been done in primary prostate cancer which may not accurately reflect the mCRPC state, as the disease may change as cancer progresses (22,23,24).

As previously mentioned, the aim of this study is to determine the prevalence of a group of pre-specified biomarkers with a potential impact in the configuration of the tumour microenvironment in mCRPC, the correlation between the expression of these biomarkers, and to determine their association with OS.

Breast cancer gene 2 (BRCA2), ataxia telangiectasia mutated (ATM), Partner and localizer of BRCA2 (PALB2), phosphatase and tensin homolog (PTEN), PD-L1, TcellinfGEPscore, tumour mutational burden (TMB), mismatch repair deficiency (dMMR), cyclin-dependent kinase 12 (CDK12), histology features of neuroendocrine differentiation, SOX2 and TP53 were selected as biomarkers potentially related to the tumour microenvironment and mCRPC prognosis. Their characteristics and the rational to justify their potential to modulate TME in mCRPC will be addressed later in this manuscript, after an introductory description of the TME cells and characteristics.

1.4 TME cells and characteristics

TME is defined as the cellular environment in which the tumour grows and expands (25). The TME is comprised of blood and lymph vessels, endothelial cells, fibroblasts, immune cells, signalling factors and the extracellular matrix (25) (**Figure 2)**. There is a permanent interaction between the tumour and its TME, during which tumour cells release extracellular factors trying to promote immune tolerance and tumourigenesis (25). These interactions between malignant and nonmalignant cells modulate a TME that promotes cancer growth and progression with the non-malignant often playing a pro-tumourigenic function (25).

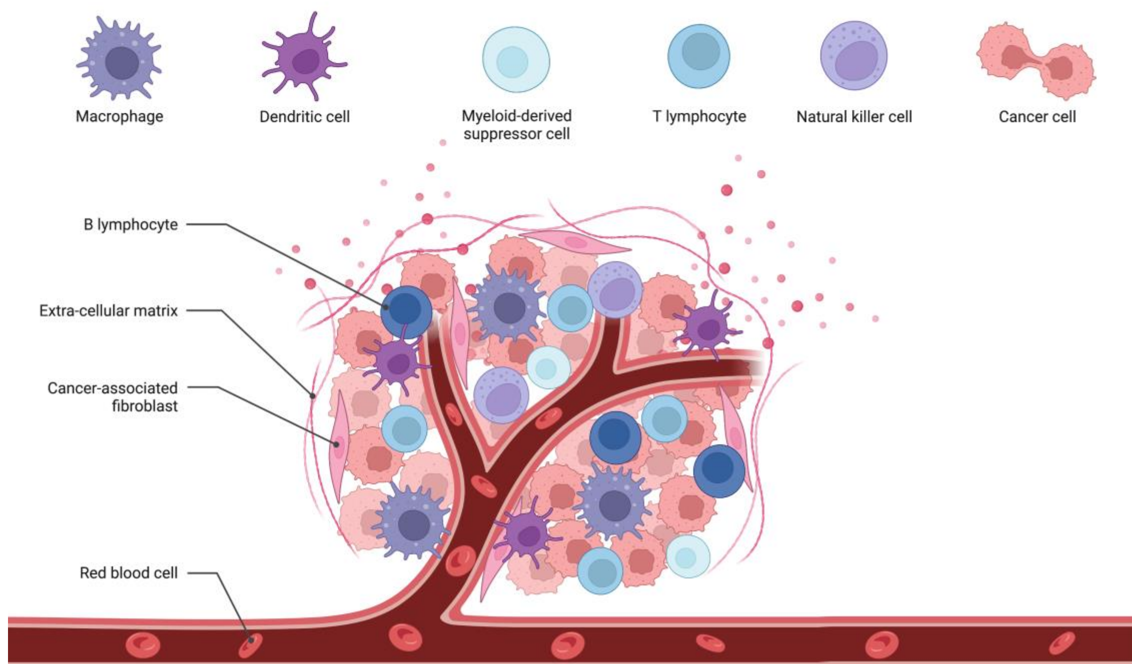


Figure 2 illustrates the different components of the tumour microenvironment. TME is composed by tumour cells, the tumour stroma, blood vessels, infiltrating inflammatory cells and a variety of associated tissue cells. Immune cells present in the TME include T and B lymphocytes, dendritic cells, macrophages,

polymorphonuclear leukocytes and other myeloid cells, as wells as natural killer cells.

In the following lines, the different cells and components of the TME will be described.

1.4.1 Endothelial cells

Although the growth of new blood cells is required for the physiologic healing of injured tissues, this process can also promote tumour growth by inducing neovascularisation (26). New endothelial cells originate through differentiation from endothelial progenitor cells (EPCs) or proliferation of mature endothelium in pre-existing vessels (27). EPCs incorporate into sites of active tumour neovascularisation promoting the growth of tumours (27). Excessive proliferation of endothelial progenitor or mature differentiated endothelial cells contributes to numerous cancer disorders, with the endothelial cells offering nutritional support for tumour growth and development (27) (**Figure 3)**. These cells also play a key role in tumour cell protection from the immune system (27). When compared to normal endothelial cells (NECs), tumour endothelial cells (TECs) have a markedly altered morphologic and genetic phenotype (26). Pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGFB) can also induce genetic reprogramming of TECs and their mode of interacting with immune cells (28). In particular, the up-regulation of angiogenic receptors, as well as the close interactions with tumour cells and pro-inflammatory immune cells, results in an inflamed and activated TEC state that induces a highly proliferative phenotype with increased tendency for migration (28).

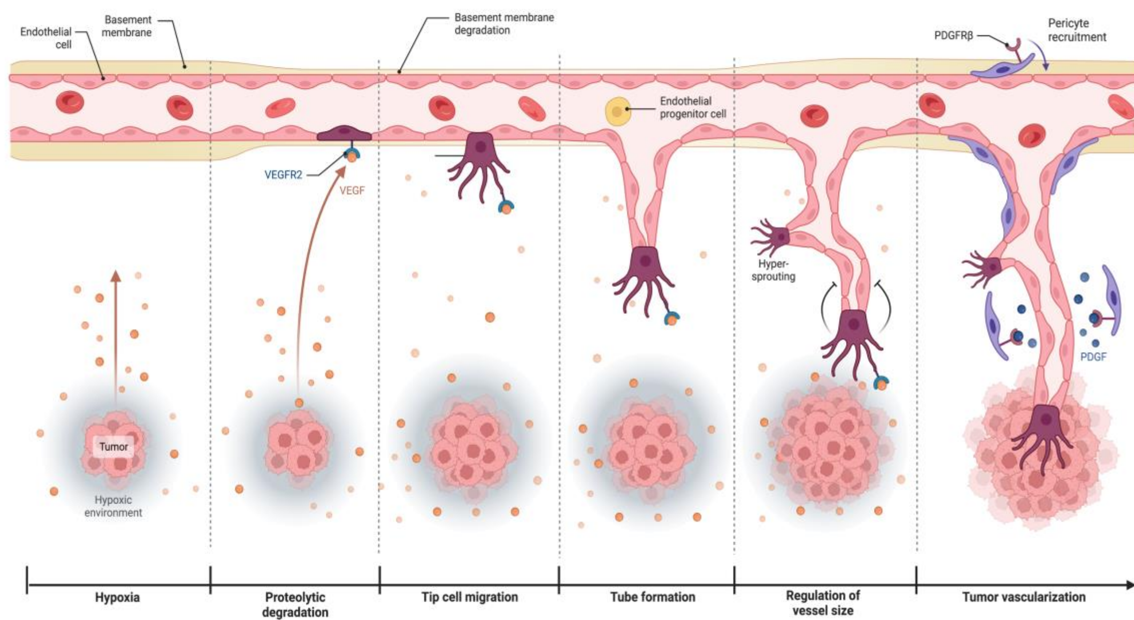


Figure 3 illustrates the different steps in tumour neovascularization. Vasculature in tumours is immature and affects the tumour microenvironment, resulting in hypoxia, acidosis, glucose starvation, immune cell infiltration, and decreased activity, all of which promote cancer progression, metastasis, and drug resistance. VEGFR2 = vascular endothelial growth factor receptor 2, PDGFRB = platelet derived growth factor receptor B.

1.4.2 Immune cells

Myeloid and lymphoid cells are involved in inflammatory reactions which can either promote or inhibit cancer survival, development and progression (29) (**Figure 4)**. These cells can also either trigger or suppress anti-tumour immune responses and mediate or antagonise the anti-tumour activity of irradiation, cytotoxic agents, and checkpoint inhibitors (29). The different subtypes of immune cells that characterise the TME are described below.

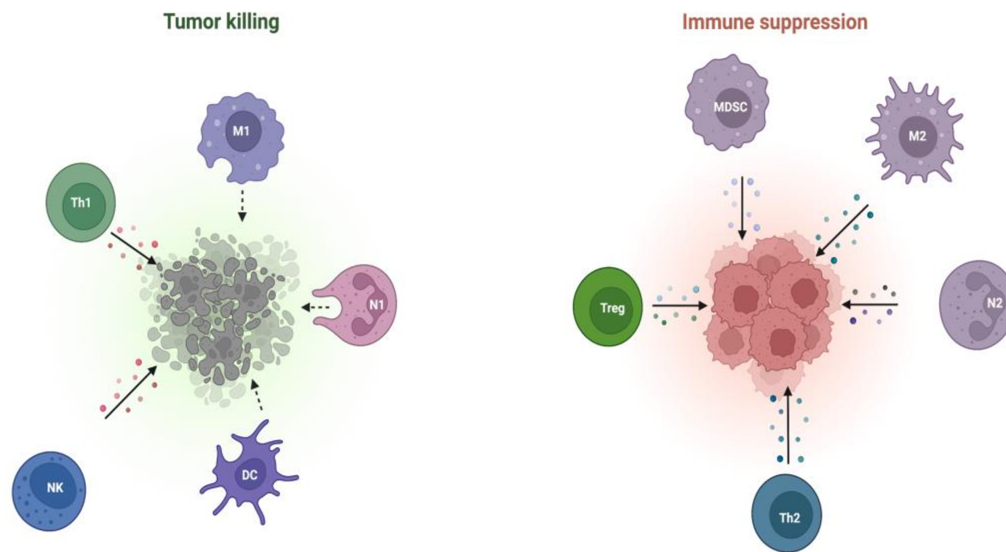


Figure 4 illustrates pro and anti-tumourogenic cells inhibiting or promoting cancer growth in the tumour microenvironment. The cellular nature of the tumour microenvironment influences disease outcome by altering the balance of suppressive versus cytotoxic responses in the vicinity of the tumour.

Myeloid cells

Myeloid derived suppressive cells

Myeloid-derived suppressor cells (MDSCs) are one of the major components of the tumour microenvironment (30). The main feature of these cells is their potent immune suppressive activity by supporting tumour growth and survival, and also by promoting angiogenesis, invasion of healthy tissues and metastasis (30). There are two different types of MDSCs, as identified in studies in both mice and humans: polymorphonuclear MDSCs (PMN-MDSCs), morphologically and phenotypically similar to neutrophils, and monocytic MDSCs (M-MDSCs) similar to monocytes (30). MDSCs accumulate in peripheral lymphoid organs and tumour tissues, and available data suggests that MDSCs in peripheral lymphoid organs and the tumour have different functional specialization (30). This suggests

that the differences in the mechanisms regulating MDSCs function in tumours and peripheral lymphoid organs may affect therapeutic targeting of these cells.

Tumour associated macrophages

Tumour-associated macrophages (TAMs) are one of the main tumour-infiltrating immune cell types in the TME (31). TAMs are generally categorised into two functionally contrasting subtypes, namely M1 macrophages and M2 macrophages (31) **(Figure 5).** M1 TAMs typically exert anti-tumour functions, whereas M2 TAMs inhibit T cell-mediated anti-tumour immune response and promote tumour angiogenesis, leading to tumour metastasis and progression (31). Both M1 and M2 TAMs have a high degree of plasticity and can be converted into each other upon tumour microenvironment changes or therapeutic interventions (31). Therefore, TAMs functional phenotype is tamed by molecules in tumour microenvironments, becoming promoters of tumour growth.

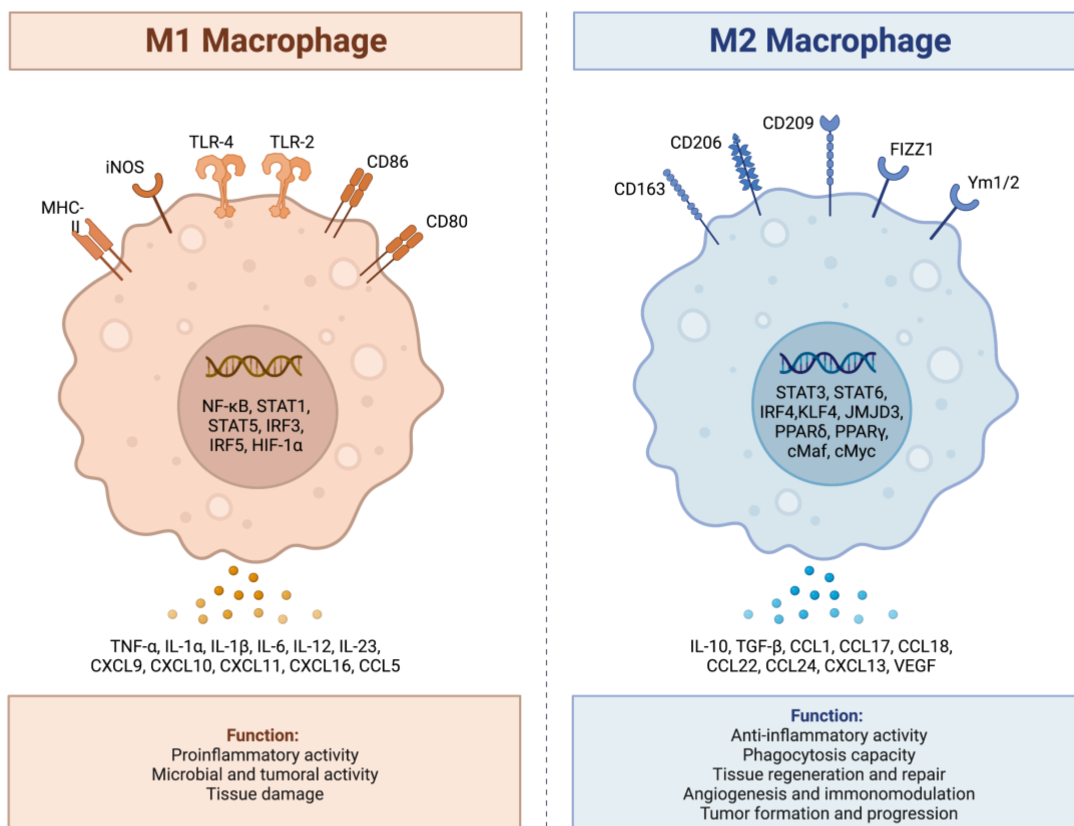


Figure 5 illustrates the characteristics of M1/ M2 TAMs polarisation to promote or stop cancer growth. M1 TAMs play an anti-tumour role, while M2-polarised TAMs contribute to pro-tumourigenic outcomes through angiogenic regulation, immune suppression, hypoxia induction, tumour cell proliferation, and metastasis.

Tumour associated neutrophils

Neutrophils make up a substantial proportion of the immune infiltrate in the TME of a wide variety of cancer types (32). Previous studies suggest that tumour-associated neutrophils (TANs) can have anti-tumour properties, including direct cytotoxicity towards tumour cells and inhibition of metastasis (32). Conversely, other studies suggest that TANs are also capable of supporting tumour progression by promoting angiogenesis, stimulating tumour cell motility, migration, and invasion, and modulating other immune cells (32). Similar to TAMs, these cells are also able to retain functional plasticity, resulting in an anti-tumour (TAN1) or pro-tumour (TAN2) TAN phenotype when exposed to different molecules in the TME (32). Interestingly, data from previous studies comparing the presence of intratumoural, peritumoural or stromal neutrophils suggest that neutrophils in different locations can have different prognostic implications (32).

Tumour associated dendritic cells

Dendritic cells (DC) are a heterogenous group of innate immune cells that infiltrate tumours and process and present tumour-derived antigens to naïve T cells (33) (**Figure 6)**. These immune cells play a critical role in priming anti-tumour T cell immunity (33). The anti-tumour function of DC can be neutralised by suppressive signals present in the tumour microenvironment (33). In addition, DC can also be tamed by tumour cells in the TME to elicit T cell tolerance and tumour growth (33).

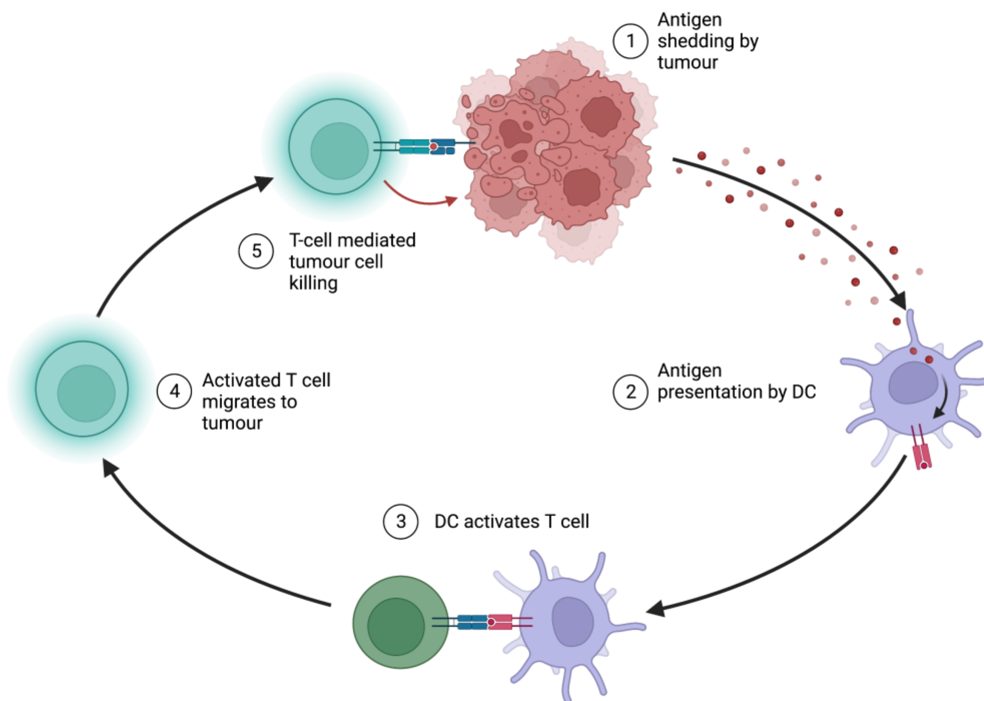


Figure 6 illustrates the process for tumour antigen presentation. Tumour antigens are displayed on the surface of cells by class I human leukocyte antigens (HLA-I). To elicit an effective anti-tumour response, cancer antigens have to be processed by dendritic cells and cross-presented for $CD8^+$ T cell priming. The antigens must also be directly presented by the tumour for recognition by primed $CD8^+$ T cells and killing.

Plasmacytoid dendritic cells

Plasmacytoid DC (pDC) are recognised as major producers of type I interferons (IFN-I) (34). In cancer, pDC derived IFN-I can promote anti-tumoural immunity by secreting inflammatory cytokines and chemokines, and also act as antigen presenting cells. However, pDC are also able to secrete tolerogenic factors and engage inhibitory receptors on T cells by expressing several of their ligands, including PD-L1 (34). The presence of pDC infiltration has been described as predictive of poor prognosis in numerous studies (34).

Conventional dendritic cells

Conventional dendritic cells (cDC) are potent antigen-presenting cells and inducers of T cell-mediated immune responses (34). These cells are a target for tumour immunosuppressive mechanisms that impair their development and function. Of note, some immune checkpoint receptors are also expressed by cDC, contributing to an impairment in their function (34).

Inflammatory dendritic cells

The role of inflammatory DC (inf-DC) in cancer is not well defined, with limited studies reporting conflicting results (34). Inflammatory DC, differentiate from monocytes during inflammation, infection and cancer (34). Humans inf-DC undertake cross-presentation and seem to be inducers of Th17 differentiation. Tumour-associated inf-DC have been detected in several solid tumours (34).

So far, the myeloid components of the TME have been described. In the following lines, the most common lymphoid cells infiltrating the TME will be described.

Lymphoid cells

Tumour-infiltrating lymphocytes (TILs) are specific killing lymphocytes which infiltrate the TME (35). However, their tumour-killing ability can be inhibited by immunosuppressive factors secreted by the TME (35). TILs mainly consists of T-cells, B-cells and natural killer (NK) cells, which are heterogeneous, have different phenotypes tamed by the TME, and can inhibit or promote cancer growth depending on the conditions where they grow (35). The specific characteristics of these cells are described below.

T-cells

The characteristics of CD4+ T helper cells, CD8+T cytotoxic cells, B cells and NK cells will be described below.

In normal conditions, CD4+ T helper cells interact with dendritic cells to stimulate CD8+T cytotoxic function (36) (**Figure 7)**. In cancer, however, different T helper subtypes tamed by the TME engage in molecular crosstalk with multiple immune signalling pathways, which can inhibit or promote tumour growth and dissemination depending on the TME conditions (36). The characteristics of the different CD4 T helper cells subtypes are summarised in the following lines **(Figure 8).**

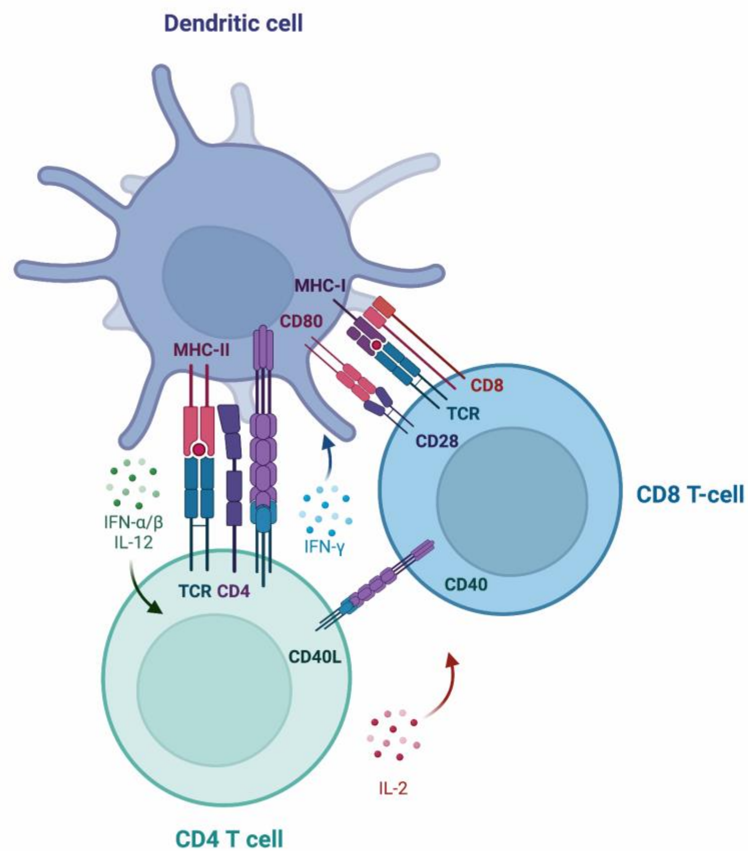


Figure 7 illustrates the process of antigen presentation and recognition by dendritic cells and CD4+ T helper cells, to modulate CD8+ T cell activation. The differentiation of CD4+ T helper cells requires a specific set of signals during antigen presentation, orchestrated by dendritic cells, IFN α/β and interleukin 12 (IL-12) among other IL.

CD4+ Th1 cells

T helper type 1 (CD4+ Th1 cells) are characterised by the production of interferon gamma (IFN-g) (37). In physiologic conditions, IL-12 recruits NK cells to produce IFN-g, and together they drive CD4+ Th1 differentiation by activating the signal transducer and activator of transcription-1 (STAT1) and STAT4 signalling pathways (37). Positive feedback regulation by IFN-g secreted by these CD4+ Th1 cells support further Th1 differentiation (37). IFN-g also upregulates major histocompatibility complex (MHC) class I and II expression to make them more susceptible to immune recognition and influences the stromal cells in the TME including TAMs, MDSCs and DC to enhance proinflammatory functions and tumouricidal activity (38,39). CD4+ Th1 cells are also a source of IL-2, a key molecular signal critical for imprinting the secondary responsiveness on CD8+ T cells, promoting their expansion (40). Additionally, the positive feedback from CD4+ Th1 produced IL-2 plays a crucial role in the recruitment of activated cytotoxic NK and CD8+ T cells to the TME (41). Conventional DC are also involved in CD4+ Th1 differentiation and recruitment of NK cells through IL-12 production (41).

CD4+ Th2 cells

CD4+ Th2 cell differentiation has been shown to be dependent on IL-4 via STAT6 signalling and transcriptional upregulation (42). Apart from CD4+Th2 cells, IL-4 can also be secreted by B cells and NK cells among others (42). Binding of IL-4 to IL-4 receptors on immune cells leads to STAT6 phosphorylation, nuclear translocation, and expression of GATA3 transcription factor (42). This results in CD4+ Th2 secretion of IL-4, IL-5, IL-10, IL-13, and IL-17 among other cytokines, enhancing M2 macrophages differentiation and promoting tumour growth, inflammation and metastasis (42,43). Interestingly, elevated CD4+Th2 cytokines and decreased CD4+Th1 cytokines seem to correlate with poorer prognosis in solid tumours (44). In this setting, CD4+Th2 cells are responsible of the increase in population of tumour infiltrating M2 macrophages in the TME by upregulating Transforming Growth Factor Beta (TGF-b) secretion and immunosuppressive responses (44). This Th2-induced tumourigenesis is also driven by their

expression of IL-7, a pro-angiogenic interleukin that promotes a leaky vasculature to allow the tumour microenvironment to grow and migratory tumour cells to enter the surrounding tissue (44). Previous studies also suggest that CD4+Th2 cells increase MDSCs infiltration within the TME, preventing TILs infiltration and inhibiting the immune response (44).

CD4+ Th17 cells

CD4+Th17 differentiation is driven by STAT3 activation and expression of the transcription factor RORgt (42). CD4+Th17 cells are characterised by the production of IL-17, IL-21, IL-22, IL-10, IL23, and CCL20 among others (42). During tumour development, CD4+Th17 promoting cytokines like IL-6, IL-23, and TGF-b are expressed within the TME (42). Although CD4+Th17 exhibits anti-tumour immune responses, the release of these molecules by TAMs can also enhance tumour growth (42). This can also be explained by the role of IL-17 in angiogenesis by increasing VEGF, IL-6 and MDSCs production, resulting in immunosuppression within the tumour (42). Furthermore, DC are also able to produce IL-6, TGF-b and IL-23 among other cytokines to support CD4+Th17 differentiation.

CD4+ Treg cells

CD4+ regulatory T cells (CD4+ Treg cells) play a crucial role in cancer progression by regulating immune surveillance and suppressing anti-tumour immune response (42). Previous studies have suggested that the efficacy of checkpoint inhibitors like cytotoxic T- lymphocyte-associated antigen 4 (CTLA4) or PD1/PD-L1 can be reduced in the presence of elevated levels of CD4+Treg cells, promoting poor survival in patients with different cancer types (42). These cells can also release immune suppressive cytokines such as IL-10, IL-35 and TGF-b, regulating CD8+ cytotoxic function and reducing anti-tumour immunity (42).

CD4+ T follicular helper cells

CD4+ T follicular helper (CD4+Tfh) cells support B cell proliferation and immunoglobulin class switching to generate high-affinity antibody responses (42). The polarisation to the Tfh effector class is driven by the production of IL-6 and IL-21, that induce the expression of the Bcl-6 transcription factor through STAT3 signalling (42). These cells have been found to be the principal component of the TILs across different cancer types (42), but their implication in mCRPC outcome is still unclear.

With the CD4+Tfh cells we finish the description of the CD4+ T helper cells subtypes. The description of CD8+ T cells, B cells and NK cells will follow in the coming lines.

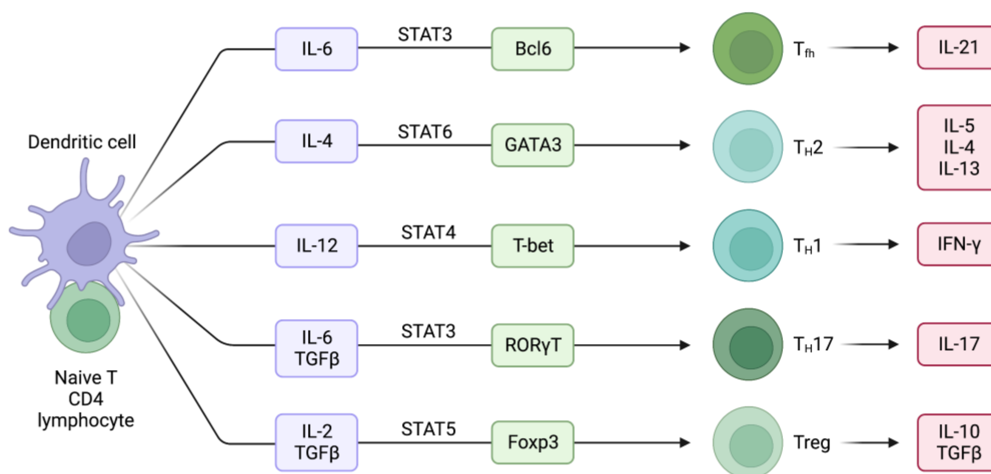


Figure 8. Th cells differentiate into distinct subtypes, including Th1, Th2, Treg, Th17, or Tfh. Each of these subtypes secretes a different panel of interleukins that drive the immune response in a specific direction. The Th differentiation into subtypes is driven by the signalling patterns from the DC.

CD8+ T cells

Cytotoxic CD8+ T cell response is the principal component of immunity but, as previously discussed, it requires help from CD4+ T cells for priming and expansion (45). CD4+ and CD8+ T cells encounter antigens presented by different subsets of DC (45). Interaction between CD40 costimulatory protein on DC and its ligand CD40L on CD4+ T cells is the key step to enhance antigen presentation on DC and to allow their interaction with CD8+ T cells (45). After that, DC act as a common platform where both CD4+ and CD8+ T receive cross antigen presentation, enhancing molecular help from CD4+ T cells to CD8+ T cells (45). Previous research suggests that this step is crucial to enhance anti-tumourigenic immune responses, since 'helped' vs 'non-helped' CD8+ T cells seem to have a differential expression of genes associated with lymphocyte activation (45). In this setting, 'helpless' CD8+T cells express high levels of co-inhibitory immune receptors which render them unable to kill tumour cells (45). This suggests that CD4+ T cells target the intrinsic function of CD8+ T cells by altering their gene expression profile and function.

B cells

Although T cells are considered the major immune cells of the tumour microenvironment to induce tumour regression, recent publications have suggested that B cells may also have a prominent role in anti-tumour immunity (46). B cells can indeed directly present tumour-associated antigens to T cells or produce antibodies that promote antigen presentation (46). Immune complexes can also increase inflammation, angiogenesis, and immunosuppression via macrophage and complement activation **(Figure 9).** Pre-clinical models have demonstrated that B-cells play an important role in CRPC. Androgens can cause damage to the stromal cell compartment of the tumour microenvironment, eliciting the recruitment of B lymphocytes among other immune cells (47). Androgen ablation can also induce the expression of the chemokine CXCL13, which recruits B cells to produce the cytokine lymphotoxin (LT). This LT leads to the activation of STAT3, which promotes the survival and proliferation of prostate

cancer cells (47). These molecular events explain one of the B cell-dependent castrate-resistant growth of the prostate cancer cells, inducing CRPC.

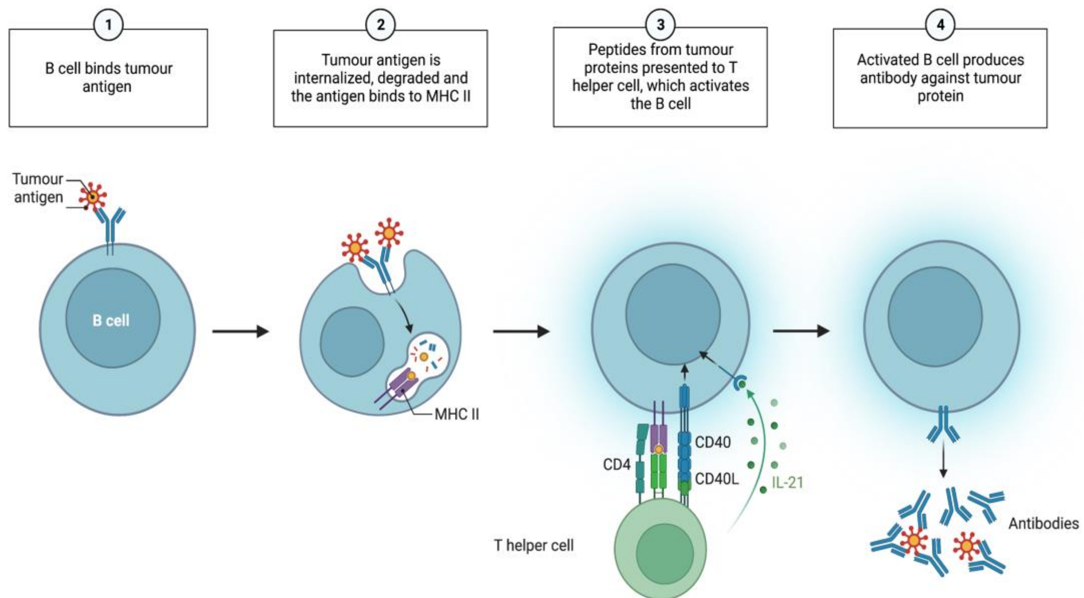


Figure 9. Tumour processing and presentation mediated by B cells. B cells can inhibit tumour development through the production of tumour-reactive antibodies, promoting tumour killing by NK cells, phagocytosis by macrophages, and the priming of CD4+ and CD8+ T cells.

NK cells

Although NK cells do not require prior antigen exposure to mediate their effector functions, priming by other factors like soluble factors released by other innate immune cells is needed for them to reach their full effector potential (48). NK cells mediate anti-tumour responses by directly killing cancer cells and indirectly improving the responses mediated by antibodies and T cells (48). NK cells also regulate dendritic cells, macrophages and neutrophils to influence T and B cell responses (48) **(Figure 10).**

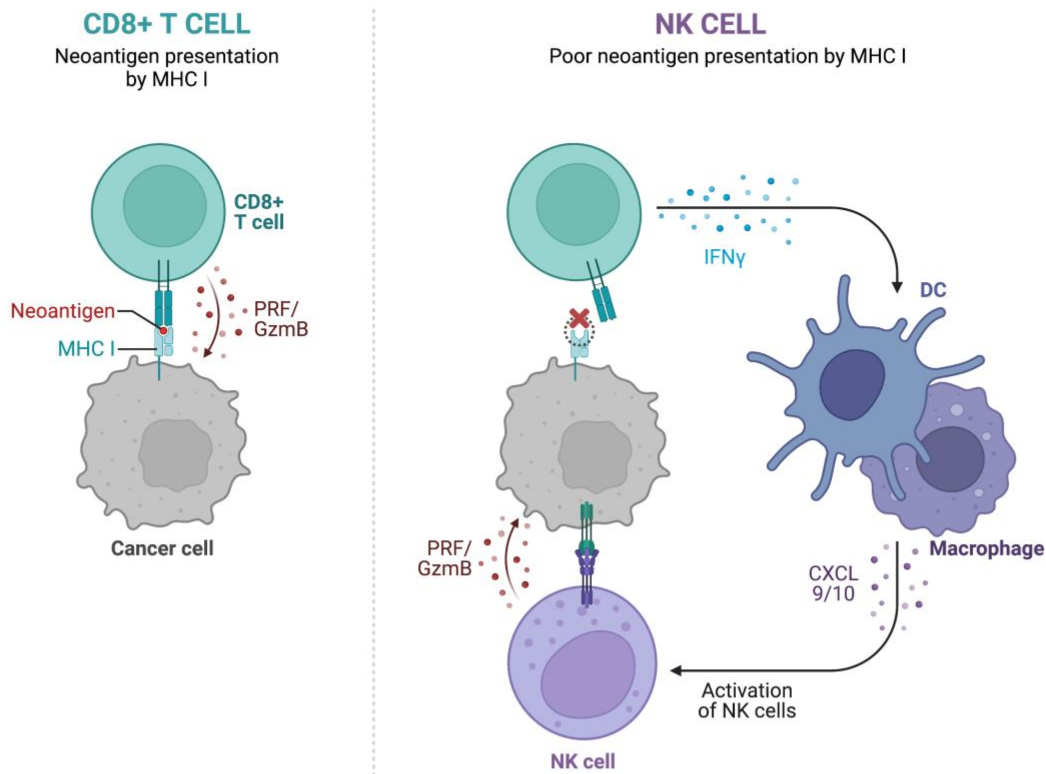


Figure 10 illustrates the differences in CD8+ T cell vs NK antigen presentation and activation. PRF = perforin, GzmB = granzyme

With NK cells, we finish the description of the immune cells which most frequently infiltrate the TME. In the following lines, we will briefly talk about the last two components of the TME (fibroblasts and extracellular matrix) before starting the characterisation of the pre-specified biomarkers selected for this study.

1.4.3 Fibroblasts

One final major cell type in the TME is the cancer associated fibroblast (CAF). Fibroblasts support cancer cell migration from the primary tumour location into the bloodstream for systemic metastasis (49). Furthermore, fibroblasts provide a reliable passage for endothelial cells undergoing angiogenesis in the tumour (49). CAFs are involved in cancer progression through their interactions with both tumour cells and other cells in the TME (49). These cells also contribute to the structure and function of the tumour stroma by generating extracellular matrix and

release metabolites that influence tumour angiogenesis, immunology and metabolism (49) **(Figure 11).**

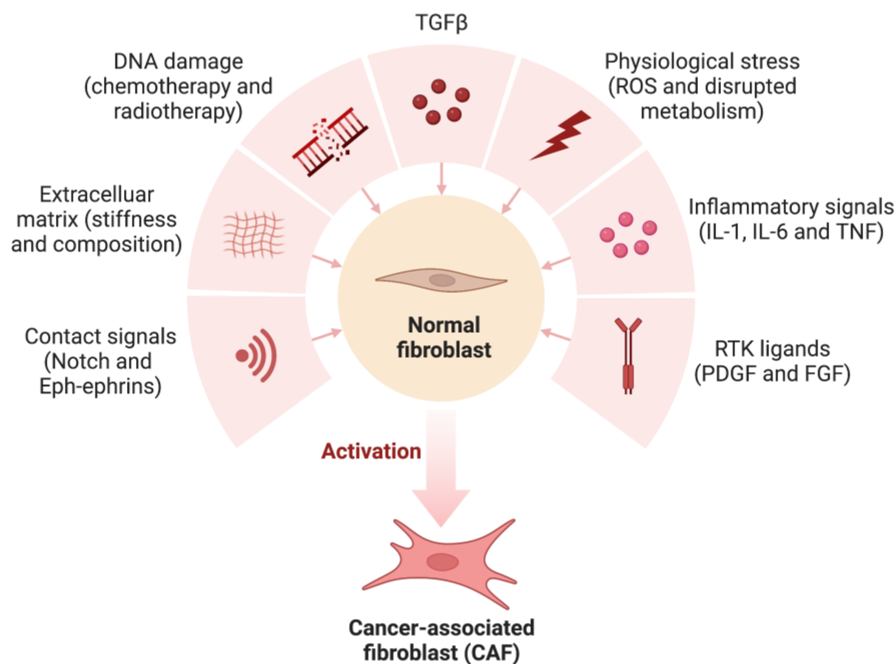


Figure 11 illustrates the different biological functions mediated by CAFs.
ROS= reactive oxygen species, TNF= tumour necrosis factor, RTK = receptor tyrosine kinase, FGF= fibroblasts growth factor.

1.4.4 Extracellular Matrix

The extracellular matrix (ECM) is the non-cellular component of tissues (50). In cancer, the ECM is defined as the network macromolecules that modulate many of the events that drive the metastatic cascade (50). This includes collagens, proteoglycans, and glycoproteins that form the physical and chemical scaffold to provide cancer progression **(Figure 12).**

The process of metastasis needs certain conditions for the tumour to grow and progress (50). These conditions include invasion of adjacent tissues, the bloodstream and the lymphatic nodes, cancer cell survival during this transit, extravasation out of vessels, and finally secondary organ colonization (50). The

ECM is key for these events by modulating the behaviour of tumour and non-malignant stromal cells along the metastatic cascade **(Figure 12).**

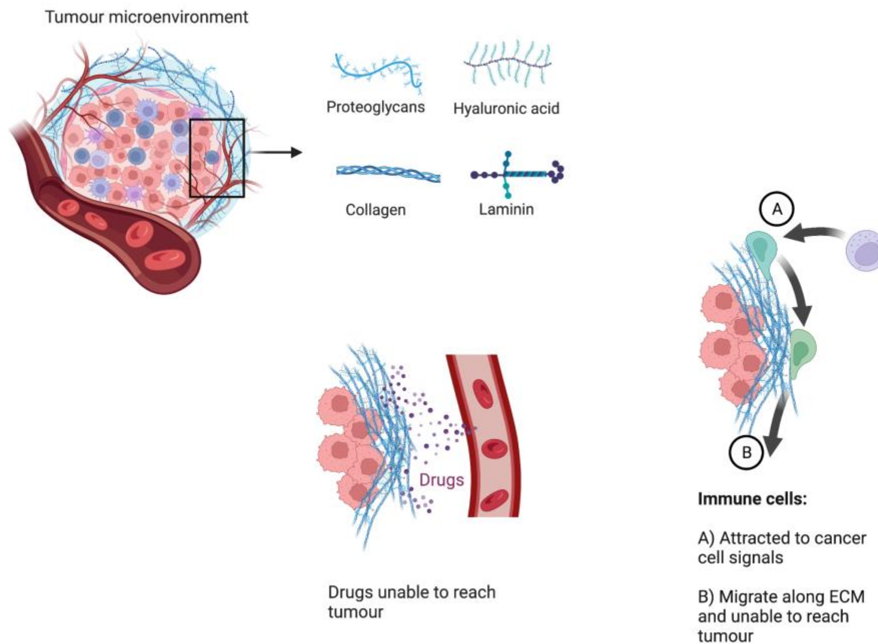


Figure 12. The ECM has the capacity to store growth factors and cytokines, establishing concentration gradients and regulating their bioavailability. There is cellular crosstalk between TME and cell-to-ECM communication. This interaction induces the release of soluble factors responsible for immune evasion and ECM remodeling, which further contribute to therapy resistance.

Taking into account the characteristics of the cells and molecules specified above, while modelling their TME, tumours can be broadly categorised as inflamed or non-inflamed (51) **(Figure 13).** Inflamed TME are characterised by genomic instability, the presence of TILs with high density of IFNg-producing cells, expression of PD-L1, and a pre-existing anti-tumour immune response (51). In contrast, non-inflamed tumours are poorly infiltrated by lymphocytes, rarely express PD-L1, have low mutational burden and low expression of antigen presentation machinery markers (51). Immune check inhibitors (ICI) are indeed most effective in inflamed tumours, acting by reinvigorating pre-existing anti-tumour T-cell (51). Other tumours can also create an immunosuppressive

microenvironment by inducing the expression of extracellular matrix genes and suppressing the expression of chemokines and cytokines required to facilitate T-cell infiltration into tumours (51). This reactive stroma creates a barrier to the infiltration of immune cells, which manifests into an excluded infiltrate phenotype with peri-tumoural or stromal T-cell localisation often associated with poor outcomes (51).

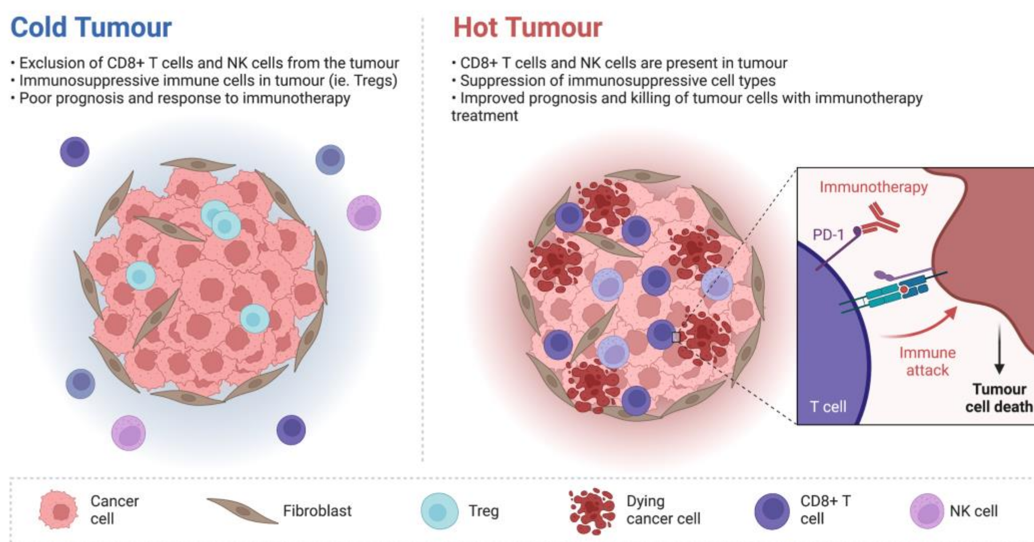


Figure 13. Simplified cartoon depiction of cold tumours vs hot tumours TME characteristics.

The majority of prostate cancers appear to have a cold tumour microenvironment with a low mutational load (52). This may be the reason why ICI have to date had limited efficacy against the majority of advanced prostate cancers, although durable responses have been observed in a small subset of patients with mismatch repair defective disease indicating that patient selection is critical to optimising benefit (53).

In this section, we have discussed the main cells and molecules that characterise the TME. In the next section, we will discuss the pre-specified biomarkers for this

study and the rational to justify their potential relation to TME configuration and mCRPC prognosis.

1.5 Pre-specified selected biomarkers

1.5.1. BRCA2, PALB2, ATM

BRCA2, PALB2 deleterious alterations and ATM loss of protein expression were selected as biomarkers representative of the DNA damage repair pathway.

Alterations in DNA repair that arise during tumour development can make some cancer cells reliant on a reduced set of pathways for survival (54). Drugs that impact these pathways have proven to be useful as single-agent therapies in different cancer types (54) **(Figure 14).** These DNA damage and deficits in its mechanisms of repair drive the development of cancer by inducing deleterious genetic alterations (54). In this situation, DNA damage response proteins activate inter-related molecular pathways like homologous recombination repair (HRR) to recognise DNA damage, mediate DNA repair and maintain the integrity of the genome (54). Poly(ADP-ribose) Polymerase 1 and 2 (PARP1 and PARP2) enzymes are key to DNA damage repair (DDR), acting as DNA damage sensors and signal transducers to repair DNA lesions (54). Around 20-25% of mCRPC harbour defects in DNA repair genes, and these defects, when bi-allelic, confer increased sensitivity to PARP inhibitors (PARPi) (54).

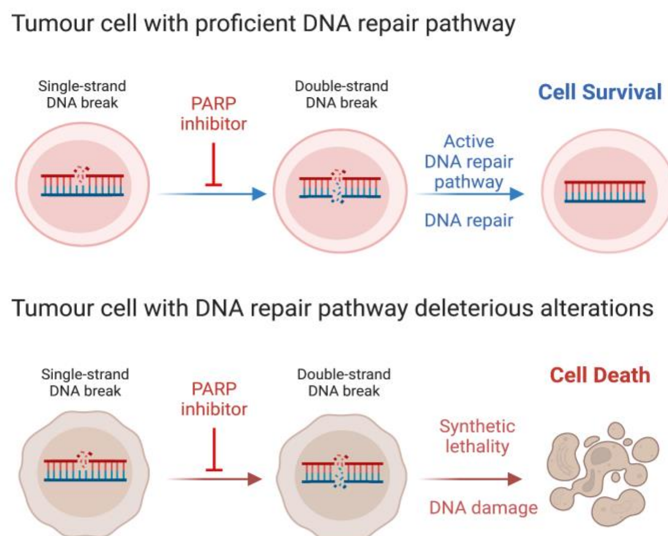


Figure 14. PARP1 binds damaged DNA at single strand DNA breaks (SSBs) and other DNA lesions. This event leads to the recruitment of DNA repair effectors and the remodelling of chromatin structure around damaged DNA as part of the DNA repair process. In tumours with deleterious alterations in the DNA repair pathway, PARP inhibition induces cell death by prompting double-strand DNA breaks (DSBs).

At present, two PARPi (olaparib and rucaparib) are approved by the FDA for use in mCRPC with HRR germline or somatic mutations (55,56). However, despite promising results, successful differential responses and resistance also prevail for these treatments, probably due to intra-patient genomic heterogeneity. Alterations in the ATM gene are the second most common DNA repair defects after BRCA2, being present in up to 10% of prostate cancers (57). Interestingly, ATM loss seems to confer sensitivity to ataxia telangiectasia and Rad3-related protein (ATR) inhibition in preclinical models (57), and other preclinical studies have shown that olaparib-resistant cancer cells with or without ATM loss may be re-sensitised to olaparib when combined with ATR inhibitors (ATRi) (58). This has provided rationale to use ATRi, either alone or in combination with PARPi, in phase 1 and phase 2 clinical trials, currently ongoing.

As discussed, sensitivity to PARPi seems particularly high in tumours harbouring Bi-allelic BRCA2 and/or ATM aberrations, but mutations in other HRR do not seem to confer such sensitivity and most patients will become resistant to these therapies (54). For that reason, further investigations are needed in this scenario, and there is rational to justify that DDR deficient tumours could promote an inflamed TME more likely to benefit from ICI (59). One potential explanation is that DDR deficient tumour cells are able to reinstate HRR via reversion mutations that restore the native reading frame of each gene, leading to the production of neoantigens that can be recognised by the immune system (60). As another example, enhanced IFN-related gene expression and higher abundance of TILs have been observed in DDR deficient tumours (59), and previous studies have also demonstrated that DDR deficiencies play important roles in upregulating the expression of PD-L1 (59).

1.5.2. PTEN

PTEN loss of protein expression was selected as the biomarker representative of the PTEN/ phosphatidylinositol 3-kinase (PI3K) /AKT pathway **(Figure 15).**

Genomic aberrations in the PI3K-AKT axis are common in primary prostate cancer and enriched in mCRPC (approximately 17% and 50% respectively) (61). This pathway is mainly activated due to mutations in the tumour suppressor gene PTEN (61). PTEN loss is associated with adverse outcomes such as increased tumour grade and stage, earlier biochemical recurrence after radical prostatectomy, metastasis, prostate-cancer-specific death, and androgen-independent progression (61).

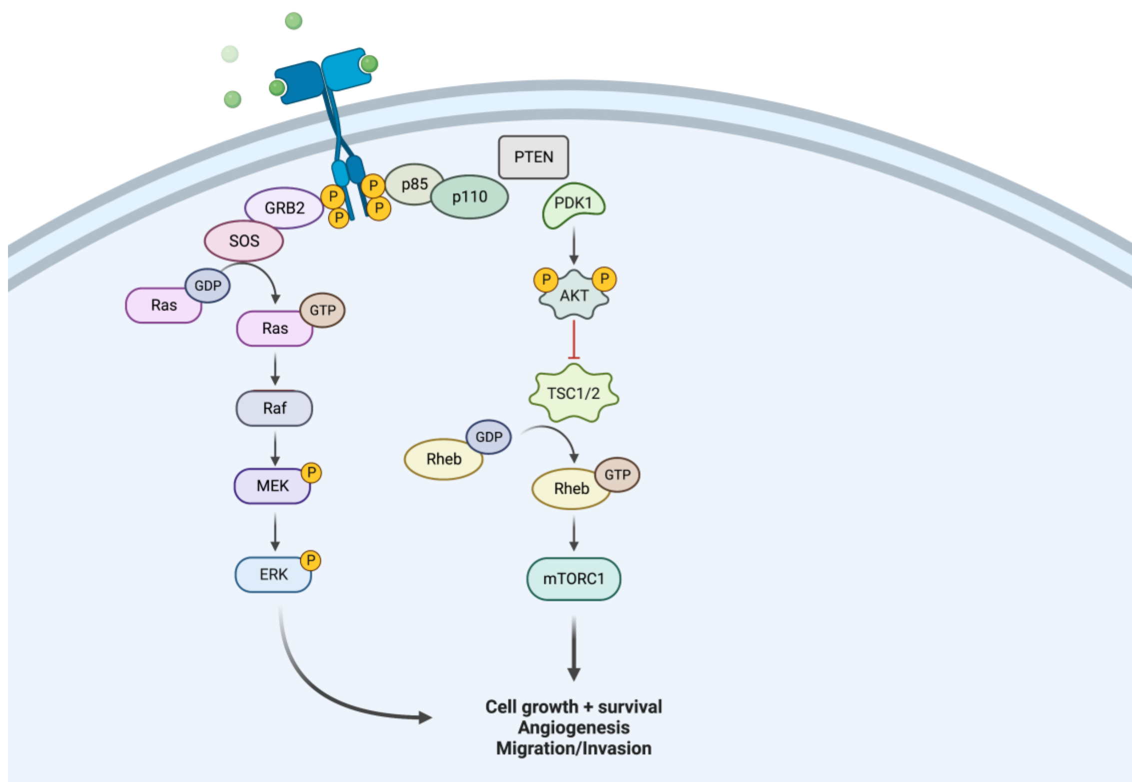


Figure 15. The PI3K/AKT pathway is regulated by upstream signaling proteins and it regulates many downstream effectors by collaborating with various compensatory signaling pathways, primarily with RAF/MEK/ERK and TSC1/2 pathways.

The PI3K/AKT pathway has also been implicated in resistance to anti-androgen therapy, as AR inhibition is associated with an increase in AKT pathway activation, suggesting that the tumour compensates for the loss of one pathway with another (62). Previous randomised phase 3 trials have shown that the combination of AKT inhibitors and abiraterone as first-line treatment resulted in significantly improved radiographic progression free survival (rPFS) and anti-tumour activity compared to abiraterone and placebo in mCRPC patients with PTEN-loss (18.5 vs 16.5 months, HR: 0.77; 95% CI: 0.61, 0.98; p = 0.0335) (62). However, the potential implication of this biomarker in the configuration of mCRPC TME is still scarce.

Previous studies in cell lines showed that PTEN knockdown increased cell-surface PD-L1 expression and PD-L1 transcripts, and this increased PD-L1 cell

surface expression led to decreased T cell proliferation and increased apoptosis (63). However, these results have not been replicated in prostate cancer cells so far.

1.5.3 PD-L1, TcellinfGEP score, TMB, dMMR and CDK12

PD-L1

PD-1 is widely expressed in a range of immune cells, such as T cells, dendritic cells and other components of the innate immune system (64). PD-L1 was the first discovered ligand of programmed cell death protein 1 (PD-1). Under physiological conditions, the PD-1/PD-L1 pathway maintains peripheral tolerance and regulates autoimmune responses (64). In cancer, PD-1 promotes immune tolerance and immunosuppression by binding to PD-L1 on tumour cells, which leads to tumour immunosuppression and immune tolerance **(Figure 16).** In recent years, ICI targeting the PD-1/PD-L1 axis have become the gold standard in cancer immunotherapy to reverse immunosuppression and to restore activity of the immune system against several tumours (65).

PD-1/PD-L1 axis

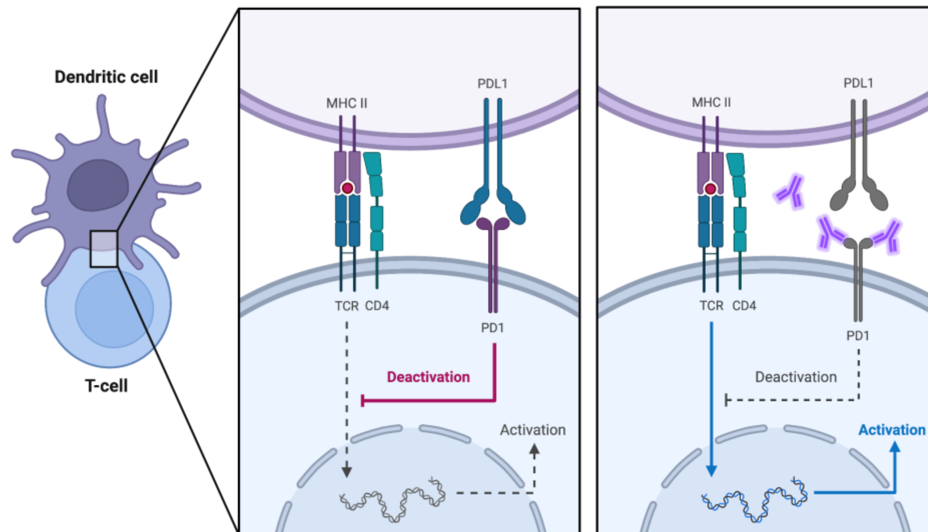


Figure 16 illustrates the characteristics of the PD1/PD-L1 axis. PD-L1 is a trans-membrane protein considered as a co-inhibitory factor of the immune response. PD-L1 combines with PD-1 to reduce the proliferation of PD-1 positive cells, inhibit their cytokine secretion and induce apoptosis. PD-L1 also attenuates the immune response to tumour cells. PD1/PD-L1 inhibitors re-activate the immune system by blocking this pathway.

Anti PD-1/PD-L1 ICI has emerged as a standard of care for several cancer types after demonstrating prolonged responses and improved OS in tumours considered to be “immune hot” due to increased PD-L1 expression, high TMB or dMMR among other biomarkers (65). Although responses to PD-1/PD-L1 blockade are thought to correlate with increased expression of PD-1/PD-L1 in the tumour microenvironment, prostate cancer is largely considered a “cold” tumour with very limited response to single-agent checkpoint inhibition, as demonstrated in previous clinical trials (20,21,52).

So far, translational studies assessing the prognostic implications of PD-L1 expression in human prostate cancer are sparse. In this setting, it is important to remark that most prostate cancer studies have evaluated primary prostate specimens, with scarce data about PD-L1 expression on metastatic lesions (22,23,24).

TcellinfGEP score

Tumours are able to adapt to take advantage of the balance between positive and negative immune signaling factors that allow the cancer to grow and progress (25). In response, an orchestrated innate and adaptive anti-tumour immune response is initiated. This leads to the production of cytotoxic cytokines to activate T cells, as well as NK cells, in the tumour microenvironment (25). However, the same cellular signals can also inhibit this anti-tumour immunity by upregulating PD-L1 and PD-L2 in the tumour, immune infiltrate, and stromal cells (25,66). In addition, other key immune suppressive molecules can also be expressed within the tumour microenvironment (25).

The TcellinfGEP score was developed by examining the gene expression profile in the tumour microenvironment using RNA isolated from formalin-fixed paraffin-embedded (FFPE) tumour tissue samples (67,68). Samples were obtained at baseline from patients undergoing treatment with pembrolizumab in clinical trials using multiple distinct tumour types (67,68). Signatures related to IFN-g signalling and activated T cell biology were initially delineated in a small pilot melanoma cohort, then confirmed and refined in a larger independent cohort of patients with melanoma. The cross-tumour predictive value of these signatures was demonstrated in head and neck squamous cell carcinoma (HNSCC) and gastric cancer cohorts, followed by a modelling exercise to determine a final T cell–inflamed gene expression profile and scoring algorithm that predicted response across 9 different cancer cohorts to arrive at a final signature (67,68).

The 18 different genes tested in this panel and their biological functions are specified below **(Figure 17).**

CCL5

CCL5 belongs to the CC subfamily of chemokines (69). T cells and monocytes are the most common immune CCL5-expressing cells (69). While CCL5 can bind to CCR1, CCR3, CCR4 and CCR5, it has the highest affinity to CCR5. The downstream pathway of CCL5/CCR5 including PI3K/AKT, nuclear factor kB (NF-kB), hypoxia-inducible factor a (HIF-a), RAS-ERK-MEK, Janus kinase (JAK) - STAT and TGF-b-smad pathways are associated with cell proliferation, angiogenesis, apoptosis, invasion, division, metastasis and inflammation (69).

CCL5/CCR5 axis promotes tumourigenesis of different types of cancers by creating a more suitable microenvironment for tumour cell survival. These signalling pathways regulate growth factors and inflammatory factors to remove barriers for tumour metastasis and invasion, and also promotes the recruitment of Tregs, MDSCs and TAMs to induce immunosuppression of the tumour (69).

CD27

Treg cells suppress CD8+ T cell priming by DCs by limiting co-stimulation through the CD70/CD27 axis (70). CD27 is generally found on naive T and memory B and T cell populations and subsets of NK cells (70). On the other hand, CD70 is only transiently expressed on antigen-activated B and T cells, NK cells and mature dendritic cells (70). In oncology, CD70 is aberrantly expressed on malignant cells without (solid tumours) or with CD27 co-expression (haematological malignancies), facilitating immune evasion through the TME and tumour progression (70). This immune evasion, driven by the CD70-CD27 axis, can be mediated by promoting apoptosis of lymphocytes, T cell exhaustion or decrease of Treg apoptosis (70).

CD274 (PD-L1)

The characteristics of the PD-1/PD-L1 axis have been described above.

CD276

CD276 (B7-H3) is an immunoregulatory protein key to trigger immune responses (71). B7-H3 is expressed on cancer cells, T and B cells and myeloid cells including monocytes/macrophages and dendritic cells (71). This protein can act as both a co-stimulatory/co-inhibitory molecule and is functional in both membrane-associated and soluble forms. When released, soluble B7-H3 (sB7-H3) can stimulate the proliferation of both CD4+ and CD8+ T-cells and activate T-effector cells by inducing IFN-g production (71). However, B7-H3 can also induce an immune evasive response when deregulated in cancers, leading to poorer outcomes (71).

CD8A

CD8A encodes the CD8 alpha chain of the αβT cells and has been described as an indicator for cytotoxic CD8+T lymphocytes recruitment (72). In this setting, cytotoxic CD8+T lymphocytes present a predominant expression level of CD8A which could be a direct indication of pre-existing anti-tumour immunity with tumour infiltrating cytotoxic CD8+T lymphocytes in the TME (72). However, recent studies also suggest that CD8A can be implicated in forming an immunosuppressive environment through T cell exclusion in the TME (72).

CMKLR1

Chemerin chemokine-like receptor 1 (CMKLR1) is a G-protein coupled receptor which participates in triggering adaptive and innate immunity (73). This protein can be expressed in plasmacytoid dendritic cells, macrophages and endothelial cells among others (73). Increased activity of CMKLR1 strongly correlates with dysregulation of angiogenesis and release of pro-inflammatory cytokines (73). The activity of CMKLR1 has also been associated with the stimulation of tumour invasion (73).

CXCL9

The CXCL9/CXCR3 axis regulates immune cell migration, differentiation, and activation (74). In vivo studies suggest this axis plays a tumourigenic role by increasing tumour proliferation and metastasis (74). CXCL9 is produced in the tumour site and associated with directing the migration of CXCR3+ effector CD4+, cytotoxic CD8+ T cells and CXCR3+ NK cells to the tumour site (74). Interestingly, CXCL9 can also be secreted by stromal cells (74).

CXCR6

CXCR6 is a chemokine receptor with its known ligand being CXC chemokine ligand 16 (CXCL16) (75). CXCR6 expression has been described to be a marker of T cell differentiation, with this protein being expressed on some subpopulations of T cells, natural killer T cells (75). CXCL16, on the other hand, is mainly expressed on dendritic cells, monocytes, and tissue cells such as fibroblasts and endothelial cells (75). CXCR6 is preferentially expressed on CD8+ T cells infiltrated in the tumour and is essential for CD8+ T cells to trigger anti-tumour response (75). Currently, the role of CXCL16 in tumour is still controversial.

HLADQA

HLA-DQA belongs to the HLA class II alpha chain paralogues. The class II molecules are expressed in antigen presenting cells, and HLA-DQA is a heterodimer consisting of an alpha (DQA) and a beta chain (DQB) (both anchored in the membrane). These play a central role in the immune system by presenting peptides derived from extracellular proteins (76).

HLADRB

HLA-DRB belongs to the HLA class II beta chain paralogs, consisting of a heterodimer of an alpha (DRA) and a beta chain (DRB), both anchored in the membrane. Similarly to HLA-DQA, it plays a central role in the immune system by presenting peptides derived from extracellular proteins (76).

HLAE

HLA-E belongs to the HLA class I heavy chain paralogues. This class I molecule is a heterodimer consisting of a heavy chain and a light chain (beta-2 microglobulin), with the heavy chain being anchored in the membrane. HLA-E binds a restricted subset of peptides derived from the leader peptides of other class I molecules (76).

IDO1

The expression of the enzyme indoleamine 2,3-dioxygenase 1 (IDO1) in cancer cells is induced by IFNγ, secreted by activated T cells infiltrating the TME (77). In this setting, IDO1 catabolizes tryptophan to generate metabolites along the kynurenine pathway to suppress T cell immunity (78). Previous research in mouse models suggest that IDO1 drives immunosuppression in both tumour cells and stromal immune cells (78).

LAG3

Lymphocyte activation gene 3 (LAG-3) is a cell surface inhibitory receptor expressed by T cells, some activated B cells and plasmacytoid dendritic cells that regulates T-cell effector functions (79). Elevated LAG-3 expression is considered a T-cell exhaustion marker, and LAG-3 and PD-1 co-expression in T cells is considered a biomarker of T-cell dysfunctionality associated with resistance to anti-PD-1/anti-PD-L1 immunotherapies (79).

NKG7

Natural Killer Cell Granule Protein 7 (NKG7) is an intrinsic membrane protein highly expressed in NK cells, CD8+ T cells and CD4+ T cells that translocate to the plasma membrane upon target cell induced degranulation, facilitating cytotoxic activity (80). Previous mouse and human models have demonstrated that loss of NKG7 indeed impairs both NK and CD8+ T cell-mediated anti-tumour activity, promoting cancer progression (81).

PDCD1LG2

PDCD1LG2 (PD-L2) is a second ligand for PD-1 that inhibits T cell receptor (TCR)-mediated proliferation and cytokine production by CD4+ T cells (66). The PD-L2–PD-1 axis can also inhibit B7-CD28 signals, playing a key role in regulating T cell responses (66).

PSMB10

The proteasome B-type 10 (PSMB10) gene encodes a member of the proteasome B-type family, that is a 20S core beta subunit (82). The expression of this gene is induced by gamma interferon and has an essential function in the processing of class I MHC peptides for antigen presentation (82).

STAT1

The IFN-g JAK/STAT1 pathway plays a crucial role in antigen processing and the subsequent dynamic changes of downstream signals, including MHC class I (83). STAT1 can be activated by various ligands including interferon-alpha, interferon-gamma and IL6, mediating the expression of a variety of genes implicated in promoting immune responses (83).

TIGIT

T cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT) is an inhibitory receptor expressed by Tregs, activated T cells, and NK cells which regulates T cell-mediated tumour recognition (84). Directly or indirectly, TIGIT activation creates a tolerogenic microenvironment which prompts cancer progression (84). TIGIT has been found in the cellular microenvironment of several tumours, with its overexpression being correlated with poor prognosis (84).

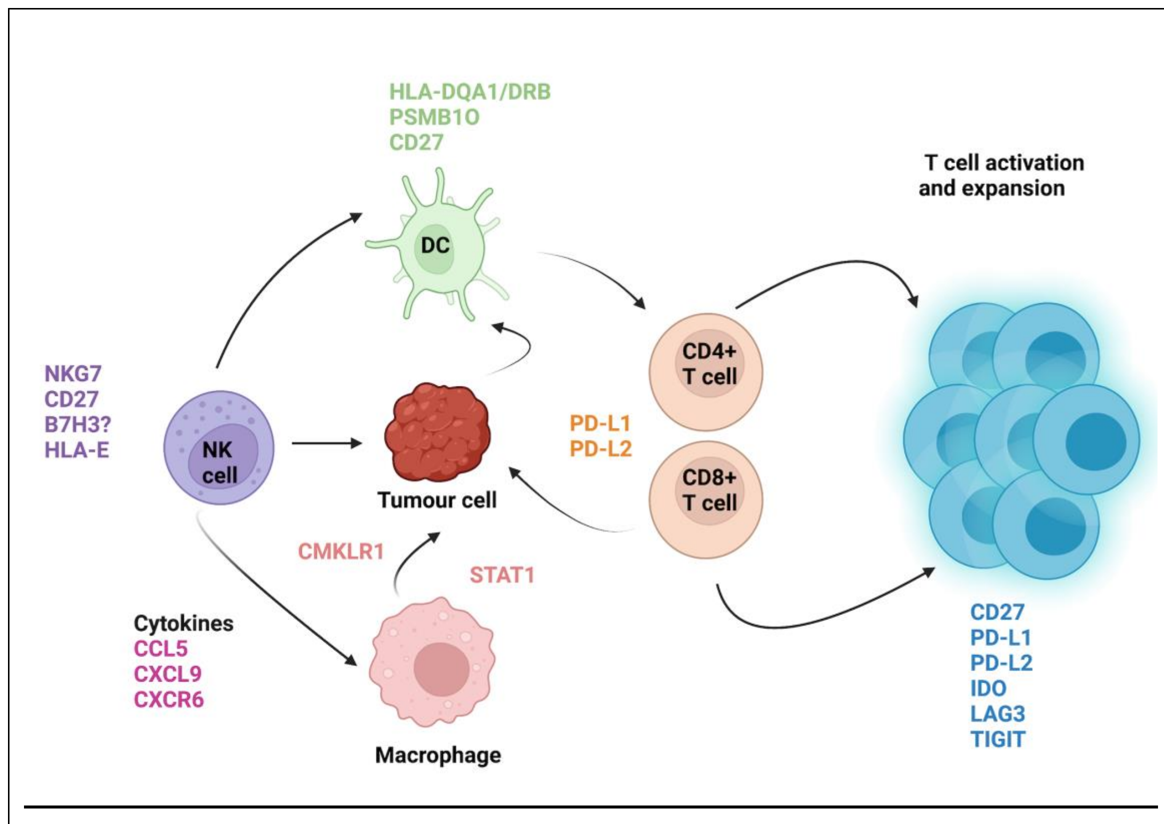


Figure 17 illustrates the different genes included in the TcellinfGEP panel and their relationship with the different steps in the anti-tumour immune response, including cytolysis, antigen processing, presentation and expansion.

TMB

Tumours with high mutational burden tend to have a better and more sustainable response to immune checkpoint inhibitors (85). This is probably because tumours with an elevated number of non-synonymous alterations are able to create more mutation-associated neoantigens (MANAs) to be recognised by the immune system, inducing cytotoxic responses (85). On average, prostate cancer is not considered to be a cancer with a high mutational burden (85). However, mCRPC can harbour either germline or somatic mutations in DNA repair genes, and defects in these pathways may also be associated with an increased neoantigen load (59,60). For that reason, there is rational to think that HRR mutations may be associated with high expression of PD-L1 and increased tumour-infiltrating

lymphocytes in this setting, which could potentially impact checkpoint inhibitor responses (59,60).

dMMR

Microsatellite instability (MSI) is characterised by the accumulation of insertions or deletions of nucleotides in microsatellites, which are continuous repetitions of 1–9 nucleotides in the DNA (86). MSI is caused by loss of function of members of the DNA mismatch repair system (MMR), which in normal conditions allow the repair and correction of DNA mismatches (86).

Depending on the frequency of mutations, MSI can classified into three subtypes: high microsatellite instability (MSI-H), low microsatellite instability (MSI-L) and microsatellite stability (MSS) (87). There are at least seven MMR proteins, of which four have the most clinical relevance in human cancer biology—MLH1, MSH2, MSH6 and PMS2. These four proteins are arranged as heterodimers which recognise and repair mismatched nucleotide base pairs (87). It has been recently described that up to 5–12% prostate cancer may have an hypermutated genomic profile due to underlying mismatch repair gene mutations and MSI-high phenotypes, and published literature has confirmed that some of these tumours can present significant and prolonged responses to ICI (88).

CDK12

CDK12 is a transcription-associated CDK implicated in DNA repair, splicing, and differentiation (89). Genomic analysis of primary and metastatic castration-resistant prostate cancer has identified deleterious CDK12 alterations in 2%–4% of primary prostate cancers and in 4.7%–11% of mCRPC (89). Previous genomic analyses have also shown that CDK12-mutated prostate cancer is prone to tandem duplications (TD), genomic rearrangements, high neoantigen burdens, and increased tumour-infiltrating lymphocytes (89).

The cellular and molecular components of the TME and the biological rational to justify that BRCA2, ATM, PALB2, PTEN, PD-L1, TcellinfGEPscore, TMB, dMMR

and CDK12 could have an impact in TME configuration have been described above. To finish this introduction, the biomarkers representative of neuroendocrine prostate cancer and other aggressive histologies are described below.

1.5.4 Neuroendocrine prostate cancer and aggressive histologies.

SOX2, neuroendocrine histology features and TP53 deleterious genomic aberrations were selected as biomarkers representative of these entities.

Despite developing castration resistance, most prostate cancers are still dependent on AR signalling through acquired AR gene mutation, amplification, or other means to re-activate the AR (90,91). However, approximately 15–20% of CRPC tumours will lose dependence on AR signalling, and the identification of AR-independent disease in the clinic remains challenging (90,91). One apparent clinical manifestation is histologic transformation from an AR-expressing prostate adenocarcinoma to an AR-negative, poorly differentiated spectrum ranging from adenocarcinoma with neuroendocrine differentiation, mixed histology, and in extreme cases complete transformation to small cell carcinoma (91). In these cancer phenotypes AR expression is typically low, less dependent (or indifferent) to canonical AR signaling and with a particularly aggressive and atypical spread of progression with low or non-rising PSA levels (90,91). These tumours may harbour genomic features common in other small cell carcinomas such as TP53 and Rb1 loss (90,91). Chemotherapy has been considered as a suitable treatment in this setting, but despite initial responses, resistance and disease progression occurs in most patients (90,91). For that reason, further research is warranted in this setting.

The sex-determining region on the Y chromosome-related high mobility group box (SOX) family is an important group of transcription factors involved in tumourigenesis (92). This family comprises a number of transcriptional regulators that critically control cell differentiation and are involved in cancer progression and metastasis (92). These proteins may act as tumour suppressor genes, oncogenes or both, depending on the cellular environment, and can be stimulated

or incapacitated through diverse genetic and epigenetic mechanisms, including DNA methylation, DNA copy number alterations and abnormal micro RNA (miRNA) expression (92). Tumourigenic deregulation occurs on transcriptional, translational and post-translational levels, and studies on different members of the SOX gene family revealed member specific mechanisms of action (92). Regarding the involvement of SOX family members in tumourigenesis, SOX2 is the most thoroughly investigated transcription factor and has been correlated with the tumourigenicity of a number of cancers (92).

The overexpression of SOX2 is a frequent driver of tumour growth and propagation (92). SOX2 overexpression correlates with the tumourigenicity of several cancer cells in vitro and in vivo and can be initiated by various mechanisms including amplification of the SOX2 gene, DNA hypomethylation and enhanced transcription by growth factors and/or other members of the SOX family (92). At a translational level, alterations to the number of distinct miRNAs have an influence on SOX2 overexpression (92). At the post-translational level, a balance between methylation and phosphorylation determines the stability and degradation of SOX2 (92).

In prostate cancer, previous studies have observed that SOX2 expression begins before the emergence of the neuronal expression signature, highlighting its potential crucial roles in driving neuroendocrine prostate cancer (NEPC) trans differentiation (93). Recent studies have indicated that SOX2 levels are significantly higher in CRPC tumours with neuroendocrine-like histology than in adenocarcinomas, and that SOX2 and the neuroendocrine marker chromogranin-A are primarily co-expressed in both prostate cancer and lymph node metastases (93). Previous research also suggests that Rb1 or TP53 depletion are likely to facilitate prostate cancer lineage plasticity and to establish an NEPC phenotype once cells transdifferentiate (94,95). In this NEPC predisposing genetic context, treatment with potent AR pathway inhibitors may release previously AR repressed neuroendocrine master regulators, such as SOX2 (94,95). However, although SOX2 seems to promote lineage plasticity and antiandrogen resistance in TP53 and Rb1 deficient prostate cancer, its role

during the transformation from adenocarcinoma to NEPC is still unclear, and further research is needed (94,95).

SOX2 is also known to be involved in recruiting tumour-associated macrophages of the M2 phenotype to the TME in certain cancer types (96). As previously discussed, these macrophages display high plasticity and constitute a heterogeneous population of myeloid cells of the innate immune system able to adapt their phenotype in response to different environmental stimuli (31). In prostate cancer, high numbers of M2 macrophages are associated with poor clinical outcome (97). Recent findings have also suggested that SOX2 can transcriptionally activate the PD-L1 gene, providing a potential intrinsic mechanism for the induction of PD-L1 expression in tumour cells (98). Therefore, SOX2 expression in mCRPC could be not only a marker of NEPC trans differentiation, but also a modulator of the mCRPC microenvironment and a promoter of PD-L1 expression associated with not only more aggressive disease but also possibly ICI sensitivity.

In summary, mCRPC is a disease characterised by lack of durable responses to the current approved therapies, probably due to its heterogeneity and genomic instability. Some of these genomic variations, alone or in combination, can result in tumour antigens that can be recognised by the immune system, triggering immune responses. However, cancer cells are able to action different mechanisms to escape immune surveillance, resulting in tumour growth and progression despite anti-tumour immune responses.

The molecular rational to support why our pre-specified biomarkers could have an impact on the configuration of the mCRPC TME and its prognosis has been justified below. In the next sections of this manuscript our hypothesis and results are disclosed.

2. Hypothesis

- Selected, clinically relevant, immune biomarkers associate with clinical outcomes in mCRPC

3. Materials and Methods

3.1. Primary objective

- To determine the association of our pre-specified biomarkers with clinical outcomes in mCRPC

3.2. Secondary objectives

- To identify the prevalence our pre-specified biomarkers in mCRPC biopsies.

- To determine the correlation among the expression of our pre-specified biomarkers

3.3. Patient selection

The study population included 100 patients with mCRPC who had prospectively had a fresh mCRPC biopsy at the Royal Marsden Hospital (RMH; London, United Kingdom (UK)) between October 2014 and July 2019. The date of biopsy was considered the index date for the study analyses. Patient selection was done according to the inclusion criteria for the study, prespecified in the study protocol. All patients were 18-year-old or older and had histologically or cytologically confirmed metastatic adenocarcinoma of the prostate without small cell histology. All patients were treated with at least one second-generation antiandrogen therapy and at least one regimen of chemotherapy that contained docetaxel in the mCRPC or metastatic hormone-sensitive prostate cancer setting. In addition to biomarker data, demographics, clinical characteristics, and outcome data for each patient were extracted from patient records.

3.4. Specimen characteristics

Archived tissue samples from all identified patients were retrieved, and slides (including one hematoxylin and eosin slide) were cut for biomarker testing. All mCRPC biopsies were prospectively acquired using an approved protocol for prostate cancer molecular characterization at the RMH (04/Q0801/60). All patients provided written informed consent.

3.5. Assay methods for biomarkers of interest

Metastatic CRPC biopsies were assayed by WES, RNA seq, targeted NGS, NanoString and IHC using previously described methods as described below (55,68, 88, 99, 100, 101, 102, 103). A pathologist with prostate cancer expertise reviewed tissue blocks. The IHC slides were then digitized at high resolution (200x) using the ZEISS Axio Scan Z1 digital slide scanner (Carl Zeiss AG, Oberkochen, Germany). A pathologist-supervised machine learning algorithm (HALO AI, Indica Labs, New Mexico, USA) was trained to recognise prostate cancer cells and surrounding benign stroma. Color deconvolution for DAB and hematoxylin stains were performed. Cell recognition and nuclear segmentation was optimised for neoplastic cells. A visual threshold was set for positive staining. The analysis algorithm was adjusted to provide continuous data on the percentage of neoplastic cells staining positively, separately for each automatically annotated tumour region to facilitate distinguishing between staining in neoplastic cells and stromal positivity

3.5.1. PD-L1

Tumour PD-L1 expression was measured by IHC on a Dako Autostainer Link 48 system (Agilent Technologies) using the Dako PD-L1 IHC 22C3 pharmDx kit (Agilent Technologies) with the EnVision FLEX visualisation system. A negative control was included for each subject and stain. PD-L1 positivity was defined as Combined Positive Score (CPS) ≥1, where CPS is the number of PD-L1 staining cells (tumour cells, lymphocytes, and macrophages) divided by the total number

of viable tumour cells multiplied by 100. A qualified pathologist performed cell counts and CPS categorization at Neogenomics.

3.5.2. TcellinfGEP score

The TcellinfGEP was previously derived across several solid tumours (67,68). Tumour RNA extracted from formalin-fixed paraffin-embedded slides were analysed on the NanoString nCounter system (Seattle, WA), and TcellinfGEP score was calculated as a weighted sum of normalised expression values for the 18 genes, as described previously (67,68). Messenger RNA was extracted at Almac and the NanoString assay was done at MSD. The cutoff for samples to be considered to have a high TcellinfGEP score was >-0.318.

3.5.3. ATM, PTEN, SOX2, neuroendocrine histology features and dMMR

Immunohistochemistry was conducted at the ICR and performed on 4µm-thick FFPE tissue sections with antibodies against ATM (clone Y170), MLH1 (clone ES05), PMS2 (clone EP51), MSH2 (clone FE11), MSH6 (clone EP49), PTEN (clone 138G6) and SOX2 (clone D6D9). Assays are detailed in *Table 1* and all cases were assessed by pathologists specialising in prostate cancer (DNR and BG). For ATM, PTEN and SOX2 assessment, nuclear and/or cytoplasmic protein expression was evaluated using histo-score (h-score) (99). ATM and PTEN loss were defined as samples with h-score <10. SOX2 expression was considered positive when the percentage of cells with SOX2 expression was higher than 5%. Presence of neuroendocrine features was determined according to visual morphology. For visual determination of a probable neuroendocrine morphology on hematoxylin and eosin (H&E) sections, we used the morphologic classification for prostate cancer with NE differentiation by Epstein et al (101).

dMMR was defined as loss of protein detected by IHC and/or a pathogenic genetic alteration in PSM2, MSH2, MSH6 or MLH1 detected by NGS associated with features of dMMR including high mutation load. Expression of MLH1, PMS2, MSH2 and MSH6 by IHC was assessed by segregating cases in a binary fashion; cases with positive nuclear staining were considered to have protein present and

cases with no nuclear staining were regarded as having absent protein expression (88).

Marker	Code / catalogue number	Supplier	Species	Staining platform	Retrieval buffer and method	Dilution and incubation time	Staining assessment
ATM	ab32420	Abcam	Rabbit monoclonal	BioGenex i6000	pH 9 Target Retrieval Solution Pressure cooker	1:400 1 hour	Nuclear histo-score
MLH1	M3640	Dako	Mouse Monoclonal	BioGenex i6000	pH 8.1 Tris/EDTA Solution Pressure cooker	1:100 1 hour	Binary fashion between absent and present
PMS2	M3647	Dako	Rabbit monoclonal	BioGenex i6000	pH 8.1 Tris/EDTA Solution Pressure cooker	1:100 1 hour	Binary fashion between absent and present
MSH2	M3639	Dako	Mouse Monoclonal	BioGenex i6000	pH 8.1 Tris/EDTA Solution Pressure cooker	1:50 1 hour	Binary fashion between absent and present
MSH6	M3646	Dako	Rabbit monoclonal	BioGenex i6000	pH 8.1 Tris/EDTA Solution Pressure cooker	1:500 1 hour	Binary fashion between absent and present
PTEN	9559	Cell Signaling Technology	Rabbit monoclonal	BioGenex i6000	pH 6 Citrate with Tween Microwave	1:250 1 hour	Binary fashion between absent and present
SOX2	3579	Cell Signaling Technology	Rabbit monoclonal	Bond RX	pH 6 Epitope Retrieval 1	1:100 15 minutes	Nuclear histo-score

Table 1. IHC assays methods for ATM, MLH1, PMS2, MSH2, MH6, PTEN and SOX2

3.5.4. Targeted NGS and WES

Targeted NGS was performed as described previously (55) using a custom Generead (Qiagen) 113 genes panel. DNA was extracted using the AllPrep DNA kit (cat# 80224, QIAGEN). DNA quantity and quality were assessed using Agilent 4200 TapeStation (Agilent, USA) for DINe (DNA Integrity Number equivalent). Libraries for WES were performed using Kapa Hyper Plus Library Prep Kits and the Agilent SureSelectXT V6 target enrichment kit as described previously. Paired-end sequencing was performed using the NovaSeq 6000 S2 flow cell (2x100 cycles; Illumina).

FASTQ files were generated from the sequencer's output using Illumina bcl2fastq2 software (v.2.17.1.14, Illumina) with the default settings. All sequencing reads were aligned to the human genome reference sequence (GRCh37-hg19) using the BWA-MEM algorithm (v. 0.7.12). Picard tools (v.2.1.0) were used to remove PCR duplicates and to calculate sequencing metrics for quality control check. The Genome Analysis Toolkit (GATK, v. 3.5-0) was applied to realign local indels, recalibrate base scores, and identify genetic variants. Somatic point mutations and small indels were called using paired tumour-normal design using MuTect2 with stand_call_conf 30 and stand_emit_conf 30. Somatic variant was further filtered by quality PASS, coverage depth > 10 and allele frequency >5%. By comparing tumour DNA to its matched germline DNA control, copy number estimation was obtained through modified ASCAT2 package using 1) BAF data matrix derived from GATK variants calling and 2) LogR data matrix of sequencing coverage at GATK variant location from Picard CalculateHsMetrics.

3.5.5. BRCA2, CDK12, PALB2 and TP53

BRCA2, CDK12, PALB2 and TP53 pathogenic status was defined as samples with deleterious genomic alterations detected by NGS. Mutation calls from targeted NGS were reviewed manually using Integrative Genomics Viewer (55,102). Mutation annotation was based on available data from public databases

(ClinVar, COSMIC, Human Genome Mutation Database, IARC TP53 Database), published literature, and in silico prediction tool).

3.5.6. TMB

Using the WES data, TMB was defined as the sum of somatic non-synonymous mutations/exome and was calculated using MuTect (version 1) and Variant Effect Predictor (68,103). The cutoff of 175 mutations/exome for TMB-high versus low as calculated from WES corresponds to the FDA approved clinical cutoff for TMB-high of 10 mutations per megabase (mut/mb) using the FoundationOne CDx (F1CDx) assay (68,103).

3.5.7. mRNAseq

Tumour RNA-Seq libraries were prepared according to the manufacturer's protocol using NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina® NEB (#E7760) and ribo depletion using the NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB #6310). All sequencing was performed on the Illumina NextSeq 500 platform (Illumina) with 2 × 75bp read length. FASTQ files were generated using the BCL2FASTQ software.

Sequencing reads were aligned to (human GRCh37/hg19) using Tophat2 (v2.0.7). Gene expression, Fragments Per Kilobase of transcript per Million mapped reads (FPKM), was calculated using Cufflinks.

3.5.8. mRNA signatures

Ten RNA expression signatures representative of key tumour biology and microenvironment elements were derived as previously described (103). RNA signatures included Angiogenesis, Hypoxia, Glycolysis, Proliferation, MYC, RAS, Granulocytic and Monocytic Myeloid-derived Suppressor cells, Stroma/Epithelial to Mesenchymal Transition (EMT)/Transforming Growth Factor Beta (TGFβ), and WNT.

3.6. Study design

This was a single-centre cohort study. The study was performed at the RMH, and an analysis was carried out at the Institute of Cancer Research (ICR). A retrospective approach was taken to collect demographic and clinical data as well as clinical outcomes from electronic medical records. The collected data span the period from October 2014 to July 2019, whereas data collection started in August 2019 and was completed in June 2020. The last day of clinical follow-up was June 2, 2020.

3.7. Statistical analysis

Patient characteristics are reported as median plus first and third quartiles, or as absolute and relative frequencies depending on their level of measurement. The prevalence of each biomarker is reported with its 95% confidence interval (CI). The association between two binary biomarkers was assessed by the phi coefficient. Venn diagrams illustrate expression of biomarkers in combination with each other. The median follow-up from the index date was calculated using the reverse Kaplan-Meier (KM) method. OS was defined as the time from the date of mCRPC biopsy (index date) to death from any cause. Patients without documented death at the time of the last follow-up were censored at the date last seen. KM plots investigated the association between each biomarker and OS. Cox regression provided adjusted hazard ratios with corresponding 95% CIs controlling for potential confounders and known prognostic factors (at diagnosis: Eastern Cooperative Oncology Group and Gleason score >7; at the index date: age, log transformed prostate-specific antigen, and presence of liver metastasis). Six patients had not been treated with docetaxel before the index date for clinical reasons but were included in the final analysis. No measures were taken to impute missing data; complete case analysis was used instead. Statistical analysis was performed using R version 4.0.0.

4. Results

4.1. Population characteristics, biomarker prevalence, and correlations

A total of 100 patients were included in the analysis; the median follow-up from the index date was 56 mo. At that point, 99 patients have died. The median age at the index date was 68 yr. The median between mCRPC diagnosis and index date was 25.5 mo, with Q1 being estimated as 15.75 mo and Q3 as 36.1 mo. Of the 100 mCRPC biopsy sites, 54 (54%) were nodal, 29 (29%) bone, nine (9%) soft tissue, seven (7%) visceral, and one (1%) other; 46/84 (55%) patients had metastatic prostate cancer at diagnosis, and 24/100 (24%) patients had metastatic liver disease at the time of mCRPC biopsy. The median number of treatments was 5 (4, 6), and the median number of treatments before the index date was 3 (2, 4). Sixty patients did not have any chemotherapy line after the index date of biopsy, while 34 patients had one line of chemotherapy and six had two lines of chemotherapy. Cabazitaxel was given to 26 of these patients, docetaxel to five patients, and carboplatin-based chemotherapy to 15 patients. When looking at PD-L1, TcellinfGEP, and SOX2, there were no remarkable imbalances in treatment with cabazitaxel after the biopsy (risk ratio 0.93 [95% CI: 0.37–2.36], 0.86 [0.39–1.89], and 0.63 [0.27–1.51], respectively). A total of 19 cases were classified as visually neuroendocrine (NE; 1% of tumour cells had NE features). Employing a cutoff of 20% of tumour cells having NE features yielded a total of nine cases with a significant number of NE tumours in the biopsy sample. Clinical characteristics by selected biomarkers are presented in Table 2.

	Overall population (n = 100)	**PD-L1 (n=23)**	**GEP (n=24)**	**TMB (n=12)**	**SOX2 (n=27)**	**NeuFea (n=9)**	**dMMR (n=7)**	**ATM loss (n=14)**	**BRCA2 (n=11)**	**CDK12 (n=4)**	**PALB2 (n=1)**	**PTEN loss (n=31)**	**p53 (n=25)**
ECOG at index date													
0	13 (13.3)	1 (4.5)	4 (17.4)	1 (9.1)	4 (14.8)	1 (11.1)	1 (14.3)	2 (14.3)	1 (10.0)	1 (25.0)	0	3 (10.0)	1 (4.0)
1	79 (80.6)	20(90.9)	18(78.3)	10(90.9)	23(85.2)	8 (88.9)	5 (71.4)	11(78.6)	8 (80.0)	3 (75.0)	1	25(83.3)	22(88.0)
≥2	6 (6.1)	1 (4.5)	1 (4.3)	0	0	0	1 (14.3)	1 (7.1)	1 (10.0)	0	(100.0)	2 (6.7)	2 (8.0)
Missing	2	1	1	0	0	0	0	0	1	0	0	1	0
Age at index date in years (IQR)	68.8 (64.8; 73.0)	67.0 (63.5; 72.5)	68.5 (65.5; 72.0)	67.0 (64.5; 70.5)	68.0 (64.0; 74.5)	58.0 (54.0; 71.0)	70.0 (64.5; 73.5)	68.0 (65.0; 73.8)	66.0 (61.5; 70.5)	67.0 (63.8; 69.2)	69.0 (69.0, 69.0)	67.0 (64.5; 73.5)	71 (63.0; 74.0)
Gleason score at diagnosis >7	64 (68.8)	6 (69.6)	18 (85.7)	7 (63.6)	19 (76.0)	6 (85.7)	5 (71.4)	57 (70.4)	8 (80.0)	4 (100.0)	1 (100.0)	18 (60.0)	19 (79.2)
Missing	7	0	3	1	2	2	0	2	1	0	0	1	1

T stage at diagnosis													
1-2	12 (14.0)	2 (9.0)	1 (4.8)	4 (40.0)	3 (14.3)	0	2 (28.6)	2 (14.4)	3 (30.0)	0	0	2 (7.6)	0
3-4	59 (68.6)	17(77.3)	15(71.5)	5 (50.0)	16(76.2)	5 (83.3)	4 (57.2)	7 (53.9)	6 (60.0)	2 (50.0)	1	23(88.5)	18(81.8)
X	15 (17.4)	3(13.69)	5 (23.8)	1 (10.0)	2 (9.5)	1 (16.7)	1 (14.3)	4 (30.8)	1 (10.0)	2 (50.0)	(100.0)	1 (3.8)	4 (18.2)
Missing	14	1	3	2	6	3	0	1	1	0	0	5	3
N stage at diagnosis													
0	29 (35.8)	8 (36.4)	7 (36.8)	5 (45.5)	7 (31.8)	1 (14.3)	3 (42.9)	3 (25.0)	5 (45.5)	1 (33.3)	0	15(57.7)	6(28.6)
1	35 (43.2)	11(50.0)	8 (42.1)	4 (36.4)	11(50.0)	4 (57.2)	2 (28.6)	7 (58.3)	4 (36.4)	1 (33.3)	1	8 (30.8)	11(52.4)
X	17 (21.0)	3(13.69)	4 (21.1)	2 (18.2)	4 (18.2)	2 (28.6)	2 (28.6)	2 (16.7)	2 (18.2)	1 (33.3)	(100.0)	3 (11.5)	4 (19.0)
Missing	19	1	5	1	5	2	0	2	0	1	0 0	5	4
M stage at diagnosis													
0	35 (41.7)	7 (31.8)	6 (31.6)	6 (50.0)	8 (38.1)	3 (42.9)	4 (57.1)	5 (35.7)	7 (63.6)	0	1	13(50.0)	7 (31.8)
1	46 (54.8)	14(63.6)	13(68.4)	5 (41.7)	12(57.1)	4 (57.1)	3 (42.9)	9 (64.3)	4 (36.4)	2(100.0)	(100.0)	12(46.2)	14(63.6)
X	3 (3.6)	1 (4.5)	0	1 (8.3)	1 (4.8)	0	0	0	0	0	0	1 (3.8)	1 (4.5)
Missing	16	1	5	0	6	2	0	0	0	2	0	5	3

Table 2 shows the clinical characteristics of the target population by selected biomarkers. Of note, biomarkers are not mutually exclusive, and this affects sample size for clinical variables.

PD-L1 protein expression, TcellinfGEP score, and TMB results were available for 70, 93, and 85 samples, respectively. PD-L1 was expressed in 23/70 (33%) mCRPC biopsies; 24/93 (26%) had high TcellinfGEP scores and 27/99 (27%) had SOX2 IHC expression (Fig. 1). **Figure 18** depicts the prevalence of the other evaluated biomarkers.

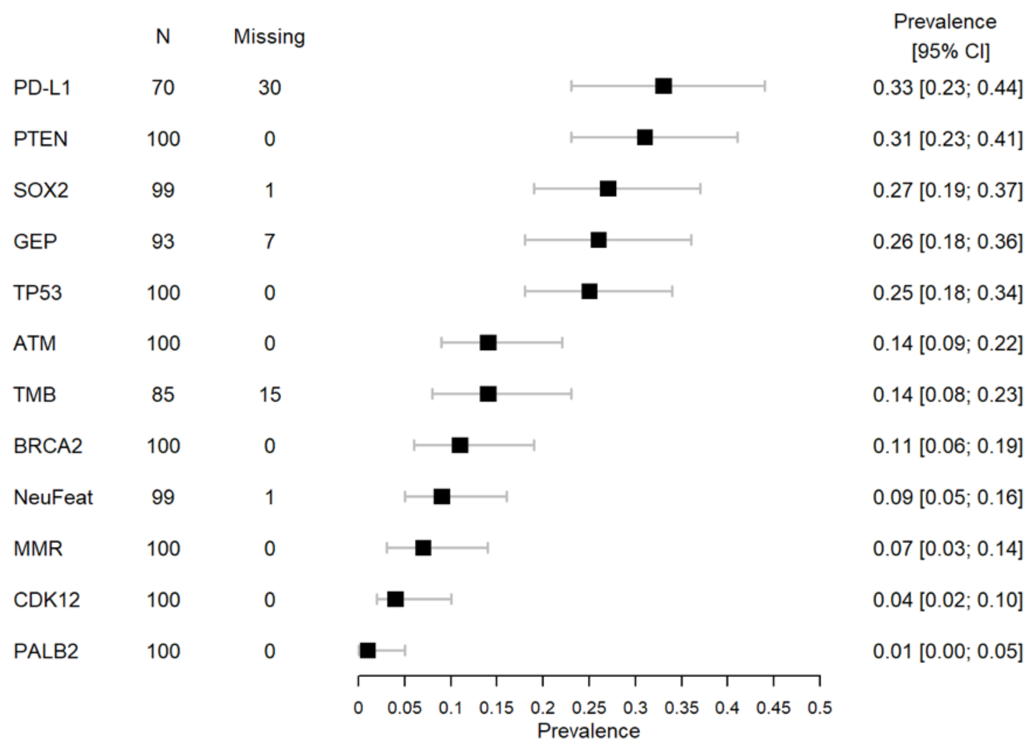


Figure 18 – Forest plot of the prevalence of biomarkers of interest. Number of available samples (N), number of missing values, and the prevalence of the biomarkers of interest, calculated as the number of patients with a biomarker expressed divided by the number of patients with available data for this biomarker, are shown. A 95% confidence interval (CI) for the prevalence is given.

PD-L1 expression and high TcellinfGEP score were positively correlated (phi 0.63 [0.45; 0.76]). The percentages of biopsy sites by PD-L1 and TcellinfGEP are presented in Table 3. Among samples that were negative for PD-L1 expression (n=47), 8 of these biopsies were from soft tissue (17%), whereas in samples that were positive for PD-L1 expression (n=23), 1 was from soft tissue (4.3%). For samples positive for TcellinfGEP expression (n=69), 19 were from bone (27.5%) and 8 from soft tissue biopsies (11.6%). This is in contrast to samples that were positive for TcellinfGEP expression (n=24), where 10 were from bone biopsies (41.7%) and 1 was from soft tissue (4.2%).

	PD-L1 neg. (n=47)	PD-L1 pos. (n=23)	GEP neg. (n=69)	GEP pos. (n=24)
Bone	**0 (0%)**	**0 (0%)**	**19 (27.5%)**	**10 (41.7%)**
Nodal	**34 (72.3%)**	**20 (87%)**	**36 (52.2%)**	**12 (50%)**
Other	**1 (2.1%)**	**0 (0%)**	**1 (1.4%)**	**0 (0%)**
soft tissue	**8 (17%)**	**1 (4.3%)**	**8 (11.6%)**	**1 (4.2%)**
Visceral	**4 (8.5%)**	**2 (8.7%)**	**5 (7.2%)**	**1 (4.2%)**

Table 3. Percentages of biopsy sites by PD-L1 and TcellinfGEP

There was a positive correlation between SOX2 IHC expression and NE features in the histology (phi 0.52 [0.36; 0.65]). No other biomarkers had strong correlations; however, TMB and dMMR were moderately correlated (phi 0.49 [0.31; 0.64]), and 71% of dMMR samples had high TMB (Fig. 2 and Table 4).

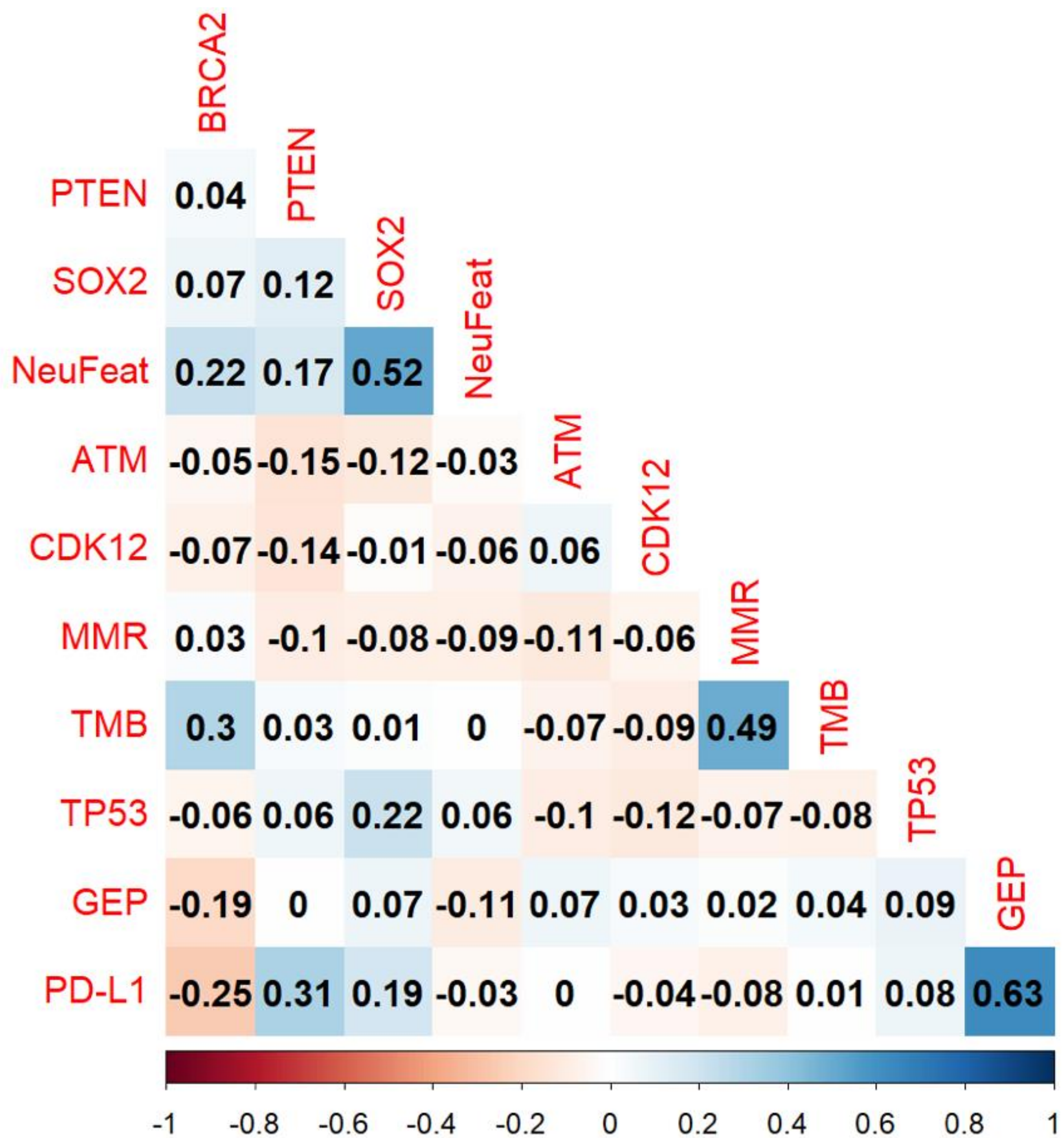


Figure 19 – Phi coefficient to assess the correlation between the expression of different biomarkers. Strong positive correlation is represented by dark blue squares, and strong negative correlation is represented by dark red squares.

Patient ID	MSH2 deleterious alteration (Yes/No)	Subtype of deleterious alteration	MSH2 IHC loss (Yes/No)	MSH6 Deleterious alteration (Yes/No)	Subtype of deleterious alteration	MSH6 IHC loss (Yes/No)	Germline mutation (Yes/No)	Bi-allelic hit (Yes/No)	High TMB (Yes/No)	PD-L1 expression (Yes/No)
059	No	N/A	Yes	Yes	p.I225fs*22	Yes	Yes	Yes	Yes	No
060	Yes	Deep deletion	Yes	No	N/A	Yes	No	Yes	Yes	Yes
062	Yes	Deep deletion	Yes	Yes	Deep deletion	Yes	No	Yes	Yes	No

010	No	N/A	Yes	No	N/A	Yes	No	No	Yes	No
032	No	N/A	No	Yes	p.T128Nfs*	No	Yes	No	No	No
035	No	N/A	No	Yes	p.S580L	No	Yes	No	No	No
039	No	N/A	Yes	No*	N/A	Yes	No	Yes*	Yes	No

Table 4. Correlations of dMMR, high TMB, PD-L1 and mutation origin (germline/ somatic) within the target population.

Co-expression of selected biomarkers is presented in Fig. 20A and 20B. Among the five samples with MMR loss for which PD-L1 was available, one (20%) had detectable PD-L1 1; one of the three CDK12 altered samples also had detectable PD-L1 1 (33%), but none (0%) of the six BRCA2 mutated mCRPC cases expressed PD-L1 (Fig. 20A). Of 14 samples with PTEN loss for which PD-L1 was available, eight (57%) had PD-L1 1; four of the ATM IHC loss samples had PD-L1 1 (33%), and nine of the 19 mCRPC cases that expressed SOX2 showed PD-L1 expression (47%) (Fig. 20B).

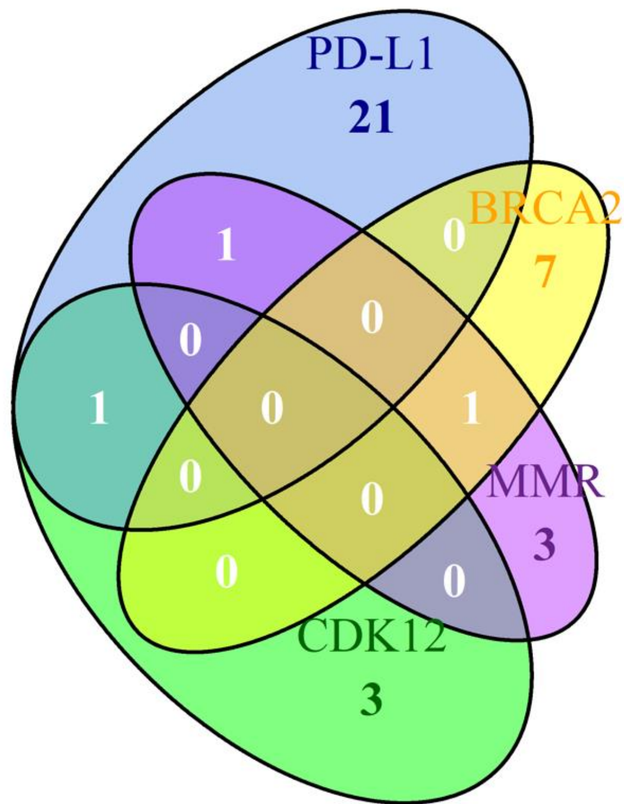


Figure 20 – (A) Venn diagram representing the overlap of PD-L1 expression BRCA2/CDK12 deleterious genomic alterations, and MMR deleterious genomic alterations and/or loss of protein expression. Absolute numbers of biomarker expression and co-expression are shown. The analysis is restricted to patients with available data for all four biomarkers (N = 70).

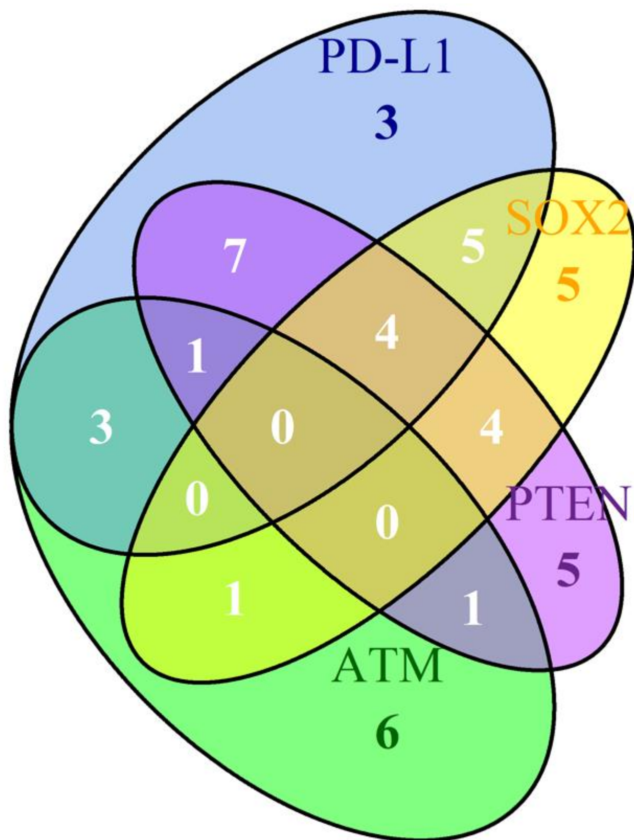


Figure 20 – (B) Venn diagram representing the overlap of PD-L1 expression, ATM loss of protein expression, SOX2 expression, and PTEN loss of protein expression. Absolute numbers of biomarker expression and co-expression are shown. The analysis is restricted to patients with available data for all four biomarkers (N = 70).

4.2. dMMR and TMB characterisation

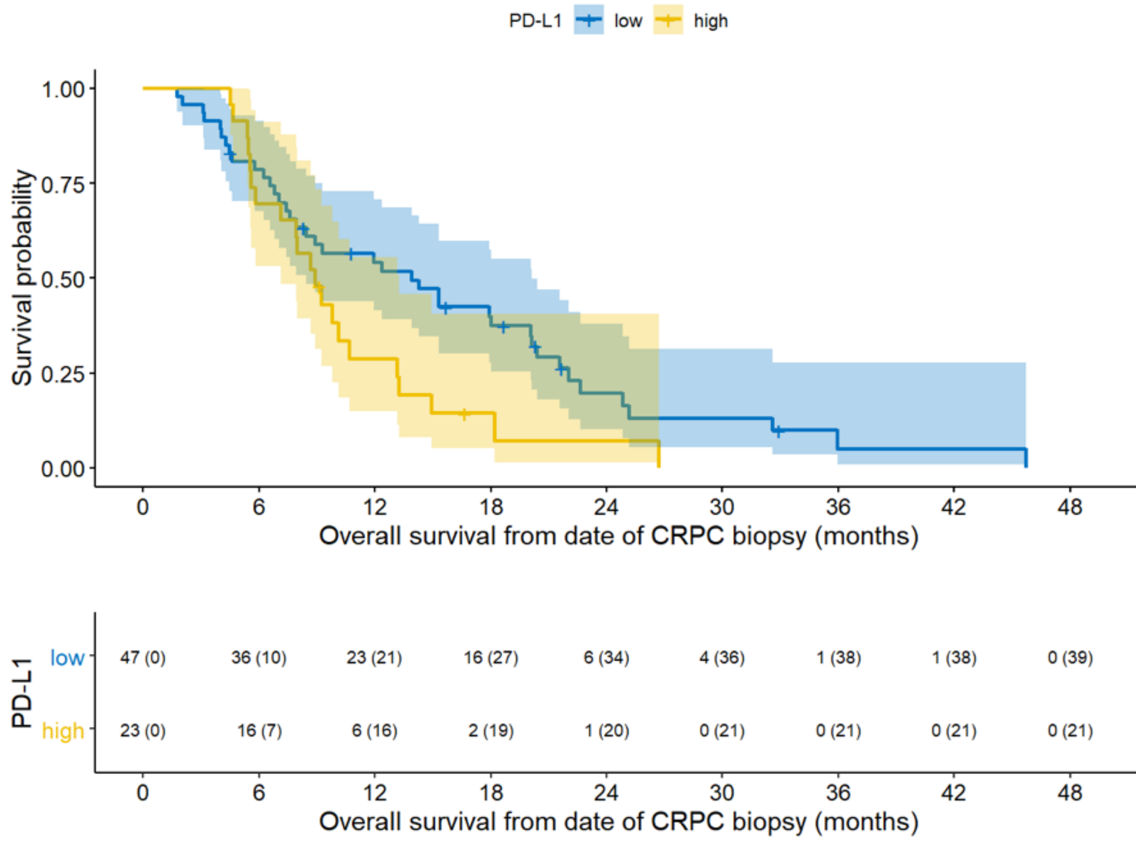
Overall, seven biopsies had dMMR; five of these had deleterious genomic alterations, and two had loss of MSH6 and MSH2 proteins without detectable deleterious genomic alterations (71% and 29%, respectively). Of the five samples with deleterious genomic alterations, three (60%) had germline alterations, three (60%) had biallelic hits, and three (60%) had concomitant MMR protein loss by IHC. This suggests that MMR protein deleterious mutation does not always result in IHC loss of expression (Table 4). Patient 039 had mCRPC with biallelic alteration with a mutation of uncertain significance in MSH6 (S241G); this sample

had MMR protein loss by IHC. Five (71%) of these seven samples with dMMR had high TMB (Table 4). Patients with high TMB had a tendency to worse OS (aHR 1.58 [CI: 0.79; 3.16]).

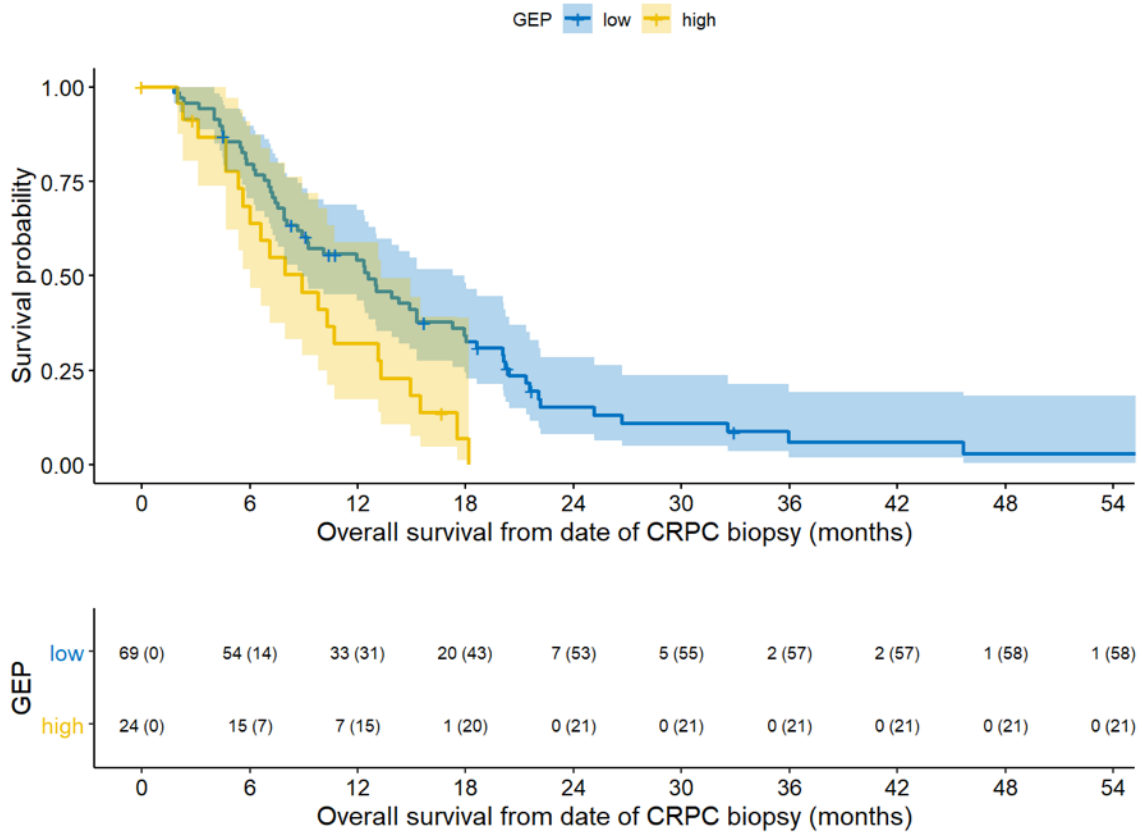
4.3. Correlation between biomarkers of interest and survival outcomes

KM plots revealed worse OS for patients whose mCRPC samples were PD-L1 positive or SOX2 positive, or who had high TcellinfGEP (**Figure 21A, 21B and 21C**). None of the other pre-specified biomarkers showed correlation with OD (**Figure 21 D-K**) PD-L1 expression was associated with an aHR of 1.90 (1.04; 3.45), high TcellinfGEP score with an aHR of 1.86 (1.04; 3.31), and SOX2 positive expression with an aHR of 2.09 (1.20; 3.63) (Figure 22). Although the precision of the estimate was lower due to more unbalanced groups, Cox regression also indicated worse prognosis for patients with ATM loss (aHR 1.72 [0.88; 3.37] (**Figure 22**) and those with high TMB (aHR: 1.58 [0.79; 3.17], (**Figure 22**) and improved OS for patients with BRCA2 deleterious alterations (aHR: 0.48 [0.21; 1.10] (**Figure 22).** Of note, of the 11 patients with BRCA2 deleterious alterations, eight were treated with olaparib and/or carboplatin.

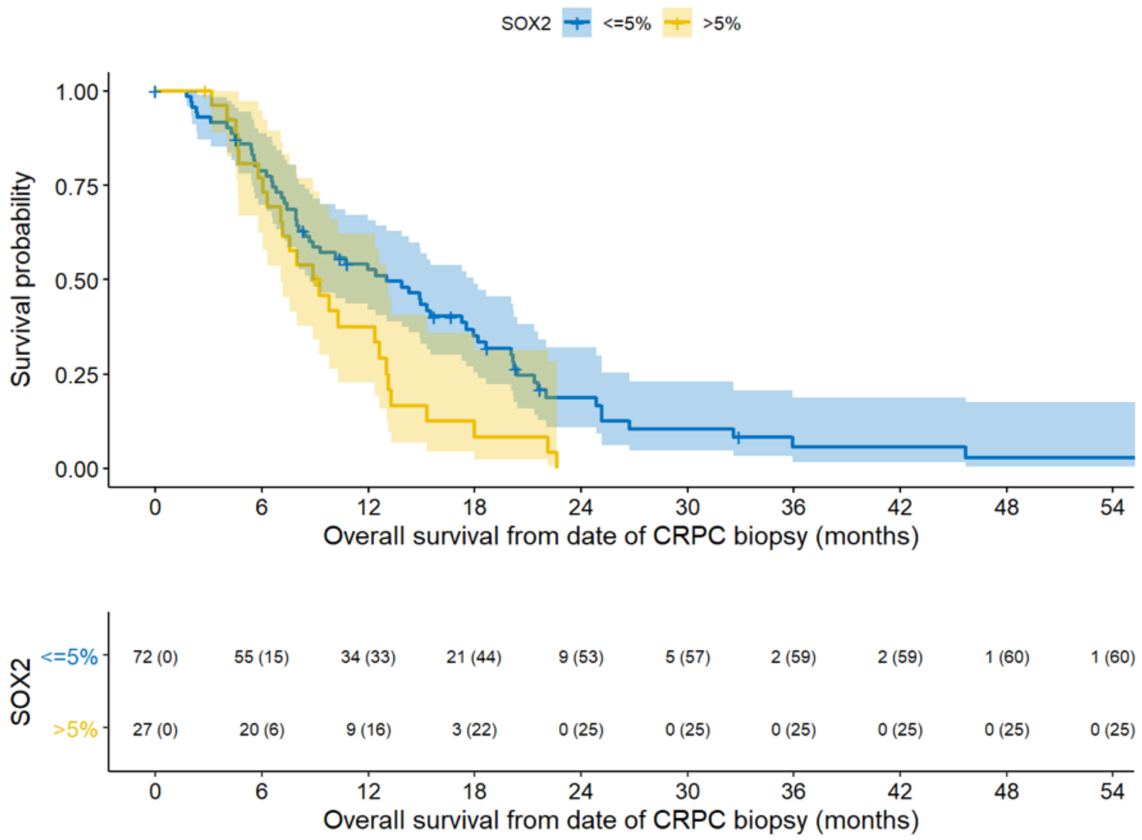
A)



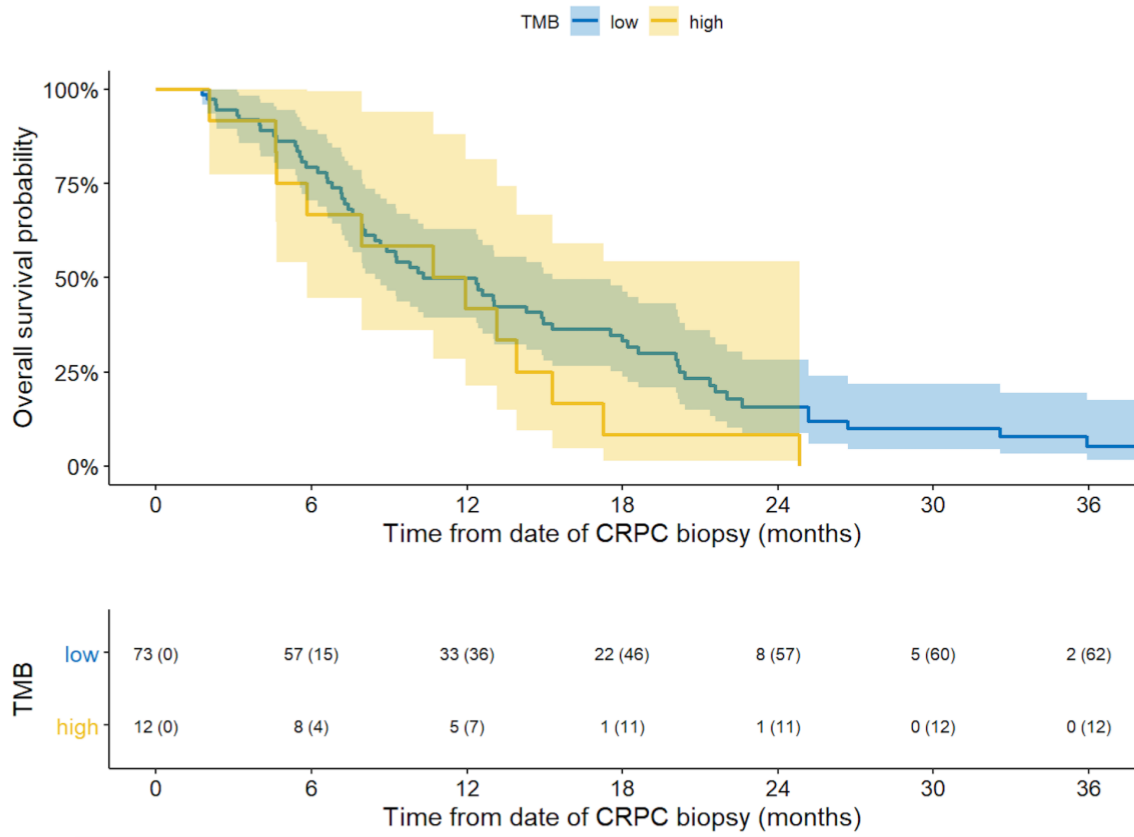
B)



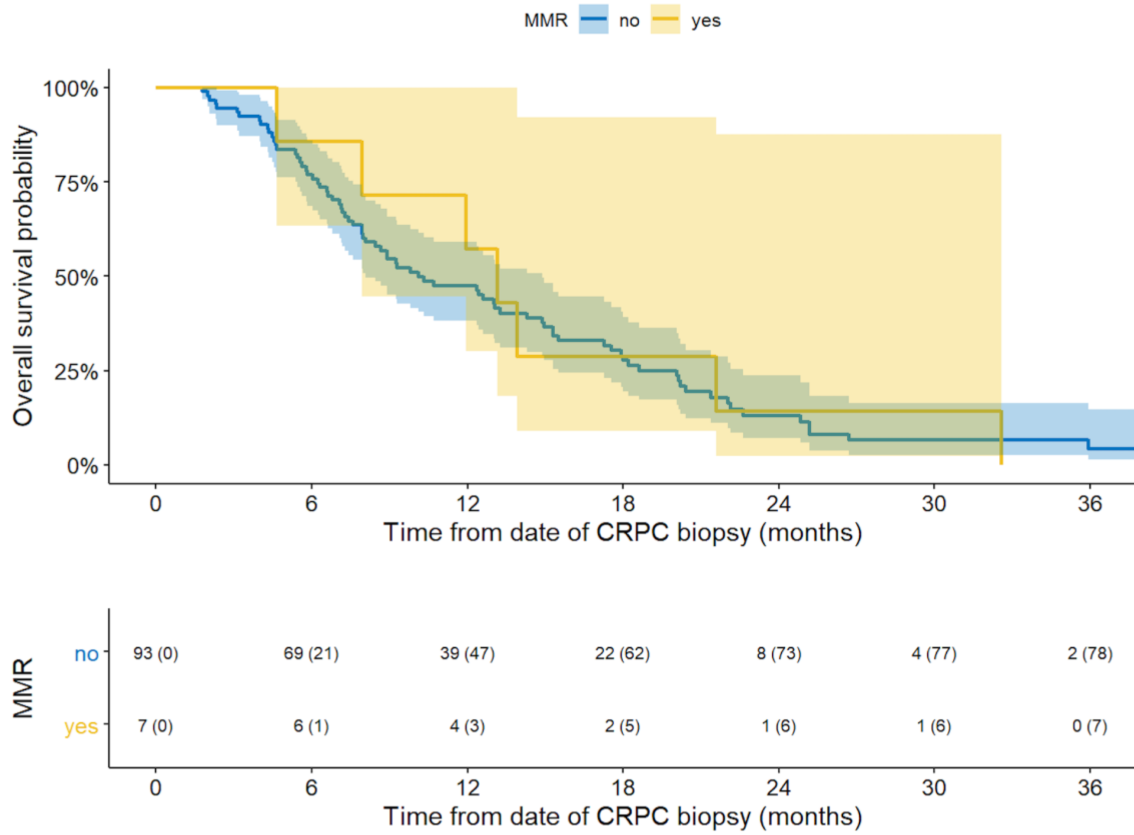
C)



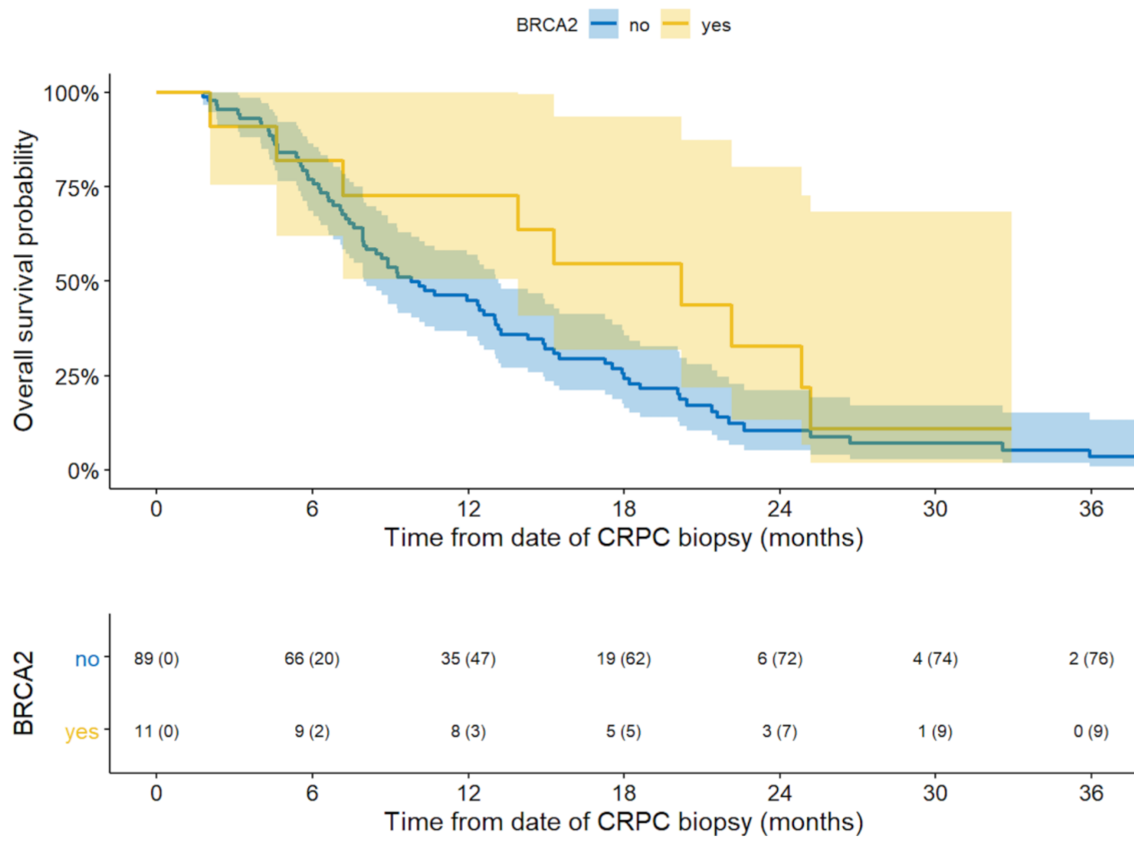
D)



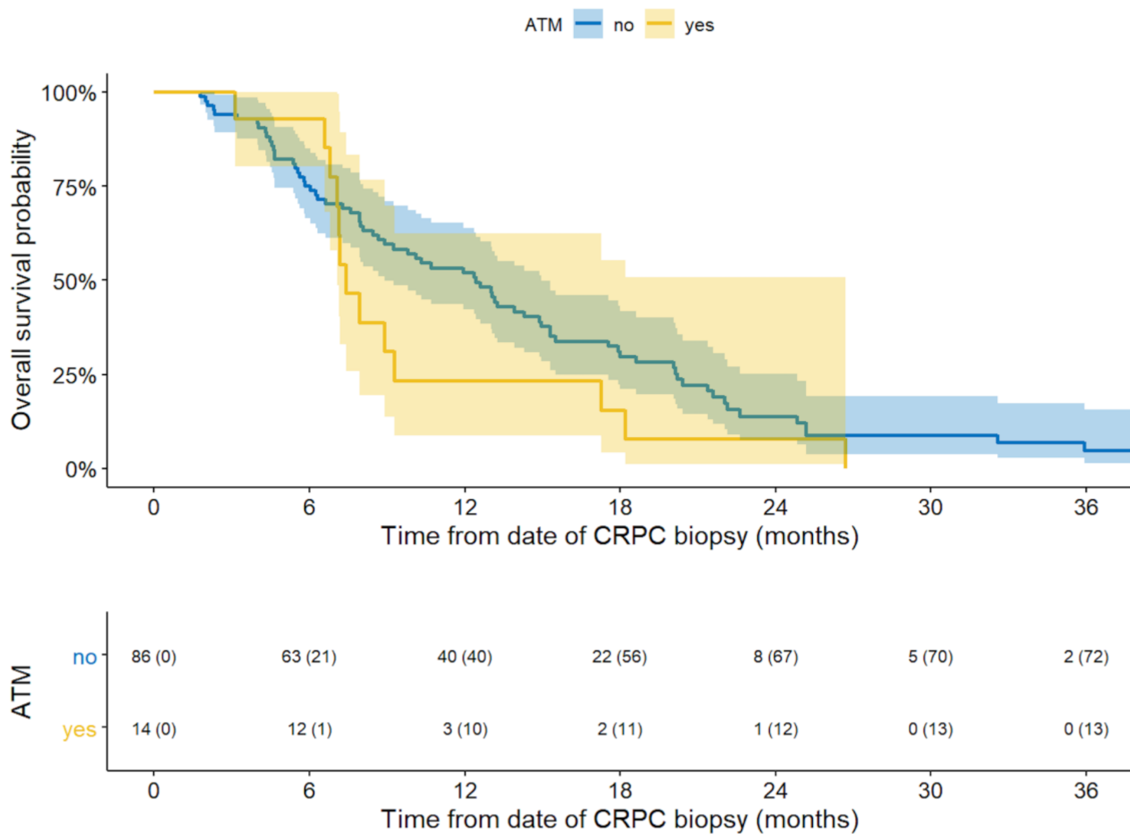
E)



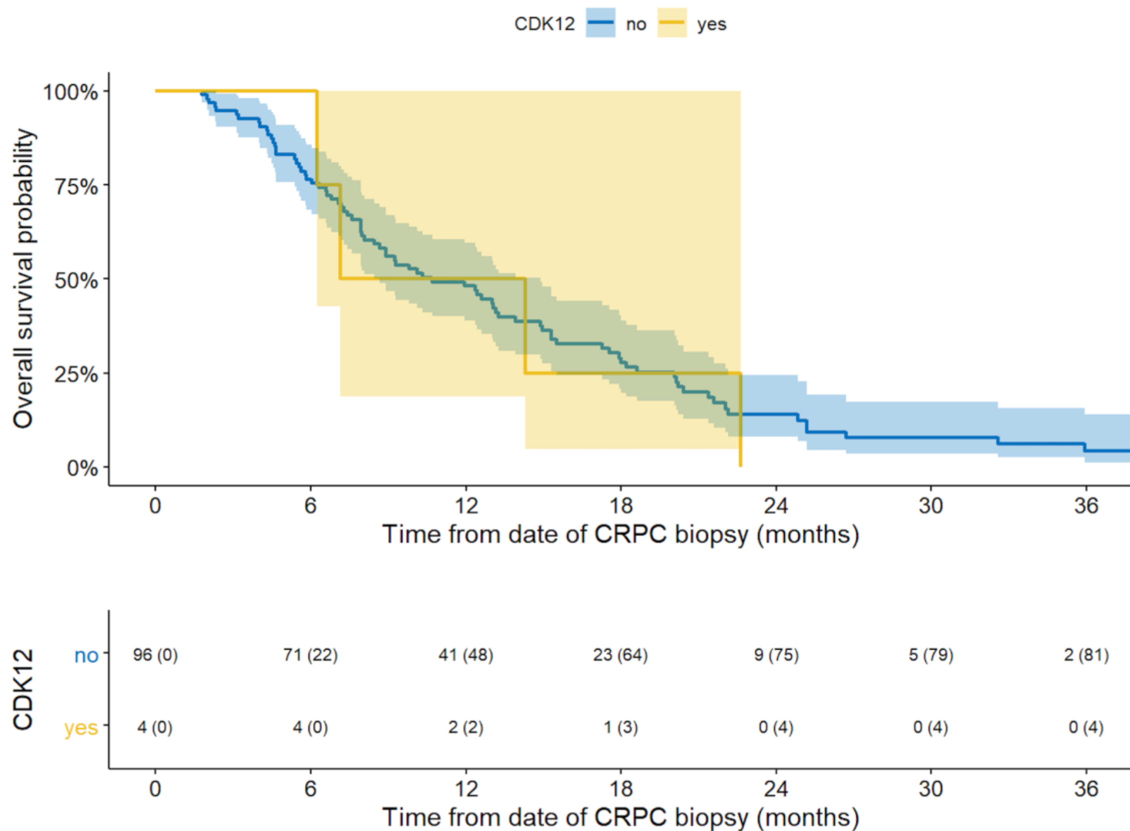
F)



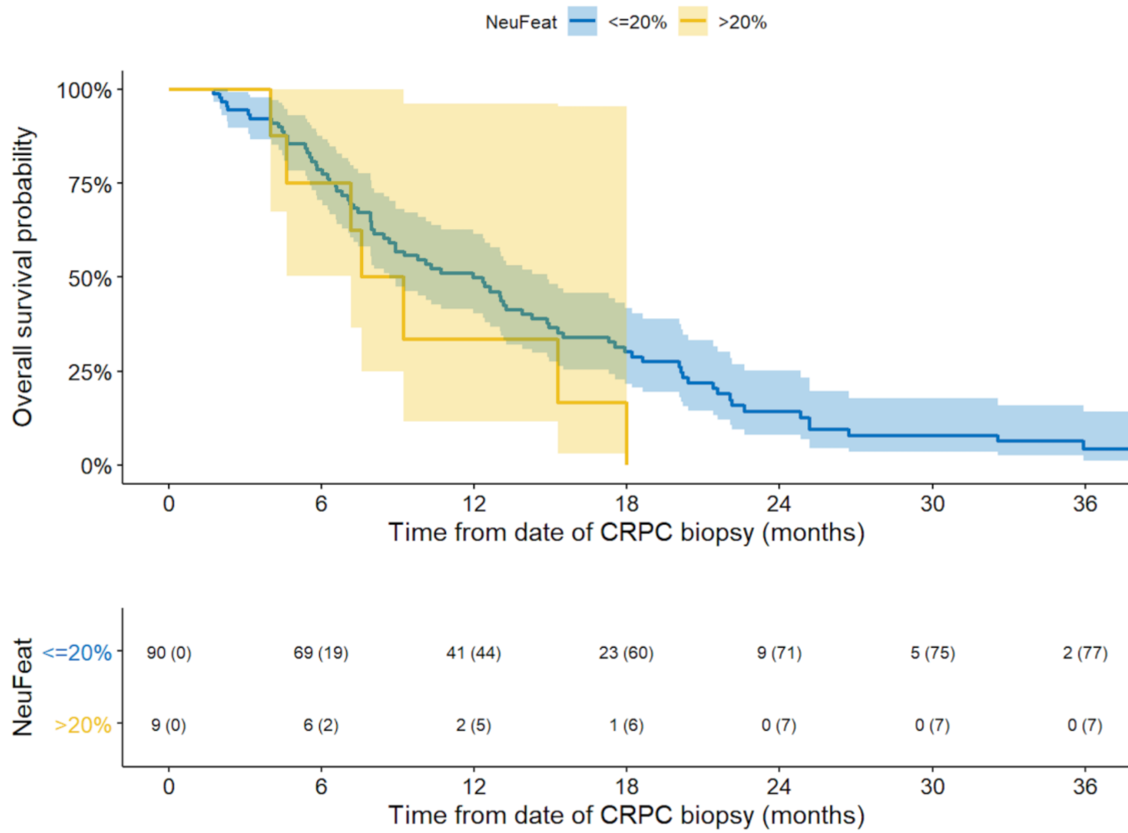
G)



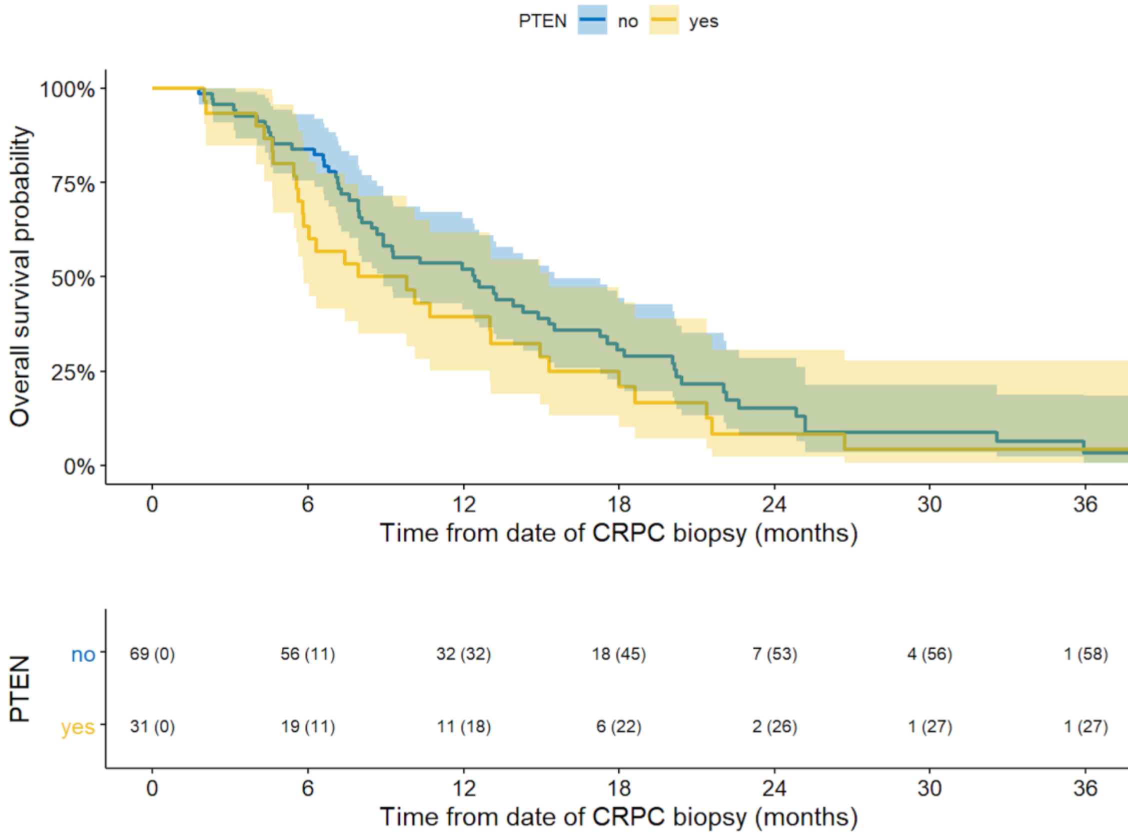
H)



I)



J)



K)

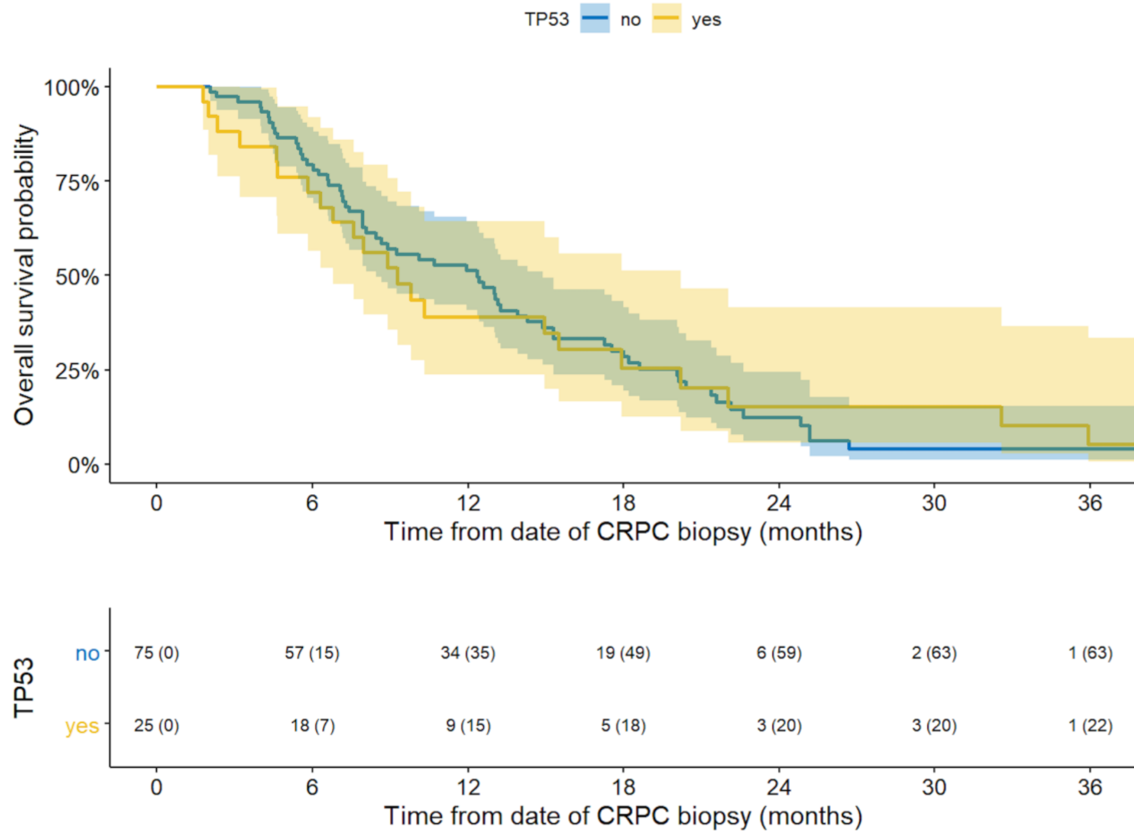


Figure 21 – Kaplan-Meier plots showing OS by biomarker of interest. (A) High PD-L1 expression defined as a combined positive score of 1 versus low PD-L1 expression. The median OS was shorter for patients with high PD-L1 expression (9 mo) than for patients with low PD-L1 expression (14 mo). (B) A high TcellinfGEP score, defined as a TcellinfGEP score of > –0.318, versus a low TcellinfGEP score. The median OS was shorter for patients with a high TcellinfGEP score (9 mo) than for patients with a low TcellinfGEP score (13 mo). (C) High SOX2 expression, defined as a percentage of cells with SOX2 expression of >5%, versus low SOX2 expression. The median OS was shorter for patients with high SOX2 expression (9 mo) than for patients with low SOX2 expression (13 mo). Note that survival curves have been truncated at 36 mo due to sparse data. (D-K) None of the other pre-specified biomarkers showed correlation with OS.

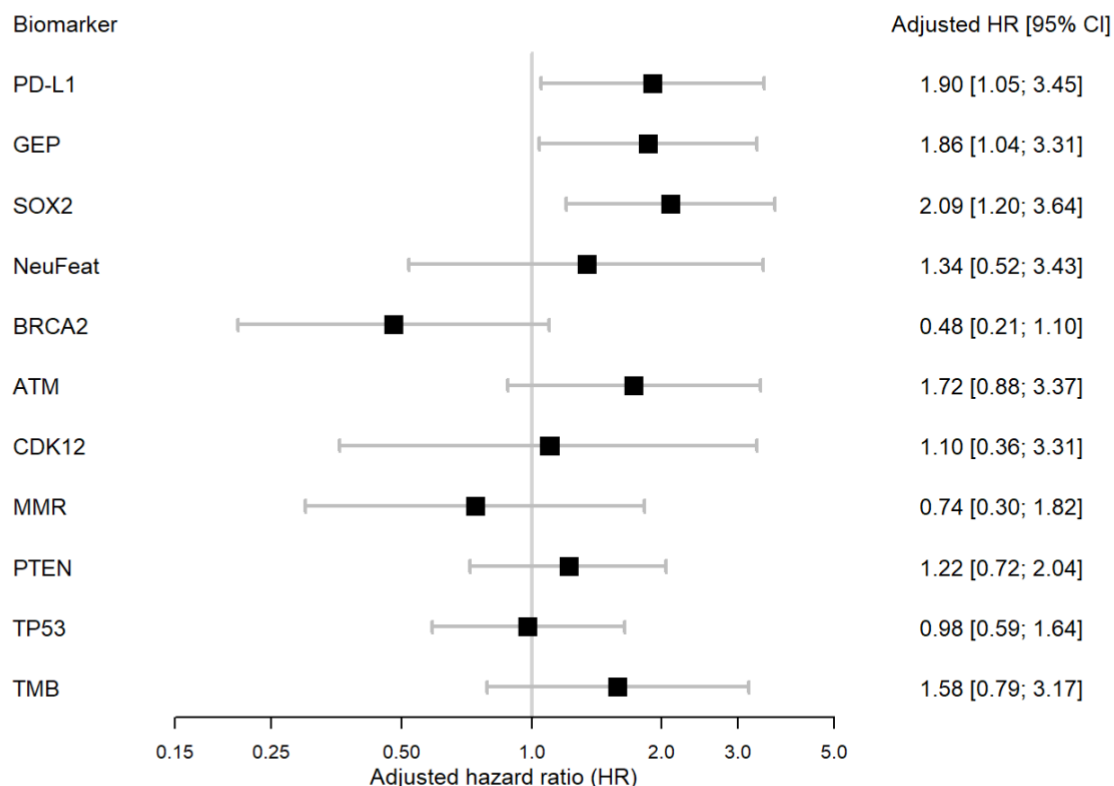


Figure 22 – Forest plot of adjusted hazard ratios for biomarkers of interest. Hazard ratios and their respective 95% confidence intervals are from a Cox regression model with the respective biomarker of interest as explanatory variable and controlling for diagnostic ECOG and diagnostic Gleason score >7 as wells as age, log-transformed PSA, and presence of liver metastasis (all at the index date). ECOG = Eastern Cooperative Oncology Group; PSA = prostate-specific antigen.

5. Discussion

In this study, we report that PD-L1, SOX2 IHC expression, and a high TcellinfGEP score are detected in 33%, 27%, and 26% of mCRPC biopsies, respectively, and that they are associated with shorter OS. This is the first study reporting that these biomarkers, when studied in the mCRPC setting, are associated with OS. We also found a positive correlation between PD-L1 expression and a high TcellinfGEP score, and between SOX2 expression and neuroendocrine histology features. Prospective studies should validate these findings, which are relevant to their study as putative biomarkers predictive of prognosis in phase 3 trials.

PD-L1 and TcellinfGEP are biomarkers representative of immune inflammation, and both of them have been associated with poorer outcomes in this study. The rational to justify why these biomarkers could have an impact in the cells infiltrating mCRPC TME has been extensively discussed in this manuscript. The aim of this study was not to assess how these biomarkers correlate with the immune cells infiltrating the mCRPC TME, but our results support the hypothesis that PD-L1 and TcellinfGEP could promote an immunosuppressive infiltrate, prompting cancer growth, progression and worse clinical outcomes. In fact, previous studies have already demonstrated a relationship between tumoural PD-L1 expression and changes in macrophage response in prostate cancer tumours, with PD-L1 promoting a M2 polarization (104).

One of the novelties of this study is the characterisation of PD-L1 as a likely biomarker of prognosis in mCRPC. As discussed, PD-L1 was detected in 33% of our mCRPC patients. Although previous research has suggested that PD-L1 expression may indeed be a biomarker predictive of prognosis in prostate cancer, these reports mainly assayed primary tumours, while our study analysed mCRPC biopsies. Two studies showed that PD-L1 expression could be an independent indicator of biochemical recurrence, whereas another study reported that PD-L1 expression is associated with a higher risk of clinical progression in men with node positive prostate cancer. A limitation of such data published based on localised disease from primary prostate tissue, however, is that biomarkers may change as cancers progress to the metastatic setting. In addition, these studies did not assess the association between PD-L1 and OS in the metastatic setting. Of note, most patients in our target population did not receive treatment with ICI, so the potential of PD-L1 as a biomarker predictive of response to anti-PD1/anti-PD-L1 therapies in mCRPC could not be evaluated in this cohort. However, as previously discussed, the results of anti-PD1/anti-PD-L1 therapies in mCRPC have been disappointing so far in molecularly unselected patients and looking at the results of previous clinical trials, there is not enough evidence to support PD-L1 as a biomarker predictive of response to ICI.

We detected a high TcellinfGEP score in 27% of our patients, and this is another of our pre-specified biomarkers that showed to be prognostic in this cohort. As previously discussed, this panel evaluated the gene expression profile of eighteen genes implicated in different steps of the immune response, including cytolysis, antigen processing and presentation, and T cell response. Although this panel had previously been validated to predict tumour responses to anti-PD1 therapies, its characterisation as a potential biomarker of prognosis in mCRPC is another of the novelties of this study. Of note, the biological implications of a high expression of the genes assessed in this panel were only tested at an RNA level in this study. In future, elucidating how a high TcellinfGEP score could correlate with the immune infiltrate in mCRPC TME, or how a high expression of these genes correlate with their protein expression is guaranteed. In fact, a better understanding of how some of these molecules could tame the TME in mCRPC to promote or inhibit cancer growth and progression is a promising approach to improve the outcomes of these patients. In this setting, previous studies have demonstrated that TAMs infiltration increases with prostate cancer progression, and in vitro experiments indicate that TAMs are able to secrete CCL5 and to promote prostate cancer stem cells (PCSCs) self-renewal and metastasis via activating β-catenin/STAT3 signaling (105). Other in vivo experiments have also shown that CCL5 silencing in TAMs significantly inhibit prostate cancer xenografts growth, bone metastasis as well as prostate cancer stem cells PCSCs self-renewal and tumourigenicity (105). This TAMs/CCL5 axis can also promote drug resistance through the STAT3 signalling pathway, and in vitro studies have shown that the blockade of STAT3 can efficiently re-sensitise prostate cancer models to treatment with chemotherapy (105). It has also been reported that that dendritic cell–based vaccines and anti-CD27 antibodies can have a synergistic activity against prostate cancer (106), so future studies in this setting should assess how the expression of this protein in mCRPC TILs could interact with dendritic cells in mCRPC TME. B7-H3, another gene assessed in the TcellinfGEP panel, is an important modulator in prostate cancer immunity (107). Although the function of B7-H3 in cancer is controversial, the majority of clinical data suggest a positive correlation with tumour progression (107). B7-H3 is weakly expressed in normal prostate epithelium, but more than 93% of prostate cancer express this protein (108). In vitro and in vivo experiments have shown that B7-H3 can

promote MDSCs accumulation in TME and promote prostate cancer progression (109). For that reason, a better understanding of how B7-H3 can modulate mCRPC TME is guaranteed in prospective studies.

CMKLR1, CXCL9 and CXCR6 were also assessed in the TcellinfGEP panel. In vitro experiments have shown that the activation of CMKLR1 induces the migration of macrophages and dendritic cells, suggesting a pro-inflammatory role (73). Controversially, in vivo studies using CMKLR-deficient mice suggest an anti-inflammatory role for this receptor, possibly due to the recruitment of tolerogenic dendritic cells (73). Previous studies in prostate cancer cell lines have demonstrated an inverse relationship between PTEN and PD-L1 expression mediated by CMKLR1, and CMKLR1 has also shown to improve T-cell–mediated cytotoxicity in prostate cancer cells (110). These results should be replicated in vivo an/or in mCRPC biopsies. High expression of CXCL9 showed to inhibit T cell infiltration into TME in a prostate cancer mouse model (74). The CXCR6/CXCL16 axis significantly contributes to prostate cancer cell metastasis and subsequent bone invasion (111).

IDO1, LAG3 and TIGIT are other three genes included in the TcellinfGEPpanel. As previously discussed, IDO1 affects differentiation and proliferation of immune cells, especially Tregs. In prostate cancer, the upregulation of IDO1 seems to increase the number of Tregs and to be associated with EMT, prompting immune evasion and tumour progression (112). LAG3 is a marker of T cell exhaustion well characterised in several solid tumours, but its role in mCRPC is still unclear. As previously discussed, TIGIT plays an essential role in regulating NK cells proliferation and cytokine production, disrupting their cytotoxicity activity. Moreover, TIGIT signalling has been associated with CD8+ lymphocytes and NK cells exhaustion, although these results have not been specifically replicated in mCRPC yet.

SOX2 was expressed in 26% of our patients and is the third biomarker associated with worse clinical outcomes in this study. As previously discussed, SOX2 is associated with lineage plasticity, with its levels increasing in CRPC with NE-like or basal disease emergence. In our cohort, SOX2 IHC expression and NE

histological features were positively correlated, and 47% of the SOX2 positive samples co-expressed PD-L1, although we did not find a positive correlation between SOX2 and PD-L1 expression. SOX2 indeed promotes the transcription of PD-L1 as per previous studies in non-prostate tumours, and also promotes adaptive immune resistance and impairment of the innate immunity by degradation of interferon genes (STING) (98, 113). Research in other hormone-dependent tumours suggests that the JAK1-STAT3-SOX2 signalling axis may upregulate the expression of PD-L1 and promote treatment resistance, with the activation of this pathway being mediated by the interleukin 20 receptor subunit alpha (IL20RA) (114). Notably, IL20RA is also reported to be a binding partner for B7-H3, which as previously discussed is highly expressed in prostate cancer and may promote prostate cancer progression by inducing MDSCs accumulation in the tumour microenvironment (109, 115). Overall, our results and this biological rational support that SOX2 expression and lineage plasticity to a basal phenotype may associate with increased PD-L1 expression in mCRPC, a strategy that merits further evaluation in prospective studies. Future studies should also test how NE changes impacts PD-L1 upregulation and sensitivity to PD-1/PD-L1 blockade in mCRPC, to address how these observations tie in with the evolution of NE prostate cancer as the disease transitions following treatment failures.

TMB is a consolidated biomarker predictive of response to PD-1/PD-L1 targeting therapies in a variety of tumours, and pembrolizumab is approved by the Food and Drug Administration for TMB-high solid tumours. Prostate cancer is known to have lower TMB than many other solid tumours, and the findings from this cohort in which 14% of tumour samples had high TMB are consistent with previous studies. In this cohort, patients whose samples showed high TMB tended to have worse OS, although 95% CIs did not confirm this tendency (aHR 1.58 [CI: 0.79; 3.16]). Previous reports in this aspect are controversial, since high TMB has been reported to correlate with both better and worse OS in prostate cancer (116,117). However, those studies assayed primary biopsies instead of metastatic biopsies.

Although the biological rational to justify why tumours with PD-L1 expression, DDR, dMMR and/or CDK12 deleterious alterations could have a high TMB has

been discussed above, we did not find a positive correlation between these biomarkers and a high TMB in our cohort. However, 71% of patients with dMMR had a high TMB, and high TMB and dMMR were moderately correlated. These finding merit further assessments in future studies.

The prevalence of dMMR and CDK12 biallelic alterations in our cohort were 7% and 3% respectively. None of these biomarkers presented correlation with OS or with the expression of the other pre-specified biomarkers, although previous reports have suggested that CDK12 altered prostate cancers have worse outcomes (89). Twenty and 33% of the dMMR and CDK12 mutated samples respectively had detectable PD-L1 by IHC. Despite small numbers and the lack of a positive correlation, the overlap expression between PD-L1 and dMMR in this cohort seems higher compared to previous studies and requires prospective validation (118). Although CDK12 biallelic loss prostate cancer has been previously associated with a high TMB and neoantigen load, we were unable to replicate this finding in our study.

As previously discussed, defects in DDR proteins can induce genomic instability and upregulate PD-L1 expression by producing tumour-associated neoantigens. Notably, deleterious DDR gene alterations have recently been reported to be associated with improved clinical outcomes in patients with bladder and renal cancer when treated with immune checkpoint inhibitors, but the success of this approach has not been demonstrated in mCRPC so far (119). In our cohort, 43% and 8% of the samples with BRCA2 deleterious genetic alterations and ATM loss, respectively, had high TMB, but none of these biomarkers presented a positive correlation with a high TMB, or presented a positive correlation with PD-L1 expression. Cox regression suggested worse prognosis in patients with ATM loss, unlike in previous reports (57). However, the precision of our estimate was lower due to unbalanced groups and small numbers, so this finding needs validation in prospective studies. Of note, the Cox regression analysis also suggested that BRCA2 patients had a better prognosis. However, this finding can be explained by the fact that most of these patients received treatment with PARP inhibitors or carboplatin.

PTEN could be another biomarker implicated in PD-L1 regulation in prostate cancer. However, we did not find a positive correlation between PTEN loss and PD-L1 expression in our biopsies. This may be due to the fact that we only assessed PTEN status at a protein level, without taking into account other potential genomic deleterious alterations in other components of the PI3K pathway.

This study has inherent limitations related to its retrospective, single-center design, including the heterogeneity of treatment regimens administered to the patients and the availability and completeness of treatment response data. However, all patients received at least one novel hormonal agent, and 94/100 patients received docetaxel. While the sample size was relatively small, strengths of this study include the deep and novel analyses of mCRPC biopsy specimens including a wide range of biomarkers potentially relevant to immune therapy, and the ability to assess OS on all patients. These findings would benefit from replication in other prospective mCRPC cohorts. Of note, our study population included patients whose disease had already progressed to the most effective therapies. This could limit the generalizability of our findings.

6. Conclusions

From this study, we can derive the following conclusions:

1) PD-L1 expression, high TcellinfGEP scores, and SOX2 expression associate with poorer prognosis in mCRPC. These results could impact the design of future phase 3 trials if validated in prospective cohorts.

2) PD-L1 expression and a high TcellinfGEP score are likely biomarkers predictive of a T-cell–inflamed tumour microenvironment in mCRPC. Future studies should assess how these biomarkers correlate with TME immune infiltrate in mCRPC, and how the gene expression assessed in the TcellinfGEP panel correlates with the protein expression of these molecules.

3) BRCA2, ATM, dMMR and CDK12 deleterious aberrations did not show any statistically significant correlation with a high TMB or PD-L1 expression in mCRPC. High TMB and dMMR had a moderate positive correlation which needs to be better characterised in future studies.

4) High TMB and ATM loss may associate with worse outcomes in mCRPC, although these results need validation and are discordant with our previous studies; the characteristics of our target population may limit the generability of these results.

5) SOX2 expression and neuroendocrine histology features were positively correlated in this cohort. Although a positive correlation between SOX2 and PD-L1 expression was not detected, 47% of the samples with SOX2 expression had PD-L1 expression. Prospective studies should test how SOX2 and PD-L1 expression could impact neuroendocrine mCRPC evolution, and the role of PD1/PD-L1 targeting therapies in this setting.

7. References

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