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‘’STUDIES ON THE MOLECULAR AND FUNCTIONAL PROPERTIES OF EXOSOMES IN THE METASTATIC PROSTATE CANCER MICROENVIRONMENT’’

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“Studies on the molecular and functional properties of exosomes in the metastatic prostate cancer bone microenvironment’’

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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This PhD thesis is for my parents

Because they are always there for me, supporting me in everything I decide to do. They are always encouraging me, and they never let me to give up.
This milestone of my life could never be achieved without them.

Το Διδακτορικό μου το αφιερώνω στους γονείς μου που πάντα με στηρίζουν σε όλες μου τις αποφάσεις. Πάντα με εμψυχώνουν και δίδαξαν να μην τα παρατάω. Αυτός ο στόχος δεν θα μπορούσε να επιτευχθεί χωρίς την συνεισφορά τους.

Abstract

Prostate cancer is the most common cancer type and the second leading cancer related cause of death worldwide in men. Prostate cancer patients initially respond to standard treatment (e.g., hormonal, surgery/irradiation) but about 30% of them will develop resistance and progress to metastatic Castration Resistant Prostate Cancer (mCRPC). The cornerstone therapy selection for these patients is chemotherapy (e.g., taxanes). Bone is the most common metastatic site in prostate cancer (mCRPC) and the most frequent cause of death in mCRPC. One of the main bone targeted therapies for patients with bone metastatic CRPC is Radium-223, an alpha emitter that has been shown to prolong survival as a single agent. Monitoring the bone tumor microenvironment (bone-TME) is challenging and is based on invasive bone biopsies that cannot be readily performed longitudinally. Liquid biopsies are an attractive approach to monitor the bone-TME including circulating tumor cells, ctDNA and extracellular vesicles (EVs). EVs have a lipid bilayer, contain nucleic acids and proteins. They have been shown to play an important role in homotypic and heterotypic intercellular communication, as well as serve as a source of biomarkers for response and resistance to therapy. In this thesis, the molecular properties of EVs were studied, co-clinically (in vitro, in vivo and in patient samples).

In paper I, we characterized the EV transcriptome changes in response to Radium-223 in vivo and patients' samples. We identified changes in genes related to bone, DNA repair and immune in both the pre-clinical models and patient samples. Treatment with Radium-223 showed a downregulation of bone related transcripts and an upregulation of DNA repair pathways (pharmacodynamic measure). Furthermore, changes in the immune system that are associated with immunosuppression and immune checkpoint activation were identified in patients with unfavorable overall survival. The data obtained indicate that EVs can detect changes in the bone-TME that were functionalized by combining Radium-223 with immunotherapy that improved treatment efficacy.

In paper II, we characterized the EV transcriptome for patients treated with Cabazitaxel. Pathway and gene enrichment analysis identified several pathways and associated genes that were enriched in patients that did not respond to Cabazitaxel. Furthermore, at baseline EVs derived from the plasma of Non-responders (NR) were enriched in transcripts encoding genes that are related to oncogenesis, cytoskeleton and immune regulation. Two genes identified to be enriched in NR are Stathmin-1 and ITSN-1 both of which have been previously associated with resistance to Cabazitaxel. Further studies are needed to determine whether longitudinal monitoring of these and other genes identified in the EVs correlates with treatment response and clinical outcome.

Taken together, our studies demonstrate that plasma derived EVs could be a useful tool in monitoring the bone TME as well as treatment responses and acquisition of resistance that correlate with clinical outcome.

LIST OF SCIENTIFIC PAPERS

I. IOULIA VARDAKI, Paul Corn, Emanuela Gentile, Jian H Song, Namrata Madan, Anh Hoang, Nila Parikh, Leah Guerra, Yu-Chen Lee, Song-Chang Lin, Guoyu Yu, Elmer Santos, Marites P Melancon, Patricia Troncoso, Nora Navone, Gary E Gallick, Eleni Efstathiou, Sumit K Subudhi, Sue-Hwa Lin, Christopher J Logothetis, Theocharis Panaretakis.
Radium-223 Treatment Increases Immune Checkpoint Expression in Extracellular Vesicles from the Metastatic Prostate Cancer Bone Microenvironment.
Clinical Cancer Research, 2021 jun 1;27(11):3253-3264

II. IOULIA VARDAKI, Seda Sabah Özcan, Pedro Fonseca, Sue-Hwa Lin, Christopher J Logothetis, Jeffrey Yachnin, Anders Ullen, Theocharis Panaretakis.
Transcriptomic analysis of plasma exosomes provides molecular information of response to cabazitaxel treatment in men with metastatic castration-resistant prostate cancer.
The Prostate, 2023 Jul;83(10):950-961

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ADT	Androgen deprivation therapy
ALP	Alkaline Phosphatase
APCs	Antigen presenting cells
AR	Androgen receptor
AR-R7	Androgen receptor variant 7
AR-R9	Androgen receptor variant 9
ATBF1	AT motif-binding transcription factor 1
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia telangiectasia and Rad3-related protein
AXL	Anexelekto
B7-H3	B7 homolog 3 protein
Bcl-xL	B-cell lymphoma-extra large
BCL2	B-cell lymphoma 2
bmCRPC	Bone metastatic Castration Resistant prostate Cancer
Bmp3	Bone morphogenic protein 3
BMP4	Bone morphogenic protein 4
Bmp6	Bone morphogenic protein 6
Bmpr1	Bone morphogenic protein receptor 1
BMPs	Bone morphogenic proteins
BPH	Benign Hyperplasia
BRCA1	Breast cancer type 1 susceptibility gene
BRCA2	Breast cancer type 2 susceptibility gene
BRIP1	BRCA1-interacting protein C terminal helicase1
BSP II	Bone sialoprotein II
C2GnT	Core2 β-1,6-N-acetylglucosaminyltransferase
CAFs	Cancer Associated Fibroblasts
CAR-T cells	Chimeric antigen receptor T-cells

CCL2	Chemokines C–C motif chemokine ligand 2
CD28	Cluster of Differentiation 28
CD4	Cluster of differentiation 4
CD44	Cluster of differentiation 44
CD54	Cluster of Differentiation 54
CD63	Cluster of Differentiation 63
CD8	Cluster of differentiation 8
CD81	Cluster of Differentiation 81
CD9	Cluster of Differentiation 9
CgA	Chromogranin A
cGAS/STING	Cyclic GMP-AMP synthase/ Stimulator of interferon genes
CgB	Chromogranin B
CHEK2	Checkpoint kinase 2
CK14	Cytokeratin 14
CK18	Cytokeratin 8
CK5	Cytokeratin 5
CK8	Cytokeratin 8
CRPC	Castration resistant prostate cancer
CTCs	Circulating tumor cells
ctDNA	Circulating cell-free tumor DNA
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DKK1	Dickkopf-1
DNA	Deoxyribonucleic acid
DRE	Digital rectal examination
ECM	Extracellular matrix
EDCs	Endocrine disrupting chemicals
EGFR	Epidermal growth factor receptor
EIF2	Eukaryotic translation initiation factor 2A
ELAC2	ElaC Ribonuclease Z 2
EMT	Epithelial to Mesenchymal Transition

EPCAM	Epithelial cell adhesion molecule
EPHB2	Ephrin Receptor B2
ERG	Erythroblast transformation-specific (ETS)-related gene
ERK	Extracellular signal-regulated kinase
ESCRT	Endosomal sorting complex required for transport
ETS	Erythroblast transformation-specific
EVs	Extracellular vesicles
FAK	Focal adhesion kinase
FasL	FS-7-associated surface antigen ligand
FasL	FS-7-associated surface antigen
FGF	Fibroblast growth factor
FGFR	Fibroblast Growth Factor Receptor
FOXM1	Forkhead box M1
FOXP3	Forkhead box P3
Fstl3	Follistatin-related protein 3
G2-M	Gap 2 - Mitosis
gDNA	Genomic DNA
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
HIFU	High-intensity focused ultrasound
HOXB13	Homeobox B13
HPV	Herpes Simplex Virus
HSC	Hematopoietic stem cells
ICOS	Inducible Costimulator Protein
ICT	Immune checkpoint therapy
IDO	Indoleamine 2,3-dioxygenase
IGF	Insulin growth factor
ILVs	Intraluminal vesicles
ITGA3	Integrin Subunit Alpha 3

ITSN-1	Intersectin-1
KLF6	Kruppel-like factor 6
KRAS	Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LAG3	Lymphocyte-Activation Gene 3
LAMP1	Lysosomal associated membrane protein 1
LC3	Microtubule-associated proteins 1A/1B light chain 3B
LDELS	LC3-dependent EV loading and secretion
lncRNA	Long non-coding RNA
M-phase	Mitosis phase
MAPK	Mitogen-activated protein kinase
mCRPC	Metastatic castration resistant prostate cancer
MDR1	Multidrug resistance gene 1
MDSCs	Myeloid derived suppressor cells
MFG-E8	Milk-fat globule EGF factor 8
MHC	Major histocompatibility complex
mHSPC	Metastatic hormone sensitive prostate cancer
mitRNA	Mitochondrial RNA
MLH1	MutL homolog 1
MMP9	Matrix metalloproteinase 9
mp-MRI	Multiparametric magnetic resonance imaging
mRNA	Messenger RNA
MSC	Mesenchymal stem cells
MSH2	MutS homolog 2
MSH6	MutS homolog 6
MSR1	Macrophage Scavenger Receptor
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
MVBs	Multivesicular bodies
NBS1	Nijmegen Breakage Syndrome 1

NEK2	NIMA related kinase 2
NIMA	Never-In Mitosis, gene A
NK cells	Natural killer cells
NKX3.1	NK3 Homeobox 1
nSMASE	Neutral Sphingomyelinase
NTA	Nanoparticle tracking analysis
ONC	Osteonectin
OPG	osteoprotegerin
OPN	Osteopontin
OS	Overall survival
P-gp	P-glycoprotein 1
p53	Protein p53
PALB2	Partner and localizer of BRCA2
PAP	Prostatic-specific acid phosphatase
PARP	Poly-ADP ribose polymerase
pATM	phosphorylated ATM
PCA3	Prostate cancer antigen 3
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand 1
PDX	Patient derived xenograft
PET	Positron emission tomography
PFS	Progression free survival
piRNA	Piwi-interacting RNA
PLP	Proteolipid protein
PMN-MDSCs	polymorphonuclear MDSCs
PMS2	Post-meiotic segregation increased 2
PON1	Paraoxonase 1
PSA	Prostate specific antigen
PSCA	Prostate stem cell antigen
PSMA	Prostate-specific membrane antigen
PTEN	Phosphatase and TENsin homolog deleted

PTHrP	Parathyroid hormone-related peptide
RANKL	Receptor activator of nuclear factor kappa-B ligand
RAS	Rat sarcoma
RB	Retinoblastoma protein
RBPs	RNA binding proteins
RNASEL	Ribonuclease L
rRNA	Ribosomal RNA
Runx2	Runt-related transcription factor 2
SDF-1	Stromal-derived factor 1
SPOP	Tumor suppressor gene Speckle Type POZ protein
SRD5A2	Steroid 5-alpha-reductase
STDs	sexually transmitted diseases
STMN1	Stathmin-1
TAMs	Tumor associated macrophages
TANs	Tumor associated neutrophiles
TCF4	Transcription factor 4
TCR	T cell receptor
TGFβ	Transforming growth factor-β
Th	T helper
TIL	Tumor-infiltrating lymphocytes
TMPRSS2	Transmembrane serine protease 2
TNM	Tumor, nodule, metastasis
Tregs	T regulatory cells
tRNA	Transfer RNA
TSAP6	Tumor suppressor-activated pathway 6
TSG101	Tumor susceptibility gene 101
VEGF	Vascular endothelial growth factor
vtRNAs	Vault RNA

WNT	Wingless/Integrated'
ZEB1	Zinc finger E-box-binding homeobox 1

1 INTRODUCTION

Prostate cancer is the most common cancer type and the second leading cancer related cause of mortality in men (according to 2022 statistics, Siegel R.L., ACS Journals, 2022)[1]. Standard of care includes surgery as well as androgen deprivation therapy. About 30% of the patients after initial response to standard treatment will progress to metastatic Castration Resistant Prostate Cancer (mCRPC). For these patients' chemotherapy treatment with the taxanes Docetaxel and Cabazitaxel is recommended. Cabazitaxel acts by causing cell cycle arrest and eventually cell death. This happens by stabilizing microtubules and is efficient in patients that have already developed resistance to Docetaxel. In addition to this, Cabazitaxel inhibits AR translocation to the nucleus.

Bone is the most common site of metastasis of prostate cancer and is associated with increased mortality. The bone microenvironment has proven to be attractive for cancer cells and this interaction promotes disease progression. Radium-223 is a bone targeting radiopharmaceutical that improves OS in contrast with other bone targeting therapies.

Exosomes are a unique type of extracellular vesicles with a specific and regulated mechanism of biogenesis and secretion. It has been shown that they are key players in cellular communication, both in close and distant sites. They are present in biological fluids and secreted by all types of cells. They play a role both in health and disease, including cancer. Exosomes have size of 30-150nm, have a lipid bilayer and contain nucleic acids and proteins. Because of their molecular composition they are a valuable source of biomarkers.

In this thesis, we studied the molecular properties of a subtype of extracellular vesicles, the exosomes in the metastatic prostate bone microenvironment. The aim was to determine whether these EVs are a suitable tool to monitor the bone tumor microenvironment and gain information for biomarkers that could assist in monitoring treatment response and acquisition of resistance to therapies targeting the bone metastatic Castration Resistant Prostate Cancer (bmCRPC) (Docetaxel and Radium-223). Overall, our goal is to exploit EVs as a tool that will allow us to monitor longitudinally mechanisms of action and resistance and thereby allow for earlier intervention and rational treatment combinations.

2 LITERATURE REVIEW

2.1.1 Prostate

Prostate is the biggest gland in males' reproductive system. It is located below the bladder and in front of the rectum. In healthy adults prostate has dimensions about 44.4 mm width, 31.2mm height and 37.5 mm length, weighs about 20 grams, has a walnut shape and elasticity[2–4]. The size of prostate is age related and older males have bigger prostates[5].

The primary **function** of prostate is to produce the prostatic fluid of the semen; it produces 20-30% of the fluid of total semen volume. The rest is produced from the seminal vesicles and a small amount from the testicles. Prostatic fluid contains nutrients crucial for the survival of the semen. Importantly, it contains prostate specific antigen (PSA) which facilitates the maintenance of correct semen viscosity. Another function of the prostate is that during ejaculation, it contracts and moves the prostatic fluid into the urethra, where it is combined with the sperm cells and fluid with from the seminal vesicles, in order to produce the semen. During ejaculation prostatic contraction, causes the closing of space between urethra and bladder and this leads the semen to pass through with speed. Finally, prostate contains 5-alpha reductase, which converts testosterone into the biologically active form dihydrotestosterone (DHT)[6].

Prostate anatomically is divided in **lobes**: The **anterior lobe** is consisted of fibromuscular tissue, and it is located in front of the urethra. The **median lobe** is located among the two ejaculatory ducts and the urethra. **Lateral lobes** (right and left) consist of the main mass of the prostate and are continuous posteriorly and are separated from the prostatic urethra. Finally, the **posterior lobe** is the part of the lateral lobes that can be palpated during the digital rectal exam[7] (**Figure 1**).

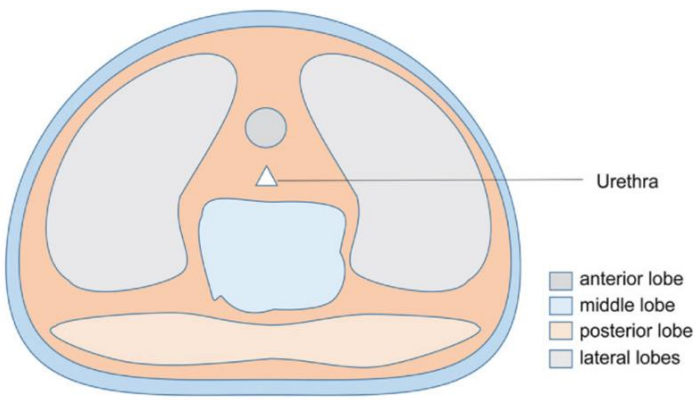

***Figure 1**: **Schematic representation of Prostate five lobes** (From Xudong Yu et al., Frontiers in Oncology, 2023).*

Histologically, prostate is divided into **five anatomic regions**: the **central zone,** which surrounds the ejaculatory glands and accounts for 25% of normal prostate volume. The central's zone glandular glands that diagonally emptying in the prostatic urethra, so it's protected from the urine

reflux. The **periurethral** zone is a very narrow area with short glands adjacent to urethra[8]. The **transitional zone** locates in the center and surrounds 5-10% of normal prostate volume. The glands of this zone are the ones that usually give rise to benign hyperplasia (BPH). **Peripheral zone** makes up the main body of prostate (about 65% of prostate volume) and is located posteriorly. Most cases of prostate cancer originate from this zone. Finally, there is also the **fibromuscular stroma** which is located anteriorly of the gland. It merges with the tissue of the urogenital diaphragm (**Figure 2a**)[4,9].

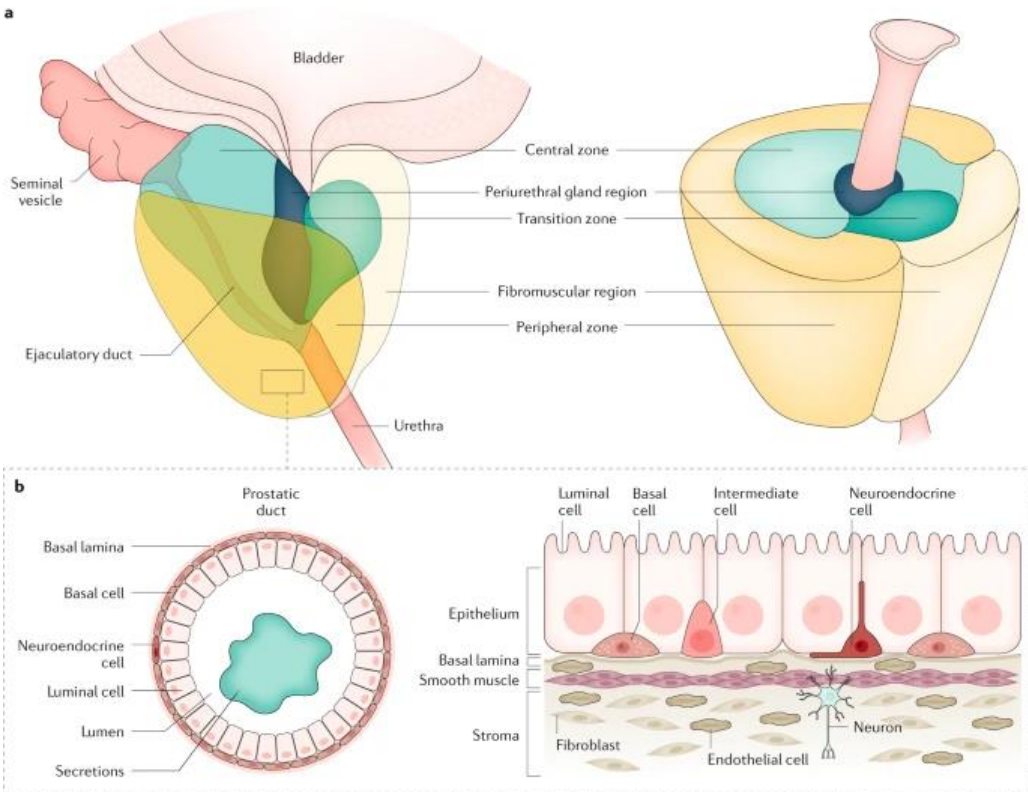

***Figure 2: Prostate structure**. a. Prostate's anatomic regions b. cell composition of prostate's regions. (Taken from Rebello R.J. et al., Nature Reviews 2021).*

The normal prostate gland consists of ducts and acini inside the stroma. The ducts and the acini built a monolayer of columnal epithelial cells, which is surrounded by a monolayer of basal epithelium that is responsible for the production of the basement membrane, in which stromal cells harbor. Those cells are mainly smooth muscle myocytes, whose function is to contract unconstrainedly in order to prevent fluid immobility. Fibroblasts are also located in the stroma and have a role in normal prostate development. They also have a role in prostate cancer development, since exhibit pro-tumorigenic capabilities (epithelial transformation, promoting tumor cells' survival) (**Figure 2b**)[9].

2.2 PROSTATE CANCER

2.2.1 Incidence, Mortality and 5-year survival

Prostate cancer is the most frequently cancer type in men, followed by lung cancer[1,10,11]. In men, it is estimated that the new cases in 2022 account for about 27% of all the new cancer cases[1] and it's the second cause of cancer related deaths after lung cancer **(Figure 3)** [1,9,10].

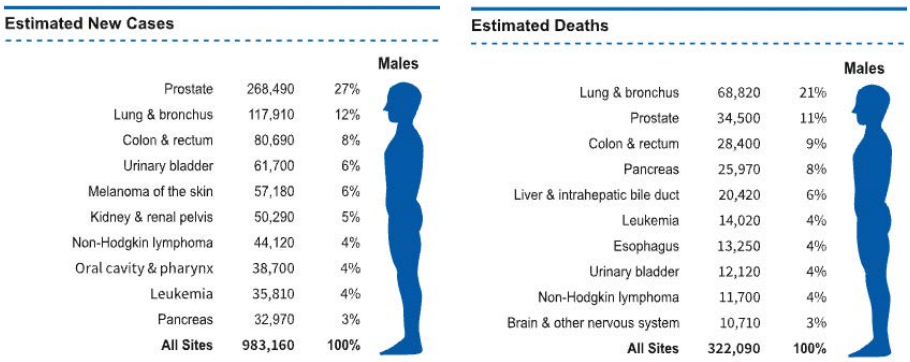

Figure 3: Leading Cancer Types for estimated Cases and deaths in men. *(Siegel R.L., ACS Journals, 2022).*

Age is a factor that contributes to higher incidence and mortality with the average age of prostate cancer diagnosis is 66 years[12]. The incidence rates differ among different countries. Northern and Western Europe (83.4 per 100,000 people and 77.6 respectively), followed by Caribbean (75.8), Australia/New Zealand (75.8) and Southern Africa (65.9). These differences in the incidence rates is proposed to be due to combination of genetic, environmental and social factors[12]. In addition to the incidence rates, mortality rates also differ in different regions: the highest rates are in The Caribbean (75.8 per 100,000 people), Sub-Saharan (22.0) and Micronesia/Polynesia (18.8) [12].

The 5-year survival rate in USA for men diagnosed with prostate cancer for localized and regional disease is almost 100%, whereas the 5-year survival in men with distant metastatic disease drops at 30%. and finally, the overall survival rate for the disease is about 98% [13]. In Europe the 5-year survival ranges from 76-88%. In Sweden, prostate cancer was also the most frequent cancer in men and has the highest mortality for 2021[14,15]. In 2020, prostate cancer incidence was 10949 which was the eighth highest worldwide[16] and the third age standardized incidence in Europe[15]. Interestingly, pandemic had an effect in incidence of prostate cancer in Sweden, causing a drop to 8900 in the year 2020[15]. Due to early detection and advances in therapies, the last 20 years, age standardized mortality has decreased by one third and the mortality in elderly people (80 year and above) has dropped by half[15].

2.2.2 Etiology and Risk Factors of prostate cancer

There are a lot of factors that contribute to the development of prostate cancer, a complex and very heterogeneous disease. The risk factors for the development of this malignancy can be divided

into two main categories: the exogenous and the endogenous, with some additional factors that can be included in both categories.

2.2.2.1 *Exogenous risk factors:*

Diet plays a major role in prostate cancer. Elevated levels of saturated animal fat have been associated with prostate cancer because it increases testosterone levels. Dairy products (milk, cheese, etc.) can increase saturated fat, thus they contribute to prostate cancer initiation[17]. Dairy products also contain levels of calcium which it has been associated with prostate cancer since intracellular pools of calcium can control cell growth and apoptosis in prostate cancer cell lines[18]. The consumption of red meat has been linked to prostate cancer in various studies[19–21] and in addition to this, cooking in high temperatures can lead to the generation of mutagenic heterocyclic amines which have been linked with carcinogenesis[20,21]. Finally, alcohol consumption is generally considered a risk factor for cancer, including prostate cancer[22].

Obesity is considered a risk factor for aggressive prostate cancer. Factors related to metabolism (insulin, Insulin growth factor (IGF), androgens and estrogens) contribute to prostate cancer development [23].

Metabolic syndrome (high blood pressure, high blood sugar, unhealthy cholesterol levels and abdominal fat), which is related to health and diet habits has also been associated with increased risk for developing prostate cancer[24].

Smoking has been linked with tumorigenesis in general and also with prostate cancer[17] and especially lethal prostate cancer[25].

Several epidemiologic studies have shown that there is a correlation of **sexually transmitted diseases (STDs)** (HPV, Herpes Simplex Virus 1 and 2, Human Herpes Virus 8, Cytomegalovirus, Trichomonas, Gonorrhea, etc.) and prostate cancer[17,26]. For bacterial infections the underlying mechanism that leads to prostate cancer is related to prostate inflammation and atrophy; for viral infections the tumorigenic mechanisms are related to transforming capabilities of these viruses[17,26].

In addition to those, there are also **environmental agents** such as endocrine disrupting chemicals (EDCs). These agents can affect hormone activity, leading to alterations in reproduction, tumorigenesis and developmental processes. One example of EDC is the presence in pesticide residues in chemicals and foods [27].

2.2.2.2 *Endogenous risk factors:*

Family history has been associated with increased risk for prostate cancer. Familial prostate cancer is when two first degree relatives are diagnosed with prostate cancer at any age or when one first degree and two or more second degree relatives are diagnosed with prostate cancer at any age. Men with one first degree relative with prostate cancer have 2.1-2.8 fold elevated risk for developing

the disease and having two relatives with the disease increases the risk 3.5 times[17,28]. These cases of familial prostate cancer account for about 5-10% of all cases of prostate cancer worldwide[29].

Genetic mutations that contribute to familial prostate cancer are rare (less that 5% of the population) but they have high penetrance (80-90% possibility of expressing this specific phenotype). Genes with mutations linked with familial cases of prostate cancer are already known to be related with other familial cancers such as breast and ovarian cancer (BRCA1, BRCA2, ATM, CHEK2 and PALB2), mismatch repair genes that are linked to Lynch syndrome (MLH1, MSH2, MSH6 and PMS2) and deletion of epithelial cell adhesion molecule-EPCAM), HOXB13, RNASEL, ELAC2, and MSR1[29–33,] .

Breast cancer predisposition gene 2 (BRCA2) germline mutations are associated with the highest risk for prostate cancer (8.6-fold for men bellow 65 years old) and mutations in BRCA1 increase the risk for 3.5 times even though a very small proportion (0.44%) of prostate cancer patients have mutations. BRCA1 and 2 are tumor suppressors and they are autosomal dominant genes but with incomplete penetrance. BRCA1 controls DNA damage response, transcription and chromatin remodeling. BRCA2 regulates DNA recombination and repair process; thus loss of one of those genes results in impaired repair of double strand breaks[34].

Lynch syndrome is one of the main causes of inherited colorectal cancer but there is also increased incidence of prostate cancer in families with Lynch syndrome. This syndrome is autosomal dominant which is derived from variants in DNA mismatch repair genes (MLH1, MSH2, MSH6 and PMS2). Finally, deletion of EPCAM which leads to promoter hypermethylation and loss of MSH2 expression is a cause of Lynch syndrome in 1-3% of the cases[33].

The variant G84E of HOXB13 gene found to be present 20 times more frequent in prostate cancer patients compared with healthy individuals and more common in patients with family history of prostate cancer. HOXB13 interacts with androgen receptor (AR) and plays a crucial role in cell growth and differentiation during the development of prostate[33].

ATM and CHECK2 mutations are linked with inherited prostate cancer. They both are tumor suppressor genes encoding proteins related to DNA damage signaling pathway. ATM is a serine-threonine kinase that upon DNA-damage phosphorylates downstream target proteins related the activation of DNA repair mechanisms[33]. CHEK2, one of the target proteins of ATM, is a cell cycle checkpoint protein kinase that triggers DNA repair or cell cycle arrest through p53[33].

There are other DNA repair related candidate genes that have been suggested to play a role in familial prostate cancer and have been reported in various studies and their alterations account for a small percentage of prostate cancer. One of these genes is PALB2 which acts as a linker between BRCA1 and 2 and loss of function alterations have been linked with Fanconi anemia and other types of cancer. BRIP1 (BRCA1-interacting protein C terminal helicase1) is a helicase that plays a role in double strand DNA break repair mechanism by binding directly to BRCA1 gene during homologous recombination process. Carriers of the variant c.657del5 of NBS1 gene has been associated with a threefold increase for prostate cancer in men bellow 60 and these patients have more aggressive

disease; thus NBS1 gene has been proposed as a candidate susceptibility gene for familial/hereditary prostate cancer[33].

Finally recently with the use of genome wide association studies a lot of (~170) low penetrance loci have been identified to associate with familial prostate cancer[33].

Somatic alterations

In addition to germline mutations, there is a wide variety of somatic alterations that promote

tumorigenesis in prostate. These included **both mutations/deletions/polymorphisms gene fusions and copy number variations**. A critical role for prostate cancer development, have the genetic alterations in TMPRSS2/ETS, MYC oncogene, PTEN/p53 and later, in advanced disease AR genes[9].

Androgen receptor: Prostate is dependent on androgens which play a very important role in prostate tumorigenesis since their levels are associated with the disease. Androgens are synthesized in the testis (testosterone) and adrenal glands (androstenedione and dehyndroepiandrosterone-DHEA). Sex hormone binding globulin binds serum testosterone leaving 1-2% free to be converted to its active form dihydrotestorone (DHT) (which binds to AR) by 5-alpha-reductase in prostatic cells. Androgens produced in the adrenal are converted to testosterone by 17-beta-hydroxysteroid dehydrogenase in testis[35]. DHT binds to AR which translocate from the cytoplasm to the nucleus and activates the expression of AR responsive genes. Most of the mutations in AR gene affect its function and are base substitutions[36]. Mutations on AR are one of the mechanisms of resistance to hormone therapies, the first line therapy for prostate cancer patients. Also a variant of AR, AR-V7 has been correlated with advanced disease[37] and resistant to treatment (Enzalutamide and Abiraterone)[38].

Translocations involving androgen regulated promoters and the ETS family of transcription factors: Translocation involving these genes are the most common genomic alterations in prostate cancer. TMPRSS2:ERG fusion is present in about 50% of localized prostate cancers[36], which promote tumorigenesis and disease progression[39].

MYC: Genomic amplifications in *Myc* proto-oncogene are very frequent in prostate cancer. It has been shown that 40% of primary and 90% of metastatic tumors carry Myc gene amplifications [40], which correlate with increased expression of MYC protein in the nucleus. Copy number amplification is more frequent in patients after receiving androgen deprivation therapy (ADT) and copy number alternations correlate with the rate of tumor cell proliferation[36].

PTEN: Is a tumor suppressor gene and the deletion of this region is very common in prostate cancer. There have been found also somatic mutations (frameshift mutations, implicated in aggressive prostate cancer[36].

p53: a tumor suppressor whose mutations are implicated in many tumors and it's the most frequent mutated gene in cancers. Mutated p53 is not degraded and accumulates in tumor cells. Mutations on p53 gene are more frequent in higher tumor stage and grade, metastatic tumors and androgen-independent tumors. It is common more than one mutation is present in the same primary

tissue thus contributing to tumor heterogeneity. It has been suggested that p53 mutations dysregulate genomic stability which leads to AR gene amplification, during hormone therapy[36].

SPOP: tumor suppressor gene Speckle Type POZ protein (SPOP) abnormalities (mutations or overexpression) have been found in a variety of tumor types (endometrial, breast, kidney cancer). SPOP is a E3 ubiquitin ligase substrate binding subunit of the proteasome complex[41]. In prostate cancer a variety of loss of function mutation have been identified. It has been proposed that they represent a new subset of prostate cancer, in which patients demonstrate more durable results in first line ADT compared to genomically non-selected patients[41]. SPOP localized in nucleus, targets AR for upcoming degradation[42] . Interestingly overexpression of mutant SPOP in animal models lead to overexpression of AR[43].

Other genes' alterations that have linked with prostate cancer:

ATBF1: This is a tumor suppressor gene which encodes for a transcription factor. It regulates cell proliferation by decreasing its rate, by upregulating p21 (another tumor suppressor) and by downregulating the oncoprotein AFP. Frameshift/truncating mutations have been reported in prostate cancer[36].

BRCA1: Except from the role in familial prostate there is association with mutations in those genes with sporadic prostate cancer, at earlier ages[36].

EPHB2: Encodes for a tyrosine kinase, is tumor suppressor and frameshift mutations are detected in 10% of sporadic cancer[36].

KLF6: is a tumor suppressor involved in cell proliferation. There have been reported somatic mutation that lead to loss of heterozygosity[36].

MSR1: Macrophage scavenger receptors (MSRs) are trimeric membrane glycoproteins, and their function is to mediate binding, internalization and the processing of macromolecules that are negative charged. Truncating mutations in this gene have been linked to prostate cancer[36].

mtDNA: Somatic mutations including deletions in mtDNA have been detected in prostate cancer. In cancer it is common the increased production of reactive oxygen species (ROS) and mutation on mtDNA can increase ROS production by inhibiting oxidative phosphorylation resulting in enhancement of tumorgenicity[36,40].

NKX3.1: Chromosomal deletions in chromosome 8 (8p21.2) that include NKX3.1 gene is the most frequent chromosomal deletion in prostate cancer. NKX3.1 is a prostate specific, tumor suppressor gene and it is expressed in higher levels in adults but expression is reduced in prostate cancer[36,40].

PON1: Serum paraoxonase function is related to eliminating the tumorigenic lipid soluble radicals. One polymorphism l102V is associated with decreased levels of this protein in serum and this has been linked with increased risk for prostate cancer[36].

RAS: Point mutations in RAS oncogenes (K-RAS, N-RAS, H-RAS) that encode RAS transform the protein into oncoprotein which leads to uncontrolled cell proliferation and carcinogenesis. Mutations in codons 12, 13 or 61 are associated with higher stage and grade of prostate cancer in some populations [40,44].

SRD5A2: Steroid 5-alpha-reductase is responsible for the conversion of testosterone to its active form DHT and it very important for the development of the prostate gland. Polymorphisms in this gene that lead to increased conversion to DHT have been found to significantly increase the risk for prostate cancer[40].

Finally, epigenetic deregulation such as methylation of DNA, histone modification, nucleosome remodeling can promote carcinogenesis in general, including prostate cancer [36].

Age

Age is an important factor for the prostate cancer. Less that 1% of the cases are below 50 years old and about 85% are older than 65[45].

Ethnicity:

The incidence of prostate cancer varies greatly across different ethnicities, with Asian people (China, India, Japan) having the lowest rates. In US, African Americans have the highest incidence rate and interestingly, when Asian people migrate to USA their risk for developing prostate cancer increases, possibly due to environmental and cultural changes [45]. Prostate cancer in men of African origin is the leading cause of mortality. Africans with good social status have poorer prognosis compared to European patients and African patients without good social status demonstrate even worse outcomes with higher mortality rates[46].

2.2.3 Prevention of Prostate Cancer

As discussed above there are a lot of factors that contribute to the development of prostate cancer. Fortunately, there have been identified some factors that could help the prevention of the disease. These include soy and phytoestrogens, lycopene that could be found in tomatoes, green tea, supplements (Vitamin E, D, Selenium). Finally, exercise plays also a very important role in prevention of prostate cancer[17].

2.2.4 Origin of prostate cancer

Prostate cancer associated with the accumulation of somatic mutation in prostate epithelium over the years and is characterized by increased heterogeneity, since patients have a number of different genetic variations. Identifying the cellular origin of the prostate cancer is very important, because there is a theory that relates the tumor aggressiveness with cellular origin (in addition to the genomic alterations). As described above (**Figure 2**) prostate is composed of a variety of cell

populations including luminal, basal, neuroendocrine and intermediate cells. The cells from which the prostate adenocarcinoma arises, initially thought to be basal or luminal cells. However, experiments in which those cell populations were mutated resulted in the generation of high-grade tumors that histologically represented adenocarcinoma but not luminal. Luminal origin of tumors was linked to *TMPRSS2-ERG* fusion which also present in patient tissue biopsies, confirming those experimental findings[9]. Luminal cells are the major cell type of the prostatic epithelium and have secretory function. Cytokeratins CK8/18 are specific markers and AR signaling is important for their survival. Basal cells are located between the luminal and basement membrane cells and express CK5/14 and p63. These cells are independent of androgens. Neuroendrocrine cells account for a very small percentage and express synaptophysin and chromogranin A. Intermediate cells express both basal and luminal markers. It has also been suggested that intermediate cells could be the cells of origin due to the fact that they express prostate stem cell antigen) (PSCA) which has been found to be upregulated in prostate cancer[47].On the other hand, basal cells are androgen independent and this is a feature of recurrent prostate cancer, thus it has been suggested they are the origin of prostate cancer[47]

2.2.5 Types of prostate cancer

In prostate cancer, the most common type is **adenocarcinoma** (95-99% of cases), which develops from glandular cells and can be divided in acinar (more common) and ductal adenocarcinoma[48]. Other types of prostate cancer include: **squamous cell carcinoma** which is an aggressive form of cancer with median survival after diagnosis of 14 months[49]. **Transitional cell carcinoma or urothelial** cancer begins from structures around the prostate. **Neuroendocrine Tumors** are slow in growth and do not affect PSA levels. Finally, **Sarcomas** develop inside soft tissues (muscles or nerves) and do not affect PSA levels as well and are found in younger men (between 35-60 years old).

2.2.6 Screening, diagnosis and staging of prostate cancer

Screening for prostate cancer is the best way to detect the disease in early stages, when the patients are asymptomatic, and the disease is mostly curable. Screening for prostate cancer includes PSA measurement after the age of 40. Some trials have shown that screening can reduce mortality from prostate cancer by 20%[9], but in others the survival benefit of screening was not evident and there is always the risk for overtreatment[24]. Therefore there are specific guidelines for screening which include informed decision making after explaining the benefits and potential harms from screening and testing[9,24].

For the **diagnosis** of prostate cancer a lot of different tests can be done including a blood test for measuring PSA, digital rectal examination (DRE) and/or imaging tests and biopsy sample may be evaluated[9]. A disadvantage of PSA for the diagnosis of prostate cancer, is that it has poor specificity (only 25% of men with PSA higher than 4ng/ml had confirmed cancer confirmed by biopsy) and high percentage of false over-diagnosis of indolent tumors (50%) that leads to over-treatment[50].

PSA is a glycoprotein expressed by both normal and malignant prostate tissue. Even though it is not cancer specific it is still considered the gold standard for diagnosing prostate cancer. There is an overlap with some non-malignant diseases (benign prostate hyperplasia or prostatitis) and prostate

cancer. Normal rates of PSA are 0-2.5ng/ml for 40-49 years, 0-3.5ng/ml for 50-59 years, 0-4.5ng/ml for 60-69 years and 0-6.5ng/ml for 70-79years. By increasing the reference rate for older men there is an increase in specificity and positive predictive value but there is also the risk for reducing the sensitivity of the method. In addition to these, PSA values are affected by ethnicity, weight and some medications. Finally, PSA is also used for monitoring the disease in response to treatment, as it represents the tumor volume, but it is not provide any info regarding therapy selection[51].

If DRE, imagining tests are abnormal or elevated PSA is obtained, then a biopsy should be performed. Samples are taken from the suspicious areas of peripheral zone of prostate. Lately, multiparametric MRI (mp-MRI) guided biopsies have been more successful in diagnosis for clinically significant disease and early detection. For the detection of potential lymph node metastases in high risk patients PET with traceable prostate specific membrane antigen (PSMA) is used[9].

2.2.7 Staging of prostate cancer

After the diagnosis of prostate cancer, staging is important because it defines treatment selection. For the complete staging of prostate cancer, two distinct types should be determined: the **clinical** and the **pathological** staging. For the clinical staging examinations such as digital rectal exam (DRE) and PSA levels in plasma should be evaluated. In addition to those x-rays, bone scans, CT scans, or MRI are also used when needed. For the pathological staging the collected biopsy samples are examined.

Grading of Prostate cancer

For the staging of prostate cancer both TNM and Gleason scores are used.

TNM grading system for prostate cancer

The TNM system describes the spread of the tumor cells in different locations and modified recently (2016) to fit the most recent clinical observations. It is based on the following parameters:

- The **size** of the primary tumor (T)
    - There are two different types of T for prostate cancer:
        - Clinical T (cT), which consists from information from physical exam, prostate biopsy or imaging tests
        - Pathologic T (pT), in the case of prostatectomy
- Any **lymph node metastasis** (N)
- If there are any **distant metastasis** to other sites (M)

The numbers after the letters provide information regarding how advanced the tumors are, the higher the number, the higher the disease progression. There are four stages of prostate cancer, from I until IV and stages are split in A, B, C, etc[52].

Gleason score

For the diagnosis of prostate cancer, histologically, the basal cells layer must be lost. The structural pattern and the differentiation of the cells play a role in prostate cancer scoring (**Figure 4**). The histological grading also reflects some of the biological properties of the malignant cells. The Gleason score became a part of TNM staging system as an important prognostic factor. It is based on the degree of glandular differentiation and the pattern of cancerous cells in the stroma of the prostate. Specimens from needle biopsy or prostatectomy are graded through this system from 1 until 5; 1 is for the most differentiated and 5 the least differentiated. For each sample two patterns are recorded due to heterogeneity in the tumor histology: a primary pattern (Gleason 1 to 5) and a secondary pattern (Gleason 1 to 5). The final Gleason score is the summary of those two and has a range from 2 to 10[53]
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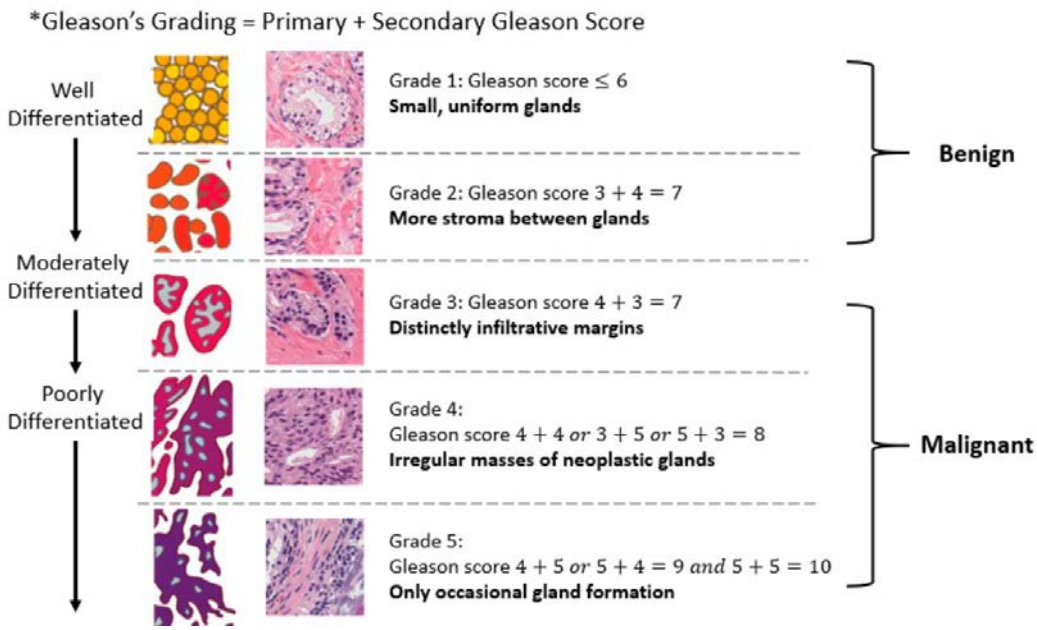

Figure 4: The Gleason grading system *(From Cho-Hee Kim et al, Cancers 2021).*

2.2.8 Treatment Strategies for Prostate Cancer

For the clinical management of prostate cancer patients' multiple parameters should be taken into consideration. Such parameters are the clinical characteristics (localized disease, advanced or metastatic disease if the cancer is castrated resistant or sensitive) histopathological characteristics, molecular characteristics and patient characteristics (age, overall health, family history)[9].

For the subset of patients with **very low risk disease** (Table 1) the strategy is to monitor if there is any progression (watchful waiting) [54,55].

Very low risk	Clinical stage of T1c, Gleason score of 6 or less, prostate-specific antigen (PSA) level of less than 10 ng/mL, less than 3 biopsy cores with cancer presence of 50% or less in each core, and PSA density of less than 0.15 ng/mL/g
Low risk	Clinical stage of T1 to T2a, Gleason score of 6 or less, and PSA level of less than 10 ng/mL
Intermediate risk	Clinical stage of T2b to T2c, Gleason score of 7, or PSA level of 10 to 20 ng/mL
High risk	Clinical stage of T3a, Gleason score of 8 to 10, or PSA level greater than 20 ng/mL
Very high risk	Clinical stage of T3b to T4, primary Gleason pattern 5, or greater than 4 biopsy cores with Gleason score of 8 to 10

Table 1: Stratification of prostate cancer patients' risk'. *(Adopted from J.L. Monhel et al. Official Journal of the National Comprehensive Cancer Network, 2016)*

For patients **with low risk local disease**, the standard treatment for non-metastatic prostate cancer is **androgen deprivation therapy**, which causes G0-G1 cell cycle arrest, since androgens has been shown to enhance cellular proliferation[48]. In addition to this, more targeted testosterone inhibition is possible with the use of novel Androgen receptor inhibitors or antagonists (Enzalutamide, Apalutamide, Daralutamide). Furthermore, Androgen biosynthesis inhibitors are also used such as Abiraterone[56].

For patients **with low to intermediate disease patients are treated with focal therapy[57–59]. These are treatments less invasive than prostatectomy that target tumor areas of prostate gland, without removing it. Focal therapies include cryosurgery or High-Intensity Focused Ultrasound (HIFU)[57].**

For patients with **localized high risk disease** with at least 10 years life expectancy, prostatectomy is usually the choice of treatment[48]. Inhibition of Androgen biosynthesis prior to

surgery has been suggested that improve therapeutic outcomes, but results in clinical trials are not consisted[60]

There is a subset of patients treated with androgen deprivation therapy that develop metastatic hormone-sensitive prostate cancer (mHSPC); those are treated with Docetaxel and abiraterone[61]. Most of the patients (80-90%) respond well to the therapy, which is accompanied in reduction in PSA levels, but within 5 years in most patients recurrent tumors are developed, which are termed castration resistant prostate cancer (CRPC)[48,62].

Castration resistant prostate cancer (CRPC) is a heterogenous disease and is characterized by clinically nonvisible disease in prostate, no disease in distant sites, but rising of PSA. Treatment options include **Enzalutamide**, which was the first second generation nonsteroid AR agonist to used. It binds direct to AR which results in the inhibition of AR translocation to the nucleus and expression of AR responsive genes. **Apalutamide** is a nonsteroidal competitive AR inhibitor which inhibits the overexpression of AR. It binds the AR's binding domain which also results in AR translocation to the nucleus and binding to DNA. **Daralutamide** is another nonsteroidal AR inhibitor which also results in AR translocation to the nucleus and binding to DNA but has fewer side effects than Enzalutamide and Apalumatide because it exhibits lower blood-brain barrier penetration. Unfortunately, patients with CRPC are at great risk to develop metastasis and metastatic castration prostate cancer[62,63].

Despite advances in therapies for prostate cancer, when the disease transitions from CRPC to mCRPC is uncurable and is main mortality cause in prostate cancer patients. This highlights the need for new treatments in order to prolong life but more importantly to improve quality of life of patients with mCRPC. Currently for choosing the treatment for an individual, a lot of factors are taken into consideration such as symptoms, disease state, patient preference and clinician's preference[64].

The first line of treatment for mCRPC patients are Abiraterone and Enzalutamide, which **inhibit AR signaling**[65]. **Abiraterone** acts by inhibiting CYP17, a cytochrome P450 enzyme which is responsible for biosynthesis of glucocorticoids (cortisol) and sex hormones (androgen), has hydroxylase and lyase activity and located in testis and in adrenals[66]. **Enzalutamide** is an AR inhibitor which binds to ligand-binding domain of the receptor which results in inhibition of AR translocation in the nucleus. In clinical trials, Enzalutamide, showed greater benefit in patients that had not received chemotherapy and delayed skeletal events. Compared to placebo, it prolonged Overall Survival (OS) and Progression Free Survival (PFS) and improved the quality of life of patients[67]. Unfortunately, resistance to these agents occurs and it is related with AR signaling and AR variants AR-V7 and AR-V9, (which serve as prognostic marker to Abiraterone and Enzalutamide in circulating tumor cells and EVs)[68,69].

Chemotherapeutic agents, such as **taxanes** are widely used in mCRPC patients. Taxanes are semisynthetic derivatives. Their mechanism of action is that they inhibit the depolymerization of microtubules by binding in the β-subunit of tubulin heterodimers which results in cell cycle arrest in M-phase. Microtubules assemble the mitotic spindle and play critical role in a variety of cellular processes that affect cancer cell proliferation and migration (cell signaling, cell division)[70]. In addition to those, because AR uses cytoskeletal components for its translocation, taxanes also inhibit its translocation to nucleus[71]. Finally except from the effect on cytoskeleton, it also induce apoptosis by reducing the expression of the anti-apoptotic gene BCL2 gene[72]. **Docetaxel** was very high

affinity for β-tubulin which makes it very successful at inhibiting tubulin assembly[70]. After initial response, eventually resistance to docetaxel will be developed. One of the mechanisms of resistance is the high affinity for the ATP-binding cassette (ABC) drug efflux pump MDR1; cells expressing it become resistant to taxanes[73].

Cabazitaxel, another taxane is currently used after patients show signs of Docetaxel resistance. It has been also shown to have a distinct safety profile compared with Docetaxel, but in general is considered well tolerated with the possibility of reducing Cabazitaxel dose, with similar efficacy [74,75]. It has reduced affinity for MDR1 which is responsible for drug resistance. In addition to that, it has prolonged intracellular retention and higher tubulin affinity, thus making it superior to Docetaxel. It also exhibits prolonged G2-M arrest and is more efficient in inducing apoptosis[76]. It has been shown that it also it disrupts actin fibers which results to inhibition of invasion of tumor cells[77].

2.2.8.1 Mechanisms of Resistance to Taxanes

Several mechanisms have been proposed as the source of resistance to taxanes, some of which involve the cytoskeleton components; alteration of microtubule regulatory proteins, different tubulin isotypes and altered microtubule dynamics are some. Mitotic spindle is required for appropriate segregation of chromosomes and consists from microtubules, a direct target of Cabazitaxel[78]. Proteins that are involved in the regulation of mitotic spindle (such as NEK2 and its transcriptional regulator LIN2) have been found elevated in taxane resistant cells, indicating that they may have a role in taxane resistance. Stathmin 1 which is a microtubule depolymerizing protein has also implicated in chemo-response and response to taxanes in ovarian cancer [79]. Other mechanisms of resistance include multidrug efflux pumps, DNA repair mechanisms and hypoxia (which leads to activation of a variety of survival genes, ROS related proteins and DNA repair mechanisms)[77]. Furthermore, upregulation of pro-survival pathways, Epithelial to Mesenchymal Transition (EMT) and upregulation of anti-apoptotic proteins contribute to induce proliferation and contribute to resistance to taxanes [70]. In addition the these, prostate cancer cells that are resistant to docetaxel, have a more efficient metabolism using oxidative phosphorylation[78]. Moreover, the level of AR activity is also a mechanism of taxane resistance in prostate cancer and Notch-1 has been found to be upregulated in prostate cancer cells resistant to docetaxel and in many other cancer types resistant to various taxanes[80,81]. Other pathways have been linked with taxane resistance in various tumor types are WNT signaling, members of EIF2 signaling which play role in translation initiation[82–84].

2.2.9 Bone metastatic prostate cancer and therapeutic strategies

2.2.9.1 Bone

Bone is the most common site of prostate cancer metastasis (85-90)[85]. Patients with bone metastasis experience pain and develop skeletal fractures which reduces their quality of life and their

overall survival. Bone consists of cortical and trabecular bone. The cortical bone surrounds the bone marrow and the trabecular bone encloses bone marrow[86]. Bone is a dynamic organ in which constant renewal by communication of osteoblasts (bone forming cells) and osteoclasts (bone resorbing cells) occurs. Osteoblasts are cells of mesenchymal origin that produce non-calcified bone matrix (called osteoid) which later becomes mineralized to form bone tissue. Osteoclasts differentiate from monocyte-macrophage precursor cells and are responsible for bone resorption[87]. In normal bone tissue constant cycles of matrix renewal are occurring and this process is called '**bone remodeling**'; this is a very well-regulated procedure in order to maintain a balance between bone deposition and bone resorption. This starts from a signal (could be either molecular or mechanical) that initiates periosteal or endosteal surrounding cells to take distance from each other in order to expose the bone surface to osteoclasts to degrade bone. This process results in the secretion of various growth factors and chemokines that attract macrophage-like cells to phagocytose debris and promote osteoblast differentiation and activation. The last step is to recruit bone lining cells again to the newly formed bone[88].

2.2.9.2 Bone metastasis

It has been shown that prostate cancer cells located in primary site can prepare metastatic sites by affecting bone marrow cells before metastasis occurs [89]. That is possible via extracellular vesicles that play critical role in bone metastasis by altering the microenvironment in order to support the future growth of tumor cells via altering the osteoblasts to establish a pro-tumorigenic phenotype[90,91]. Another feature of prostate cancer cells is that they are capable of acquiring an osteoblast like phenotype (osteomimicry) via expressing bone matrix proteins and markers, such as osteopontin (OPN) and its receptor CD44, bone sialoprotein II (BSP II), osteonectin (ONC), alkaline phosphatase (ALP), and the osteoblastic specific factor Runx2 [90,92]. Finally, adipocytes increase the migration of prostate cancer cells and as well as their proliferation. Additionally, they increase osteoblast differentiation, proliferation and induce their mineralization [89].

Bone is an attractive environment for prostate cancer cells. Bone marrow is abundant in vasculature which assists cancer cell migration[86]. Bone metastasis is facilitated by interactions of migrating cancer cells, osteoblasts and osteoclasts. Other factors contributing to metastasis include extracellular vesicles, cancer associated fibroblasts (CAFs) and MSCs[89].

During bone metastasis the interplay among cancer cells, osteoblast and osteoclasts can cause an osteolytic, osteoblastic or mixed response. Pure osteoblastic response is the deposition of new bone formation and there less bone resorption activity. Pure osteolytic is when the destruction of normal bone which is caused by the inactivation of osteoblasts and the degradation of the bone by recruited osteoclasts. In prostate cancer, bone metastasis is accompanied by tumor-induced new bone formation (osteoblastic) and those newly generated lesions are the result of imbalanced function between osteoblasts and osteoclasts[93,94]. The process of prostate cancer metastasis and new bone formation in the bone may be explained via ''vicious cycle'' hypothesis[87] (**Figure 5**). Tumor cells enhance a non-physiological osteoblast mediated bone resorption through cytokines and growth factors[95]. Parathyroid hormone-related peptide (PTHrP) upregulates receptor activator of nuclear factor-kappa B ligand (RANKL) and downregulate osteoprotegerin (OPG) (inhibitor of RANKL) in osteoblasts.

RANKL acts on RANK of osteoclasts, which in turn resorbs bone; this leads to TGFβ secretion inside the bone TME that promote tumor growth. It has been shown that inhibiting TGFβ signaling resulted in inhibition of new osteoblastic bone formation and decreased tumor incidence in mice injected prostate cancer cells thus making TGFβ a target of therapy[93,96].

New bone in prostate cancer is mechanically unstable (prone to fractures) and called woven bone[85]. Those lesions are detected by bone scans, radiography, biopsy, CT scans, MRI and increased levels of alkaline phosphatase in plasma, but do not provide any information regarding future resistance to treatment[94]. Hematopoietic stem cells in the bone can differentiate into osteoclasts by cancer cells' stimulation and promote MSCs differentiation into osteoblasts. MSCs, in turn can control the HSC niche, produce DKK-1. Cancer associated fibroblasts have the ability to promote the prostate cancer cells growth inside the bone. Adipocytes can increase osteoblast differentiation, proliferation and induce their mineralization. Moreover, they increase the migration of prostate cancer cells and their proliferation. Nervous system also has a role where it can increase RANKL expression on osteoblasts[89].

There are evidence suggesting than Dickkopf-related protein (DKK1), an inhibitor of Wnt pathway expressed in initial stages of bone metastasis can promote a switch towards osteoblastic response instead of an osteolytic one[85]. In addition to this, Bone Morphogenic Proteins (BMPs), endothelin 1, FGFs, IGFs secreted by tumor cells can promote the differentiation of mesenchymal cell towards osteoblasts and activate them[85]. Finally, it has been shown that BMP4 secreted from prostate cancer cells has a major role in new bone formation via inducing the differentiation of endothelial cells to osteoblasts in bone marrow. This is supported by the presence of hybrid endothelial-osteoblast cells in areas with newly formed bone in bone metastatic specimens[97].

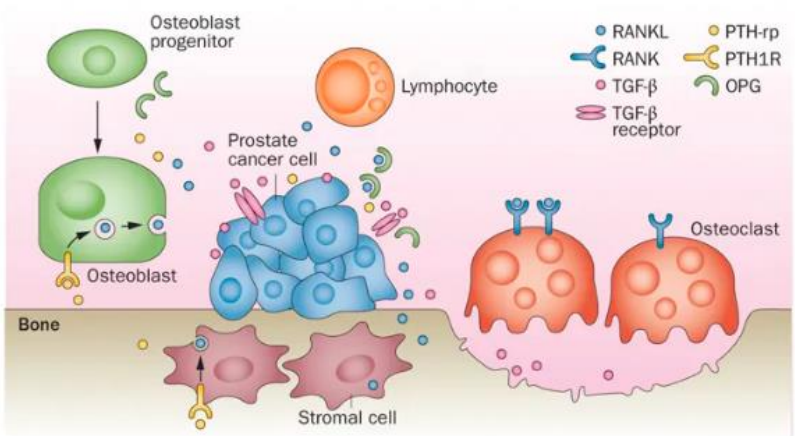

Figure 5: The vicious cycle during prostate cancer bone metastasis *(B.A. Gartrell et al., Nature Reviews Clinical Oncology, 2014).*

2.2.9.3 Prostate cancer bone microenvironment and crosstalk with the immune system

Bone marrow is considered a hematopoietic organ. However, growing evidence shows that a variety of immune cells are located and actively interacting inside the bone marrow, thus it is considered an immune organ[98]. The immune microenvironment plays a very important role in the establishment of tumor cells inside the bone microenvironment, where macrophages, myeloid derived suppressor cells (MDSCs), dendritic cells and T- cells interact with components of the vicious cycle.

Healthy bone is composed from naïve myeloid cells with no immunosuppressive capabilities that differentiate into mature macrophages, granulocytes and neutrophils. When tumor cells are present in the microenvironment, they secrete factors such as VEGF, SDF-1 and IL-3 which inhibit this differentiation process and promote the development of **MDSCs** which are immunosuppressive.

MDSCs are heterogenous, immature**,** activated myeloid cells that can suppress T-cells, NK cells and promote tumor growth[89]. They are divided in two main subsets: polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (MDSCs), which have morphological and phenotypic similarities with neutrophils and monocytes respectively[99]. Also, MDSCs that reside in the bone marrow have the ability to directly affect the vicious cycle by differentiating into osteoclasts. In addition to this they secrete TGFβ which promotes the production of PTHrP which is cancer promoting[89].

Macrophages are mononuclear myeloid lineage cells; signaling from the bone tumor microenvironment polarizes naïve macrophages into different differentiative fates towards anti- (M1) or pro-tumorigenic (M2)-like macrophages. In the tumor microenvironment, cancer cells release factors which recruit macrophages and polarize them towards an M2-like phenotype, the Tumor associated macrophages (TAMs), which have a very important role in promoting cancer progression and tumor metastasis by secreting anti-inflammatory cytokines (IL-10)[100,101]. TAMs also express MMP-9 which promote angiogenesis. There is also a specific type of macrophages in the bone microenvironment, the *osteomacs* which are involved in the process of bone healing[89]. In metastatic lesions, they have been linked to woven bone[102]. In addition to that, prostate cancer cells express CCL2 which attract TAMs as well as osteoclast percussors to the bone microenvironment. Moreover bone marrow macrophages express osteogenic genes, such as Cathepsin K, thus promoting tumor progression [89,103].

T-cells (CD4+, CD8+) differentiate from HSCs inside the bone marrow and become active in the thymus gland and migrate back to the bone (and other locations). Inside the bone microenvironment they contribute in homeostasis, both in normal and pathological conditions, where they promote or inhibit cancer progression[89]. T-cells recognize tumor-antigen-MHC-I complex presented by APCs and can eliminate cancer cells through apoptosis. This anti-tumor activity can be inhibited by TGFβ secreted by the osteoclastic activity on the bone[101], as well as cytotoxic CD8+ T cells[101]. CD4+ (T helper) lymphocytes play a very important role in regulating immune responses by controlling CD8+ (cytotoxic) lymphocytes, B cells, NK cells, macrophages and dendritic cells. There are several categories of Th cells, among those Th1, Th2, Th17 and Tregs.

Tregs are immune suppressors and have been found in increased numbers in bone marrow from prostate cancer with bone metastasis. In addition to these FOXP3+ Tregs produce large amounts of RANKL which promotes the differentiation of osteoclasts and bone metastasis[101].

Dendritic cells presenting antigens to adaptive T-cells, consist of two categories: the myeloid and the plasmacytoid, which regulate osteolysis[89]. Dendritic cells function as antigen presenting cells (APCs) to cytotoxic CD8+ T-cells, but it has also been shown that tumor infiltrating dendritic cells suppress these cytotoxic T-cells by producing TGFβ, nitric oxide, IL-10, VEGF and arginase. In addition to this, they also recruit other immunosuppressive cell types (Tregs, MDSCs)[101].

CD4+ Th17 cells also have a role in osteoclastogenesis and bone metastasis through RANKL[101]. Th17 cells are characterized by the secretion of high levels of IL-17 and other inflammatory cytokines and have different functions in autoimmunity, infection and cancer where they promote or inhibit the immune system. This fate is determined by the ratio of chemokines and cytokines inside the microenvironment[104].

Neutrophils primarily compose a component of inmate immune and thereby have tumor attacking properties but recently it has been shown that there are also tumor infiltrating neutrophils that are divided in two categories: N1 with anti-tumor function and N2 with tumor promoting function, with TGFβ playing again a role to this shift towards N2[101].

NK cells which belong to innate part of immune have tumor killing activity through apoptosis. It has been shown that cancer cells express a ligand (C2GnT) which inhibits the anti-tumor immune response mediated by these cells[101].

2.2.10 Bone targeting therapeutic agents

There are a few available therapeutic strategies for bone metastatic prostate cancer which target new bone formation.

Biphosphonates (zoledronic acid, ibandronate, neridronate and risedronate) act by inhibiting the osteoclast mediated resorption of bone matrix and they are commonly used in managing osteoporosis. In cancers that metastasize to the bones are used in order to reduce skeletal related events. It has been found that except from their effect on osteoclasts they can also affect the immune system and they inhibit angiogenesis, induce apoptosis of tumor cells and reduce the cancerous cell adhesion to the bone stroma[87].

Denosumab is a monoclonal antibody that acts as an inhibitor of RANKL by binding and neutralizing it, resulting in inhibition of osteoclast development. In addition to this some cancer cells express RANKL so it may target those to inhibit bone metastasis[87]. In prostate cancer patients previously treated with ADT Denisumab treatment increased bone mineral density and is currently approved for increase bone mass in non-metastatic prostate cancer patients on ADT[87,105]

Radium-223 is an alpha-emitting radiopharmaceutical that targets new bone formation during metastasis. It is a calcium mimetic which can form complex with hydroxyapatite and half-life of 14 days. Alpha particles have the ability to cause DNA double strand breaks due to high linear energy transfer resulting in a short effective range, smaller than 100μm, which is much smaller than other radiopharmaceuticals thus making Radium-223 more targeted with less side effects on surrounding tissue[106]. Except for targeting osteoblasts and osteoclasts, it also targets cancer cells, which results in tumor growth and abnormal bone growth inhibition[107]. In phase 3 clinical trial ALSYMPCA for patients with metastatic prostate cancer patients with more than 2 bone metastases Radium-223 showed 30% reduction in mortality; median OS was 14.9 in Radium-223 treated vs 11.3 in Placebo group. In addition to this, Radium-223 prolonged significantly the time to the appearance of first skeletal event. Moreover patients experienced less pain and had improved quality of life, ALP reduction whereas no PSA reduction was observed [106,108]. Additionally, it has been suggested that since Radium-223 can cause lethal DNA double strand breaks it could be less potent in the development of resistance, compared to treatments such as chemotherapy where escape mechanisms exist[107]. Due to Radium-223 mechanism of action and its relatively safety it seemed reasonable investigating its efficacy in combination with other treatments such as CYP17 and AR inhibitors, Microtubule inhibitors, immunotherapy and PARP inhibitors (DNA damage response)[107]. Surprisingly the combination of Radium-223 with Abiraterone showed unexpected toxicity and caused more bone fractures and deaths in patients with mCRPC than abiraterone[109].

2.2.11 Immunotherapy for prostate cancer

The last years immunotherapy revolutionized the way several cancers are treated. The first immunotherapy agent for prostate cancer approved in 2010 was the vaccine sipuleucel-T[110]. Cancer vaccines activate immune system in order to recognize tumor-associated antigens and promoting T-cell responses[110]. Sipuleucel-T is generated from autologous CD54+ dendritic cells isolated from patients and incubated with the recombinant fusion protein of PAP and GM-CSF. PAP is a protein expressed on prostate cells [111].

Treatment with immune checkpoint monoclonal antibodies has been very successful for a wide variety of cancers such as melanoma, lung cancer and renal cell carcinoma[112]. Immune checkpoint therapy targets cytotoxic lymphocyte antigen-4 (CTLA-4), programmed cell death protein 1 (PD-1) or programmed cell death ligand 1 (PD-L1) in order to activate T-cells (CD4 Th1 and CD8 cytotoxic) for targeting tumor cells[113,114]. Unfortunately, prostate cancer is not among those types of cancer that respond to immunotherapy. Only a small portion of patients respond to therapy and the response is less in patients with bone metastasis[110]. For example, in a clinical trial comparing ipilimumab (anti-CTLA-4) versus placebo in prostate cancer patients, PSA reduction was observed in 4% of patients with bone metastasis whereas in patients without bone metastasis the PSA reduction was observed in 18% of the patients[115]. Prostate cancer is considered as a ‘’cold’’ tumor, which means that is highly immunosuppressive. It has been suggested that inside the tumor microenvironment of prostate cancers, tumor infiltrating lymphocytes (TIL) could contribute by promoting T-cell activity

inhibition. It has been shown that in biopsies from prostate cancer patients, TILs have acquired phenotypes of T-regs and Th17 that have immunosuppressive capabilities[112,116]. Also the interaction of T-regs, TAMs, MDSCs, the immunosuppressive cytokines secreted from tumor stromal cells and fibroblasts and adenosine production also contributes to the immunosuppressive environment of prostate cancer TME [117,118].

Thus, combination immunotherapies could be beneficial in these ''cold'' microenvironments. There are current studies that introduce a combination of immune checkpoint antibodies (anti-PD-1/PD-L1, anti-CTLA-4/PD-1) to prostate cancer patients and in a small numbers of patients there are durable responses in terms of disease progression [112]. Interestingly it has been found that anti-CTLA-4/PD-1 combination therapy had great benefit in patients with homologous repair deficiencies compared with patients that did not had these defiencesies[119]. Also a number of clinical trials combining immunotherapies with vaccines, CAR-T cell therapies are also ongoing, as well as clinical trials testing bispecific antibodies, some of them yielding promising results[120].

In addition to this, combination with other type of treatments could be beneficial. There are evidence in pre-clinical models and patients, that Enzalutamide resistance can lead to upregulation of PD-L1, PD-L2, PD-1 and CTLA-4[121]. This was tested in clinic where anti PD-L1/PD-1 and androgen receptor blockade were combined and a small number of patients showed complete responses whereas others partial[122]. In addition to that it has been shown that targeting TGF-β in combination with anti-CTLA-4 and anti-PD-1 in a bone metastatic prostate cancer model showed regression of bone mCRPC and prolonged survival[114].

Finally, immunotherapies targeting other immune checkpoints are being investigated for prostate cancer such as B7-H3 which is overexpressed in prostate cancer and has correlated with bad prognosis[123].

2.2.12 Other treatment for prostate cancer

As mentioned above, genomic alterations in DNA repair mechanisms have been linked with prostate cancer and for some of those alterations there are already approved therapies (poly(ADP-ribose) polymerase (PARP) inhibitors for instance)[107]. An example is Olaparib that has been approved for mCRPC patients with BRCA1,2 or ATM mutations and Niraparib[107]. Moreover since Radium-223 causes double strand breaks combination with PARP inhibitors could be beneficial and indeed there are such studies, since PARP inhibitors act on single strand breaks[107]. An overview of treatments for prostate cancer are seen in **Figure 6**.

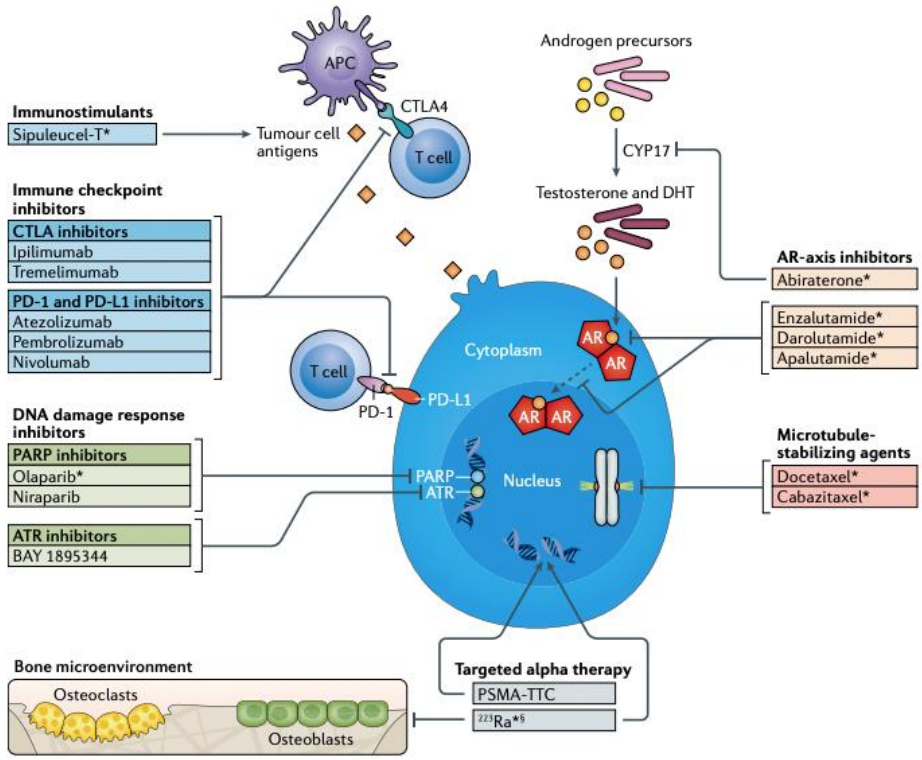

Figure 6: Mechanism of action of anticancer agents used in prostate cancer (M.J. Morris and al, Nature Reviews Urology 2019).

2.2.13 Prostate cancer as a progressive disease-Reclassification of prostate cancer

Despite extensive research, there are no specific biomarkers to guide therapy selection for prostate cancer patients. Patients are categorized based on their disease stage, therapy and their response to therapy. This does not provide any information regarding therapy selection based on molecular characteristics, but only on morphological features. Also, there are clinical observations that specific treatments have different outcomes and that outcomes are disease stage specific (e.g., androgen targeted therapy is more beneficial at early stages of prostate cancer, but chemotherapy at later stages of prostate cancer). Based on this information, Dr. Logothetis and his colleagues, suggested a new model for prostate cancer, which is built on molecular information, rather than on morphological characteristics of prostate cancer. This model of prostate cancer could serve as guide for therapy selection. According to this proposed model, prostate cancer is a progressive disease that undergoes evolution and can be divided into three categories: endocrine driven, microenvironment dependent and tumor cell autonomous. The most crucial factors contributing to the progression of prostate cancer are the androgen receptor (AR), which plays a role in all stages of the disease progression, oncogenes (Src, MET, Axl and FGFR), tumor suppressor genes (PTEN, p53 and RB) and the microenvironment. In early stages of the prostate cancer, where it is still endocrine driven, DHT depletion influences AR signaling. Paracrine-driven progression is termed when tumors escape

from this DHT dependence, but still AR signaling is crucial. This transition, from endocrine to paracrine is the step that cancer has the potential to lead in lethality. In this step, prostate cancer enters to the ‘’progression spiral’’, where alterations in AR signaling coexist with changes in microenvironment and oncogene activation. In the last step of prostate cancer, tumor becomes AR independent, exit from ‘’paracrine progression spiral’’ and transformed to cancer cell autonomous (**Figure 7**)[124].

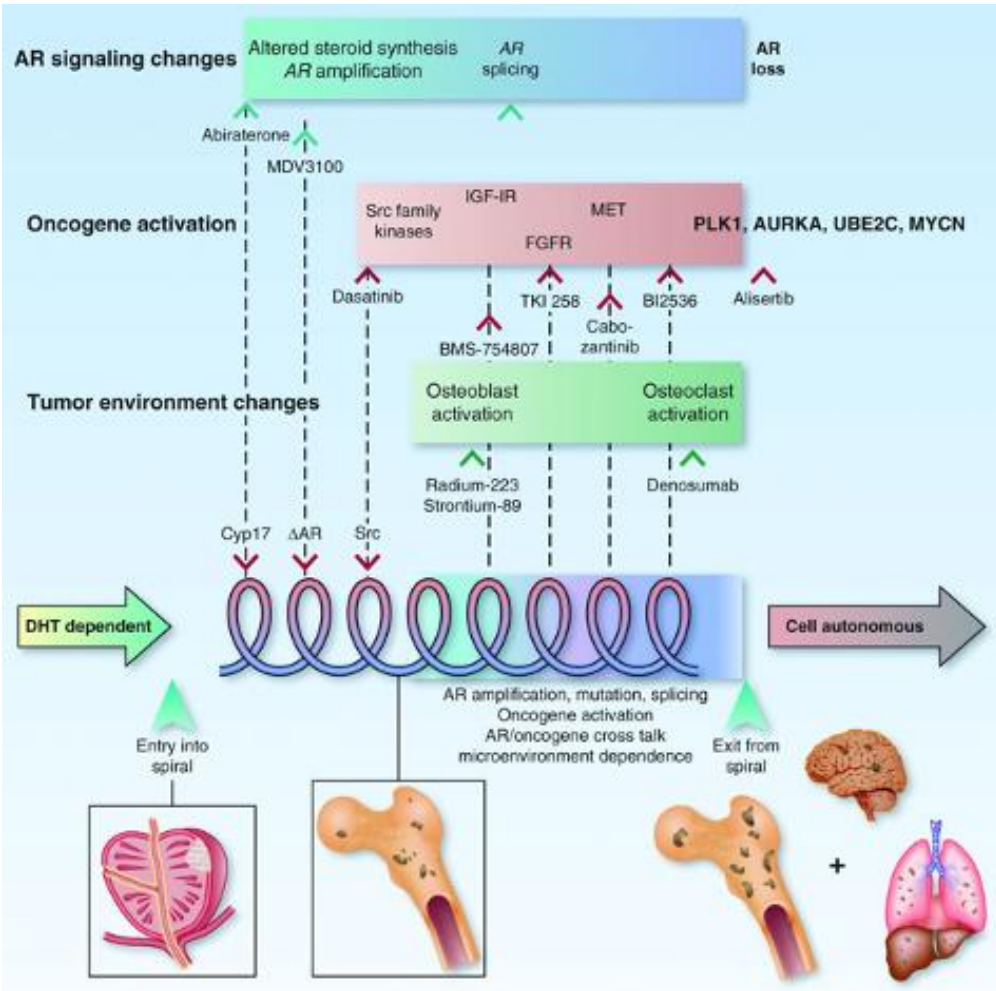

Figure 7*: *Proposed spiral Model for PCa progression *(C.J. Logothetis et al., Cancer Discovery, 2013).*

2.3 BIOMARKERS AND LIQUID BIOPSY

The last decade there is a lot of ongoing research for identifying in bodily fluids prognostic and predictive biomarkers for prostate cancer patient stratification and therapeutic strategy selection. Such a strategy has a number of advantages over tissue biopsies due to the feasibility of easily accessible repetitive sampling (which can be very useful for monitoring disease progression during treatment), minimal risk and pain from the procedure. Biomarkers from liquid biopsy could be from plasma, circulating tumor cells (CTCs) and circulating cell-free tumor DNA (ctDNA), extracellular vesicles and lately with the use of immunotherapy there is a need to also analyze circulating T-cells[125].

2.3.1 Liquid biopsy specific for prostate cancer

Liquid biopsy is sampling of various biofluids (blood, saliva, urine, etc.) and analyzing the molecular content of their components (circulating tumor cells, nucleic acids and extracellular vesicles)[126]. Liquid biopsy is noninvasive, rapid, and precise. Moreover since its systemic it can solve the problem of tumor heterogeneity that affects a lot of diagnostic biopsies[125]. The last years liquid biopsy has been considered as a promising way to diagnose, monitor disease, and predict response in particular treatments.

As already mentioned, the diagnosis of prostate cancer is currently based on histopathological features of tissue sections, PSA measurements, and imaging. PSA in particular is used for monitoring the disease as well. However, PSA is not informative regarding therapy selection. Another disadvantage of PSA for the diagnosis of prostate cancer, is that it has poor specificity and high percentage of false over-diagnosis of indolent tumors that leads to over-treatment, as mentioned before[50].

EVs have a lot of advantages as source of biomarkers for liquid biopsy compared with other sources (e.g., ctDNA). It is multiplex with proteins, RNA and DNA, a molecular content that is protected by the lipid bilayer and make them stable in circulation. They are present in high number and even more in patients with prostate cancer. Also exosomes from prostate cancer patients have smaller size compared with healthy men [127]. This finding could be proved useful in potential screening the population for cancer. Their bioavailability in the plasma makes repetitive sampling is feasible, making them ideal source of biomarkers [126].

2.3.2 Extracellular vesicles

Extracellular vesicles represent a heterogenous group of cell-derived vesicles. They are secreted by all cells, are composed from lipids, proteins, DNA and RNA. Initially, when they first discovered more than 30 years ago, it was thought that they contain cellular waste, but now they are considered as a way of distant cell communication, since they travel through the body and a valuable source of biomarkers after it was shown that they can enhance adaptive responses [128–130]. Extracellular vesicles have been linked with a lot physiological and pathological functions such as stem cell maintenance, repair in tissues, immune related functions, cancer progression and chemotherapy resistance [129,131].

There are different types of extracellular vesicles, based on their biogenesis and cellular compartment of origin which share common protein thus making their identification challenging[132] Based on their **biogenesis process**, EVs are divided into 3 main categories: **macrovesicles**, **apoptotic bodies and exosomes**[132,133] . **Macrovesicles** are budding off from the cell membrane and have a size which range from 30-2000nm. **Apoptotic bodies** are released by cells undergoing apoptosis, during their final steps when the cells are dividing into a variable number of apoptotic bodies and can contain many different cellular components (organelles, chromatin remaining, fragmented DNA, proteins and degraded proteins etc.)[129,134]. **Exosomes** have a unique biogenesis process, through Multivesicular Bodies (MVBs). Newer guidelines suggest the use of small extracellular vesicles S-EVs and L-EVs from vesicles <200 nm and < 200 nm respectively, unless there is molecular confirmation (knockout genes responsible for exosome biogenesis for example)[132]. In this thesis we use the term **exosomes** for extracellular vesicles with size until ~150nm.

2.3.3 Exosomes

2.3.3.1 Biogenesis

Exosomes' size ranges from 30-150nm and are cup shaped particles enclosed by a bilayer composed of phospholipids. They represent a unique type of extracellular vesicles due to their mechanism of biogenesis; they derived from multivesicular bodies (MVB) membrane by two distinct pathways. The first one characterized is the endosomal sorting complex required for transport (ESCRT) dependent. This pathway is composed mainly by four complexes that are responsible for the transport of lysosomes (ESCRT 0-III) and accessory proteins such as Alix and VPS4. The proteins that are loaded in exosomes via ESCRT dependent mechanism are selected via a ubiquitination mechanism (EGFR, MHC-I) or by sumoylation (a-synuclein)[135–137]. Then ESCRT-I and ESCRT-II are recruited in the outer site of endosomes and promote the inward invagination of the endosomal membrane. The scission of the intraluminal vesicles (ILVs) is promoted by ESCRT-III (ALIX, TSG101 are mainly responsible for this) complex; these ILV containing endosomes are now called MVBs.

The second pathway of MVB formation is ESCR-independent, first identified in mouse oligodendroglia cells, where the sorting of proteolipid protein during MVBs generation in ILVs was studied. They found that ESCRT machinery did not affect PLP secretion whereas sphingomyelinase and ceramide did. Finally, in an experimental setting where all ESCRT subunits were depleted, MVBs could still be generated[138,139]. In this ceramide dependent biogenesis pathway ceramide found to induce curvature of endosomal membranes promoting the inward budding[129,140].

After the generation of MVBs, there are two distinct routes the first one is degradation and recycling upon fusion with the lysosomes and the second one is the fusion of MVBs with the plasma membrane, where ILVs are released to the extracellular space now termed as exosomes. Studies suggests that there are various subpopulations of MVBs, some are more likely to be degraded and others are more likely to fuse with the cell membrane[141].

Finally, there are prostasomes, a cell type specific vesicle. These are released from prostate epithelial cells. Their size ranges from 30-200nm and they are of intracellular origin and are generated through multiple invaginations of the of the membranes of endosomes (called MVBs) which are generating intraluminal vesicles, which after MVB's fusion with plasma membrane released as prostasomes [142]. Prostasomes function as intercellular communicators between the prostate acinar epithelial cells and the spermatozoa and assist in fertilization[142]. Regarding their content, previous work of our team showed that prostasomes contain different peptides of Chromogranin (Cg) family; peptides of CgA detected in on prostasomes whereas CgB were detected on prostate cancer derived EVs. This finding could be used in assays for detection of EVs or even could reveal a possible target for prostate cancer treatment [143].

2.3.3.2 Loading of Cargo into Exosomes

The exact process of loading cargo inside exosomes is not fully understood but there are studies related to this.

2.3.3.2.1 Loading of DNA

It has been previously shown that genomic DNA (gDNA) is present in exosomes. It was recently shown that there is a relationship between gDNA localization in exosomes and senescence as well as the inflammatory pathway cGAS/STING, in order to maintain cellular homeostasis[144]. gDNA is detected mostly in exosomes derived from malignant rather than normal cells. The majority of DNA found into exosomes is double stranded[145] and it has been proposed one mechanism for the loading of gDNA in exosomes, which is related to micronucleoli (markers of genomic instability). Micronucleoli are created when the nucleus is uncapable of segregating nuclear material and are cytoplasm structures surrounded by nuclear membrane[146,147]. Induction of MN generation has also been shown to promote the activation of cGAS/STING pathway[146]. Another type of DNA found in exosomes is mitochondrial DNA (mtDNA) from various cells (astrocytes, glioblastoma etc.)[148–150]. Until now, there are not many evidence regarding the mechanism for loading mtDNA into exosomes, but it is hypothesized that is due to oxidative damage [151].

2.3.3.2.2 Loading of Proteins

Exosomes are loaded with a variety of proteins which are functional, and they can also be used as biomarkers. For some proteins, the sorting is related to ESCRT machinery, for example the transferrin receptor loading into exosomes in reticulocytes is dependent on binding with ESCRT protein ALIX[152]. Also proteins that belong to syndecan family are sorted in exosomes by the protein adaptor syntetin which binds to ALIX[153]. Heparanase plays a role in this process by promoting the binding of syndecan to syntetin, promoting ALIX-ESCRT mediated loading into exosomes. In addition to this heparanase also plays a role in syntetin-dependent loading of CD63 in exosomes[153,154]. Exosomes are enriched in tetraspanins and sorting of other membrane proteins in exosomes is related to their interaction with tetraspanins. Networks of tetraspanins and those associate proteins are stabilized by protein modification such as palmitoylation[138]. In addition to this lipids also play a role in the generation of these networks; this is the reason for enrichment of cholesterol and glycosylceramides in exosomal membrane and the major regulatory role of sphingomyelinase

(nSMASE) in exosomal generation[138,155]. In addition to this sorting mechanism, others that are not related to heparanase, such as flotillin, CD9 and CD81 exist. Finally, lipid rafts or detergent resistant membranes represent membrane areas that are enriched in cholesterol, sphingolipids, and GPI-anchored proteins [156,157]. This has been observed in many studies on exosomes derived from many different cell lines (reticulocytes, myelogenous leukemia cells, Burkitt lymphoma, breast cancer, retinal pigment epithelial cells)[156–158]. Lipid raft mediated invagination of cell membrane is a well-studied mechanism of endocytosis, so it has been suggested that proteins related to this mechanism, also are related and regulate loading proteins into exosomes[147]. That means that inside a cell there are several sorting mechanisms operating at the same time that create heterogeneity in the molecular content of EVs. This would support the hypothesis that different sorting mechanisms produce distinct EV sub-populations[153,154].

2.3.3.2.3 Loading of RNA

There is a variety of RNA molecules loaded in exosomes (mRNAs, rRNA, tRNAs, micro RNAs, lncRNAs and circular RNAs, small nuclear RNAs, small nucleolar RNAs, piRNAs, mitRNAs, Y RNAs vtRNAs)[159–163]. Inside the exosomal cargo there are transcripts that represent the parental cell to a certain extent. There are a few models that may explain how these different RNAs are loaded into exosomes. RNAs can be targeted to the endosomal compartment in the ILVs and thus released by exosomes. RNA sequence motifs and secondary configuration have also been suggested to be related for the packaging into exosomes[159–161,163], as well as RNA affinity for membrane lipids and interaction with RNA binding proteins (RBPs) (AGO2 and others)[164]. Notably, it has been recently shown that RNA loading into exosomes has been linked with autophagy, by an LC3 dependent mechanism termed LC3-dependent EV loading and secretion (LDELS)[165]. Finally, another study regarding miRNA loading into exosomes has shown that it is a sequence-related process, where EXOmotifs selectively drive RNA species into exosomes, opposed to CELLmotifs that are responsible for cellular localization. EXOmotifs are sorting sequences and interestingly are cell derived specific sequences thus defining the EXO-miRNA profile[166].

2.3.3.3 Secretion of Exosomes

The mechanisms of exosome release require MVBs' fusion with the plasma membrane, where Ca^{2+} dependent exocytosis via SNARE complex is involved which is a highly regulated process. VAMP7 has been shown to control exosome secretion on maturing reticulocytes but in MDCK cells seem to regulate only secretion of lysosomes and not exosomal secretion. R-SNARE Ykt6 on the other hand is related with exosomal secretion of exosomes carrying Wnt. Rab GTPases are also implicated in exosomes secretion[167]. Rab11 is required for exosome secretion in reticulocytes[168]. Rab27a and Rab27b play a role since when depleted from cells, exosomes amount secreted from cells is highly decreased. Rab27 is related with the secretory lysosome related organelles. Rab27a and Rb27b control different steps in the process of exosomal secretion. Silencing of Rab27a in HeLa cells resulted in secretion of bigger sized particles, whereas Rab27b silencing resulted in redistribution of MVBs close to perinuclear region. In addition to this, it has been shown, in mouse models that Rab27a

blockade in breast cancer cells resulted in decreased exosomal secretion and decreased primary tumor growth and lung metastasis[169,170]. Moreover, depletion of two proteins that act as Rab27 effectors: Slp4 and Slac2b inhibited as well exosome secretion[169]. Additionally, Rab GTPase activating proteins TBCD10A, B and C and interaction with Rab35 has been shown to control exosome secretion[171]. It is important to mention here that even that Rab11, Rab27 and Rab35 play a role in exosome secretion. They have complementary roles (when only one of them is depleted this results in a reduction and not complete inhibition of exosome secretion). This means that the roles of these GTPases role are not redundant, different cells use different Rab GTPase for their exosome secretion. In prostate cancer cell lines it has shown that knock out of Rab27a gene via CRISPR/Cas9 mediated mutagenesis resulted in depletion of exosomes[172].

Finally, another mechanism that leads to exosomal secretion is by lysosomal exocytosis. As described before MVBs can be fused with lysosomes that usually leads to degradation. However, it has been described in cancer cells, that the EVs present in the lysosomes are protected from degradation and are later released in the extracellular space by lysosomal exocytosis in a oversyalated LAMP1 dependent manner[173,174]

2.3.3.3.1 Factors affecting exosome secretion

There are several factors that can affect exosome secretion. For example the metabolic state of tumor cells, such as hypoxia, starvation, acidosis, oxidative stress, thermal stress, radiation, shear stress[175]. In lung cancer it has been shown that γ-radiation promotes exosome secretion, *in vitro* and *in vivo* by TSAP6, a p53 regulated gene product[176,177]. In addition to that, cellular stress is tightly linked with exosome secretion since ceramide is mediating the stress responses from various signals (lipopolysaccharide, interleukin 1β, TNF-α, deprivation of serum, irradiation and cytotoxic drugs)[178]. Another example is that low pH is able to promote the fusion of MVBs with the cell membrane. Moreover hypoxia and ROS affect exosome secretion by leading to loss of vacuolar-protein sorting associated protein VPS4B, which is involved in MVB maturation[179].

In addition to these mechanisms, autophagy also plays a role in exosome secretion. Autophagy is a highly regulated process that leads to degradation of proteins and organelles in a mechanism involving the formation of autophagosomes which are double membrane vesicles. There is a close relationship between the pathways of autophagy and EV biogenesis. It has been shown that autophagy can play a role in biogenesis and degradation of exosomes and exosomes could promote autophagy. In the context of cancer, it is known that exosome release and autophagy share common pathways and can have an effect of drug resistance[180]. The close relationship between exosomes and autophagy is also demonstrated by molecules inhibiting the lysosomal function (bafilomycin A and cloroquine) promote exosome secretion[181].

2.3.3.4 Biodistribution of Exosomes by different sites

As already mentioned, exosomes mediate cellular communication in close and distant sites[182]. Research has shown that exosomes from cell lines and body fluids can reach the majority of organs

such as liver, lung, kidney, pancreas, spleen, ovaries, colon, and brain when there were orally administrated but when exosomes administrated intravenously there was a different pattern, where the predominant organ of biodistribution was liver, followed by spleen, lungs and organs of gastrointestinal track[183,184].

2.3.3.5 Cell-exosome interactions

When exosomes arrive to their destination, they can traduce signal by interacting to surface cellular receptors or they can enter the cell by various ways.

Surface Interaction: Exosomes can interact with their target cells via membrane proteins on their cell surface and transduce signals. This is a very important function of exosomes, since they can modulate a lot of functions including immune reactions and apoptosis. Dendritic cell derived exosomes can activate T cells since they express MHC-peptide complex on their surface and can also bind bacterial Toll like receptor ligands, as well as enhance anti-tumor activity[185,186]. On the other hand it has been shown that tumor derived exosomes express PD-L1 on their surface that can induce immunosuppression by suppressing the function of $CD8^+$ T cells[187]. In addition to this exosomal membrane FasL which can interact with the FAS receptor on $CD8^+$ T cells and induce apoptosis[188]. Another example of signal transduction by exosomes is raft related; rafts are known that they play important role in signal transduction via modification of phosphorylation in raft localized proteins by kinases and phosphatases. Exosomal transferrin receptor 2 (TfR2) present in rafts, could mediate signaling cells about iron status[189]. Moreover exosomal integrins from B-cells has been shown that they can transduce signaling to ECM and cell surface adhesion molecules[190].

Internalization of exosomes and release of the intravesicular cargo into the recipient cells can occur in several ways. Uptake process in different cell types have different uptake rates, but usually is quick (about 15 minutes). It is an energy requiring process, as well as temperature sensitive with low temperatures decreasing the rate[191–194]. Endocytic pathways in recipient cells are responsible for exosomal uptake. **Clathrin mediated endocytosis, caveolin-depended on endocytosis macropinocytosis**, **phagocytosis, internalization via lipid rafts** are involved in the exosomal uptake. **Fusion with plasma membrane** in which the two membrane merge via direct contact of the outer leaflets, is another way of exosome uptake[194]. **Receptor mediated** internalization of exosomes has been also described. One of the mechanisms is EGFR depended micropinocytosis by EGFR in cancer cell lines[195]. Moreover receptor mediated endocytosis has been described in many cells and a lot of different receptors have been identified to promote exosome internalization in various cells lines[196]. Moreover, a lot of different proteins (**protein-protein interactions**) have been found in different studies to be involved in endocytosis of exosomes, similar with the process of recognition and internalization of viruses, liposomes and nanoparticles[196]. Finally, our group has identified a unique mechanism of uptake of stroma derived exosomes by hematological malignant cells. We found that active caspase-3 inside exosomes, drives the cleavage of Bcl-xL on exosomes surface and this cleavage is required for their uptake by recipient cells. We also showed that inhibition of this cleavage molecularly or chemically, reduced the uptake of the exosomes[197].

2.3.3.6 Functions of Tumor derived exosomes

Exosomes are mediators of cellular communications and upon interaction or internalization in recipient cells, they can alter their characteristics. This ability is dependent on their molecular composition (enzymes, proteins, receptors and different types of RNAs). They affect several key functions of cancer cells like promoting carcinogenesis and tumor growth, modulate response to different therapies, modulate immune responses, they mold the tumor microenvironment and they promote angiogenesis (**Figure 8**) [198].

As already known, tumor microenvironment (TME) is a complex environment which consists of a variety of cell types (stromal cells, immune cells, cancer cells, cancer associated fibroblasts, endothelial cells) extracellular matrix, that interact with each other via direct interaction and soluble factors. TME regulates a lot of critical functions in cancer development, progression metastasis and treatment outcomes[199]. Exosomes are important mediators of communication inside TME. It has been shown that tumor derived exosomes, can increase other cancer cells' cell growth and survival[200]. Also it has been showed that tumor derived exosomes can alter the composition and characteristics of non-transformed cells, which is attributed to their RNA or protein content[201,202].

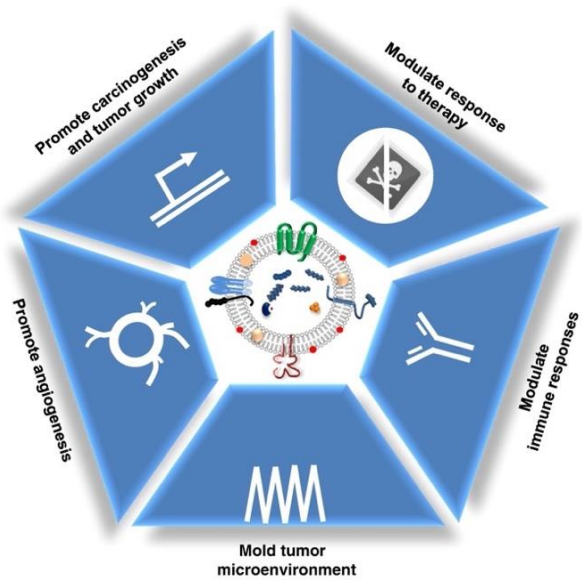

***Figure 8**: **Functional properties of tumor derived exosomes** (Kharaziha et al, Biochim Biophys Acta 2012).*

Moreover previous work from our team has showed in the breast cancer setting exosomes from metastatic cells could have the potential to affect the migration rate of recipient non-metastatic breast cancer cells[203]. Also in leukemia it has been shown that exosomes could can induce the migration pf endothelial cells, which is a hallmark of tumor angiogenesis[200].

Communication between fibroblasts and cancer cells is also controlled by exosomes, as it has been shown that fibroblast derived exosomes can enhance metastasis[204]. Additionally, exosomes from malignant cells in turn they modulate CAFs which results to remodeling of the extracellular matrix and TME in order to support tumor growth[200].

Immune cells are an important compartment of TME that are also affected by cancer exosomes. It has been shown that Tregs (immunosuppressive) expansion, proliferation, tumor promoting function and resistance to apoptosis is promoted by tumor derived exosomes[205]. Moreover it has been demonstrated that non-small cell lung cancer derived exosomes, harboring mutant KRAS, can induce a phenotypic switch of $CD4^+$ T-cells into $FOXP3^+$ Treg-like cells that have immune-suppressive function[206]. Furthermore, exosomes can suppress the NK mediated cytotoxicity as well as inhibit the maturation and differentiation of monocytes. Finally, cancer derived exosomes can promote the immune suppressive function of MDSCs[207] and in addition to this, exosomes derived from cancer cells can recruit inhibitory immune cells such as tumor associated macrophages (TAMs), tumor associated neutrophiles (TANs), Tregs and MDSCs[208].

Homing of tumor cell derived exosomes to the TME has been shown to be mediated in an organotropic manner. Breast cancer derived exosomes were shown to prepare the pre-metastatic organ specific metastasis in an integrin depended manner; where Integrin α6β1 and α6β4 dictate lung tropism, αvβ5 dictate liver tropism [209].

2.3.3.6.1 Functions of Prostate cancer derived exosomes

2.3.3.6.1.1 Role of prostate cancer derived exosomes inside prostate microenvironment

Inside TME, exosomes from prostate cancer cells promote cancer growth, tumor cell proliferation, inhibit apoptosis, induce angiogenesis promote an immunosuppressive microenvironment and induce therapy resistence[199]. EV-derived soluble TGF-β can promote the differentiation of fibroblasts into myofibroblasts (a key characteristic of a tumor reactive stroma) via activation of TGF-β/SMAD pathway, which also promotes tumorigenesis and immunosuppression[210–213]. It has also been shown that tumor derived exosomes contain proteins (c-SRC tyrosine kinase, IGF-1R and FAK) and mRNAs that promote pro-angiogenic activity[214]. Angiogenesis is very important for tumors since nutrients and oxygen can reach to them, as well as it assists with metastasis [214].

2.3.3.6.1.2 Role of prostate cancer derived exosomes in preparing metastasis

Prostate cancer derived exosomes can prepare the microenvironment for subsequent establishment of cancer cells in distant sites via promoting epithelial to mesenchymal transition (EMT), a key mechanism for metastasis, via a TGF-β dependent manner. Furthermore, prostate cancer derived exosomes, contain Integrin Subunit Alpha 3 (ITGA3) and Integrin Subunit Beta 1 (ITGB1)

proteins which can promote invasion[215]. Bone is the preferred site of metastasis in prostate cancer as already mentioned, which is pre-conditioned in advance of metastasis from released soluble factors and EVs from primary tumor[216]. Prostate cancer derived exosomes remodel the extracellular matrix, in distant sites, in order to promote adhesion of bone marrow derived cells which are important players of pre-metastatic niche. Furthermore, prostate cancer exosomes are educating osteoblasts towards acquiring premetastatic phenotype[90,199]. Moreover exosomes from prostate cancer cells communicate with bone marrow via cholesterol metabolism leading to osteoclast differentiation via NF-κB signaling [199,214]. It has been shown that exosomal miR141-3p from prostate cancer cells also plays a role in promoting osteoblastic activity and inhibiting osteoclasts' activity in a p38-MAPK38 depended manner[217]. Finally, exosomal derived hs-miR-940 showed to promote osteogenic differentiation from human MSCs[218]. All these underlines the very important role of exosomes in bone-tropism of prostate cancer and in preparing the environment to host cancer cells and support their growth. But exosomes have also other roles that contribute to survival and spread of cancer cells.

2.3.3.6.1.3 Role of prostate cancer derived exosomes in modulating the immune system

Cancer cells can modulate the immune system in order to escape from its antitumor responses. Exosomes derived from prostate cancer cells has been shown to have similar properties as well. They can alter the cytotoxic function of lymphocytes and induce apoptosis of T cells. Programmed death receptor 1 (PD-1) is mostly expressed by macrophages, activated T-cells, and B-cells whereas programmed death ligand 1 (PD-L1) is mostly expressed on cancer cells. When PD-1 interacts with PD-L1 , an inhibitory signaling pathway is activated which leads to inhibition of T-cell activation, proliferation and survival, as well as reduced cytokine secretion[208,219]. This inhibitory signaling results in attenuation of TCR/MHC and CD28/B7-1 interactions that have T-cell activating roles[220]. Prostate cancer derived exosomes has been shown to express PD-L1 even though parental cell have low or no levels at all[172]. Exosomal PD-L1 is located on the surface and has the same functions as cellular PD-L1, inhibiting the function of CD8+ T-cells *in vitro,* limiting the infiltration of T-cells in lymph nodes and in spleen and promotes tumor progression *in vivo* [172].

Except from their role in transmitting inhibitory signals towards T-cells, prostate cancer derived exosomes target also dendritic cells by induction of CD73 on DCs[221]. They can also induce M2 macrophage polarization by milk-fat globule EGF factor 8 (MFG-E8), which is known for its M2 polarization capacities in vitro[222].

Those evidence show the importance of exosomes as immune modulating factors and key players in immune escape of prostate cancer and in the generation of the immune 'cold' microenvironment of prostate cancer, that leads in the failure of ICT therapy in most patients. But prostate cancer derived exosomes have additional roles in mediating resistance in other therapies as well.

2.3.3.6.1.4 Role of prostate cancer derived exosomes in resistance to therapy

As mentioned above, chemotherapy is one of the main treatments used in prostate cancer patients and unfortunately resistance can occur. Enzalutamide and Abiraterone resistance is mediated by AR-V7[38] and AR-V7 has been found in exosomes[223]. In addition to these, it has been shown that resistance in Enzalutamide is mediated by BRN4 and BRN2 (neural transcription factors) mRNAs via exosomal transfer to recipient cells causing oncogenic reprogramming towards neuroendocrine state. Moreover, exosomes from prostate cancer cell lines and patients resistant to Docetaxel, can transfer their resistance to recipient cells[224,225]. Those exosomes also contain multidrug resistance protein 1/P-glycoprotein (MDR-1/P-gp), which except from Docetaxel can transfer resistance to other drugs [224] . In addition to that there are evidence suggesting that prostate cancer cells resistant to docetaxel, secrete Docetaxel resistant exosomes that contribute to transfer of resistance in recipient cells[226]. Furthermore, bioinformatic analysis revealed that exosomal miRNAs from Paclitaxel resistant cell lines regulate AR, PTEN and TCF4 genes in recipient cells contributing to drug resistence[227].

Regarding immune checkpoint therapy, as already discussed is not efficient in most patients. In tumor types, such as melanoma where ICT is efficient PD-L1 is present in the cells as well as exosomes[187]. Also in melanoma resistant to immunotherapy patients have higher levels of exosomal PD-L1[187]. This is very interesting finding because it could be one of the reasons why immune checkpoint therapy in prostate cancer is less effective than other solid tumors in which cellular PD-L1 is in high levels. This could lead to new therapeutic approaches that could target exosomal PD-L1 in prostate cancer.

2.3.3.7 Prostate cancer cell derived exosomes as a source of biomarkers

As mentioned before prostate cancer derived exosomes carry a variety of nucleic acids, protein and lipids and they can transfer information to distant sites. Usually their content reflects their cellular origin and this makes them as a good source of biomarkers[228]. They can be used as biomarkers for the early diagnosis of the disease, in therapy selection and in prognosis[229].

RNAs considered highly valuable as biomarkers in prostate cancer. It has been shown that exosomes have the ability to protect miRNAs, which makes them ideal source of biomarkers[230,231]. A meta-analysis confirmed that plasma derived exosomal miRNAs have high diagnostic value for prostate cancer patients[232,233]. This lead to the development of ExoDx, an assay that uses urine derived exosomes for the detection of PCA3 and TMPRSS2:ERG fusion mRNA, that are prostate cancer related. It has been shown that this signature in combination with standard of care (SOC) (prostate-specific antigen level, age, race, and family history) can predict with statistical significance more accurately prostate cancer of Gleason score of 7 (GS7) from GS 6 and benign disease than SOC alone. This assay aims to reduce the invasive sampling for biopsis[234]. Moreover, recently it has been shown

that the addition of GATA2 mRNA (which is expressed in aggressive prostate cancer) enhance the detection of high risk prostate cancer[235].

Except from the development of ExoDx, other studies have identified exosomal miRNAs that could serve as biomarkers for disease. Increased levels of exosomal miR-21 in plasma and urine are associated with prostate cancer. Additionally, plasma derived exosomal miR-574 and miR-107 have identified as biomarkers for prostate cancer [236].

For prognostic purposes exosomal amount in plasma from patients with CRPC is increased compared with healthy donors and are associated with worse overall survival[237]. An example of this, is plasma exosomal miR-1290 and miR-375 in high levels were associated with worse OS in prostate cancer patients[238]. Another example, is a signature composed from urine derived exosomal miRNAs (miR-151a-5p, miR-204-5p, miR-222-3p, miR-23b-3p and miR-331-3p) plus serum PSA, which found to predict biochemical recurrence after curatively radical prostatectomy in prostate cancer patients[239].

On the protein level, plasma exosomal PSA was showed to be increased in prostate cancer patients compared to healthy men or men with BPH and is more accurate in detecting cancer than regular PSA test[240]. Another prostate related protein, STEAP-1 (six-transmembrane epithelial antigen of the prostate 1) was detected in higher levels in exosomes from prostate cancer patients compared to healthy individuals[241].

Exosomes have been proved to be informative also in predicting response to therapy. In particular, plasma exosomal derived AR-V7 mRNA found to be present in 36% of patients with CRPC and this expression is correlated with lower progression free survival and OS[223]. In addition to this, miR-34a has been shown that could be used as a biomarkers for response to Docetaxel and has also clinical relevance to the progression of the disease[236]. In particular, decreased levels of miR-34a are related with poor survival and regulates Bcl-2 and there is evidence of regulating response to Docetaxel[242]. Moreover as previously mentioned Docetaxel resistant prostate cancer derived extracellular vesicles carry MDR1/P-gp protein that can be used as biomarkers of resistance to therapy[225].

2.3.3.8 Exosomes as delivery vesicles

Except from the exosomal functions already extensively discussed, exosomes have been proposed as excellent drug delivery vehicles. Exosomes derived from patients have excellent biodistribution capabilities and biocompatibility, which significantly reduces the clearance from phagocytes[140]. This reduced immunogenicity allows the loading into exosomes a wide variety of therapeutics that can be delivered to their targets and have increased half-life and reduced toxicity. A variety of agents can be incorporated into exosomes such as exogenous siRNA and various chemotherapeutic drugs. Another approach involves the genetic manipulation of donor cells in order to produced exosomes with specific proteins or DNA, mRNA [140].

3 RESEARCH AIMS

The aims of this thesis are to:

1. Determine whether EVs can provide molecular information on the bone tumor microenvironment in co-clinical studies.
2. Identify EV-derived biomarkers that inform of response and resistance to therapies for mCRPC.

In Paper I: We investigated for potential biomarkers that could predict the response for the bone targeting agent Radium-223. We performed molecular profiling of exosomes from preclinical models and compared the molecular profile in patients with favorable and unfavorable overall survival. In addition to this we showed that Radium-223 alters the TME, and those changes are reflected in the exosomal molecular and proteomic content. We detected changes in DDR in exosomes, as a pharmacodynamic measure of Radium-223 treatment. Moreover, we could detect changes in immune system upon treatment with Radium-223.

In Paper II: We search for exosomal biomarkers that could predict response to the taxane Cabazitaxel in patients (Non-responders vs Responders) with mCRPC. At baseline Non-responders' exosomes were enriched in transcripts encoding genes that are related to oncogenesis, cytoskeleton, and immune regulation. STMN-1 and ITSN-1 genes (associated with Cabazitaxel resistance) found to be statistical significantly enriched in Non-responders compared to Responders. We also investigated the early effect of Cabazitaxel treatment (Cycle 1) in the molecular content of exosomes, and this analysis showed differences in pathways related to response to treatment.

4 MATERIALS AND METHODS

4.1 CELL LINES AND CELL CULTURE:

The murine prostate cancer cell line MyC-Cap was purchased from ATCC. MyC-Cap-Luc^{+}-RFP cells were kindly provided by Guocan Wang (MDACC, Houston, TX). These cell lines were maintained in DMEM at 37^{o}C and 5% CO2 as the vendor recommended. The DMEM was supplemented with 10% FBS and 1% penicillin/streptomycin. Identification of cells was confirmed by fingerprinting analysis at IDEXX Laboratories Inc.

Murine TRAMPC2-BMP4 cells were kindly provided by Dr. Sue-hwa Lin and maintained at 37oC and 5% CO2 in DMEM supplemented with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 0.005 mg/ml bovine insulin and 10 nM dehydroisoandrosterone, 90%; fetal bovine serum, 5%; Nu-Serum IV, 5%.

Routine testing for Mycoplasma contamination was performed.

4.2 EXOSOME ISOLATION FROM PLASMA

Plasma samples were thawed on ice and gently mixed by rotating for 2 minutes at 4C. Then the samples were spun down at 500g for20 seconds at 4C. Continuously, 500 mL of plasma sample was mixed by inversion with 500 mL of cold PBS and centrifuged at 12,000 g for 45 minutes at 4C to remove cellular debris. The supernatant was transferred to an ultracentrifuge tube, and 8 mL of cold PBS was added and ultracentrifuged for 2 hours at 120,000 g for 2 hours. The supernatant was discarded. For RNA isolation, the exosome pellet was resuspended directly in Qiazol Lysis Reagent and proceeded immediately to RNA extraction. For Western blot analysis were lysed in 1XRIPA buffer, whereas exosomes used for Nanosight analysis were resuspended in conditioned medium or PBS.

4.3 RNA EXTRACTION FROM CELLS, CELL DERIVED EXOSOMES AND PLASMA DERIVED EXOSOMES.

The miRNeasy Micro Kit (Qiagen, cat: 217084) was used to extract RNA from plasma derived exosomes, according to the manufacturer's instructions, with minor modifications. The number of cells used to extract RNA was $\leq 1 \times 10^{6}$. In the cell pellet or exosome enriched pellet, 700µL of Qiazol Lysis Reagent was added and homogenized by vortexing. The homogenate was incubated for 15 min at room temperature, and 140µL of chloroform was added. The samples were mixed vigorously for 15 sec, incubated at room temperature for 3 min, and centrifuged for 15min at 4^{o}C. Continuously, the upper aqueous phase containing the RNA was transferred to another tube and 1.5 volumes of 100% ethanol was added, mixed, and transferred to a RNeasy MinElute spin column and centrifuged at

8000 g for 15 sec, at room temperature. Then, 700µL of RWT buffer was added to the RNeasy MinElute spin column and centrifuged at 8000g for 15sec at room temperature, and the flowthrough was discarded. In the RNeasy MinElute spin column, 500µL of RPE buffer was added and centrifuged at 8000g for 15 sec at room temperature, and the flowthrough was discarded. Next, 500 µL of 80% ethanol was added, and the samples were centrifuged at 8000g for 2 min at room temperature, and the flowthrough was discarded. The spin column was then dried by centrifuge at 20800g for 5 min at room temperature. Finally, 14µL of RNase free water was added to the spin column membrane and centrifuged at 20800g for 1 min at room temperature. The RNA quantification was performed with an Implen NanoPhotometer.

4.4 EXOSOMAL RNA-SEQ

Illumina-compatible libraries were prepared using the Ovation® RNA-seq System V2 (Nugen) and the KAPA Hyper Library Preparation Kit (Kapa Biosystems, Inc). Briefly, exosomal RNA samples treated with DNase-1 were assessed for size distribution and quantity using the Fragment Analyzer High Sensitivity RNA Analysis Kit (Advanced Analytical) and the Qubit® RNA HS Assay Kit (ThermoFisher), respectively. One and a half nanograms of RNA was converted to double-stranded cDNA, then amplified using Nugen's proprietary single primer isothermal (Ribo-SPIA) protocol. Five hundred nanograms of the resulting cDNA was fragmented to an average size of 200 bp, and libraries were constructed using the KAPA Hyper Library Preparation Kit, followed by two cycles of PCR library enrichment. Following cleanup, the libraries were mixed (three libraries per pool), then quantified by qPCR using the KAPA Library Quantification Kit (KAPA Biosystems) and sequenced with one pool per lane on the Illumina HiSeq4000 Sequencer using a 75 bp paired-end format.

4.5 GENE EXPRESSION ARRAY DATA ANALYSES

The Affymetrix GeneChip Human Transcriptome Array 2.0 (HTA 2.0) was utilized by the core gene expression analysis unit as described by the manufacturers. Affymetrix CEL files were processed, and quantile normalized with GC-RMA background correction using the Expression Console and Transcriptome Analysis Console (TAC) 2.0 (Affymetrix). Differential expression of genes was statistically evaluated using TAC 2.0. $P < 0.05$ was considered significant.

4.6 BEAD BASED ISOLATION OF PLASMA EXOSOMES/ LUMINEX ASSAY

Conjugation of Streptavidin beads and biotinylated antibodies conjugation

For each well, 2.5 µL of streptavidin beads/antibody was used. The beads were washed twice in wash buffer containing 0.01% BSA in PBS, and 0.0625 µg of biotinylated antibody was added to the beads and incubated for 2 hours at 4^{o}C. The streptavidin beads/biotinylated antibody conjugate was washed 3 times in wash buffer, and the antibodies conjugated to the beads were resuspended in wash buffer.

Luminex-based arrays

For the analysis of the levels of various proteins in exosomes, exosomes were captured from the plasma of patient samples (50 µL plasma/well in a 96-well plate) using magnetic beads conjugated with antibodies against exosomal markers (CD9, CD63, and CD81). Exosomes were lysed with 1 x RIPA buffer, and 25 µL/well was used for the Luminex arrays, according to manufacturer's instructions. Samples were run in duplicates and data were normalized to total exosomal protein concentration, measured by Bradford assay (Bio-Rad, cat: 5000006). The Luminex assays used in this study were purchased from ThermoFisher Scientific (Immune Monitoring 65-Plex Human ProcartaPlex™ Panel, cat: EPX650-10065-901; Immuno-Oncology Checkpoint 14-Plex Human ProcartaPlex™ Panel 1, cat: EPX14A-15803-901).

4.7 NANOPARTICLE TRACKING ANALYSIS (NTA)

For the secretion study, NS500 (NanoSight Limited, London, UK) was used, equipped with an 8-megapixel camera (Andor Technology, Tokyo, Japan) and a 405 nm laser, in order to measure exosome size and concentration. NTA v3 software (NanoSight Limited) was used for both data acquisition and analysis. The camera level was set at 14 and the detection threshold was 3. Thirteen samples from the favorable group and four from the unfovarable group of patients were used and their average was calculated. The duration of each video was 5 x 1 minutes.

4.8 WESTERN BLOT

Exosome pellets were lysed using 1 x RIPA buffer (containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL, 1% glycerol), supplemented with Complete Protease Inhibitor Cocktail (Roche, Basel, Schweiz), PhosSTOP Phosphatase Inhibitor Cocktail (Roche, Basel, Schweiz), 100 mM vanadate (Invitrogen Life Technologies, Paisley, UK), and 1 mM

dithiothreitol (DTT) (Sigma Aldrich, St. Louis, MO, USA). Protein concentration was determined with Bradford assay using Bradford solution (Bio-rad). For each Western blot, 50 µg of protein was boiled at 90°C for 5 min with LDS (Invitrogen Life Technologies, Paisley, UK) and DTT. Proteins were separated in 10% or 12% Bis-Tris gels (Invitrogen Life Technologies, Paisley, UK) in 1 x MES running buffer (Invitrogen Life Technologies, Paisley, UK) containing antioxidant (Invitrogen Life Technologies, Paisley, UK) or 4% to 12% Criterion TGX (Bio-Rad, Hercules, CA) in 1 x Tris-glycine-SDS running buffer, according to manufacturer's instructions. Proteins were transferred to PVDF membranes (PerkinElmer, Santa Clara, CA, USA) in transfer buffer (Invitrogen Life Technologies, Paisley, UK) containing 20% methanol, or using the Trans-Blot Turbo System (Bio-Rad, Hercules, CA). Membranes were blocked in 5% (w/v) non-fat milk in 1 x TBS containing 0.1% Tween-20 for one hour at room temperature and then incubated with primary antibodies in the appropriate dilutions (1/500-1/1000) overnight at 4°C. HRP-conjugated antibodies in 1/2000 dilution were used as secondary antibodies and the membranes were incubated for one hour at room temperature. Washing steps between incubations with antibodies were performed with 1 x TBS containing 0.1% Tween-20. Enhanced chemiluminescence (ECL) or ECL+ (PerkinElmer, Santa Clara, CA, USA) was used for developing the X-ray film (CL-Exposure Film, Thermo Scientific, Bremen, Germany).

4.9 IMMUNOHISTOCHEMISTRY (IHC)

Four-micron paraffin sections were cut and placed in the oven at 60oC for one hour. The primary antibodies were stained with standard DAB IHC on Dako AutoStainer Plus (Dako, Carpinteria). The sections were deparaffinized and rehydrated with deionized water. Endogenous peroxidases were blocked with a 3% hydrogen peroxide/MeOH solution for ten minutes, followed by three rinses in deionized water. Tissue sections were pretreated in citrate buffer (pH 6.0) (cat: S1699, Dako) and heated in a Pascal pressure cooker to unmask the epitopes. The pressure cooker was set to boil at 125°C for 30 seconds. The slides were rinsed three times in deionized water.

Following removal from the instrument, the slides were counterstained for one minute with CAT Hematoxylin (Biocare Medical), followed by two water rinses. The nuclei were blued with Bluing reagent (Richard Allen Scientific) for one minute and then dehydrated through xylene and increasing percentages of EtOH (95% EtOH 3X, 100% EtOH 3X, xylene 3X). The slides were then cover-slipped using a xylene-based mounting media and air-dried at room temperature.

4.10 TREATMENT OF MYC-CAP CELLS WITH RADIUM-223

For testing Radium-223 toxicity, MyC-Cap cells expressing luciferase-RFP were cultivated in a 12-well plate (30000 or 60000 cells/well, Vf = 1mL) and treated with 300 or 600 Bq/well. The growth was measured by bioluminescence signal using an automatic plate reader (PerkinElmer, EnVision 2104 multilabel plate reader) after the addition of luciferine solution (50 µg/mL) at 5 and 7 days.

4.11 ANIMAL STUDIES

MDA PCa 118b cells (1 million per site) were implanted subcutaneously in SCID mice. The mice were killed when tumors reach 8 to 10 mm in size and the plasma was collected for exosome isolation. For the Radium-223 study, mice with MDA PCa 118b tumors were treated with or without Radium-223 (300 kBq/kg). Plasma was collected two weeks after Ra 223 treatments.

FVB mice were purchased from Taconic BioSciences, NY, and the mice were housed at the animal facility of Experimental Radiation Oncology, Department of Veterinary Medicine & Surgery, MD Anderson Cancer Center. All animal studies were conducted in accordance with the current regulations and standards of the US Department of Agriculture, the US Department of Health and Human Services, and the National Institutes of Health and were approved by The University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee. The mice used in this experiment were five to six weeks old and all were surgically injected with 1 x 10^6 MyC-Cap cells in the right femur with 5 µL of phosphate buffer saline (PBS). The cells used for femoral injection were at 60% to 70% confluency. All animals were regularly monitored for changes in their health status and weight. Tumor growth was measured by MRI every two weeks after initiation of treatment, and tumor volume was calculated using ImageJ. Mice were euthanized when tumor growth was found outside of the femur or if they had weight loss exceeding 30%. Both femurs were collected and fixed in formalin buffer-saturated with 10% PBS. H&E and IHC staining were conducted.

5 RESULTS

5.1 PAPER I

5.1.1 Detection of bone-related markers in plasma exosomes from a bone-metastatic patient- derived xenograft

We used the MDA-PCa 118b PDX model, an osteogenic PCa model in order to investigate whether the molecular content of plasma derived exosomes is enriched in bone-related markers compared to the EVs from mice without tumor. We isolated exosomal RNA and performed RNA sequencing. In order to be able to distinguish tumor (human) vs host (mouse) transcripts we performed species-specific comparative analysis. Ingenuity pathway analysis revealed that the top enriched pathways in the Host transcriptome, were junctions, cytoskeletal remodeling and bone formation (TGFβ, Wnt/β catenin and calcium signaling) related. Specific genes related with bone formation were also enriched (*fstl3* and genes encoding Bmpr1b, BMP3, and 6). Importantly, those pathways and genes weren't identified in the tumor transcripts. The top pathways identified in Tumor EVs that were identified are related to tumor growth (mTOR, Ephrin, ERK/MAPK and CXCL4 signaling cascades), which are consistent with the cellular origin of the tumor (human).

In the host transcriptome we found an enrichment in pathways associated with new formation (TGFβ, Wnt/b-catenin, and calcium) and decreased pathways related to osteoclasts. *Fstl3* found to be the top enriched host gene, which promotes bone formation and inhibits osteoclast differentiation. In addition to this, *Bmpr1b, bmp3, and bmp6* which also promote bone formation were detected in host transcriptome.

5.1.2 Detection of pharmacodynamic changes induced by Radium-223 within the bone tumor microenvironment and plasma exosomes from a bone-metastatic patient-derived xenograft

For this purpose, tumor bearing mice were treated with Radium-223. Exosomal RNA from these mice compared with exosomal RNA from untreated mice and we found that bone related pathways are downregulated upon treatment with Radium-223. Then we confirmed those results with IHC in mice tissues and we found that the expression of osteocalcin (osteoblast secreted marker) was reduced in treated mice.

In addition to these, exosomes from Radium-223 treated mice were enriched in DDR related pathways compared with exosomes from untreated mice. This finding was also confirmed by IHC of pATM protein, which is in accordance with the fact that Radium-223 induces DNA damage.

5.1.3 Plasma exosomes from patients detect pharmacodynamic changes in Radium-223 and detect biomarkers associated with prolonged OS in patients treated with OS.

To confirm if the above results have clinical relevance, we also used plasma-derived EVs from patients with mCRPC treated with Radium-223. Interestingly the results confirmed the PDX model data regarding bone related and DDR pathways.

In patients, plasma derived exosomal RNA was isolated and HTA was performed. Analysis showed that there was enrichment in patients with unfavorable OS in pathways related to tumor growth, DDR and immune responses compared to patients with favorable OS.

Regarding bone related markers, in order to validate these results, we also checked in protein levels by Luminex using a multiplex bone panel and we found that in EOT proteins known to promote osteoclast differentiation were enriched in patients with unfavorable OS.

Regarding immune related pathways, 3 out of top 10 altered after Radium-223 treatment were related with immune regulation and immune checkpoint activation. In patients with unfavorable OS there was an upregulation of PD-L1. We validated and confirmed the presence of PD-L1 by Western blot, where the patients with unfavorable OS had higher levels of PD-L1 in baseline compared with the patients with favorable OS. In addition to this, in patients with unfavorable OS there was an enrichment of immune checkpoint proteins (PD-L1, LAG3, and IDO) at the end of treatment compared to the patients with favorable OS.

5.1.4 Using Radium-223 to target new bone formation plus immune checkpoint blockade in an immunocompetent prostate bone cancer model.

We performed *in vivo* study, in which we targeted the new bone formation and administrated immune checkpoint blockade (anti-PD1 and anti-CTLA-4) at the same time. FVB mice were used in which Myc-Cap cells were injected and treated. There were four treatment groups Degarelix (Deg), Deg plus Radium-223, Deg plus ICB (anti-PD-1 and anti-CTLA4), and Deg plus Radium-223 plus ICB. The most important finding of this experiment was that in mice treated with both Radium-223 and ICB the size of the tumors was significantly more reduced that the monotherapies. Moreover, mice treated with Radium-223 had higher levels of PD-L1 protein than the ones with Degarelix alone in their plasma derived exosomes. Finally in tissues from mice we performed IHC, and we showed that in the Deg plus Radium-223 and Deg plus Radium-223 plus ICB groups, there was an increase of PD-L1 in tumor cells, and an increase in ICOS was evident only in Deg plus Radium-223 plus ICB. These results could be interpreted as that PD-L1 is increased upon treatment with Radium-223 and combining Radium-223 with immune checkpoint therapy can have better therapeutic outcomes.

5.2 PAPER II

5.2.1 Plasma exosomes from Non-responders are enriched in pathways that are known to be associated with resistance to Cabazitaxel.

Patient samples from patients with mCRCP treated with Cabazitaxel (from ConCab clinical trial) were used in order to perform HTA 2.0 transcriptome analysis from plasma derived exosomal RNA in order to search for biomarkers to resistance in Cabazitaxel. We categorized patients in two groups (Responders vs Non-responders) based on their Radiographic and PSA responses.

At baseline, we searched and identified pathways that are already known to play a role in Cabazitaxel's resistance. Indeed, in Non-responders there was an enrichment in Cytoskeleton, Oncogenic Signaling, Cell cycle/DDR, Hormone Signaling and Metabolism related pathways. Interestingly, there was also an enrichment in Immune related pathways.

5.2.2 Exosomal profiling from Non-responders are enriched in genes that are known to be associated with resistance to Cabazitaxel.

With regard to specific genes that were enriched in Non-responders at baseline, in the Top 20 genes was STMN1 which is cytoskeleton related gene. In addition, TAOK, a cell cycle/DDR related gene and FOXM1, a cell cycle regulator gene were significantly upregulated in Non-responders.

5.2.3 Cabazitaxel-induced alterations in Cytoskeleton, Cell cycle/DDR and immune related pathways can be detected in exosomal RNA after one cycle of treatment.

Then we searched for changes in exosomal transcriptome as indicators of early response, after one cycle of treatment and we saw that Cabazitaxel induced upregulation of pathways associated with Cytoskeleton, Cell cycle/DDR, Cell Death and Immune related pathways. Finally, we wanted to see if there was the feasibility of detection of differences that could reflect response to treatment in the two groups of patients (Non-responders vs Responders). Cell Death related pathways found to have higher induction in Responders compared to Non-responders after exposure to Cabazitaxel. In immune related pathways we saw different responses between the two groups: Natural Killer Cell Signaling was downregulated in Non-responders and upregulated in Responders. On the other hand, PD-1/PD-L1 pathway that has immunosuppressive properties, were downregulated in higher degree in Responders compared to Non-responders.

6 DISCUSSION

6.1 PAPER I

The identification of predictive biomarkers is very important because they can provide information on the acquisition of resistance to therapy. In this study we used Radium-223 which targets the new bone formation that is one of the main characteristics of bone metastatic prostate cancer [107]. So far bone metastatic disease is monitored with serum ALP concetration[243], which had specificity and sensitivity limitations; For example, only 4 out of 25 patients had ALP levels above pathologic threshold and the decline of ALP (more or less that 30%) did not correlate well with OS trend; thus for this study we stratified the patients in two groups according to their OS.

In our study we showed that there are differences in exosomal cargo (RNA and protein) between mice bearing tumors or not, related to bone formation and these changes are affected by the bone targeted agent Radium-223. One important finding is the decreased osteoblasts related pathways and increased RANK signaling in osteoclasts after Radium-223 treatment in mice bearing Myc-Cap tumors. This shows that exosomes reflect changes in TME upon treatment and they may be used as a pharmacodynamic measurement.

Radium-223 is known to cause DNA double strand breaks[107]. Genes involved in DDR found to be upregulated both in mice and patient plasma exosomal RNA (ATM, ATR-interacting protein (Atrn), XRC family members, and ZEB, which shows that these changes in exosomal content can be used as pharmacodynamic measure of treatments efficacy. To this end, they are evidence from mCRPC treated with Radium-223 that showed patients with DDR alteration had survival benefit, completed all cycles of Radium-223 therapy and time to next treatment had a trend to be longer. This explains the rational for combing Radium-223 treatment with DDR targeting agents, which could result in enhanced Radium-223 efficasy[244].

Regarding immune related system and bone formation, it is known that those two are closely related, not only due to the homing (in the bone marrow) of immune cells but also because the lineage background of osteoclasts. This could explain the changes in immune related pathways upon Radium-223 treatment. Our results are in accordance with a study in which mCRPC patients treated with Radium-223 had longitudinal changes in immune cells[245]. Exosomal PD-L1 found to be upregulated upon treatment with Radium-223 and thus we designed an experiment in which we combined Radium-223 and immune blockade therapy and indeed the combination therapy showed greater effect in reducing tumor size.

6.2 PAPER II

In the second study, we utilized exosomal RNA in order to search for biomarkers for response to the taxane Cabazitaxel. Taxanes bind to tubulin across the microtubules, which results in stabilization of cytoskeleton, thus causing mitotic arrest (G2/M), apoptosis, inhibition of intercellular trafficking and AR signaling. In addition, Cabazitaxel has lower affinity for the drug efflux pump p-glycoprotein-1 in comparison with other taxanes[70]. We utilized samples from patients from ConCab clincial trial, in which they compared two different treating schedules (Cabazitaxel every three weeks, 25 mg/m2 versus weekly, 10 mg/m2 5 of 6 weeks) in order to avoid dose delays, reductions or terminations due to toxicity[246].

For the identification of exosomal markers predicting response to treatment with Cabazitaxel, we performed exosomal transcriptome analysis, at baseline. Patients were stratified in Non-responders and Responders according to their PSA decrease and Radiological Response. Non-hierarchical clustering revealed the differential enrichment of those two groups of patients. Thus, we also searched for differences in cytoskeleton related pathways, and we showed that Non-responders had enrichment in actin cytoskeleton related pathways compared to Responders at Baseline. Except this, cytoskeleton related genes found to be significantly enriched in Non-responders: STMN1 (which encodes the protein Stathmin 1) was found to be among the Top 20 upregulated genes in Non-responders. Stathmin promotes the depolymerization of microtubules and inhibits the polymerization of tubulin heterodimers[247]. Moreover, it has been shown that protein and RNA levels are elevated in aggressive prostate cancer[248]. These existed data together with our findings generate the rational for further studying Stathmin's role in Cabazitaxel resistance and also investigate the functional properties of exosomal Stathmin in mCRPC. Additionally, ITSN1 (which encodes for intersectin-1), also actin-cytoskeleton related, was found to be upregulated in Non-responders at baseline, as well.

Moreover, we showed an enrichment in pathways related to cancer progression, cell cycle and DNA damage response pathways, as well as specific genes: TAOK1 (in Top 20 list) which is related with DNA damage response and cytoskeleton stability. Finally FOXM1 which regulates PSA transcription, is a cell proliferation transcription factor and in breast cancer is linked with paclitaxel resistance also was upregulated in Non-responders [249,250].

Moving further we looked and found pathways related to hormone signaling to be enriched in Non-responders at baseline and in particular AR signaling pathway. AR pathway is related both to prostate cancer progression and taxane resistance[71,251].

Cell death pathways were enriched in Responders compared to Non-responders after the first cycle of treatment, showing the cytotoxic effect of Cabazitaxel in Responders.

Finally, we searched for changes in immune related pathways since they are data showing that Cabazitaxel has an effect in immune system and specifically in macrophages population [252,253]. Moreover it has been shown that in prostate cancer animal model, Cabazitaxel induced PD-L1 upregulation [254]. Here we found that components of immune system, with role in tumor immunity, found upregulated in Non-responders compared to Responders at baseline, which indicates that

Cabazitaxel resistance in prostate cancer patients could be partially due to immunosuppression. Finally, we looked for potential effect of Cabazitaxel in immune profiling after one cycle of treatment, where we detected an effect in pathways that have differential roles in cancer immunity. Surprisingly, PD-1/PD-L1 immunotherapy pathway was downregulated after one cycle of treatment and this reduction was higher in Responders. Also, there were different level of response between the two groups of patients and interestingly, there was a dramatic differential response in NK signaling in that this pathway was highly upregulated in Responders, whereas Non-responders had a marginal decrease in this critical pathway. These specific pathways should be investigated further because they might play a role in Cabazitaxel resistance and could be potentially targetable.

Taken these data together we showed that exosomes have a distinct molecular content in Non-responders, compared to Responders at baseline. Pathways related to cancer progression and therapy resistance are increased in Non-responders compared with Responders. We also provide data that could be further explored in functional studies; exosomal STMN1's role in resistance to Cabazitaxel, as well as exosomal STMN1 and ITSN1 levels could be further confirmed as a potential signature for response to Cabazitaxel in large cohorts. Finally, we have interesting data regarding the role of immune related factors in Cabazitaxel resistance that also could be further explored.

7 CONCLUSIONS

7.1 PAPER I

From our results from the first study, we showed that there, is the possibility of longitudinally monitoring the progression of prostate cancer inside the bone microenvironment, by molecular profiling of plasma derived exosomes. Moreover, we showed changes in immune related markers. The changes we detected in DDR can serve as pharmacodynamic measurement and proof of principle. Our data from pre-clinical and clinical studies showed that exosomes provide valuable information on the bone tumor microenvironment and the ability to identify the mechanism of action and resistance to bone targeting agents.

7.2 PAPER II

In this study, we showed that Cabazitaxel related changes can be detected exosomal RNA isolated from plasma of CRPC patients. We found a number of significant differences in gene expression between Responders and Non-responder patients at baseline, including immune related pathways that require further investigation. Cytoskeleton related genes were found to be upregulated in Non-responders and more research is required to evaluate their prognostic value in response to Cabazitaxel. In addition to those, cell cycle related genes are also of interest. Finally, we showed initial data that support the notion that immune system could also play a role in resistance to Cabazitaxel.

8 POINTS OF PERSPECTIVE

During recent years, extracellular vesicles have gained the attention of scientific community as it became evident that they are important mediators of intracellular communication and affect a lot of physiological and pathological processes, including cancer initiation and progression[128–130].

Here we used exosomes in order to search for biomarkers in response and resistance to therapies for mCRPC and how therapies affect the TME, and these changes are reflected in the exosomal content.

From our first study we were able to identify differences in regard to bone formation, cancer progression, immune related and DDR related pathways in exosomes from patients with Favorable versus Unfavorable OS at baseline and after treatment with Radium-223. This finding could be confirmed in larger cohorts of patients. Moreover, in the same study we showed evidence that exosomal PD-L1 could play a role in resistance to Radium-223. This could be further investigated in studies were exosomal secretion is diminished and/or pharmacologically targeted *in vitro* and later *in vivo* and see if tumor growth is reduced. Finally, since we provided evidence that DDR is also affected by Radium-223, studies combining Raddium-223 treatment with PARP inhibitors could be performed to confirm the possible synergistic effect, reveal the exact mechanism of action and if there would be any resistance mechanisms. This would be very insightful in selecting the proper patients that could benefit from those combinations, as results from clinical trials are shifting to this direction, as seen in the Phase 1 trial where Radium-223 was combined with Olaparib [255].

In our second study we were able to identify differences in exosomes from Non-responders vs Responders to Cabazitaxel treated patients with mCRPC. These differences included transcripts related to Cytoskeleton Cell, Cycle/DDR, cell death, and immune related pathways. From these data, we could further study the role of exosomes in driving resistance to Cabazitaxel. We could perform functional studies where exosomes from Cabazitaxel resistant cell lines could change the phenotype pf Cabazitaxel sensitive cells; then we could try to reverse those effects by targeting exosome secretion.

Moreover, from the finding that Cabazitaxel Non-responders have a distinct exosomal immune profile, we could identify which from which immune cell population exosomes leads to this Cabazitaxel resistance. Then we could target their exosomal secretion to confirm the finding, as well as try to target those cell populations pharmacologically, having as ultimate goal in suggesting the next line of treatment for patients that develop resistance to Cabazitaxel.

Another future direction could be confirming the potential signature for response to Cabazitaxel (STMN and ITSN1) in larger patient cohorts. Then, the role of exosomal Stathmin as a mediator of Cabazitaxel metastasis could be explored. Finally, since there is evidence for early responses to Cabazitaxel, further studies with longitudinal sampling until the End of

Treatment could be performed in order to study the potential of using exosomes for monitoring disease progression during the course of treatment.

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