School of Life, Health and Chemical Sciences

**EZH2 as a therapeutic target for aggressive prostate cancer**

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Thesis submitted in partial fulfilment of the requirements for the degree of Master of Philosophy

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Abbreviation

A
acetylation
(Ac) ................................................................................................................. 37
Aggressive variants of prostate cancer
(AVPC) ............................................................................................................. 9
albumin standard
(BSA) .................................................................................................................. 48
American Type Culture Collection
(ATCC) ................................................................................................................ 46
Androgen deprivation therapy
(ADT) ................................................................................................................ 20
androgen receptor
(AR) ..................................................................................................................... 9
androgen-response-elements
(ARE) ................................................................................................................... 19
arginine
(R) ......................................................................................................................... 38
B
Basic Local Alignment Search Tool
(BLAST) ................................................................................................................ 46
Benign prostatic hyperplasia
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bicinchoninic acid
(BCA) .................................................................................................................... 48
C
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(CRPC) .................................................................................................................. 18
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cell viability
(IC50) ...................................................................................................................... 52
chloride
(Cl−) ....................................................................................................................... 26
chromobox proteins
(CBX2, 4, 6, 7, 8) .................................................................................................. 38
Chromogranin A
(CHGA) ................................................................................................................... 12
complete response
(CR) ....................................................................................................................... 24
cuprous ion
(Cu1+) .................................................................................................................... 48
cyclin D-dependent kinases
(CDK4 and CDK6) ........................................................................................................ 39

D cyclin-dependent kinase inhibitor 2A
(CDKN2A) .................................................................................................................... 39

D dehydroepiandrosterone
(DHEA) ................................................................................................................................ 19

D Diffuse Large B-Cell Lymphoma
(DLBCL) ................................................................................................................................ 44
dihydrotestosterone
(DHT) ..................................................................................................................................... 20
disease stabilisation
(DS) .......................................................................................................................................... 24
DNA binding domain
(DBD) ........................................................................................................................................ 19

E embryonic ectoderm development
(EED) ........................................................................................................................................ 9

E Embryonic Ectoderm Development
(EED) ......................................................................................................................................... 38
enhancer of zeste homologue 2
(EZH2) ....................................................................................................................................... 9
epigenetic regulators
(EpRs) ......................................................................................................................................... 37
epithelial-to-mesenchymal transition
(EMT) ......................................................................................................................................... 33
Erythroblast transformation specific
(ETS) .......................................................................................................................................... 33

F FDA
(Food and Drug Administration) ............................................................................................ 25
F Fetal Bovine Serum
(FBS) ......................................................................................................................................... 46
follicle-stimulating hormone
(FSH) .......................................................................................................................................... 19
four parameters logistic
(4PL) ......................................................................................................................................... 53

G GSK-2816126
(GSK-126) ............................................................................................................................. 42
histone 3 lysine 27 di- and trimethylation
(H3K27me2 and H3K27me3) ................................................................. 38

histone 3 lysine 4 di- and trimethylation
(H3K4me2 and H3K4me3) ....................................................................... 38

histone 3 lysine 9 di- and trimethylation
(H3K9me2 and H3K9me3) ....................................................................... 38

histone 3 lysine 9 mono-methylation
(H3K9me1) .................................................................................................. 38

histone 3 tail at lysine 4
(H3K4) ........................................................................................................ 34

histone H2A at lysine 119
(H2AK119Ub1) .......................................................................................... 38

histone H3 Lys27 tri-methylation
(H3K27me3) ............................................................................................... 9

histone methyltransferases
(HMTs) ........................................................................................................ 9

Histone post-translational modifications
(PMTs) ........................................................................................................ 9

intermediate cells
(EM) ............................................................................................................ 34

keratins 8
(K8) .............................................................................................................. 12

luteinizing hormone
(LH) ............................................................................................................ 19

luteinizing hormone-releasing hormone
(LHRH) ........................................................................................................ 19

lysine
(K) ................................................................................................................ 38

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(KMT2C) ...................................................................................................... 34

Lysine (K) specific methyltransferase 2D
(KMT2D) ...................................................................................................... 34

mesenchymal
(M) ............................................................................................................. 34

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(M) ............................................................................................................. 16
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Prostate cancer (PCa)

Prostate specific antigen (PSA)

prostatic acid phosphatase (PSAP)

Prostatic intraepithelial neoplasia (PIN)

Radiotherapy (RT)

recommended Phase II dose (RP2D)

S-adenosylmethionine (SAM)

sex hormone-binding globulin (SHBG)

Small Cell Carcinoma (SCC)

streptavidin horseradish peroxidase (HRP)

suppressor of zeste 12 (SUZ12)

synaptophysin (SYP)

Tazemetostat (EPZ-6438)

Trans urethral resection of the prostate (TURP)

transfer buffer (TBS)

transmembrane protease serine 2 (TMPRSS2)

trimethylation of histone H3 at lysine 27 (H3K27me3)

Tumour (T)

Tumour, Node, Metastasis (TNM)
Abstract

Background

Prostate cancer (PCa) is a malignant neoplasia of the prostate gland. Aggressive variants of prostate cancer (AVPC) are a subtype of metastatic castration resistant PCa (mCRPC), which display reduced or absent androgen receptor (AR) -dependent signalling. AVPCs show early and extensive visceral metastases, and much worse prognosis than other PCas. Neuroendocrine prostate cancer (NEPC), often with Small Cell Carcinoma (SCC) features, is one of the AVPC subtypes. Anaplastic PCa is the other main type of AVPC. AVPCs are generally treated with platinum-based chemotherapy, but therapeutic responses are short-lived.

Epigenetic gene regulation comprises all heritable phenotypic alterations that are not caused by changes in DNA primary structure. Epigenetic alterations are attractive therapeutic targets since they are reversible and targetable by small molecule inhibitors. Some epigenetic alterations are measurable in biological fluids and could be therefore used for treatment monitoring. The main mechanisms of epigenetic regulation are histone modifications, DNA methylation, and chromatin remodelling. Histone post-translational modifications (PTMs) play a key role in the regulation of gene expression and promote tumour initiation and progression. Methylation on lysine residues has been shown to modulate gene expression. Histone methylation is catalysed by histone methyltransferases (HMTs) such as enhancer of zeste homologue 2 (EZH2). EZH2 acts in complex with suppressor of zeste 12 (SUZ12) and embryonic ectoderm development (EED) to catalyse histone H3 Lys27 tri-methylation (H3K27me³) and silence target genes. H3K27me³ levels positively correlate with PCa progression and metastasis. EZH2 overexpression promotes cell viability and invasion in vivo. Three small molecule EZH2 inhibitors (Tazemetostat, GSK126, and CPI-1205) are currently in clinical trials.

Even though EZH2 has been proposed as an attractive therapeutic target in PCa, no study has explored the efficacy of combining EZH2 inhibitors with platinum-based chemotherapy in AVPC.

Materials and Methods

The expression and gene amplification of EZH2, EED and SUZ12 in clinical PCa samples were analysed by using an interactive open-source platform (cBioPortal for Cancer Genomics, http://cBioportal.org). This analysis allowed this research to correlate genetic alterations and gene expression profile of the aforementioned genes with PCa clinical features (NEPC differentiation, AR activity) and with patients’ prognosis. AVPC cells were exposed to different concentration of the three EZH2 inhibitors and their effect on cell viability were measured by cell counting; H3K27me³ levels were measured via western blot from nuclear extract and via Nu.Q kit from cell supernatant. Also, the effects of combining EZH2 inhibitors with platinum agents were measured (cell viability).

Results

It was found that in PCa tissue samples, EZH2 has a higher percentage of gene amplification (i.e., gene copy number increase) compared to SUZ12 and EED. It was found that EZH2 mRNA expression had a positive correlation with NEPC features and a negative correlation with AR activity. Higher EZH2 expression also predicts a poorer prognosis in PCa patients. EZH2
inhibitors reduced intracellular H3K27me$^3$ levels by western blot but induced modest growth inhibition in AVPC cells. GSK-126 was the most effective EZH2 inhibitors among the ones tested in this study. H3K27me$^3$ levels were measurable in cell supernatant at all the selected timepoints and were reduced upon treatment with EZH2 inhibitors. Adding GSK-126 inhibitor to platinum agents reduced the IC$$_{50}$ of this chemotherapy drug.

**Conclusions**

The **EZH2** gene is overexpressed in AVPCs. This study suggests that EZH2 inhibitors, when used in combination with platinum agents, could be an effective therapy for advanced stages of PCa. This hypothesis needs to be corroborated further in other models (e.g., patient-derived xenografts) and in clinical studies.

*Key words:* Aggressive prostate cancer, epigenetic, EZH2 inhibitors, H3K27me$^3$. 
I owe sincere and earnest thankfulness to my supervisors; Dr Francesco Crea, Dr Cheryl Hawkes, and Dr Sushila Rigas; specially Dr Francesco Crea whom this project would not been possible without his guidance and support.

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1 Introduction

1.1 Prostate cancer: clinical aspects

1.1.1 Anatomy of the prostate

The prostate is the largest exocrine gland in the male reproductive system. This organ is located in the pelvic cavity (ventral position in relation to the rectum, caudal position in relation to the bladder) (Figure 1). This walnut shaped gland is about the size of a chestnut and weighs about 30 grams (Tindal and Scardino, 1999 and Lee et al., 2011).

The prostate is composed of a connective tissue (or stroma) and an epithelium (Figure 1). The latter is the origin of most prostatic neoplasms. The stroma contains a range of different cells such as nerves, fibroblasts, infiltrating lymphocytes and macrophages, endothelial cells, and smooth muscle cells (Issacs and Coffey, 1989 and Hudson et al., 2000). A basement membrane separates the stroma from the epithelium.

The prostate pseudo stratified epithelium contains luminal epithelial cells, basal cells, and a small component of neuroendocrine (NE) cells which are scattered throughout the epithelium. The luminal cells are the main cell type, and express prostate specific antigen (PSA), prostatic acid phosphatase (PSAP), AR, and keratins 8 (K8) and K18 (Xue et al., 1998). The basal cells express K5, K14 (members of the keratin family that are structural components of cell filaments) and p63 which is a member of the p53 gene family. NE cells constitute less than 1% of the epithelial cells, and express Chromogranin A (CHGA), synaptophysin (SYP) and neuropeptides (Crea F. et al., 2016).

![Figure 1. Schematic representation of prostate epithelium cellular structure.](image)

This Figure illustrates the macroscopic structure of the prostate (top left) and the detailed structure of prostatic epithelium and stroma (bottom) (modified from Rybak et al., 2015).
1.2 Prostate cancer: epidemiology and prevalence

Prostate cancer (PCa) is a malignant neoplasia of the prostate gland. PCa is the second cause of cancer-related deaths in men, and one of the most common male cancers worldwide (Ferlay et al., 2010).

The prevalence of PCa has been rising between the late 1980s and mid-1990s due to the introduction of the prostate-specific antigen (PSA) blood test for screening. A PSA test measures the concentration of PSA in the patient’s serum. The PSA level depends on the age and the normal range is less than 4.0 ng/ml in men older than 50. In men younger than 50, the normal PSA level is less than 2.5 ng/ml (Hass et al., 2007). As shown in Table 1, a positive PSA test does not always mean that the individual has PCa. At the same time, around 30% of patients with PSA lower than 4.0 ng/ml developed PCa (Gretzer and Partin, 2003). Despite these limitations, the PSA test is still generally considered to be the most useful and accurate PCa biomarker to date.

The cases with a high level of PSA (Table 1) are further investigated through a biopsy to confirm the presence of PCa.

Table 1: This table shows the association between the individual’s risk of PCa and serum PSA levels (ng/ml).

<table>
<thead>
<tr>
<th>Serum PSA level (ng/ml)</th>
<th>Probability of cancer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;2.5</td>
<td>&lt;2</td>
</tr>
<tr>
<td>2.5 – 4.5</td>
<td>~ 18</td>
</tr>
<tr>
<td>4.0 – 10.0</td>
<td>~ 25</td>
</tr>
<tr>
<td>&gt;10.0</td>
<td>~ 67</td>
</tr>
</tbody>
</table>

1.2.1 Prostate cancer grading

PCa grading is one of the most important factors to determine the patient’s prognosis and to guide therapeutic decisions. Grading is normally performed on biopsy samples, or in post-operative samples. This technique can identify different alterations that may be associated with higher PSA levels as discussed in the previous paragraph:

I. Benign prostatic hyperplasia

Benign prostatic hyperplasia (BPH) is a benign enlargement of the prostate gland. The enlarged prostate gland may affect urination due to the squeezing of the urethra (Weinreb et al., 2016). Histologically, BPH is characterised by increased proliferation of the basal epithelial cells and by stromal hyperproliferation. Notably, the hyperproliferative cells maintain a normal phenotype and do not invade adjacent tissues. None of these alterations are linked to carcinoma (McNeal, 1978).
II. PIN

Prostatic intraepithelial neoplasia (PIN) represents the first stage of prostate epithelium transformation. PIN is a pre-malignant lesion and the precursor of PCa. Histologically PIN is characterised by hyperplasia of luminal cells, decreasing basal cells, enlarged and atypical nuclei and nucleoli, cytoplasmic hyperchromasia (Bostwick, 1989, Epstein et al. 1995 and Haggman et al., 1997). The main difference between PIN and PCa is that PIN does not cross the basement membrane, therefore not invading the stroma (Tomlins et al., 2008).

III. Prostate cancer

Conventional prostatic adenocarcinoma shows luminal phenotype with no expression of basal cell markers such as p63 (Tomlins et al., 2008). PCa has all the characteristics of PIN, plus the invasion of the surrounding stroma. PCa is a heterogeneous group of malignant tumours. Approximately 96% of these cancers are adenocarcinomas originating from luminal cells.

Grading of the adenocarcinoma using the Gleason score helps physicians decide the type of therapy. First described by Donald Gleason, this grading system defines PCa aggressiveness (Figure 2). The score is based on the sum of the two most prevalent grades of neoplastic area. For example, a total Gleason score of 8 can result from the two most frequent patterns being both 4 (4 plus 4) or by the most frequent pattern being 3 and the second most frequent pattern being 5 (3+5). Higher Gleason score indicates a more advanced carcinoma and is associated with worse prognosis (Millinger et al., 1967, Epstein, 2010 and Gleason, 1992). Gleason score, along with cancer staging is fundamental to estimate the cancer’s aggressiveness and to direct therapeutic decisions.

![Gleason's Pattern Scale](image)

**Figure 2.** This Figure shows the Gleason score pattern with details of each grade (1 to 5) from the low grade to high grade. The Gleason score indicates how fast the cancer can spread (PCEC, 2022).
A new grading system has been proposed to replace the system provided by the International Consensus Conference of Specialised Urologic Pathologists in 2005. In this new grading system (Table 2), cancers grades from 1 to 5 group based on the Gleason scores. The lower grade means the less aggressive neoplasm. This system facilitates the understanding of Gleason scores (Epstein et al., 2016).

There are 5 grade groups, in ascending order of severity:

- Grade Group 1 (Gleason score 6) is the lowest grade and the least likely to spread.
- Grade Group 2 (Gleason score 3 + 4 = 7).
- Grade Group 3 (Gleason score 4 + 3 = 7).
- Grade Group 4 (Gleason score 8).
- Grade Group 5 (Gleason scores 9 and 10) is the highest grade and the most likely to spread.

In this system, Gleason scores 3 + 4 and 4 + 3, have been distinguished and categorised in two different groups, which are grade group 2 and grade group 3, respectively. This is to reflect the observation that Group 3 (4+3) cancers are more aggressive than group 2 (3+4) cancers.

<table>
<thead>
<tr>
<th>Gleason score</th>
<th>Grade Group</th>
<th>What it means</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gleason score 6 (or 3 + 3 = 6)</td>
<td>Grade Group 1</td>
<td>The cells look similar to normal prostate cells. The cancer is likely to grow very slowly, if at all</td>
</tr>
<tr>
<td>Gleason score 7 (or 3 + 4 = 7)</td>
<td>Grade Group 2</td>
<td>Most cells still look similar to normal prostate cells. The cancer is likely to grow slowly</td>
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<tr>
<td>Gleason score 7 (or 4 + 3 = 7)</td>
<td>Grade Group 3</td>
<td>The cells look less like normal prostate cells. The cancer is likely to grow at a moderate rate</td>
</tr>
<tr>
<td>Gleason score 8 (or 4 + 4 = 8)</td>
<td>Grade Group 4</td>
<td>Some cells look abnormal. The cancer might grow quickly or at a moderate rate</td>
</tr>
<tr>
<td>Gleason score 9 or 10 (or 4 + 5 = 9, 5 + 4 = 9 or 5 + 5 = 10)</td>
<td>Grade Group 5</td>
<td>The cells look very abnormal. The cancer is likely to grow quickly</td>
</tr>
</tbody>
</table>

### 1.2.2 Prostate cancer staging

In addition to grading, PCa staging is a fundamental diagnostic step to determine patients’ prognosis and to guide treatment. Whilst grading estimates the cancer’s potential to invade other tissues, staging is a measure of the current dissemination of malignant cells. Clinicians use information from both systems to determine the best and more feasible treatments. Staging is based upon the Tumour, Node, Metastasis (TNM) system (Woo et al., 2019).
○ Tumour (T)

Tumour (T) reflects the size of the primary cancer and ranges from T1 to T4, as illustrated in Figure 3. This is a summary of the different Ts in prostate cancer:

- T1 - the tumour is too small to be felt in the rectal examination or to be seen on a scan, but it can be diagnosed by a biopsy.
- T2 - the tumour is inside of the prostate, and it can be felt by rectal examination.
- T3 - the tumour has spread out through the capsule which covers the prostate gland.
- T4 - the tumour has invaded the close organs, such as the bladder or the pelvic wall (Woo et al., 2019).

![Figure 3. T staging (T1 to T3) based on the TNM system. T4 is not illustrated here as it describes the spread to other organs such as bladder or pelvic wall (CRUK, 2022).](image)

○ Node (N)

Node (N) defines whether the cancer has invaded the pelvic lymph nodes.

- N0 - there is no cancer in the lymph nodes near the prostate.
- N1 - there are cancer cells in the lymph nodes near the prostate.

○ Metastasis (M)

Metastasis (M) explains whether cancer has invaded distant organs.

- M0 - cancer has not invaded the other parts of the body.
- M1 - cancer has invaded other parts of the body outside the pelvis (Woo et al., 2019).

The most frequent site of metastasis for prostate cancers are the bones, lungs, and liver (Gandaglia et al., 2014).
1.2.3 Prostate cancer therapies

Grading and staging are used to help diagnose the type of PCa and to inform choices of treatment. In some cases, there is no need for treatment, because some localised PCAs grow very slowly and might never cause a problem.

Active surveillance (Table 3) is the way of monitoring localised, Gleason grade <7 PCAs through regular hospital appointments and tests such as:

- Blood tests to monitor serum PSA levels
- Prostate examination (digital rectal examination)
- MRI scans (Romero-Otero et al., 2016).

**Table 3. UK Protocol for Active Surveillance.** The National Institute for Health and Care Excellence has proposed this protocol for Prostate Cancer active surveillance (NICE, 2014).

<table>
<thead>
<tr>
<th>Time</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>An enrolment in active surveillance</td>
<td>MRI scan if not previously performed</td>
</tr>
<tr>
<td><strong>Year one</strong></td>
<td></td>
</tr>
<tr>
<td>Every 3-4 months</td>
<td>Measure PSA</td>
</tr>
<tr>
<td>Throughout</td>
<td>Monitor PSA kinetics</td>
</tr>
<tr>
<td>Every 6-12 months</td>
<td>Digital rectal examination</td>
</tr>
<tr>
<td>At 12 months</td>
<td>Prostate re-biopsy</td>
</tr>
<tr>
<td><strong>Year 2-4</strong></td>
<td></td>
</tr>
<tr>
<td>Every 3-6 months</td>
<td>Measure PSA</td>
</tr>
<tr>
<td>Throughout</td>
<td>Monitor PSA kinetics</td>
</tr>
<tr>
<td>Every 6-12 months</td>
<td>Digital rectal examination</td>
</tr>
<tr>
<td><strong>Year 5 and every year until active surveillance ends</strong></td>
<td></td>
</tr>
<tr>
<td>Every 6 months</td>
<td>Measure PSA</td>
</tr>
<tr>
<td>Throughout</td>
<td>Monitor PSA kinetics</td>
</tr>
<tr>
<td>Every 12 months</td>
<td>Digital rectal examination</td>
</tr>
</tbody>
</table>

If PCa progresses after one of these examinations, surgical, radiological, or pharmacological treatments will be considered.

1.2.3.1 Surgery

Surgery is the most effective treatment option for PCa (Linton and Hamdy, 2004). In general, tumours that are localised (within the prostate) or locally advanced (within the pelvis) are operable.
There are different types of surgery for PCa:

- Radical prostatectomy aims to remove the whole prostate to cure localised PCa. It can be done either by an open surgery or guided using a robotic system called the Da Vinci surgery.

- Trans urethral resection of the prostate (TURP) is another type of surgery. In this case, only part of the prostate will be removed. This technique is mainly used to treat BPH but sometimes it is used in locally advanced PCa. The TURP does not always remove all the cancer cells but improves some of the symptoms.

1.2.3.2 Radiotherapy (RT)
Radiation therapy uses high energy, ionising radiations, such as x-rays, to kill the cancer cells. It may be used in combination with surgery or with pharmacological therapies. Radiotherapy can be curative for localised PCas or can alleviate the symptoms of incurable cancers.

There are two main types of RT:

- External beam radiotherapy, where beams are focused on the prostate gland from a machine outside of the patient’s body.

- In Brachytherapy (internal radiation), small radioactive pellets (iodine-125 or I-125) are placed in the prostate gland by using a needle or catheter. Brachytherapy is used mainly for advanced PCa that has metastasized to the bones (CRUK, 2022).

1.2.3.3 Pharmacological therapies
There are two main types of pharmacological therapies for PCa: hormonal therapy and chemotherapy. Both types are employed in metastatic prostate cancers and aim to reduce the progression of the disease. Since hormonal therapy is the backbone of metastatic PCa therapy, these advanced tumours are divided in three main groups, each with different treatment options:

- Hormone-sensitive PCa
- Castration-resistant PCa (CRPC)
- Aggressive variants of CRPC

Each of these groups can be treated by different hormonal and/or chemotherapies. Hence, we will discuss them separately in the next paragraphs.

1.3 Hormone-sensitive prostate cancer
Most PCa cases are classified as adenocarcinomas. These malignancies are characterized by uncontrolled proliferation of luminal-like cells, which retain some glandular formation and still express androgen receptor (AR) and PSA (Sun et al., 2009). The key role of AR signalling in PCa has been first described in 1941, by a study showing that orchiectomy can cause a significant tumour regression (Huggins and Hodges, 1941).

The AR gene is located on the X chromosome; this gene encodes a 110 KD protein containing 919 amino acids. The AR protein is a member of the steroid hormone receptor superfamily.
The AR contains three functional domains, which are: the N-terminal domain (NTD), the DNA binding domain (DBD) and the C-terminal ligand binding domain, which is linked to the DBD by a flexible hinge region (Gelmann, 2002). Upon binding to androgens (the receptor’s ligands), the AR translocates to the nucleus (Figure 4) and acts as a transcription factor.

The physiological production of androgens is regulated by an endocrine system. The hypothalamus-pituitary-Leydig cell axis produces most of the endogenous androgens (Figure 4). The hypothalamus activates some pituitary cells by releasing the luteinizing hormone-releasing hormone (LHRH). As a result, the pituitary releases the luteinizing hormone (LH), and follicle-stimulating hormone (FSH). LH binds to receptors expressed by Leydig cells in the testis and stimulates the production of testosterone. FSH binds to receptors expressed by Sertoli cells in the testis and promotes the production of sperm (Figure 4). In addition, the adrenal glands produce androstenedione and dehydroepiandrosterone (DHEA) which are converted to testosterone by 17-beta-hydroxysteroid dehydrogenase in the cytoplasm of prostate cells (Radmayr et al., 2008 and El-Alfy et al., 1999). Notably, androgens also feedback to the hypothalamus and pituitary where they inhibit LHRH and LH release (Kuhl and Taubert, 1975).

**Figure 4.** This Figure shows the hypothalamus-pituitary-Leydig cell axis (left), the prostate which is a target organ for this axis (centre) and intracellular activity of androgens (right). In AR-positive cells, AR-androgen binding triggers the translocation of the AR into the nucleus, where the AR acts as a transcription factor, by binding specific androgen-response-elements (ARE). The Figure also shows how LHRH agonists and antagonists (which will be discussed in the next paragraphs) interfere with this system (Matsumoto and Bremner, 1987 and Chang et al., 2013).

The prostate is dependent on the level of testosterone to develop and grow (Figure 4). In the prostate, circulating testosterone is transformed into dihydrotestosterone (DHT), which is the...
key intracellular ligand that binds to AR, thereby activating the AR signalling pathway (Kyprianou and Isaacs, 1988 and Chang et al., 2013).

Circulating testosterone mainly binds to serum sex hormone-binding globulin (SHBG) and albumin (Figure 4). But the free form of testosterone can enter the prostate epithelial cells. In the cytoplasm of these cells, testosterone is converted to DHT, which stimulates the growth and survival of the prostate cells. DHT binds the AR with high affinity by displacing the heat shock proteins that normally keep the AR in the cytoplasm. Following the release of the heat shock proteins, the AR dimerizes, and the hormone-receptor complex enters the nucleus. The hormone-receptor complex binds DNA on the androgen response elements (AREs) regions, which are located in the promoter regions of androgen-dependent genes, such as PSA and transmembrane protease serine 2 (TMPRSS2). The activation of androgen-dependent genes stimulates biological responses such as growth and survival (Shang et al., 2002 and Jentzmik et al., 2016).

Steroidal sex hormones such as androgens (testosterone), oestrogens and progesterone, all play important roles in carcinogenesis and progression of PCas (Flier et al., 1992). Like normal prostate cells, PCA cells need AR activation for their growth. It has been shown that androgens play several oncogenic roles in PCa, including growth stimulation (Huggins, 1967), and enhance the metastatic potential of malignant cells (Koivisto et al., 1997).

Androgen deprivation therapy (ADT) is therefore the first-line hormone therapy for metastatic PCAs (mPCAs). In ADT, the level of androgen hormones is reduced either by surgery (orchiectomy) or by medications that reduce the levels of circulating androgens. For example, LHRH agonists and LHRH antagonists block the hypothalamus-pituitary-Leydig cell axis (Figure 4). LHRH antagonists bind to the LHRH receptors on pituitary cells to prevent the production of FSH and LH. On the contrary, LHRH agonists initially stimulate the production of LH. However, the prolonged presence of high levels of LHRH agonists eventually blocks the production of LH. Hence, serum testosterone levels are decreased by both types of therapies (Kalsary et al., 1995 and Iversen et al., 1996). Almost all PCas initially respond to ADT; however, ADT resistance appears in a substantial fraction of the patients through a variety of AR-dependent and AR-independent mechanisms (Vogelzang, 2012).

However, in 2018 a phase III clinical trial demonstrated that the combination of Enzalutamide and ADT can reduce the risk of metastasis or mortality compared to Enzalutamide alone in patients with metastatic hormone-sensitive PCa (Armstrong et al., 2019). Moreover, in 2022, the same research group confirmed that the combination of Enzalutamide and ADT significantly improves the survival of patients with metastatic hormone-sensitive PCa, compared to ADT alone (Armstrong et al., 2022).

According to James et al., 2017, ADT plus Abiraterone and Prednisolone showed 37% improvement in overall survival (compared to ADT monotherapy) in patients with locally advanced or metastatic PCa (James et al., 2017).

Moreover, some studies have demonstrated that Docetaxel plus ADT increased the overall survival in patients with metastatic hormone resistant PCas, especially in patients with visceral metastases or four or more bone lesions with at least one outside the vertebral bodies and pelvis (Botrel et al., 2016 and Sweeney et al., 2014).
Based on this new evidence, the standard treatment for patients with metastatic hormone-sensitive PCa is now ADT plus docetaxel or plus novel hormonal therapies (enzalutamide or abiraterone).

1.4 Castration-resistant prostate cancer (CRPC)

Despite the overall efficacy of ADT, some metastatic PCas become resistant to this therapy and progress to CRPC (Beer et al., 2014). To explain the molecular mechanisms underpinning this progression, it is necessary to briefly discuss the micro-evolutionary nature of cancer. To explain cancer initiation and progression, an evolutionary process model was introduced by Peter Nowell in 1976. This model suggests that a single cell (clone) expands into a heterogeneous population of cancer cells with acquired somatic mutations thereby generating several tumour-initiating subclones. When exposed to further challenges (e.g., specific therapies) the heterogeneous population of sub-clones will display different reproduction rates. In these conditions, rare drug-resistant sub-clones can quickly overtake the whole tumour and generate a drug-resistant neoplasm (Figure 5). In keeping with this hypothesis, metastases can originate from the drug-resistant neoplasm and/or from the acquisition of more invasive phenotypes (Caldas, 2012).

This model is illustrated in Figure 5.

**Figure 5. Clonal evolution in tumours and tumour recurrence.** A normal cell (light green) can accumulate somatic mutations (orange) that generate the founder clone in the tumour. Tumours are characterised by genetic heterogeneity (different colours). This creates a range of cells with different sensitivity to treatment. For example, hormonal treatments can act as a selective pressure and enable the survival and expansion of the resistant of hormone-resistant cells (dark green). The metastatic process can induce further selection and generate sub-clones which can give rise to recurrence due to an increased level of genetic instability (Greaves and Maley, 2012).
The clonal evolution model explains the progression of CRPC. In this type of lethal PCa, patients do not respond to first-line ADT, and they are therefore treated with chemotherapy (Figure 6) such as the anti-mitotic compound Docetaxel, or with next-generation hormonal therapies (e.g., the irreversible AR antagonists Enzalutamide and Apalutamide or Abiraterone which inhibits CYP17, an enzyme necessary for androgen synthesis) (Beer et al., 2014 and Mori et al., 2020).

A variety of AR-dependent mechanisms are involved in CRPC progression as depicted in Figure 6:

- Point mutations in the AR:
  - Point mutations increase the affinity of AR to androgens and allow its activation even in the presence of anti-androgens (Taplin et al., 1995).

- AR gene amplification:
  - AR gene amplifications increase the number of AR protein produced. So, PCa cells with AR amplification can survive even under ADT, and progress to CRPC (visakorpi et al., 1995).

- Changes in AR coactivator proteins in prostate cancer:
  - AR coactivators are transcriptional coregulator proteins that bind to AR and increase transcription of target genes by enhancing binding of AR to AR-binding site on the target gene. Amplification of these coactivators occur in ADT, resulting in castration-resistance (Qin et al., 2014).

Generally, these AR-positive CRPCs can be still treated with next-generation hormonal therapies, such as Enzalutamide, Apalutamide and Abiraterone. Enzalutamide and Apaludamide (nonsteroidal antiandrogen agent) are potent AR inhibitors that block the binding to androgens, the AR translocation, and the AR binding to DNA (Saad, 2013). Abiraterone inhibits the CYP17 enzyme, which is essential for androgen biosynthesis (Potter et al., 1995). Both these potent
inhibitors of the AR signalling are effective in men with CRPC and significantly extend their survival (Wei et al., 2021).

1.5 Aggressive variants of prostate cancer

A further subtype of CRPC has been recently identified and named "aggressive-variant" PCa (AVPC). AVPC is characterised by a combination of the following characteristics: (I) prevalence of AR/AR\(^{bw}\) cells, and consequently low PSA levels at diagnosis; (II) expression of neuroendocrine markers (CHGA, SYP); (III) small-cell carcinoma histology; (IV) exclusively visceral metastases or prevalently lytic bone metastases; (V) bulky lymphadenopathy, or bulky high-grade tumour mass (Gleason ≥ 8) in prostate/pelvis; (VI) short progression (<6 months) upon hormonal therapy (Beltran et al., 2014 and Aparicio et al., 2013). Since they are generally AR-negative, AVPCs are resistant to all hormonal therapies, including abiraterone and enzalutamide. In fact, the prevalence of AVPCs is higher in men treated with these drugs (Beltran et al., 2014). As we will discuss in the next paragraphs, the response rate to taxane-based chemo-hormonal therapy is low in AVPCs, however, platinum-based combination therapies are more active in this setting (Aparicio et al., 2013 and Culine et al., 2007). There are two main subtypes of AVPCs, which will be discussed in the following paragraphs.

1.5.1 Treatment-emergent neuroendocrine prostate cancer

Neuroendocrine prostate cancer (NEPC) is one of the AVPC subtypes. NEPC is highly resistant to all treatments and frequently metastatic, with a median survival time of 7 months (Fulton, 2017). While NEPC may rarely arise de novo, most cases result from the trans-differentiation of a typical prostate adenocarcinoma (PCa) into NEPC following ADT or enzalutamide/abiraterone treatment (Palmgren et al., 2007). Recent studies have shown that longer courses of ADT are linked to increased prevalence of NEPC (Ito et al., 2001)

The difference between NEPC and other forms of PCa is the presence of neuroendocrine cells (NEC) that express neuroendocrine markers such as CHGA and SYP. Most NECs are composed by small cells that do not express the usual PCa markers such as PSAP, PSA and AR (Schmechel et al., 1978 and Wang and Epstein, 2008). The circulating PSA and PSAP level do not rise in NEPC patients. For this reason, more than 50% of NEPC patients are diagnosed at the metastatic stage (Aggarwal et al., 2014). However, expression of PSA and AR have been observed in 25% of NEPC cases (Lotan et al., 2011). In this case, it is likely that the patients have a mixed histology carcinoma (adenocarcinoma and NEPC). Pure NEPC is diagnosed in about 50% to 60% of total NEPC cases with the remaining cases showing co-presence of NEPC cells with prostate adenocarcinoma (Schron et al., 1984 and Wang and Epstein, 2008).

1.5.2 Anaplastic prostate cancer

Anaplastic PCa is another subtype of aggressive PCa that is characterised by higher incidence of common metastases like bone metastases and uncommon metastases like lung, liver, adrenal and brain with or without neuroendocrine differentiation (Ganeshan et al., 2017). Anaplastic PCas show low PSA levels, absent or very low AR expression, but no expression of NEPC markers. Cancer cells are called “anaplastic” because they are non-differentiated. Patients with anaplastic PCa respond inadequately to ADT and to new hormonal therapies and show rapid progression. Hence, the median survival time of these patients is 6-17 months (Schwartz et al.,
1998). Platinum based chemotherapies are active on anaplastic PCas, similarly to what is observed on NEPC (Aparicio et al., 2013).

1.5.3 Prostate cancer chemotherapy
Chemotherapy drugs have been traditionally used as systemic therapies for patients with mCRPC.

Some clinical trials involving the usage of cytotoxic chemotherapy in CRPC were initiated in the 1950s with alkylating agents (anticancer drugs). The response rates to chemotherapy were as low as 8.7 % and median survival was 10–12 months. It was hard to interpret these results because of the small group of patients and the absence of a meaningful endpoint. This approach prompted scientists to suggest that PCa was resistant to chemotherapy (Yagoda and Petrylak, 1993). Due to the ineffectiveness of monotherapies, clinicians started to test combinations of two or more drugs. However, none of the combination studies presented any significant effect on response or survival time. In 1972, a series of randomised Phase II trials on either one drug or combination of two or more drugs were initiated in CRPC patients by the National Prostatic Cancer Project (NPCP). The main endpoint in those studies was response rate. Moreover, chemotherapy drugs were compared to palliative therapies such as radiation and hormonal therapies. The NPCP standard endpoints of drug efficacy were complete response (CR), partial response (PR), and disease stabilisation (DS). High rates of objective responses (OS) were presented in these studies; however, there was no improvement of survival time and very low rates of CR and PR were reported. Similarly, the combination of chemotherapy and hormonal therapy hasn’t shown significant benefits (Eisenberger et al., 1985). In the late 90s, clinical trials began to use a novel group of chemotherapy called taxanes for mCRPC (Dumontet and Sikic, 1999). Since then, chemotherapy drugs are standard therapies for mCRPC and some of the common chemotherapies will be summarised here:

1. Taxanes
2. Platinum agents

1.5.3.1 Taxanes
Taxanes are a group of chemotherapy drugs that were first extracted from the bark of the Yew tree (*Taxus brevifolia*) (Dumontet and Sikic, 1999). The first product extracted from these trees was Paclitaxel (Abraxane), which showed promising results in phase II trials on CRPC patients, both as a single agent and in combination with Estramustine (an agent that can inhibit the function of microtubules) (Roth et al., 1993 and Trivedi et al., 2000). Taxanes bind beta-tubulin, which is an essential component of the microtubules (Figure 7). Microtubules are important components of the cell cytoskeleton, involved in numerous cellular processes, such as mitosis, maintaining the cell shape, intracellular transportation, and cell signalling. Taxanes inhibit the intrinsic instability of microtubules and block their depolymerisation. This causes G2/M arrest and apoptosis (Dumontet and Sikic, 1999). Interestingly, pre-clinical and clinical results suggest that CRPC cells can modulate the expression of specific beta-tubulin isoforms to become resistant to taxanes. In particular, class III beta-tubulin has been linked to lower taxane efficacy *in vitro* and to reduced survival in CRPC patients exposed to this drug (Terry et al., 2009).
Figure 7. The mechanism of action of taxanes. Taxanes inhibit mitosis by binding β-tubulin and arresting the cell cycle and leading the cell to death (Dumontet and Sikic, 1999).

In addition to their effects on cell cycle, taxanes inhibit AR translocation to the nucleus, which is an important step in AR signalling. Microtubules facilitate the AR translocation to the nucleus, thereby inhibiting AR migration and AR activity. Taxanes stop this process by binding β-tubulin and stabilising the microtubule thereby inhibiting AR immigration and AR activity (Van Soest et al., 2013).

Two main taxanes are currently employed for CRPC treatment:

- Docetaxel (Taxotere) is a semi-synthetic analogue of Paclitaxel and can inhibit the depolymerization of microtubules almost twice as effectively as Paclitaxel in pre-clinical studies (Diaz and Andreu, 1993). After its antitumour activity and efficacy were demonstrated in phase II (Berry et al., 2001 and Beer et al., 2001) and in phase III (Petrylak et al., 2004 and Tannock et al., 2004) studies respectively, Docetaxel was approved in 2004 for the treatment of CRPC. In addition, the combination of Docetaxel with ADT has been shown to be effective in hormone-sensitive prostate cancer patients (Van Soest and de Wit, 2015).
- Cabazitaxel (XRP6258) also known as Jevtana, as a semi-synthetic third generation taxane, and is used after tumour cells lose their sensitivity to Docetaxel treatment (Mita et al., 2009). Cabazitaxel has been approved by the FDA (Food and Drug Administration) on 17th of June 2010 (FDA centre, 2010) for the treatment of Docetaxel resistant CRPC. Advantages of Cabazitaxel over Docetaxel include:
  1) The reduced affinity for P-glycoprotein, also known as multidrug resistance protein 1 (MDR1) that is important for drug efflux, a major mechanism of resistance.
  2) The capability of crossing the blood–brain-barrier and potentially reaching metastatic sites in the brain (Cisternino et al., 2003 and Mita et al., 2009).
However, Cabazitaxel works as effectively as Docetaxel which is the first line therapy. Thus, it remains an option of second-line therapy for patients with mCRPC (Oudard et al., 2017). According to recent studies, taxane drugs are affordable and clinically effective drugs for the treatment of mCRPC (Huebner et al., 2020).

### 1.5.3.2 Platinum agents

Three approved platinum (Pt) agents have been used as cancer therapies (Figure 8) globally:

1. Cisplatin
2. Carboplatin
3. Oxaliplatin

Four more platinum agents have been approved in specific countries such as Heptaplatin (Korea), Lobaplatin (China), Miriplatin (Japan), and Nedaplatin (Japan) (Oun et al., 2018).

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**Figure 8.** Chemical structure of three clinically approved platinum agents (Oun et al., 2018).

Pt agents contain a Pt ion in the centre and chloride (Cl\(^-\)) ions, or ammonia groups. Pt anticancer drugs were first described by Rosenberg and his group in 1969. They were investigating whether electric currents affect bacterial growth. They found that the electrodes activity caused the formation of Pt ions in the medium, and that these ions inhibited cell viability. They subsequently showed that numerous compounds that contained Pt ions exhibited *in vivo* anticancer activity (Rosenberg *et al.*, 1969).

The most effective of these complexes was the one now identified as Cisplatin (also known as Platinol, cis-diaminedichloroplatinum (II), or cis-DDP) which can cure testicular tumours in more than 90% of the cases (Loehrer and Einhorn, 1984). In 1978, the FDA approved Cisplatin as a treatment for other cancer types, such as ovarian, cervical, head and neck, oesophageal, and non-small-cell lung cancers (Loehrer and Einhorn, 1984 and Morris *et al.*, 1999). Cis-DDP causes important side effects, such as nephrotoxicity, gastrointestinal toxicity, and neurotoxicity; these effects often reduce the treatment length (Loehrer and Einhorn, 1984). Although Cisplatin was effective in testicular cancer treatment, its efficacy in other cancers was restricted due to acquired or inherent resistance. Also, the mechanism of Cisplatin resistance is still not completely elucidated (Kartalou and Essigmann, 2001). Once platinum agent’s anticancer potential was proved (Rosenberg *et al.*, 1969), scientists were interested to
discover the mechanism of action of Cisplatin and other Pt complexes (Figure 9). According to recent studies platinum agents generate apoptosis by four steps as follows:

1) Intracellular accumulation either by passive or active transport.
2) Activation of the platinum complex.
3) The platinum compound binds nucleic acids to make several Pt-DNA adducts that cross-link DNA strands.
4) This initiates the cellular response to DNA damage and apoptosis (Wang and Lippard, 2005 and Jung and Lippard, 2007).

In the beginning, it was assumed that Cisplatin penetrates cells by passive diffusion, because it is unsaturated and it is not inhibited by structural analogues (Gale et al., 1973 and Gately and Howell, 1993). But it has been demonstrated that the cellular internalisation happens through active cellular uptake by membrane proteins, such as the copper transporter CTR1 (Figure 9) (Ishida et al., 2002 and Holzer et al., 2004). Cisplatin is activated by a hydrolysis process, whereby chloride ligands replace the water molecules during the cell membrane crossing (Howe-Grant and Lippard, 1980). The hydrated version of Cisplatin then binds DNA at the N7 location of purine bases, to make 1,2-intrastrand adducts across guanosine residues (Cohen et al., 1980). Some cellular procedures such as DNA replication and transcription are thereby disturbed by the DNA damage caused by Cisplatin. Subsequently, the cell cycle is arrested, and apoptosis is activated. However, platinum lesions can be removed by multiple DNA repair pathways that mediate drug resistance (Cleare and Hoeschele, 1973 and Furuta et al., 2002). For example, the mismatch repair (MMR) proteins are known to recognise and repair some Cisplatin lesions (Kunkel and Erie, 2005). A major mechanism of Cisplatin resistance is via the activation of the nucleotide excision repair system (Duan et al., 2020).

Carboplatin (also known as Paraplatin; cis diamine (1,1-cyclobutane dicarboxylato platinum II)) is a second generation of Pt agent that was developed to reduce the toxicity of Cisplatin. However, Carboplatin has the same mechanism of action as Cisplatin, but it shows minor activity and slower DNA binding kinetics (Natarajan et al., 1999). With a retention half-life of 30 hours, Carboplatin is more stable in the human body than Cisplatin (retention half-life of 1.5 to 3.6 hours). Carboplatin has shown a lower excretion rate than Cisplatin (Canetta et al., 1985). However, the medical standard dose of Carboplatin compared to Cisplatin is 4 in 1; this means that the required dose of Carboplatin is four times higher than the dose of Cisplatin required to obtain the same efficacy (Go and Adjei, 1999). The advantage of Carboplatin is the reduction of side effects, such as significantly lower nephrotoxicity. Carboplatin also affects bone marrow cells and causes leukopenia and reduced platelet count (Canetta et al., 1985). The FDA approved Carboplatin for the treatment of ovarian and lung cancers in 1989 (Kelland, 2007). However, this drug has also been used for the treatment of retinoblastomas, neuroblastomas, nephroblastomas, and brain tumours, as well as cancers of the head and neck, endometrium, cervix, testes, breast, and bladder (US National Library of Medicine, 2015).
Figure 9. The mechanism of action of platinum agents. This pathway shows platinum-induced cell death. Platinum complexes penetrate the cell and bind DNA creating double-strand breaks, thereby activating DNA repair mechanisms, and initiating apoptosis.

Oxaliplatin ([oxalate (2)-O, O’] [1R,2R-cyclohexanediamine-N, N’] platinum-(II)) is a third generation of Pt agent with an oxalate group that replaces the amine groups of Cisplatin. The higher lipophilicity of Oxaliplatin facilitates its cell penetration abilities. It has been demonstrated that the organic cation transporters (OCT)1 and OCT2 are specific transporters of Oxaliplatin (Zhang et al., 2006). However, researchers found that Oxaliplatin induces fewer DNA adducts and more toxicity compared to Cisplatin (Woynarowski et al., 2000). But this drug has shown promising results particularly when it was combined with 5-FU and Leucovorin therapy in metastatic colorectal cancer (André et al., 1998). The overexpression of OCTs by colorectal cancer cells may predict effectiveness of Oxaliplatin (Zhang et al., 2006).

Also, it has been shown that Oxaliplatin is highly active in patients who have ovarian cancer and are resistant to Cisplatin (Sessa et al., 1999). Similarly, a study indicated that Oxaliplatin elicited a partial response in 15% of advanced non-small-cell lung patients who were resistant to Cisplatin (Monnet et al., 1998).
1.5.4 Summary of prostate cancer types and treatment

Figure 10 summarises the different stages of prostate cancer progression, and the main treatments used for each stage, as discussed in the previous sections. For the two AVPCs, we also summarise their main molecular features: both these cancer types are AR$^-$ or AR$^{\text{low}}$. However, NEPC expresses neuroendocrine markers, which are not present in anaplastic PCa.

![Diagram of prostate cancer progression and treatments](image)

**Figure 10. Different types of PCa and their treatment.** This flowchart describes the progression of PCa and the therapies associated with each stage. For the two aggressive variants (Anaplastic and NEPC) the main molecular features were shown. HS= hormone-sensitive.

Since AVPCs are the main focus of this research, the genetic and epigenetic characteristics will be discussed in the next part.

1.6 Genetic landscape of AVPC

The genetic correlates of AVPC have been recently described by Abida et al., (2019), who studied 444 biopsies (Figure 11) collected from 429 enrolled patients with advanced mCRPC. In this study, matched blood samples were collected for DNA analysis on non-neoplastic cells. Whole exome sequencing was carried out on 444 tumour samples. RNA sequencing was carried out on 332 samples from 323 patients.
Figure 11. The proportion of samples by tissue sites in the Abida et al. 2019 study. Proportions given as percentages out of a total of 444 samples.

All the biopsies in the Abida et al. 2019 study, were identified by pathologists as adenocarcinoma, pure small cell/neuroendocrine cancer, adenocarcinoma with neuroendocrine features, or could not be classified because of insufficient material or no tumour observable on the immunohistochemistry slides that were available for review (Figure 12).

Figure 12. Histopathologic classification of PCa biopsies. This Figure presents the percentages of samples with different histopathologic classification.

This study has shown that different genes are mutated in advanced prostate cancer. Table 4 summarises the main pathways altered in this neoplasm. The main genetic alterations will be discussed in the next sections.
Table 4. Top-ranking altered genes involved in advanced metastatic PCas. This table shows the top-ranking altered genes involve in advanced metastatic PCas with their alteration frequency and their specific pathways based on Abida et al. study in 444 samples.

<table>
<thead>
<tr>
<th>Gene(s)</th>
<th>Frequency of genetic alteration (% of all cases)</th>
<th>Most frequent alteration (case number)</th>
<th>Cellular function</th>
<th>Protein type</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETS-ERG</td>
<td>32.21%</td>
<td>Fusion (130 cases)</td>
<td>Control of gene expression</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>ETS-ETV1</td>
<td>9.46%</td>
<td>Fusion (21 cases)</td>
<td>Control of gene expression</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TP53</td>
<td>40.09%</td>
<td>Mutation (161 cases)</td>
<td>DNA repair and apoptosis</td>
<td>Tumour suppressor</td>
</tr>
<tr>
<td>PTEN</td>
<td>32.66%</td>
<td>Deep Deletion (114 cases)</td>
<td>Cell cycle control</td>
<td>Tumour suppressor</td>
</tr>
<tr>
<td>RB1</td>
<td>12.84%</td>
<td>Deep Deletion (41 cases)</td>
<td>Cell cycle control</td>
<td>Tumour suppressor</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>11.94%</td>
<td>Amplification (39 cases)</td>
<td>Cell cycle control</td>
<td>Oncogene</td>
</tr>
<tr>
<td>KMT2C</td>
<td>12.16%</td>
<td>Mutation (40 cases)</td>
<td>Control of gene expression</td>
<td>Histone lysine methyltransferase</td>
</tr>
<tr>
<td>Gene</td>
<td>Mutation Rate</td>
<td>Mutation Cases</td>
<td>Function</td>
<td>Role in Tumour Suppression</td>
</tr>
<tr>
<td>--------</td>
<td>--------------</td>
<td>----------------</td>
<td>-----------------------------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>FOXA1</td>
<td>13.96%</td>
<td>(40 cases)</td>
<td>Control of gene expression</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>KMT2D</td>
<td>8.33%</td>
<td>(37 cases)</td>
<td>Gene transcription</td>
<td>Histone lysine methyltransferase act as tumour suppressor</td>
</tr>
<tr>
<td>BRCA2</td>
<td>12.16%</td>
<td>(37 cases)</td>
<td>DNA repair</td>
<td>Tumour suppressor</td>
</tr>
</tbody>
</table>
1.6.1 Transcription factors and epigenetics

- **ETS fusion**

  One of the most common alterations in PCa genomes are fusions of androgen-regulated promoters with the erythroblast transformation specific (ETS) family genes, which encode oncogenic transcription factors (ERG and ETV1). In particular, the promoter of TMPRSS2-ERG fusion is the most frequent molecular alteration in PCa (Tomlins et al., 2005). In this case, the gene fusion results in over-expression of the oncogenic transcription factor. ERG can bind to the EZH2 promoter and induce its expression (Yu et al., 2010). In PCa, overexpression of ERG is also associated with advanced tumour stage, high Gleason score, metastasis, and shorter survival times (Hagglof et al., 2014).

- **FOXA1**

  FOXA1 is a forkhead family transcription factor that is a significant inhibitor of epithelial-to-mesenchymal transition (EMT) in cancer. EMT is a dynamic process that entails morphological and cell signalling changes in cancer cells (Figure 13) (Nieto et al., 2016). It has been demonstrated that EMT is a key driver of various tumour functions, such as tumour cell migration and invasion, intravasation, metastasis, and resistance to therapy (Craene and Berxt, 2013).

  FOXA1 upregulates E-cadherin and maintains the epithelial phenotype, thereby inhibiting metastasis. It has been shown that FOXA1 deletion drives NEPC differentiation (Kim et al., 2017). Parolia et al. (2019) found that FOXA1 is mutated in over 34% of mCRPC. This study also found that FOXA1 reprograms AR-activity in PCa. Therefore, FOXA1 and AR are co-expressed in PCa cells, in which FOXA1 activity is essential for cell survival and proliferation (Pomerantz et al., 2015).
Figure 13. Phenotypic and epigenetic state transitions during EMT (image modified from Nieto et al., 2016). The EMT process involves a progressive transformation of epithelial (E) cells, into intermediate cells (EM), and finally into mesenchymal (M) cells. While the cells convert from E to M (left to right), they gradually reduce the expression of proteins involved in apicobasal polarity and in cell-cell adhesions, whilst increasing front-back polarity and cell-matrix interactions. The coloured spectra represent theoretical conversion points (x-axis). During the EMT transition, different states of net thermodynamic equilibrium (y-axis) are achieved. Some states are stable (low energy) whilst others are metastable (high energy) and therefore reversible. A specific set of epigenetic modifications (histone modification and DNA methylation) promotes the activation or inactivation of gene expression programs in each of these states (Tam and Weinberg, 2013).

• **KMT2C and KMT2D**

Lysine (K)-specific methyltransferase 2C (KMT2C, also known as MLL3) and Lysine (K)specific methyltransferase 2D (KMT2D, also known as MLL4) are members of the mixed lineage leukemia (MLL) family of HMTs, which methylate the histone 3 tail at lysine 4 (H3K4) (Shilatifard, 2012). Recent studies have discovered frequent genetic alterations in various members of the MLL family, such as MLL3/KMT2C, and MLL4/KMT2D in solid tumours (Rao and Dau, 2015 and Gonzalez-Perez et al., 2013). Limberger *et al.* (2018) have indicated that loss of *KMT2C* increases PCa tumorigenesis in mouse models and have found the same alterations in human patient samples. So, KMT2C has been proposed as a PCa tumour suppressor.

KMT2D is critical for tumour cell proliferation and *KMT2D* loss increases chemotherapeutic sensitivity in solid tumours such as pancreatic adenocarcinoma, breast, and colorectal cancer (Ford and Dingwall, 2015). It has been discovered that *KMT2D* is frequently mutated, and its overexpression was significantly associated with metastasis and with worse prognosis in PCa patients (Lv *et al.*, 2018).
1.6.2 DNA repair and cell cycle control

Although mutations in genes that are involved in DNA repair and cell cycle control are relatively frequent in primary PCAs, the concurrent genetic alteration of several of these genes has been mainly observed in clinical samples and patient derived xenograft (PDX) models of AVPCs (Aparicio et al., 2016).

• **RB1 and TP53**

*TP53* mutations are identified in approximately 30% of PCa samples (Ecke et al., 2007). Also, the *TP53* alteration frequency in PCa tissue does not increase in correlation with higher grade and stage of PCa, as it does in other malignancies, such as bladder cancer (Sidransky and Hollstein, 1996). Therefore, *TP53* mutations per se are not highly associated with AVPC.

Recent studies show that *RB1* is one of the most frequently altered gene in primary PCa, and that this alteration is associated with uncontrolled cell cycle signalling and with increased activity of the PI3K pathway. Co-loss of two or more tumour suppressor genes such as *TP53*, *RB1*, and *PTEN* may initiate more advanced PCa (Taylor et al., 2010 and Robinson et al., 2015). For example, it has been shown that *RB1* loss triggers lineage plasticity and PCa metastasis in *PTEN*-mutant mice. Depletion of *TP53* provokes resistance to antiandrogen therapy (Ku et al., 2017). Notably, *RB1* alteration is mutually exclusive with alterations in *AR*. Moreover, *RB1* mutations were identified as a potent prognostic factor in advanced PCa patients (Abida et al., 2019). All this evidence points towards a central role of *RB1* alterations in the pathogenesis of AVPC.

• **BRCA2**

Loss of *BRCA2* is associated with the PCa cell lines. Co-deletion of *BRCA2-RB1* in human PCa cells induces EMT, which is associated with invasiveness and aggressiveness (Chakraborty et al., 2020).

• **PTEN**

*PTEN* is a frequently altered tumour suppressor gene in PCa, but mainly by deletion, rather than point mutation. A recent study suggested a significant role for *PTEN* in PCa progression. Loss of *PTEN* expression was significantly associated with shorter progression-free survival. It has been demonstrated that the *TMPRSS2: ERG* fusion is associated with deletion of *PTEN* and with reduced PTEN expression levels (Taylor et al., 2010).

• **PIK3CA**

The *PIK3CA* gene encodes the p110 alpha (p110α) protein, which is a subunit of an enzyme named phosphatidylinositol 3-kinase (PI3K). Increased activity of the PI3K pathway is regularly associated with PCa progression, therefore it can be an attractive therapeutic target. In fact, loss of *PTEN*, a negative regulator of the PI3K pathway, is expected to occur in 40% to 50% of patients with PCa (Grasso et al., 2012 and Taylor et al., 2010) and this pathway is an active area of investigation for new PCa therapies.
1.7 Summary of PCa development, some of the genetic alterations, and treatment options

The data discussed in the previous section show that PCa is a very complex disease. PCa develops from normal epithelial cells (Basal, Luminal and NE cells) to PIN, to localized PCa and then finally to invasive adenocarcinoma (Figure 14). There are different therapies for primary PCas including surgical resection, radiotherapy, and ADT. After an initial response to ADT, some patients relapse with resistance to ADT leading to CRPC with or without metastasis. These patients undergo additional treatment with the next generation of hormonal therapies, such as Enzalutamide or Abiraterone. During this treatment a fraction of CRPCs further evolve into AVPCs, which are completely androgen-independent and characterised by key genetic alterations (e.g., \textit{P53} and \textit{RB1} mutations). AVPCs are currently incurable and treated with platinum-based palliative chemotherapy (Patel \textit{et al.}, 2019).
Figure 14. A comprehensive overview of PCa progression, metastasis, drug resistance and neuroendocrine differentiation (NED). This Figure defines PCa development from early stage to the advanced PCa (modified from Patel et al., 2019). Some key genetic alterations are highlighted. On the right side, the Figure illustrates some of the therapeutic options for each stage of disease.
2 Epigenetic

Epigenetic gene regulation comprises all heritable phenotypic alterations that are not caused by changes in DNA primary structure (Waddington, 1959). Epigenetic can be defined as a bridge between genotype and phenotype. Epigenetic alterations are attractive therapeutic targets due to the fact that they are reversible (Berger et al., 2009). The main mechanisms of epigenetic regulation are histone modifications, DNA methylation, and chromatin remodelling. For the purpose of this report, histone modifications will be the main focus.

The structural unit of chromatin is the nucleosome, an octamer containing two copies of each histone core protein (H2A, H2B, H3 and H4) and a 147 bp- (base pairs) DNA strand wrapped around the core (Figure 15). Histone protein H1 links adjacent nucleosomes to maintain the chromosome structure (Luger et al., 1997). A large group of epigenetic regulators (EpRs) are responsible for the modification of histones’ N-terminal tails, thereby controlling chromatin structure and gene expression (Allfrey et al., 1964).

![Figure 15. Nucleosome structure.](image)

The nucleosome is an octamer containing two copies of each histone core protein. Epigenetic regulators are responsible for the modification of histone N-terminal tails. Here acetylation (Ac) is shown as an example of histone modification. The coloured wavy lines are the tails of the corresponding histone (Modified from Llovet et al., 2008).

The activity of EpRs is deregulated in severe diseases such as cancer. Potential for reversing epigenetic regulation makes them attractive pharmacological targets for small molecule inhibitors, some of which have been approved for clinical use (Nebbioso et al., 2012).

2.1 Histones post translational modifications

Histone post translational modifications play a key role in regulation of gene expression in tumour initiation and progression (Zhou et al., 2011). These post-translational modifications (PTMs) include methylation, acetylation, ubiquitination, and phosphorylation (Figure 15).

These PTMs define a “histone code”, which control local gene expression (Allfrey et al., 1964).
Histone PTMs are catalysed by specific enzymes, which are called “epigenetic writers” and can be removed by another class of enzymes, which are called “epigenetic erasers”. A third class of enzymes called “epigenetic readers” can bind to histone PTMs and to regulate gene expression (Jenuwein and Allis, 2001).

While acetylation (ac) of histones leads to increased gene expression, the effect of histone methylation (me) depends on the number of methyl groups, the affected residue, and its location on the histone tail. For example, histone 3 lysine 4 di- and trimethylation (H3K4me² and H3K4me³) and histone 3 lysine 9 mono-methylation (H3K9me¹) activate gene expression whereas histone 3 lysine 27 di- and trimethylation (H3K27me² and H3K27me³) and histone 3 lysine 9 di- and trimethylation (H3K9me² and H3K9me³) lead to gene repression (Schneider and Grosschedl, 2007).

2.1.1 Histone methylation

In histone tails, lysine (K) and arginine (R) residues are the main sites of methylation. Lysine can be monomethylated, di-methylated, or trimethylated, while arginine can be monomethylated or di-methylated. Methylation on lysine residues in different histone proteins have been discovered such as K27 on H3 (Zhang et al., 2003). Histone methylation is modulated by HMTs that mostly contain catalytic domains referred to as the SET domain [Su(var), enhancer of zeste, Trithorax] (Ng et al., 2002).

2.1.2 The role of Histone PTMs in aggressive prostate cancer

PCa is characterised by extensive alterations of histone PTMs. Several histone PTMs, e.g., H3K4me, H3K9me², H3K9me³, H3Ac, and H4Ac are decreased in PCa compared to normal prostate tissue. It has been demonstrated that H3K4me is a predictor of PSA recurrence after radical prostatectomy. Significant increase of H3K4me¹, H3K4me², and H3K4me³ levels were demonstrated in AVPC. (Ellinger et al., 2010). However, an increase of H3K27 methylation, which is catalysed by the methyl-transferase Enhancer of zeste homologue 2 (EZH2) is associated with PCa progression and metastasis (Varambally et al., 2002).

2.2 Polycomb group proteins

Polycomb group (PcG) proteins are amongst the most studied epigenetic regulators and are involved in cellular differentiation and development via transcriptional repression. Particularly, PcGs are involved in the progression of both solid and haematological malignancies (Crea et al., 2012). PcG function was first described in Drosophila melanogaster where these EpRs inhibit Hox gene expression. PcG proteins repress gene expression via formation of multi-subunit complexes known as “Polycomb repressive complexes (PRCs)” 1 and 2.

PRC1 complexes catalyse the monoubiquitination of histone H2A at lysine 119 (H2AK119Ub1) through RING1A or RING1B, thereby silencing the gene expression. The PRC2 complex is composed of three main core components: EZH2 (catalytic subunit), suppressor of zeste 12 (SUZ12), and Embryonic Ectoderm Development (EED). The PRC2 complex catalyses the trimethylation of histone H3 at lysine 27 (H3K27me³) through the histone methyltransferase EZH2, which binds the N-terminal chromodomain of chromobox proteins (CBX2, 4, 6, 7, 8) to silence gene expression (Kaustov et al., 2011) via H32K27 mono-di- and tri-methylation.
PRC1 and PRC2 have been widely involved in the initiation and progression of most solid cancers and are particularly important for the development of drug resistance.

### 2.3 Enhancer of Zeste homologue 2

EZH2 is overexpressed in many malignancies such as prostate, breast, bladder, ovarian, lung, liver, brain, kidney, gastric, oesophageal, pancreatic cancer, and melanoma (Bachmann et al., 2006). In various cancers, higher EZH2 expression is associated with higher cell proliferation, advanced stages, and poor prognosis. EZH2 overexpression promotes cell proliferation and invasion in vivo (Rao et al., 2010 and Crea et al. 2011). EZH2 acts as an oncogenic factor in several types of cancers and represses the tumour suppressor genes through high levels of H3K27me3, a histone mark associated with gene silencing (Bracken et al., 2007). By repressing the tumour suppressor genes, EZH2 promotes cancer growth, metastasis, and therapy resistance. EZH2 regulates the expression of a variety of genes. MHC class I (MHC-I) genes and p16 are some of the most common EZH2 targets.

p16 (p16\textsuperscript{INK4a}) also known as cyclin-dependent kinase inhibitor 2A (CDKN2A), encoded by the INK4A/ARF locus, is a tumour suppressor protein and acts as a cell-cycle inhibitor by targeting cyclin D-dependent kinases (CDK4 and CDK6) thereby arresting the cell in G1 phase (Figure 16). p16 expression is promoted by cellular stress (via P53) and DNA damage. p16\textsuperscript{INK4a} controls cell growth and proliferation, thereby acting as a tumour suppressor (Jacobs et al., 1999 and Gil and Peters, 2006). The INK4A/ARF locus encodes another tumour suppressor named p14ARF (also called ARF tumour suppressor, ARF, p14). p14 has diverse molecular function as a tumour suppressor and one of these physiologic functions show p14 acts via the p53 pathway to induce cell cycle arrest or apoptosis (Saito et al., 2016). EZH2 depletion has been shown to rescue the expression of p16 and p14 thereby promoting tumour cell growth. EZH2 inhibitors reduce the H3K27me3 levels in the p16\textsuperscript{INK4a} locus thereby increasing levels of p16 (Gil and Peters, 2006). In keeping with this evidence overexpression of EZH2 has been reported to induce INK4A/ARF gene silencing (LaPak and Burd, 2014).

EZH2-dependent transcriptional repression of MHC-I genes has been detected in a variety of cancers, including NEPC and this phenomenon has been identified as a mechanism of resistance to immunotherapy (Paulson et al., 2018).
Figure 16. Roles and interactions of the proteins (P16 and p14) encoded by the INK4A/ARF locus. (A) p16 inactivates CDK4/6, by blocking the phosphorylation of Rb, thereby arresting cell cycle in G1. (B) p14 inhibits the E3 ubiquitin ligase activity of MDM2, leading to P53 stabilization. P53 stabilization initiates cell apoptosis and/or indirectly arrest cell cycle (LaPak and Burd, 2014 and Peurala et al., 2013).

Current studies show that EZH2 is particularly important in AVPC. EZH2 is overexpressed in NEPC patient-derived xenografts and clinical samples, compared to adenocarcinomas (Beltran et al., 2016 and Clermont et al., 2015); in addition, EZH2 is overexpressed in a subset of aggressive localised PCas with an increased risk of recurrence after radical prostatectomy (Varambally et al., 2002). EZH2 has been shown to cooperate with N-Myc in increasing cancer cell plasticity, a feature that enables the trans-differentiation of prostatic adenocarcinomas into NEPC (Berger et al., 2019). Notably, EZH2 inhibitors reversed this phenotype. More recently, EZH2 has been implicated in the transcriptional modulation of stem cell and neuronal genes in PCas that are transitioning to anaplastic and/or NEPC states (Davies et al., 2021). Finally, EZH2 inhibition has been shown to reverse enzalutamide resistance in PCa cells (Bai et al., 2019). Taken together, this evidence makes EZH2 an attractive therapeutic target and biomarker for AVPC.

2.4 Histone modifications as possible cancer biomarkers

Tumour markers are molecules that can be used to demonstrate the presence of cancer, but also to detect cancer progression and to monitor the efficacy of epigenetic therapies. A wide range
of tumour markers have been studied, including tumour antigens, enzymes, hormones, receptors, growth factors, DNA mutations and epigenetic alterations (Wallner et al., 2006).

Some markers that have been produced by cancer cells, will be detected in the blood, urine, or other biological fluids. These “soluble” markers enable the non-invasive detection and monitoring of neoplasm.

Recently, it has been shown that specific histone PTM secreted by cancer cells can be detected in the blood or in other biological fluids (Rasmussen et al., 2018). It is thought that histones can be released into the circulation upon cancer cell death and fragmentation (Rahier et al., 2017). Histones can be identified as bound to circulating tumour DNA or within circulating tumour cells. In addition, activated immune cells in the tumour microenvironment can release histones due to a unique form of immune cell death called NETosis (Figure 17). Moreover, apoptotic, or necrotic cells can release histones into the blood due to decreased phagocytosis (Chen et al., 2014).

The company Volition has recently developed ELISA-based assays to detect circulating histones and histone modifications from tiny amounts of plasma and serum (between 10 to 20 microliters): https://volition.com/nu-q. These kits have been previously used for early cancer detection (Bauden et al., 2015). The company has recently developed a new kit to detect H3K27me\(^3\) level. This kit can be used to monitor the efficacy of EZH2 inhibitors in cancer patients and to tailor therapies (Kang et al., 2020). For this reason, this kit was used in the current research project to measure whether EZH2 inhibitors affect the concentrations of H3K27me\(^3\) molecules released in the medium of PCa cells, and to compare Volition’s kit with a standard western blot assay.

**Figure 17. How histones are released in response to cellular stress.** Apoptotic cells, necrotic cells and NETosis can release histones into the circulation when under stress, such as ischemia-reperfusion injury (I/R), infection, trauma, exposure to toxic agents. These phenomena are more frequent in cancers than in normal tissues (modified from Chen et al. 2014).
2.5 EZH2 inhibitors

Several compounds that suppress the enzymatic activity of EZH2 have been described and tested in pre-clinical studies. Three of these compounds have entered phase I/II clinical trials for solid cancers (Table 5). These compounds are Tazemetostat (EPZ-6438), GSK-2816126 (GSK-126) and CPI-1205.

Table 5. This table summarizes the main characteristics of the three clinically tested EZH2 inhibitors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Route of administration</th>
<th>Clinical trial phase</th>
<th>Recommended dose</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tazemetostat</td>
<td>Oral</td>
<td>Approved</td>
<td>800mg twice/day</td>
<td>Hoy, 2020</td>
</tr>
<tr>
<td>GSK2816126</td>
<td>Intravenous</td>
<td>I/II</td>
<td>N/A</td>
<td>McCabe et al., 2012</td>
</tr>
<tr>
<td>CPI-1205</td>
<td>Oral</td>
<td>I</td>
<td>N/A</td>
<td>Vaswani et al., 2016</td>
</tr>
</tbody>
</table>

2.5.1 Tazemetostat

Tazemetostat (EPZ-6438 or E7438) also known as Tazverik, is an orally bioavailable small-molecule inhibitor of EZH2. It competes with the co-substrate S-adenosylmethionine (SAM) to bind the EZH2 catalytic site (Knutson et al., 2014). Tazemetostat has been studied more than the other EZH2 inhibitors in clinical trials.

Clinical trials on lymphomas or advanced solid tumours have shown that Tazemetostat is safe and has potential antitumor activity.

I. The following phase I clinical trials have been conducted:

- The main side effects reported in this Phase I which was conducted recruiting patients with refractory B-cell non-Hodgkin lymphoma and advanced solid tumours, including epithelioid sarcoma, were generally of grade 1 and 2 severity and included asthenia (33%), anaemia (14%), anorexia (6%), muscle spasms (14%), nausea (20%), and vomiting (9%). The objective response rate was detected at 38% in patients with B-cell non-Hodgkin lymphoma and at 5% in patients with solid tumours. (Italiano et al., 2018).

- Another phase I clinical trial assessed the safety and antitumor activity of Tazemetostat in Japanese patients with relapsed or refractory B-cell non-Hodgkin-type lymphoma. The objective response rate was 57% (Munakata et al., 2021).

Their results have revealed that the Tazemetostat safety profile was acceptable, and it presented promising antitumor activity. Also, no dose-limiting toxicity and no death due to the treatment were observed on both phase I trials (Italiano et al., 2018 and Munakata et al., 2021).
• A phase Ib clinical trial has been conducted on patients with diffuse large B-cell lymphoma (DLBCL) in the combination with R-CHOP (chemotherapy used to treat non-Hodgkin lymphoma). The main side effects reported in this study were grade 3 to 4 hematologic adverse events 47% including neutropenia (47%), leukopenia (29%), anaemia (18%), and thrombocytopenia (12%) (Sarkozy et al., 2020).

II. The following phase II clinical trials have been conducted:

• In this phase II trial, the patients with follicular lymphoma were considered by EZH2 status (mutant vs wild type). The main side effects were thrombocytopenia (3%), neutropenia (3%), and anaemia (2%). Serious treatment-related adverse events were reported in 4% of 99 patients and no deaths were reported. The objective response rate was detected at 69% in the EZH2mut group and at 35% in the EZH2WT group (Morschhauser et al., 2020).

• Another phase II clinical trial of Tazemetostat was conducted in Japanese patients with relapsed or refractory B-cell non-Hodgkin lymphoma with EZH2mut including two subgroups. The first subgroup included Japanese patients with follicular lymphoma and the objective response rate of this subgroup was detected at 76.5%. The second subgroup was included Japanese patients with diffuse large B-cell lymphoma that all these patients achieved a partial response (Izutsu et al., 2021).

In conclusion, Tazemetostat monotherapy elicited strong responses and a positive safety profile in severely pre-treated patients with relapsed or refractory follicular lymphoma. Tazemetostat is an innovative therapy for patients with follicular lymphoma (Morschhauser et al., 2020).

Based on positive results in Phase III clinical trials which is median overall survival = 82.4 weeks, the FDA approved Tazemetostat (TAZVERIK, Epizyme, Inc.) for adults and paediatric patients older than 16 with metastatic or locally advanced epithelioid sarcoma in January of 2020 (Hoy, 2020). Also, the FDA has approved Tazemetostat as a novel therapy for relapsed or refractory follicular lymphoma patients whose tumours are with EZH2 mutated and who went under two complete treatments or who have not suitable treatment choices in June of 2020 (Morschhauser et al., 2020).

Some studies are also currently recruiting using Tazemetostat in:

- Subjects with moderate and severe hepatic impairment with advanced malignancies (NCT04241835) (phase I/II)
- Study of Tazemetostat with Enzalutamide or Abiraterone/Prednisone in subjects with Castration Resistant Prostate Cancer who have not received chemotherapy (NCT04179864) (phase Ib/II)
- Adult subjects with INI1-Negative tumours or relapsed/refractory synovial sarcoma (NCT02601950) (phase II)
- Combination with doxorubicin as frontline therapy for advanced epithelioid sarcoma (NCT04204941) (phase III)

Therefore, it is expected that Tazemetostat will be increasingly used in oncology.
2.5.2 GSK-126

Also known as GlaxoSmithKline 126, this is a selective EZH2 inhibitor that inhibits both wild type and mutant EZH2. It has been demonstrated that GSK126 is an S-adenosyl-methionine-competitive, small compound that inhibits EZH2 methyltransferase activity resulting in lower H3K27me3 levels and reactivation of silenced PRC2-target genes. GSK-126 prevents the proliferation of EZH2-mutant Diffuse Large B-Cell Lymphoma (DLBCL) cell lines and significantly prevents the growth of EZH2-mutant DLBCL xenografts in mice (McCabe et al., 2012). There is one terminated study on GSK2816126 in subjects with Relapsed/Refractory DLBCL, transformed Follicular Lymphoma (tFL), other Non-Hodgkin's Lymphomas, Solid tumours, and Multiple Myeloma (NCT02082977). This study had two parts to find out the recommended Phase II dose (RP2D) for GSK-126, which was given twice per week by intravenous (IV) infusion. Part 1 of this study has been conducted in adult subjects with relapsed/refractory DLBCL, transformed follicular lymphoma, other NHL, solid tumours including CRPC and multiple myeloma (MM). All patients had no less than one side effect in the study. Fatigue (53.7%) and nausea (48.8%) were the most frequent side effect. 32% of patients had a serious side effect which was grade 3 or 2, and none were related to treatment. Serious side effects included various infections (n = 7) and pyrexia (n = 3). In this study, the objective response rate was detected at 38% in patients with B-cell lymphomas and at 5% in patients with solid tumours. Only 34% of patients had stable disease after treatment. A relatively short half-life of 27 hours can explain the modest anticancer activity at the tolerable dose. This short half-life of the drug can limit effective drug exposure (Yap et al., 2019).

2.5.3 CPI-1205

CPI-1205 is an orally bioavailable, indole-based, small molecule EZH2 inhibitor. It selectively binds in its EZH2 catalytic pocket, partly overlapping with SAM (Gehling et al., 2015). CPI-1205 inhibits tumour growth in the xenograft model of EZH2 mutant DLBCL. This drug has been shown to efficiently decrease H3K27me3 levels in vivo (Vaswani et al., 2016). The phase I clinical trial of CPI-1205 (evaluating CPI-1205 in patients with B-Cell lymphomas) has been completed in September 2019 (NCT02395601). However, the results of this trial have not been published yet. A phase I/II trial to evaluate CPI-1205 with ipilimumab in patients with advanced solid tumours (NCT03525795) and a phase Ib/II trial consider the combination of oral CPI-1205 with either enzalutamide or abiraterone/prednisone metastatic CRPC (NCT03480646) are currently recruiting patients. There are no interim results on CPI-1205 presented at the time of this report.

2.5.4 Summary of EZH2 as a potential treatment

Altogether EZH2 inhibiting drugs may be potential new treatments for EZH2-mutant lymphomas, where they were first tested, but these drugs have not been extensively investigated in PCa and in other solid tumours. In particular, very few studies have tested the efficacy of EZH2 inhibitors in AVPCs: NEPC and anaplastic PCa. The combination of EZH2 inhibitors with a chemotherapy agent used in AVPC (e.g., platinum agents) can be a promising strategy to re-sensitise advanced PCa to current therapies.
3 Hypothesis, aims and ethics

3.1 Hypothesis

We hypothesise that EZH2 inhibitors can suppress cell viability and/or re-sensitise AVPC to treatment with clinically-relevant drugs. We also hypothesise that H3K27me³ levels are reduced by the EZH2 inhibitors and that these reduced levels are detectable in biological fluids.

3.2 Aims

To test the hypotheses, the following aims were conducted:

1. Evaluate the genetic alterations and gene expression profiles of PRC2 genes (EZH2, SUZ12, and EED) in AVPC clinical samples using publicly available bioinformatics datasets.

2. Expose AVPC cells to clinically achievable doses of EZH2 inhibitors (monotherapy), to measure the effects of these drugs on cell viability. Using the same AVPC cells, test whether EZH2 inhibitors can re-sensitise cancer cells to currently employed drugs (combination) using combination treatments.

3. Measure H3K27me³ methylation levels in the cellular extracts and supernatant of AVPC cells exposed to EZH2 inhibitors (supernatant will be used as a model for a biological fluid, for future in vivo applications).

3.3 Ethical considerations

There is no need for direct ethical consideration for the human cell line used in this project. Yet it was confirmed that the company of purchase followed the guidelines of the Human Tissue Act.

Regarding the murine cells used in this project, our collaborators at "Istituto Nazionale Tumori" have confirmed that they followed relevant EU regulations on animal research.

Additionally, Control of Substances Hazardous to Health) (COSHH) forms were completed according to our university’s guidelines.
4 Materials and Methods

4.1 Bioinformatic analysis

To study the genetic alterations and gene expression of main PRC2 genes in AVPC, the Cbio-portal for cancer genomics database website (cBioPortal for Cancer Genomics, http://cbioportal.org) was used.

To investigate PRC2 gene expression, the data set called Metastatic Prostate Adenocarcinoma (SU2C/PCF Dream Team, PNAS 2019) (Abida et al., 2019) was chosen because it was the most recent dataset, and it includes high number of samples. This study has been widely discussed in the introduction of this report. Expression of PRC2 genes (EZH2, SUZ12, and EED) has been detected in this study and the results are in section 5.1.

To investigate whether the mRNA expression of EZH2, SUZ12, and EED genes correlated with NEPC features and AR score, the same study was chosen which contains the genomic and transcriptomic analysis of 429 patients with mCRPC. Correlation between Polycomb gene expression and NEPC features, or AR scores (AR signalling score) were calculated using the Pearson’s correlation factor between the log2-transformed FPKM values of each gene score and a reference gene expression vector in all tumours where RNA-sequencing data were available (Beltran et al., 2016). Presence of NEPC features was estimated by experienced pathologists. AR signalling scores are based on a transcriptomic study that has identified AR-target genes in prostate cancer cells (Hieronymus et al., 2006). From this study, a score was determined to estimate the level of activation of the AR pathway, under different conditions.

For transcriptomic studies, mRNA expression was analysed as Z-score. A Z-score describes a value's relationship to the average of a group of values. Z-scores may be positive or negative, for example Z-score -3 to +3 mean 3 standard deviations below the mean and 3 standard deviations above the mean, respectively (Brase C.H. and Brase C.P., 2017).

Also, to investigate the prediction of survival time by EZH2 expression, the same study has been chosen (Abida et al., 2019).

To identify the evolutionary conservation of EZH2 between humans and mice, the Basic Local Alignment Search Tool (BLAST) was chosen.

4.2 Cells in culture

One cell line was purchased from the American Type Culture Collection (ATCC): DU-145 (Manassas, VA) and another cell line was provided by our collaborators at Istituto Italiano Tumori: OPT-7714.

DU-145 cells originated from a brain metastasis of prostatic adenocarcinoma from a 69-year-old male Caucasian. DU-145 is a model of anaplastic PCa since it is both AR-negative and NEPC markers-negative. DU-145 were cultured in media RPMI 1640 (Gibco®, Thermo Fisher) supplemented with 10% (v/v) of heat-inactivated Fetal Bovine Serum (FBS) (Thermo Fisher) and 1% of Penicillin-Streptomycin (Pen/Strep) (Gibco®, Thermo Fisher).

OPT-7714 cells (murine cell line) were provided by our collaborators; Elena Jachetti and Mario P Colombo (Istituto Nazionale Tumori, Milan, Italy). OPT-7714 is a model of NEPC. OPT-7714 cells were cultured in media DMEM, high Glucose (Gibco®, Thermo Fisher)
supplemented with 10% (v/v) of heath inactivated FBS (Thermo Fisher), 1% (v/v) of Pen/Strep (Gibco®, Thermo Fisher), Sodium Pyruvate (Gibco®, Thermo Fisher), and HBSS, no calcium, no magnesium, no phenol red (Gibco®, Thermo Fisher).

Both cell lines were cultured following their ATCC protocols under controlled conditions within the laboratory. Both cell lines were cultured in T-75 flasks and kept in humidifier at 37°C degrees with 5% CO₂. As ATCC recommends, all cell lines need to be cryopreserved in liquid nitrogen. So, both cell lines were cryopreserved in liquid nitrogen following the cryopreservation protocol.

Mycoplasma contamination was detected every few weeks by using the MycoAlert™ Detection Kit (Lonza, UK). The MycoAlert™ assay is a biochemical kit that measures the activity of the enzyme of mycoplasma basically by adding two reagents to the supernatant collected from cells in culture and ending by luminescence readings. Only mycoplasma-free cells were used for this study.

The reagents used to culture the PCa cells are given in Table 6.

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 1640 medium (DU-145)</td>
<td>Gibco®, Thermo Fisher</td>
<td>21875034</td>
</tr>
<tr>
<td>DMEM (high Glucose) (OPT-7714)</td>
<td>Gibco®, Thermo Fisher</td>
<td>11995065</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>Gibco®, Thermo Fisher</td>
<td>11360039</td>
</tr>
<tr>
<td>HBSS, no calcium, no magnesium, no phenol red</td>
<td>Gibco®, Thermo Fisher</td>
<td>14175053</td>
</tr>
<tr>
<td>Hank’s balanced salt solution (HBSS)</td>
<td>Sigma-Aldrich</td>
<td>H6648</td>
</tr>
<tr>
<td>FBS</td>
<td>Thermo Fisher</td>
<td>10099141</td>
</tr>
<tr>
<td>Pen/Strep</td>
<td>Gibco®, Thermo Fisher</td>
<td>15140122</td>
</tr>
<tr>
<td>Trypsin-EDTA (0.25%)</td>
<td>Gibco®, Thermo Fisher</td>
<td>25200056</td>
</tr>
</tbody>
</table>

**4.3 EZH2 inhibitors effects**

**4.3.1 Cell lysis for protein isolation**

To investigate the conceivable impact of EZH2 inhibitors on the expression of H3K27me³ in PCa cells, three clinically tested EZH2 inhibitors were purchased (Table 5). Both DU-145 and OPT-7714 cells were seeded (100,000 cell/ml) in flasks (T75cm²) at the same cell density. After 24 hours, they were exposed to Tazemetostat, GSK-126, and CPI-1205 at concentrations 1, 5, 10µM (Figure 18). To purify the proteins after 72 hours, cells were lysed by RIPA lysis and extraction buffer (Thermo Fisher, Cat no: 89900) containing protease inhibitor cocktail (Merk, Cat no: P2714). The cells were incubated in ice-cold RIPA lysis and extraction buffer containing protease inhibitor cocktail on ice for 5 minutes and infrequently swirled. Cells were collected by cell scrapers and transferred into ice-cold Eppendorf tubes. Next, samples were
sonicated for 10 seconds at 20% amplitude by ultrasound sonication. Following this step, samples were centrifuged at 14,000 g for 15 minutes at 4°C to separate cell pellet and supernatant. The supernatant was collected by avoiding the pellet into new microtubes and were aliquoted into different microtubes to avoid multiple freeze/thaw cycles and all were stored at -20°C.

![Diagram of experiment](image)

**Figure 18.** Schematic representation of the experiment designed for EZH2 inhibitors effects on both cell lines and followed by cell lysis for protein purification.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Supplier</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tazemetostat</td>
<td>Insight biotechnology</td>
<td>HY-13803</td>
</tr>
<tr>
<td>GSK-126</td>
<td>Insight biotechnology</td>
<td>HY-13470</td>
</tr>
<tr>
<td>CPI-1205</td>
<td>Insight biotechnology</td>
<td>HY-100021</td>
</tr>
</tbody>
</table>

**Table 7.** This table shows three EZH2 inhibitors, their suppliers, and their catalogue numbers.

**4.3.2 Protein quantification**

The protein concentration of the cell lysate was measured using the Pierce™ Gold BCA protein assay kit (Thermo Fisher, Cat no: A53225) that is a colourimetry assay based on compatibility of two reagents of A and B. The kit has been established to measure (A480nm) total protein concentration compared to a protein standard curve. The protein concentrations were completed by an extensive working range (20–2000 µg/mL).

This technique includes the biuret reaction (Figure 19) that is the reaction of protein with Cu²⁺ in an alkaline medium and creating a strong purple complex with cuprous ion (Cu¹⁺) by bicinechonic acid (BCA) that is a constant and water-soluble sodium salt (Smith *et al.*, 1985).

![Diagram of biuret reaction](image)

**Figure 19.** The biuret reaction. The first step is reaction of protein with Cu⁺² in an alkaline environment making a blue-coloured complex.

Following the protocol, a serially diluted albumin standard (BSA) and a mixture of reagents were prepared. Next, 20µL of each standard or unknown sample replicate were pipetted into a 96 well
microplate. The plate was incubated at room temperature for 5 minutes. Then the absorbance was measured at or near 480nm on a BMG Optima polar STAR plate reader (BMG Labtech, Aylesbury, UK). A standard curve was prepared by plotting the average of each standard absorbance and the protein concentrations were calculated based on the curve (Table 8).

Table 8. A guide to prepare a set of protein standards.

<table>
<thead>
<tr>
<th>vial</th>
<th>Volume of diluent (µL)</th>
<th>Volume and source of BSA (µL)</th>
<th>Final concentration (µg/mL)</th>
<th>BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>300 (Stock)</td>
<td>2000</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>62.5</td>
<td>187.5 (Stock)</td>
<td>1500</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>100</td>
<td>100 (Stock)</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>100</td>
<td>100 (vial B dilution)</td>
<td>750</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>100</td>
<td>100 (vial C dilution)</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>100</td>
<td>100 (vial E dilution)</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>100</td>
<td>100 (vial F dilution)</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>160</td>
<td>40 (vial G dilution)</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

4.3.3 Western Blot

The cell extracts were obtained using RIPA Buffer as described in 4.2 and protein quantified by BCA Assay Kit as described in 4.3.2 (Figure 18). Proteins were resolved by gel electrophoresis on reducing SDS-polyacrylamide gels on 10–20% Tris-Glycine, 1.0 mm, Mini Protein Gel (Thermo Fisher Scientific, Cat no: XP10205BOX).

In the laboratory, samples were prepared as 10µl of 15µg protein diluted in water to obtain the same amount in each well and each was mixed with 10µl of Novex Tricine SDS Sample Buffer (2X) (Thermo Fisher, Cat no: LC1676). The samples were heated at 85°C for 2 minutes to allow proteins to run directly to the bottom of the polyacrylamide gel and meanwhile, the Tris-Glycine gel was inserted in the chamber with the short side facing the middle. Moreover, 500ml of 1X Novex Tris-Glycine SDS Running Buffer (1:10 dilution from 10X buffer) (Thermo Fisher, Cat no: LC2675) comprising 500µl of NuPAGE™ Antioxidant (Invitrogen, Cat no: NP0005) was prepared and decanted into the chamber as washing off any gel obstructing wells. Following, 10µl of Novex Sharp Pre-Stained Protein Standard (Invitrogen, Cat no: LC5800) and 15µl of each protein heated samples were pipetted into the wells. At that point, the gel was run at 110 v for 2 hours.

Over the duration, 300 ml of 1X Pierce™ transfer buffer (TBS) (diluted from 20X TBS) (Thermo Fisher, Cat no: 28358) with 20% (v/v) methanol was prepared for soaking the following items:

- 5 x sponges per gel
- 2 x blotting paper per gel
- 1 x membrane per gel (0.2um nitrocellulose, Fisher Scientific, Cat no: 15249794)

To transfer the protein from the gel to the membrane, glass plates need to be detached and the gel was retrieved to create the transfer sandwich as shown in Figure 20. The sandwich was rolled out to ensure there is no bubbles within the different layers.

**Figure 20. Example of a prepared sandwich.** When the proteins are loaded on the gel, their charge is negative due to denaturing by heat. So, they move to the positive electrode as soon as the specific voltage is applied (the arrow shows this movement). Therefore, proteins are transferred to the membrane from the gel that is called electrophoretic transfer. Sponges and blotting papers are to protect the gel.

The sandwich was inserted into the chamber surrounded by solid protection and was topped up with TBS mixed with ethanol. The outside of the sandwich was topped up with cold tap water. Proteins were transferred at a constant 300mA for 2 hours and 30 minutes.

In the next step, the membrane was separated from the sandwich and was blocked in 5% (w/v) dried milk with TBST (TBS + 0.1% tween 20) at room temperature for 1 hour with moderate shaking. This step is a very important step, as it prevents non-specific antibodies to bind to the membrane.

The upcoming step was the incubation of the membrane with specific primary antibodies which are H3K27me³ antibody [Cell Signalling Technology, Tri-Methyl-Histone H3 (Lys27) (C36B11) Rabbit mAb, Cat no:9733] diluted in 5% (w/v) BSA, TBST and anti-GAPDH [1mg/ml stock concentration (1:10000 dilution) Sigma Aldrich, Cat no: G9545] diluted in 5% dried milk, TBST, at 4°C with gentle shaking overnight.

Following this, the membrane was washed 3X 10 minutes in TBST. Then the membrane was incubated in HRP-conjugated anti-rabbit secondary antibody [1µg/ml stock concentration, 1:2000 dilution (Thermo Fisher, Cat no: 31460)] liquified in TBST with 5% (w/v) milk at room temperature for one hour. After the incubation, the membrane was washed 4X 10 minutes in TBST.

Eventually, for visualisation of blot chemiluminescence, ECL western blotting detection reagents (Cytiva, Cat no: RPN2209) were added over the membrane and the membrane was visualised by Syngene Gbox with GeneTools software (Syngene, Bangalore, India)
4.3.4 Volition kits

Volition company is developing simple ELISA tests to diagnose a range of cancers and other diseases in early stages. Early detection of cancer increases the chances of treatment and enhances the survival of patients.

Volition NU.Q assay is a colorimetric ELISA 96 well plate-based assay. The ELISA method is established for the quantitation of antigens in biological samples such as serum, plasma, and CSF. ELISAs are adaptable to high-throughput screening because results are rapid, consistent, and relatively easy to analyse. In ELISA, an enzyme-labelled immunoreaction (antigen or antibody) and an immunosorbent (antigen or antibody bound to a solid support) are used for measuring the unknown concentration, such as either competitive binding between the labelled reactant and unlabelled unknown (antigen, antibody, or protein) or a sandwich method in which the unknown antigen binds both the immunosorbent and labelled antibody. The best results have been obtained with the sandwich method. The resulting absorbance provides data that is very sensitive and highly specific (Vashist and Luong, 2018).

H3K27me³ RUO kit was used in this project to measure the H3K27me³ levels in supernatants. The supernatants were collected from the experiments described in Figure 18 and Figure 21.

![Figure 21. H3K27me³ levels measurement experiment.](image)

5 of plates were prepared

Treated with Tazemetostat, GSK-126, and CPI-1205 at 10 µM

24 hrs

3 days

5 days

7 days

10 days

H3K27me³ levels measurements were performed following the manufacturer’s instructions.

All the buffers for each step of the protocol should be warmed up at room temperature before use. Likewise, all the steps should be done at room temperature. After determining the exact number of microwell strips for samples and standards in duplicate, serially standards were prepared by using positive control and control diluent buffer.
Next, the microplate was washed three times with wash buffer. Afterwards, 20 µl of standards and supernatants with 80 µl assay buffer were incubated in a 96-well plate at room temperature on a microplate shaker at approximately 700rpm for 2 hours and 30 minutes.

Following another wash step, the microplate was incubated with 100µl of diluted biotinylated anti-nucleosome detection antibody in each well as described above for 90 minutes.

After the third wash step, 100µl of streptavidin horseradish peroxidase (HRP) conjugate was dispensed to each well and incubated for 30 minutes. Following the fourth wash step, 100µl of substrate solution was dispensed to each well and incubated for approximately 20 minutes. At the last step 100µl of stop solution was added to each well. Finally, the optical densities of all microwells were read by a microplate spectrophotometer at 450nM.

The obtained optical densities were used to plot and draw the standard curve. Afterwards statistical analysis was done in statistical software which is GraphPad Prism.

The detection of circulating nucleosomes in as little as 20µl of samples show the sensitivity of the kit and nucleosome epitope-specific antibody which enables detection of only intact nucleosomes to prove the specificity of volition kits. These kits are fast because the results will be obtained in 6 hours totally. The detection limit is from 0.039 to 2.5 µg/ml (Volition brochure).

4.3.5 IC₅₀ calculation

For measuring cell viability (IC₅₀), both cell lines were seeded in 6 well plates (10,000 cell/ml). After 24 hours different concentration of EZH2 inhibitors (25, 10, 1, and 0.01 µM) and vehicle control (% of DMSO correspondent to concentration of the drug) were added. The media were changed on 5th day of the experiment with the same pattern. The experiment was stopped on 10th day and the cells were counted. IC₅₀ of both cell lines treated with three EZH2 inhibitors, have been calculated upon direct cell count. The media and cell palette have been frozen at -80 °C.

4.3.6 Combination of chemotherapy agent and EZH2 inhibitor

Carboplatin (Sigma, Cat no: C2538) as a chemotherapy Pt agent was used in the combination with GSK-126 as a EZH2 inhibitor.

To estimate the effect of EZH2 inhibitor in combination with chemotherapy, both cell lines were seeded in 6 well plates (10,000 cell/ml). After 24 hours, cells were treated with GSK-126 at 10 µM for 72 hours and re-treated with combination of GSK-126 (at 10µM) and Carboplatin at different concentrations (500, 100, 50, 10, 1, 0.1, 0.01µM) for another 72hours (Figure 22).

*Figure 22. Combination of GSK-126 and Carboplatin schematic experiment design. The experiments were stopped on 6th day of treatment and the cells were counted.*
4.4 Data analysis

All statistical analyses were performed using Graph Pad Prism 8 software. Statistical tests employed included: t test (comparison between two groups), one way ANOVA and post hoc tests i.e., Dunnett's multiple comparisons test (comparison among more than 2 groups) and log-rank test (survival data).

For the absorbance obtained from Volition assay, the data was quantified using a 4 parameters logistic curve fitting by Graph pad prism 8 software and the concentration related to each sample were interpolated in respect to the curve.

Graph pad prism 8 software was used for statistical analysis of the data for IC_{50} and combination study [Sigmoidal, four parameters logistic (4PL), X is log (concentration)]. For IC_{50} measurement, the data of counting the cells have been normalized to DMSO and transformed to X concentration to make a plot. For combination study, the data of counting the cells have been normalized to GSK-126 and Carboplatin and transformed to X concentration to make a plot.

For all experiments, three independent biological replicates were done (n = 3). Moreover, all data were shown as mean ± SEM.
5 Results
5.1 Bioinformatic analysis

The cBioPortal clinical database, called “Metastatic Prostate Adenocarcinoma (SU2C/PCF Dream Team, PNAS 2019)” has been queried to measure the frequency of gene amplifications of the three main PRC2 genes in advanced PCa patients. Moreover, the whole exome sequencing data of 444 tumours from 429 patients with mCRPC and RNA sequencing of 332 tumours from 323 patients were employed for my analysis. The level of expression estimated by Z-score in each data. The clinical and molecular characteristics of this study are summarised in Section 1.6. The results show that PRC2 genes amplifications are relatively rare (<6%). However, EZH2 has the highest percentage of gene amplifications compared to SUZ12 and EED (Figure 23).

![Figure 23. Genetic amplification of PRC2 genes in PC. This Figure shows the percentages of samples with PRC2 genes amplification in metastatic PCa. N= 429 [in cBioPortal for Cancer Genomics, http://cbioportal.org- the data set called Metastatic Prostate Adenocarcinoma (SU2C/PCF Dream Team, PNAS, Abida et al., 2019)]](image-url)

Subsequently, the mRNA expression of each PRC2 gene has been investigated in the same dataset, in this case, I have analysed the correlation between gene expression and two clinical characteristics:

1. NEPC features (panel A of Figures from 24 to 26)
2. AR activity score (panel B of Figures from 24 to 26)
Figure 24. Expression of EZH2 in samples with or without NEPC features (A). A t-test has been conducted on two groups: PCa samples with or without NEPC features (n=167) (***, p ≤ 0.0001).

Correlation between EZH2 expression and AR activity score (B). Linear regression showing that the expression of EZH2 is negatively correlated with AR activity (n=264) (Abida et al., 2019) [in cBioPortal for Cancer Genomics, http://cbioportal.org - the data set called Metastatic Prostate Adenocarcinoma (SU2C/PCF Dream Team, PNAS, Abida et al., 2019)]

The results indicate that there is a significantly higher mRNA expression of the EZH2 gene in NEPC compared to prostatic adenocarcinomas samples (Figure 24; t-test, p < 0.0001 difference) and that EZH2 expression is inversely correlated with AR activity.
Figure 25. Expression of *EED* in samples with or without NEPC features (A). A t-test has been conducted on two groups: PCa samples with or without NEPC features (n=167) (**** P ≤ 0.0001).

**Correlation between *EED* expression and AR activity score (B).** Linear regression showing that the expression of *EED* is negatively correlated with AR activity (n=264) (Abida *et al.*, 2019). [in cBioPortal for Cancer Genomics, http://cbioportal.org - the data set called Metastatic Prostate Adenocarcinoma (SU2C/PCF Dream Team, PNAS, Abida *et al.*, 2019)].

The results suggest that there is a significantly higher mRNA expression of the *EED* gene in NEPC compared to prostatic adenocarcinomas samples (Figure 25; t-test, P < 0.0001) and that *EED* expression is inversely correlated with AR activity. However, the inverse correlation with AR score is weaker for *EED* (Pearson = -0.28 for *EZH2*, Pearson = -0.17 for *EED*).

![Image](image1.png)

Figure 26. Expression of *SUZ12* in samples with or without NEPC features (A). A t-test has been conducted on two groups: PCa samples with or without NEPC features (n=167) (**** P ≤ 0.0001).

**Correlation between *SUZ12* expression and AR activity score (B).** Linear regression showing that the expression of *SUZ12* is negatively correlated with AR activity (n=264) (Abida *et al.*, 2019). [in cBioPortal for Cancer Genomics, http://cbioportal.org - the data set called Metastatic Prostate Adenocarcinoma (SU2C/PCF Dream Team, PNAS, Abida *et al.*, 2019)].

Finally, the results suggest that there is a significantly higher mRNA expression of the *SUZ12* gene in NEPC compared to prostatic adenocarcinomas samples (Figure 26; t-test, P < 0.0001) and that *SUZ12* expression is inversely correlated with AR activity. However, the inverse correlation with AR score is weaker for *SUZ12* (Pearson = -0.28 for *EZH2*, Pearson = -0.09 for *SUZ12*).

Taken together, these results indicate that all the Polycomb genes are upregulated in AVPC. *EZH2* (mRNA) seems to be the most up-regulated gene in AVPC. One limitation of this
analysis is that we could not measure the protein levels of these genes (samples were not available). Another limitation is that there was no other study with more AVPC samples, so we could not replicate our analyses.

Due to the prominent role of EZH2 in determining AVPC features, it was queried the correlation between the expression of this gene and the overall survival of advanced PCa patients. The results show that patients with higher EZH2 expression have a shorter survival time (Figure 27).

\[
\text{p-value} = 0.0272
\]

![Overall survival time based on EZH2 expression (Abida et al., 2019). The Kaplan-Meier plot is based on EZH2 expression levels higher (red) or lower (blue) than the median (p value based on Log-rank test). Median survival times: high expression = 17.58; low expression = 30.72. This study includes a subgroup of 80 patients with survival data. Time 0 was when those biopsies were collected which overlapped with diagnosis of metastasis. Ticks on the graph mean censored patients, i.e patients who did not die within the timeframe of observation. n= 80 patients [in cBioPortal for Cancer Genomics, http://cbioportal.org- the data set is called Metastatic Prostate Adenocarcinoma (SU2C/PCF Dream Team, PNAS, Abida et al., 2019)].](image)

### 5.2 Evolutionary conservation of the EZH2 coding sequence

One of the best ways to identify functional sequences is to search the conserved sequences over the species (Hardison et al., 1997 and Loots et al., 2000). To identify the evolutionary conservation of EZH2 between humans and mice, the BLAST was chosen. In BLAST, evolutionary conservation of proteins or nucleotides sequences is recognised by aligning the sequences and by analysing similarities and dissimilarities (Figure 28). The results demonstrated that the EZH2 protein is highly conserved between human and mouse, with 98% perfect identity and 99% similarity (Table 9). Similarity is defined as amino acid identity or presence of two aligned residues with similar physico-chemical properties (Basic Local Alignment Search Tool, NIH, https://blast.ncbi.nlm.nih.gov/Blast.cgi).
Figure 28. Alignment of EZH2 protein sequences between humans and mice. The green (+) shows similarity between humans and mice amino acids. The yellow highlights (++) show different amino acids between these two species. All other residues (no colour) are perfectly aligned. Query lines refer to human protein sequences and Subject (Sbjct) lines refer to mice protein sequences.
This very high level of conservation of EZH2 has allowed this project to choose one human and one mouse model for investigations in this study: DU-145 (AR+ and NEPC−) is a human cell line that has been chosen as a model of anaplastic PCa; OPT-7714 (AR+ and NEPC+) is a murine cell line that has been chosen as a model of NEPC. All the experiments below will be conducted with these two cell lines.

5.3 H3K27me3 levels in cells exposed to EZH2 inhibitors

To determine the effects of three clinically tested EZH2 inhibitors on EZH2 enzymatic activity, DU-145 and OPT-7714 cells were exposed to these compounds, and H3K27me3 levels were measured in cellular extracts, using Western blots (Figures 29 and 30). Cells have been exposed to three different concentrations of the compounds (1, 5 and 10µM) for 72h. These results show that all the EZH2 inhibitors induce a measurable reduction of H3K27me3 levels in both cell lines.

![Figure 29. H3K27me3 levels in DU145 cells exposed to different EZH2 inhibitors. DU-145 cells were treated with DMSO (as a control), Tazemetostat 1, 5, and 10µM, GSK-126 1, 5, and 10µM, and CPI-1205 1, 5, and 10µM for 72 hours; cellular protein extracts were analysed by immunoblotting using anti-H3K27me3 and anti-GAPDH antibodies. [N=3 biological replicates]. The whole membrane picture including protein standard is shown in Appendix 1. (Page 86).]
Figure 30. H3 K27me\(^3\) levels in OPT-7714 cells exposed to different EZH2 inhibitors. OPT-7714 cells were treated with DMSO (as a control), Tazemetostat 1, 5, and 10µM, GSK-126 1, 5, and 10µM, and CPI-1205 1, 5, and 10µM for 72 hours followed by immunoblotting against H3K27me\(^3\) antibody. [N=3 biological replicates]. The whole membrane picture including protein standard is shown in Appendix 1. (Page 86).

Subsequently H3K27me\(^3\) levels have been measured in cell culture supernatants collected after treatment with each of the three EZH2 inhibitors, at the same concentrations and for the same treatment duration used for Western blots (Figure 18). These measurements have been conducted using the Volition NU.Q assay. The results showed that nucleosomes are detectable in the supernatant of cancer cells, and that the levels of “soluble” H3K27me\(^3\) are significantly reduced upon treatment with the EZH2 inhibitors (Figure 31).

Figure 31. H3K27me\(^3\) levels in the supernatant of DU-145 (A) and OPT-7714 (B) cells. Cells were exposed to EZH2 inhibitors and DMSO (as a control) at the indicated concentrations for 72h. Statistical
analysis: two-way ANOVA and Dunnett's multiple comparisons test [ANOVA P-value column factor <0.0001 (DU-145 P-values: **P= 0.0012, ***P<0.0004, ****P< 0.0001)] and [ANOVA P-value column factor <0.0001 (OPT-7714 P-values: ns= 0.1898, ***P< 0.0008 and ****P< 0.0001)]. [N=3 biological replicates (error bars represent SD)].

To investigate the duration of EZH2 inhibition, H3K27me³ levels have been measured in the supernatant of AVPC cells exposed to these compounds for different durations, up to 10 days (Figure 32). The results suggest that EZH2 activity is progressively inhibited in these cells, and that this inhibition lasts for at least 10 days.

Taken together, these results indicate that pharmacological inhibition of EZH2 leads to reduced H3K27me³ both in the nucleus and in cell-free nucleosomes (supernatant). A limitation of this observation is that this effect was not confirmed in vivo. We are planning to do this in future experiments.

Figure 32. H3K27me³ reduction upon treatment with 10µM EZH2 inhibitors at different time points in DU-145 (A) and OPT-7714 (B). Both graphs show the reduction of H3K27me³ levels (supernatant) from day three to day ten after treatment with Tazemetostat, GSK-126, and CPI-1205 on DU-145 (A) and OPT-7714 (B). [N=3 biological replicates (mean ± SEM)].

5.4 Effects of EZH2 inhibition on cell viability

To investigate the anticancer activity of the EZH2 inhibitors, growth inhibitory values have been calculated in two cell lines (DU-145 and OPT-7714) treated with Tazemetostat, GSK-126, or CPI-1205 at different concentrations for 10 days. These results show that the EZH2 inhibitors induce a very modest dose-dependent growth inhibition in both cell lines, as confirmed by the fact that the growth inhibitory IC₅₀ values were not calculable (Figures 33 - 35). GSK-126 was the only compound inducing almost 100% cell death, albeit only at very high concentrations (Figure 34).
Figure 33. Growth inhibitory effects of Tazemetostat on DU-145 (A) and OPT-7714 (B) cells. DU-145 (C) and OPT-7714 (D) images at the end of the treatment (bright field microscopy). The cells were counted after exposing the cells to tazemetostat in different concentrations including 0, 0.01, 1, 10, and 25 µM for 10 days. Cell viability (IC₅₀) was calculated via Graph Pad Prism. [N=3 biological replicates (mean ± SEM)]
Figure 34. Growth inhibitory effects of GSK-126 on DU-145 (A) and OPT-7714 (B) cells. DU-145 (C) and OPT-7714 (D) images at the end of the treatment (bright field microscopy). The cells were counted after exposing the cells to GSK-126 in different concentrations including 0, 0.01, 1, 10, and 25 µM for 10 days. Cell viability (IC$_{50}$) was calculated via Graph Pad Prism. [N=3 biological replicates (mean ± SEM)]
Figure 35. Growth inhibitory effects of CPI-1205 on DU-145 (A) and OPT-7714 (B) cells. DU-145 (C) and OPT-7714 (D) images at the end of the treatment (bright field microscopy). The cells were counted after exposing the cells to CPI-1205 in different concentrations including 0, 0.01, 1, 10, and 25 µM for 10 days. Cell viability (IC<sub>50</sub>) was calculated via Graph Pad Prism. [N=3 biological replicates (mean ± SEM)]

Taken together, these results indicate that monotherapy with EZH2 inhibitors is not effective on AVPC cells. One limitation of this study is that we used only two cell lines, due to the scarcity of models available when this study began. With new AVPC cell lines available, we plan to expand those studies to a wider range of AVPC subtypes. Moreover, GSK126 seems to decrease cell number at higher concentrations, this could mean that the cells are dying or undergoing growth arrest. This phenomenon could be further investigated utilising assays that determine apoptosis (cleaved PARP, cleaved caspase 3) and cell cycle analysis (e.g., utilising FACS analysis).

5.5 Effects of EZH2 inhibitor in combination with chemotherapy

The results so far indicated that the EZH2 inhibitors are effective at reducing the histone methyltransferase activity but show modest anticancer efficacy as monotherapy. I therefore set out to investigate whether EZH2 inhibitors could increase the anticancer activity of Carboplatin, which is a chemotherapy drug currently employed in the treatment of AVPC. GSK-126 was selected for this type of treatments, as this compound was slightly more effective in reducing AVPC cancer cell viability, compared to other EZH2 inhibitors. The growth inhibitory IC<sub>50</sub> values have been
calculated on two AVPC cell lines (DU-145 and OPT-7714) treated with combination of GSK-126 plus Carboplatin vs Carboplatin alone (Figure 36). The results show that GSK-126 increases the activity of Carboplatin in AVPC cells as evidenced by a markedly reduced IC₅₀ value in both cell lines treated with the two drugs, compared to Carboplatin alone.

### Figure 36. Cell viability (IC₅₀) on DU-145 (A) and OPT-7714 (B) treated with combination of GSK-126 and Carboplatin vs Carboplatin alone.

Cells were exposed to 10 µM GSK-126 for 72 hours, followed by GSK-126 (10 µM) and Carboplatin (different concentrations as indicated by the X axis) for the following 72 hours. Cells were counted at the end of the experiment. Cell viability inhibition (IC₅₀) was calculated via Graph Pad Prism. [N=3 biological replicates (mean ± SEM)]

These results indicate that the combination between EZH2 inhibitors and chemotherapy may be effective in NEPC. These results will need to be confirmed in other cell lines and in clinically relevant in vivo models.
6 Discussion
In this work, the effects of EZH2 inhibitors on AVPCs cells were investigated, to test the hypothesis that EZH2 is a therapeutic target in this type of PCa. Since the survival time of patients with AVPC is very short, and since there is no treatment for this disease, new therapies are needed. EZH2 is overexpressed in several cancers, including AVPCs (Bachmann et al., 2006). The bioinformatic analyses have confirmed that EZH2 is the most frequently amplified PRC2 gene in AVPCs and that the overexpression of EZH2 mRNA is associated with NEPC features; notably, the expression of this gene is negatively correlated with AR transcriptional activity. The clinical datasets did not include proteomic results, and this is a limitation of this study. However, differentially expressed mRNAs tend to be highly correlated with their protein expression (Koussounadis et al., 2015). Hence, it is likely that EZH2 protein is also highly expressed in both main types of AVPC: anaplastic PCa and NEPC.

Results from the current experiment indicate that treatment with GSK-126 reduced H3K27me3 levels as detected by Western blotting and confirmed by Volition assays. These results are similar to those previously reported (McCabe et al., 2014) and GSK-126 may be offered as a new therapy for lymphoma and other malignancies. In addition, a recent study has shown that combination of two of the EZH2 inhibitors (tazemetostat and GSK-126) with AR antagonists improved efficacy of AR antagonists in CRPC cells (Kim et al., 2018). Also, it has been shown that combining GSK-126 and Enzalutamide inhibits cell proliferation and colony formation and stimulate apoptosis in Enzalutamide resistant PCa cells (Bai et al., 2019). CPI-1205 decreases the H3K27me3 and displays anti-cancer activity in lymphoma (Vaswani et al., 2016). As there is no current study of CPI-1205 on PCa, this study would be the first study of efficacy of CPI-1205 in this type of cancer. No study so far has investigated the activity of any EZH2 inhibitor in AVPC.

Based on published evidence and on the bioinformatic analysis in this study, it was decided to test the activity of three clinically tested EZH2 inhibitors in one model of anaplastic PCa (DU-145) and one model of NEPC (OPT-7714). As shown in the results, three EZH2 inhibitors induced a potent and durable reduction of EZH2 enzymatic activity (H3K27me3 levels) in both cell types. The enzymatic inhibition lasts for at least 10 days in these models. However, the EZH2 inhibitors showed only modest anticancer activity, when used as monotherapy. Interestingly, adding the EZH2 inhibitor to Carboplatin dramatically reduced the IC_{50} value of the chemotherapy drug. These results suggest that the combination of epigenetic therapies and chemotherapy is a promising strategy in AVPC.

Platinum-based complexes are a critical component of contemporary cancer intervention approaches. However, the role of platinum agents in PCa therapy has been limited. Platinum agents have been shown to be potentially active against AVPCs. For example, a phase II study has reported high response rates to platinum-based chemotherapies in patients with anaplastic prostate cancer (Aparicio et al., 2013). Similarly, a study comparing the effects of platinum agents in men with prostate adenocarcinoma vs NEPC has found that the latter group showed a significantly higher response rate to platinum agents (Humeniuk et al., 2017). No study so far has shown that platinum agents increase the survival of AVPC patients. However, because of their activity in reducing tumour burden, these drugs are currently employed as palliative treatment for AVPCs. The results in this project, support the hypothesis that a combination of
platinum agents and EZH2 inhibitors could provide substantial clinical benefit to AVPC patients. This hypothesis should be tested by future in vivo and possibly clinical studies.

Histone modifications have been described as important epigenetic mechanisms of PCa cancer progression and have been proposed as PCa biomarkers (Seligson et al., 2005). However, these modifications are not easily detectable in biological fluids and usually require invasive procedures for protein extraction (e.g., tumor biopsies). Volition has recently developed an assay that measures H3K27me3 levels from tiny amounts (a few µl) of biological fluids (https://volition.com/nu-q). We have proposed that this technology could be used in conjunction with EZH2 inhibitors for patients’ selection and for treatment monitoring (Kang et al., 2020). To gather proof-of-principle evidence that this strategy could work, the H3K27me3 levels were measured in cell supernatants collected from cells exposed to the EZH2 inhibitors. Our results are encouraging and show that H3K27me3 levels are detectable with this methodology, and that the cell supernatant readings are in agreement with Western blot measurements. However, these results will need to be confirmed in vivo, e.g., in serum samples from patient-derived xenografts, before we can propose clinical studies.

Hence, the development on EZH2 inhibitors (and companion diagnostics) represents a promising epigenetic therapy that can be translated into the clinics. Future in vivo studies will need to confirm this hypothesis. In addition, the mechanism by which EZH2 inhibition facilitates the activity of Carboplatin is currently unknown. EZH2 has been shown to silence several genes that induce apoptosis and block cell cycle progression (Hubaux et al., 2013). For example, the cyclin dependent kinase inhibitor 2A (CDKN2A) locus is a well characterised target of EZH2-dependent silencing (Duan et al., 2020). This locus encodes p16, which blocks cyclin-dependent kinases leading to cell cycle arrest, and p14, which activates apoptosis via p53. EZH2 inhibition results in the reactivation of p16 and p14 expression. Notably carboplatin is able to induce apoptosis and cell cycle arrest by modulating the same pathways (Singh et al., 2007) (Figure 37). It is therefore conceivable that EZH2 inhibitors synergise with carboplatin by activating cellular pathways that lead to the same outcomes (cell cycle arrest and apoptosis).

Our results are promising but this study has several limitations that will need to be addressed in future studies. As discussed, our results are limited to only two cell lines. Some new NEPC models have become available (Lee et al., 2016 and Okasho et al., 2021); the carboplatin-EZH2 inhibitor combination will need to be tested in these models to generalise our conclusions. In addition, we have not investigated whether the treatments under investigation induce apoptosis and/or cell cycle arrest. These analyses will elucidate the mechanism of action of the drugs under investigation. Our laboratory is also conducting RNA Seq analyses on AVPC cells exposed to GSK-126, to measure the whole-transcriptome changes in gene expression patterns induced by EZH2 inhibition. We will then conduct pathway analyses on these samples, to confirm whether modulation of cell cycle, apoptosis or other relevant pathway can explain the observed synergism with platinum agents. In the future, our results will need to be confirmed in vivo to collect crucial data on pharmacokinetic profiles and toxicity. We hope that all these efforts will lead to a new therapy for AVPC.
Figure 37. Schematic representation of a proposed mechanism of action to explain the efficacy of combining EZH2 inhibitors and platinum agents. EZH2 methylates H3K27, which leads to the silencing of p16 and p14; H3K27me3 levels are decreased upon treatment with EZH2 inhibitors. This results in the potential reactivation of genes that stop the cell cycle (p16) and induce apoptosis (p14). The expression of p16 and p14 is promoted by platinum-induced DNA damage. Hence, the concurrent activity of EZH2 inhibitors and platinum agents results in higher levels of genes that promote cell cycle arrest and apoptosis.
7 Conclusion
Based on this project’s findings and on published evidence, the following conclusions can be drawn:

- EZH2 is a key epigenetic modulator that suppresses gene expression via histone PTMs.
- EZH2 is overexpressed in many advanced solid tumours including AVPC and is linked to poor prognosis.
- EZH2 overexpression in AVPC cells and in the tumour micro-environment promotes tumour cell growth, metastasis, and drug resistance.
- These results show that a pharmacological EZH2 inhibitor can facilitate the activity of Carboplatin in AVPCs. This combination can improve the efficacy of cancer therapies and prevent the occurrence of chemo-resistance.
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Appendix 1:
DU-145:

Both images are from the same gel, just the different exposure times and different brightness to show the bands of H3K27me3 clearer.