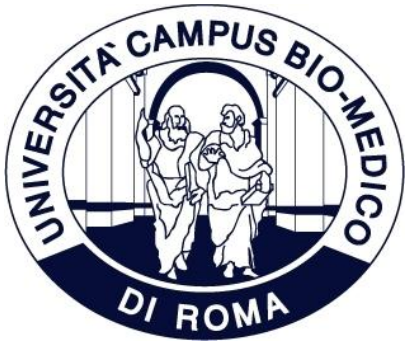

Università Campus Bio-Medico di Roma

PhD Course: Scienze Biomediche Integrate e Bioetica
Curriculum: Patologia Osteo-Oncologica
XXX cycle- 2014/2015

"ROLE OF OSTEOBLASTS IN CASTRATION RESISTANT PROSTATE CANCER (CRPC) PROGRESSION"

Giulia Ribelli

Coordinator:
Prof. Giuseppe Tonini

Tutor:
Dr Francesco Pantano

26th April 2018

"To strive, to seek, to find and not to yield".

"Lottare, cercare, trovare e non arrendersi".

-Alfred Tennyson

ABSTRACT

Prostate cancer has the propensity to metastasize to the bone forming mainly osteoblastic lesions. Although androgen ablation remains the standard of care for advanced metastatic disease an eventual tumour progression occurs and androgen-independent disease develops. Prostate cancer can be considered a microenvironment-driven disease, indeed bone and tumour cells interaction is crucial for tumour progression and bone metastasis onset. Since this complex interplay is currently not completely elucidated, this research project was focused on osteoblast (OB) role in the progression of castration resistant prostate cancer (CRPC).

We found that in direct co-cultures, OBs strongly inhibited AR receptor of CRPC cell line (C4-2B cells). Since AR activation is the driving signal for prostate cancer cell growth, we analysed the impact of AR inhibition on tumour cell proliferation. Contrary to expectations, C4-2B cells proliferated significantly more when cultured with OBs, supporting the hypothesis that OBs stimulate an androgen-independent cancer growth. Moreover, we demonstrated that OB effects were mediated by the release of osteoblastic soluble factors, since AR inhibition persists when C4-2B cells were treated with osteoblastic conditional media (OCM).

Our results showed also that OB-mediated AR inhibition is only partially restored adding an exogenous androgen, demonstrating that its repression could be in part androgen independent. In addition, the effect of Enzalutamide, an AR inhibitor was significantly lower in C4-2B treated with OCM. The lower effect of AR agonist (R1881) and inhibitor (Enzalutamide) could be a consequence of the reduced expression of AR mRNA and protein levels found in C4-2B cells after OCM treatment. Once again, together with the repression of the receptor, we observed that OBs promoted tumour cell growth also in indirect co-culture.

In order to identify the molecules involved in this process, we focused on IL-6, IL-8, WNT16, DKK-1 and CXCL12 expression, finding that OBs presented higher mRNA levels of all these factors compared with C4-2B cells. According to literature data, IL-8 and IL-6 represent the best candidates to mediate OB effects, since their protein levels were highly express in OCM.

However, further analyses are necessary to fully elucidate their role in the androgen-independent cancer cell growth.

The identification of the targets responsible of OBs pro-tumour effects could represent a strong rationale for the development of new target therapies that could support the existing strategies of CRPC treatment.

INTRODUCTION

1 The Prostate

The prostate is a tubule-alveolar exocrine gland of the male reproductive system that produces and secretes a slightly alkaline fluid important for sperm motility and viability. The most abundant proteins in prostatic secretion are PSA and prostatic acidic phosphatase (PAP) both of which are used in clinic as markers of prostate cancer. (Beer *et al.*, 2014)The prostate can be divided in four distinct regions: the peripheral zone, the central zone, the transition zone and the antero fibro-muscular zone. The majority of prostate cancers arise in the peripheral zone (McNeal et al., 1988). The development, growth and function of the prostate are strictly associated to androgens.

1.1 Androgen/Androgen receptor pathway

Androgen Receptor (AR) is a 110 KDa protein that consists in four distinct functional motifs—the aminoterminal domain (NTD), DNA-binding domain, hinge region, and ligand-binding domain (LBD)(Shafi, Yen and Weigel, 2013).
In the cytoplasm, the receptor is bound by heat-shock proteins (specifically HSP90 chaperone complex) in the inactive state and it is activated by androgen binding, specifically dihydrotestosterone (DHT) or testosterone. The bond causes a conformational change leading to dissociation of the HSP90 complex, homo-dimerization of the receptor, translocation to the nucleus, and binding to androgen-response elements (AREs) in the promoter region of androgen-regulated genes. This interaction with the promoter region is under the control of many transcriptional coregulators (corepressors or coactivators) that act to open the chromatin structure promoting the transcription (Shafi, Yen and Weigel, 2013).

The androgenic pathway activation is mediated by adrenal dihydropiandrosterone (DHEA) or by dihydrotestosterone (DHT). DHT derives from testosterone catalysis through 5 alpha-reductases (SRD5A) produced by prostatic epithelial and stromal cells (Pham and Ziboh, 2002).

Testosterone is the primary circulating androgen, with approximately 90% produced by Leydig cells in the testes and 10% produced by the adrenal cortex. Only a small portion (3%) of circulating testosterone is unbound and functionally active, while the remainder is bound and sequestered by globulin and albumin (Manni et al., 2015).

DHT and DHEA bind AR, even if DHT affinity is stronger and so it is the predominant metabolite (Krieg, Weisser and Tunn, 1995).

Androgens production is regulated by the hypothalamic-pituitary-adrenal/gonadal axis (Lamb, Massie and Neal, 2014). In particular, hypothalamus releases the gonadotropin-releasing hormone that stimulates the luteinizing hormone (LH) secretion by the pituitary gland. In turn, secreted LH stimulates testosterone release by gonads and, in minor quantity, by adrenal glands (Taplin, 2001) (Fig. 1).

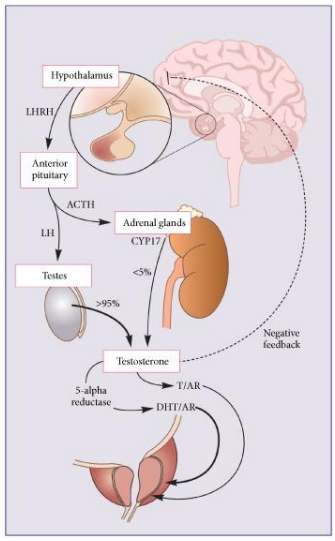

Figure 1. *Hypothalamic-pituitary-adrenal/gonadal axis*
(Lamb, Massie and Neal, 2014)

Physiologic levels of androgens are required to promote growth and prevent apoptotic death. AR influences several pathways and its major role is the production of seminal fluid proteins such as prostate specific antigen (PSA) by luminal epithelial cells.

Therefore, AR regulates multiple genes expression involved in the metabolic pathway leading to increased protein and lipid synthesis (Heinlein and Chang, 2004).

2 Prostate Cancer

Prostate cancer is the most frequently diagnosed non-skin cancer in men and the second leading cause of men death in western countries. It has estimated that 15-30% of over 50-year old men and 70% of 80-year old men present latent prostate cancers (Cauchi et al., 2011).

The increased incidence of prostate cancer in the last decades is probably due to longer life span, but also it is has been attributed to the use of serum PSA test in the clinic (Ung et al., 2002).

Despite advances in the early detection of prostate cancer and in the management of locally defined disease, a large portion of men continues to present advanced or metastatic disease (Studer et al., 2004), that represents the main cause of prostate cancer-related deaths.

Prostate cancer is a multifactorial disease and several genetic and environmental risk factors are involved in its etiology and among these, age, ethnicity, country of origin and family history (Bostwick et al., 2004).

In particular, it has known that the incidence of that disease is highest in African-American men compared to men of other origins. The higher levels of testosterone, DHT and 5-α-reductase in these men can explain the higher incidence. Moreover, black men present an alteration of androgen encoding gene length (Zeigler-Johnson et al., 2008).

A difference can also be observed in Europe, with Sweden at the top (Bray et al., 2010).

Moreover, a wide variety of nutrients are implicated in the development of prostate cancer, first and foremost polyunsaturated fats that show a strong positive correlation with prostate cancer incidence and mortality (Bostwick et al., 2004).

Aside exogenous risk factors, several endogenous risk factors are involved in prostate cancer onset. In particular, chromosomal alterations (loss of heterozygosity of

chromosome 1, 13, 16, 17), chromosome X mutations and polymorphisms (e.g. vitamin D or androgen receptor encoding genes) have been showed.

An overexpression of Ets transcriptional factor encoding genes (ERG, ETV, ETV4) was observed in bioptic tissues from metastatic prostate cancer (Furusato et al., 2010). This overexpression is associated with genic translocations: indeed, a fusion of 5' untraslated TMPRSS2 gene region and ERG or ETV1 genes was observed in prostate cancer samples (Tomlins et al., 2005).

The TMPRSS2 fusion with ETS family members is likely to be the most common rearrangement yet identified in human malignancies and the only rearrangement present in the majority of one of the most prevalent carcinomas.

Since TMPRSS2 is a prostate specific, androgen responsive, transmembrane serine protease, the fusion of these genes leads to the production of Ets transcription factors under the control of the androgen sensitive promoter elements (Tomlins et al., 2005).

Moreover, genome-wide association studies (GWAS) have successfully identified 77 common polymorphisms that are strongly associated with prostate cancer; several of these, finding in the non-coding region 8q24, affect the proto-oncogene c-MYC expression (Attard et al., 2016). In addition, rare germline BRCA2 mutations occur in men with family history of breast or ovarian cancers (Attard et al., 2016).

2.1 Diagnosis

In most cases, early prostate cancer has no clear symptoms and sometimes these are similar to those of other diseases, such as frequent micturition, haematuria (blood in urine) and dysuria (painful urination).

Elevated PSA serum levels are indicative of prostate cancer, even if this screening test has a limited diagnostic specificity and low predictive value (Djulbegovic *et al.*, 2010). After PSA positive test, digital rectal examination is performed to verify the presence of an eventual tumour and ultrasonography-guided biopsies are taken to make a diagnosis.

Tissue samples from biopsies are examined under a microscope to determine whether cancer cells are present, and to evaluate the microscopic features (or Gleason score) of any cancer found.

2.2 Classification

The most common clinical classification used is TNM (tumour, lymph nodes and metastasis) system that evaluates tumour volume, number of lymph nodes involved and the presence of distant metastasis. Regarding prostate cancer in T1 and T2 stages cancer is confined to the prostate, instead in T3 and T4 stages the tumour might have spread to other organs (Union for International Cancer Control). N and M represent respectively the involvement of lymph nodes and the presence of metastasis (Table 1) (Sobin, Gospodariwicz and Wittekind, 2009).

Table1. TNM classification of prostate cancer (UICC 2009).

Primary Tumour (T)	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
T1	Clinically unapparent tumour not palpable or visible by imaging
T1a	Tumour incidental histologic finding in ≤5% of tissue resected
T1b	Tumour incidental histologic finding in >5% of tissue resected
T1c	Tumour identified by needle biopsy (because of elevated prostate specific antigen [PSA] level)
T2	Tumour confined within prostate; tumours found in 1 or both lobes by needle biopsy but not palpable or reliably visible by imaging
T2a	Tumour involves one-half of 1 lobe or less
T2b	Tumour involves more than one-half of 1 lobe but not both lobes
T2c	Tumour involves both lobes
T3	Tumour extends through the prostatic capsule; invasion into the prostatic apex, or the prostatic capsule is classified not as T3 but as T2
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumour invading seminal vesicle(s)
T4	Tumour fixed or invades adjacent structures other than seminal vesicles (eg, bladder, levator muscles, and/or pelvic wall)
Regional lymph nodes (N)	
Nx	Regional lymph nodes were not assessed

N0	No regional lymph node metastasis
N1	Metastasis in regional lymph node(s)
Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	No regional lymph nodes(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

2.3 Histological Grade

Gleason score is recommended as international standard to classify prostate cancer histological grade. The system classifies tumours from 2 to 5 grade, where 5 is the most malignant (Humphrey, 2004).

The Gleason score is based on architectural patterns of prostate adenocarcinoma and cellular infiltrate. In particular, the classification individuates five architectural features of prostate gland to witch a score is awarded.

Following ISUP 2005 classification, these are the Gleason grades for prostate cancer (Epstein *et al*, 2015):

- Grade 1: Gleason score of 1+1=2 is extremely rare, described as single, separate, closely packed, uniform round glands arranged in a circumscribed nodule with pushing borders
- Grade 2: like grade 1, but more variability in gland shape and more stroma separating glands, less circumscribed at periphery, although no infiltration into stroma or between benign glands.
- Grade 3: Single, separate, much more variable glands, may be closely packed but usually irregularly separated, ragged, poorly defined edge, but still in circumscribed structure, looser than a nodule, slightly infiltrative, still has intervening stroma between neighbouring glands.
- Grade 4: Coalescent or fused glands, absence of intervening stroma between adjacent glands. Papillary or cribriform tumours with irregular/invasive edges.

- Grade 5: Comedocarcinoma: papillary/cribriform carcinomas with central necrosis (must have intraluminal necrotic cells or karyorrhexis) or Single-celled cancer, possibly forming cords, possibly with vacuoles (signet ring cells) but without formation of a glandular lumen.

Besides Gleason score, nuclear grading WHO can be used. Nuclear aplasia is measured as 1, 2 o 3 based respectively on lower, moderate or marked nuclear alteration (Ozdamar, Yildiz and Atilla, 1996).

The histological grade according to WHO is the following:

- **GX**: Grade cannot be assessed
- **G1**: Well differentiated (low anaplasia)
- **G2**: Moderately differentiated (moderate anaplasia)
- **G3**: Poorly differentiated (marked anaplasia)

2.4 Treatment

Men diagnosed with localized or locally advanced prostate cancer have four major treatment options: observation, active surveillance, surgery, and radiation. Other newer treatments include cryotherapy, high-intensity focused ultrasound, and primary hormone therapy (Mottet *et al*., 2017). Choosing the best treatment is generally based on the man's age, the stage and Grade of the cancer, the man's general health, and the man's evaluation of the risks and benefits of each therapy option.

Indeed, observation is chosen for low risk prostate cancer patients who have a life expectancy of <10 years and who not require treatment neither cancer monitoring.

Instead, during active surveillance, prostate cancer is monitored for signs of progression with PSA blood test and digital rectal exam (DRE) and a repeat biopsy of the prostate usually every 1-3 years. If there is evidence that the cancer is progressing, treatment might be warranted (Mottet *et al.*, 2017).

Surgery and radiation therapy remain the standard of care for localized prostate cancer, but other, less popular treatment options might be beneficial as well, even if, until now, none are chosen as standard treatments for localized prostate cancer.

The efficacy and safety of local treatments are reported in two different randomised trials that compare prostatectomy and watchful waiting (Chang *et al.*, 2014) (Kibel and Trial, 2006). The results suggest that prostatectomy improves overall survival in high-risk patients, while low-risk patients have no benefit from surgery, supporting conservative approach in this subgroup (Chang *et al.*, 2014) (Kibel and Trial, 2006).
In men with advanced disease (the state of prostate cancer that has grown beyond the prostate and is unlikely to be cured with surgery or radiation alone) who have a PSA progression after surgery or radiation, orchiectomy or hormonal therapy represent the treatment options. In particular, hormonal therapy is designed to stop testosterone secretion and the most common drugs used are LHRH agonists or LHRH analogues. Unfortunately, LHRH agonists produce what is known as a "*Flare reaction*" because of an initial transient rise in testosterone over the first three weeks after the drug administration causing a variety of symptoms, from bone pain to urinary frequency or difficulty (Thompson, 2001). To prevent this phenomenon, anti-androgens such as bicalutamide (Casodex), flutamide (Eulexin), and nilutamide (Nilandron) can help block the action of testosterone in prostate cancer cells (Thompson, 2001).
Initial responses to castration therapy are quite favourable, with a significant clinical regression and rapid biochemical responses, such as the decline in levels of PSA in 80–90% of patients with metastatic disease, but despite this good initial response, remissions with an eventual progression occur after castration (Harris WP, Mostaghel EA, Nelson PS, 2009).

3 Castration Resistant Prostate Cancer

Castration Resistant Prostate Cancer (CRPC), previously defined as hormone-refractory prostate cancer, is now understood to still be androgen dependent.

Despite other signalling pathways can contribute to CRPC onset, the majority of mechanisms identified leading to this progression are mediated by AR/ androgen axis. Indeed, in patients with CRPC there is a continuous activation of AR promoted by several events (Chandrasekar *et al.*, 2015). (Fig. 2)

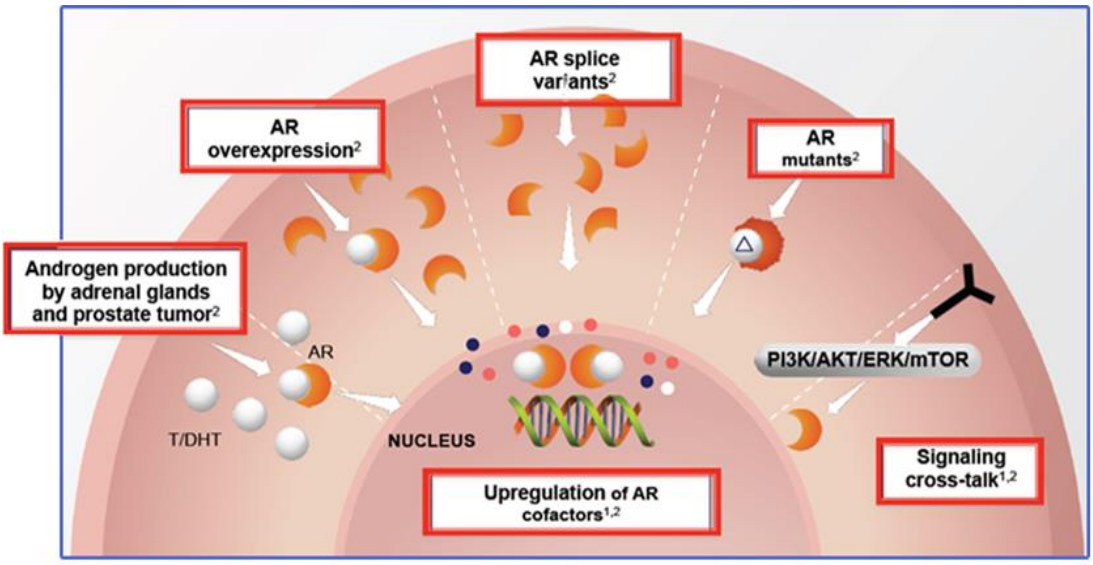

Figure 2. *Major activated mechanisms responsible of CRPC development* (Schalken and Fitzpatrick, 2016)

· AR amplification and mutations/hypersensitivity pathway/promiscuous pathway

In treated prostate cancer patients, the levels of androgens are very low and in this microenvironment, some tumour cells develop sensitivity to these levels of hormone through the amplification of AR (hypersensivity pathway) or the development of AR mutations. In CRPC, mutated AR could also be activated by molecules other than androgens (promiscuous pathway).

AR amplification, uncommon in hormone naïve prostate cancer, has been observed in CRPC and allow tumour cells to become hypersensitive to low androgens levels.

A polymorphism in the gene that encodes for 5α-reductase has been found in a subset of CRPC patients; this alteration increases the activity of the enzyme driving to the increase of DHT levels and, thus, to a higher AR activation. Moreover, several point mutations identified in the AR gene, mainly in LBD domain, lead to increased AR activity in the presence of low levels of androgens as well as other steroid hormones.

· Co-activators and co-repressors

Over 150 molecules have been identified as co-activators or co-repressors of AR, many to witch are enzymes that phosphorylate, methylate, acetylate or ubiquitinate the targets or regulate RNA splicing. One of these is FKBP51 that keeps AR in a conformation with high affinity for the ligands, while the Steroid Receptor Coactivators (SRC1, SRC2 and SRC3) enhance AR-induced transcription by promoting formation of complexes between AR-associated enhancers/ promoters and the transcription start site.

· Aberrant activation (post-translational modification)/outlaw pathway

Ligand-independent AR activation is also an important mechanism of progression to castration-resistance; indeed, several growth factors, cytokines or pathways drive to castration resistance in a ligand independent manner. One of the most important is NF-kβ, Src and PI3K pathways that are associated to CRPC development. NF-kβ, Src and PI3K signalling are activated by growth factors, cytokines, chemokines and showing a pleiotropic effect on prostate cancer cells.

Growth factor pathways, such as IGF and KGF, bind and activate AR in a castrate state. In addition, growth factor receptors, such as IGF-1R, IL-6R, and EGFR, control critical downstream growth and survival pathways of prostate cancer cells like MAPK, PI3K/AKT, and STAT signalling.

· Altered steroidogenesis

In CPRC there is an alternative androgen production: indeed, adrenal glands produce an androgen precursors, the dehydroepiandrosterone (DHEA), which can be converted in the DHT. The levels of these precursors remain in circulation also after androgen

deprivation therapies (ADT). DHEA is been converted in androstenedione and then in DHT typically through testosterone as intermediary, but in presence of ADT, the conversion occurs bypassing testosterone completely. In addition, increased expression of steroidogenic enzymes HSD3B1, HSD3B2, HSD17B3, AKR1C3, and SRD5A1 that contribute to a de novo androgen synthesis, has been observed in CRPC.

· AR variants

Recently, has been demonstrated that CRPC development is caused by AR splice variants presence. In particular, the majority of these spliced forms are characterized by the loss of the C-terminal domain LBD, which represents the target site of many ADT. One of the most well-known splice variants is AR-v7, that is located in the nucleus and thanks to the absence of LBD is constitutively active. Intriguingly, it regulates both AR-regulated genes and a unique set of AR-independent genes, suggesting an overlapping but also distinct role in prostate cancer cells compared to full-length AR.

3.1 Traditional Therapies

The first treatments approved by the US Food and Drug Administration for CRPC management, focused on the palliative benefits of pain control, were mitoxantrone, strontium and samarium. Nevertheless, these drugs did not affected overall survival (OS) of treated patients that did not exceed 12 months (Tannock *et al.*,1996).

In 2004, a phase III trial demonstrated that the chemotherapeutic docetaxel improved the overall survival compared with mitoxantrone (Tannock *et al.*, 2004) . Since then, docetaxel plus prednisone, at 3 weeks intervals, represents the standard of care for men with CRPC.

Therapeutic scenario changed again in 2010 with the publication of TROPIC trial results: this study demonstrated that a new taxan, cabazitaxel, in association with prednisone, increased significantly the survival of metastatic CRPC (mCRPC) patients

previously treated with docetaxel, adding a new second-line therapeutic agent (de Bono *et al.*, 2010).

3.2 New target therapies

Although chemiotherapy has been shown to improve overall and progression free survival, it is not well tolerated by all patients because the majority are elderly men with limited bone marrow and other comorbidities. For this reason, in the last years, several drugs have been introduced in mCRPC clinical management. These include AR inhibitors, new generation hormonal therapies, immunotherapies and drugs targeting key molecular pathways involved in tumour progression and bone metastases onset (Pantano *et al.*, 2016).

· *Anti-androgen therapies*

Abiraterone acetate is an orally administered selective androgen biosynthesis inhibitor that potently and irreversibly blocks steroidogenesis. It inhibits both the hydroxylase and lyase activities of CYP17A resulting in virtually undetectable serum and intratumoural androgen production in the adrenals, testes and prostate cancer cells (O'Donnell *et al.*, 2004). Because adrenal inhibition of CYP17A results in blockade of glucocorticoid as well as adrenal androgen synthesis, abiraterone is co-administered with prednisone to ameliorate the secondary rise in adrenocorticotropic hormone that can lead to excess mineralocorticoid synthesis (Attard *et al.*, 2015). Two randomized phase III trials showed that abiraterone improves overall survival in metastatic prostate cancer patients (Fizazi *et al.*, 2012) (Ryan *et al.*, 2013).

Enzalutamide is another promising oral AR inhibitor that targets multiple steps in the AR signalling pathway. Two large phase III trials have demonstrated the efficacy of enzalutamide in the treatment of patients with mCRPC (Beer *et al.*, 2014) (Scher *et al.*, 2012).

In the randomized Phase III AFFIRM study (Scher *et al.*, 2012), significant improvements in survival versus placebo were observed when enzalutamide was used

as a treatment for patients with mCRPC following prior treatment with docetaxel. Additional benefits included significant delay in time to first SREs and improvement in several measures of pain and health-related quality of life.
In the Phase III PREVAIL study, enzalutamide prolonged overall survival in patients with mCRPC (32.4 months in treated arm vs 30.2 in the placebo arm) (Beer *et al*, 2014),

· *Immunotherapies*

Prostate cancer is an attractive target for immunotherapies because it expresses several tumour-associated antigens (TAAs) and shows slow growth (Pardoll, 2012), which could allow the immune system to have sufficient time to induce an effective antitumour immune response. In this regard, the agent Sipuleucel-T has recently been approved in mCRPC (Higano *et al.*, 2009).
Another promising field of investigation is the possibility of interfering with the phenomenon of tumour immune escape at the cellular level. The most successful approach appears to be the inhibition of immunosuppressive cells by blocking their co-inhibitory molecules, a growing family of proteins that contribute to the regulation of T-cell mediated adaptive response towards a wide spectrum of antigens, including TAAs (Tollefson *et al.*, 2010). Among these molecules, cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) is the most studied, and represent a promising target in metastatic prostate cancer. In this regard, Ipilimumab a human monoclonal antibody against CTLA-4 receptors showed encouraging results in treated patients with advanced prostate cancer (Kwon *et al.*, 2015).

· *Sipuleucel-T* is an immune-modulating agent best described as an autologous cellular immunotherapy generated after apheresis of the patient's own immune cells. The patient's peripheral blood mononuclear cells are treated with a prostatic acid phosphatase–granulocyte macrophage colony-stimulating factor (PAP-GM-CSF) fusion protein in addition to various other cytokines to generate the final product. This treatment was FDA-approved in 2010 as treatment for patients with mCRPC, based on the results of the pivotal IMPACT study. This trial enrolled 512 patients with asymptomatic or minimally symptomatic chemotherapy-naive mCRPC randomized to

receive sipuleucel-T versus placebo in a 2:1 ratio. Although sipuleucel- T provided OS benefit compared with placebo (23.2 vs 18.9 months), no significant effect on PSA response rate, radiologic responses or time to progression were observed (Higano *et al.*, 2009).

· *Ipilimumab* is a human monoclonal antibody that enhances and prolongs T-cell activation by blocking immune checkpoint CTLA-4 receptors found on the surface of T cells (Pardoll, 2012). In a randomized Phase II trial, 108 patients with advanced prostate cancer treated with ipilimumab plus androgen-deprivation therapy showed undetectable PSA levels by 3 months compared with patients treated with endocrine therapy alone (55 vs 38%) (Tollefson *et al.*, 2010).
Recently, the results from a randomized, double-blind Phase III study (CA-184-043) comparing ipilimumab with placebo following bone-directed radiation therapy in CRPC patients previously treated with docetaxel demonstrated no improvement in OS (Kwon *et al.*, 2015). Nevertheless, a subgroup analysis suggests that ipilimumab may be most active in patients with favorable laboratory prognostic factors (e.g., decreased alkaline phosphatase or elevated hemoglobin level) or in patients without visceral disease (Higano *et al.*, 2009).

4 Bone Metastases

Prostate cancer, like breast cancer, is an osteotropic tumour, so skeleton represents its favourite metastatic site (Coleman, 2001). Indeed, 90% of patients with metastatic prostate cancer develop bone metastases. For these patients the prognosis dramatically changes, the morbidity increases and overall survival decreases. Moreover, bone metastases led to so-called *skeletal-related events* (SREs), which include pathological fractures, spinal cord compression and severe bone pain that require palliative radiotherapy and/or orthopaedic surgery impairing health-related quality of life and survival (Coleman, 2006).

Bone metastases differ depending on their tumour origin. In particular, metastases from breast and lung cancer are typically osteolytic, with a strong enhancement of bone resorption by osteoclasts that drives to bone degradation (Mundy, 2002)(Vessella and Corey, 2006). Instead, bone metastases from prostate cancer are predominantly characterized by a strong osteoblastic component that increases bone mass (Roodman, 2004).

3.3 Bone Physiology

Bone is a dynamic tissue that is undergone continuous vital process of remodelling made by bone cells: osteoblasts (OBs), osteoclasts (OCs) and osteocytes (Raisz, 1999).

Osteoblasts derived from mesenchymal stem cells (MSCs), the precursors of many cell types, like chondrocytes, fibroblasts, myoblasts, adipocytes and neuronal cells (Bianco, 2015). MSCs differentiate first in pre-OBs producing alkaline phosphatase (ALP) and then in mature OBs producing osteocalcin (OCN) and bone matrix (Lian and Stein, 2003). Osteoblastogenesis is strictly regulated by several genes and proteins; in particular, Runt-related transcription factor 2 (Runx2) controls the expression of different osteoblastic proteins like Osterix (a transcriptional factor required for osteoblast maturation), Osteopontin, Bone Sialoprotein, Type I Collagen,

OCN and the receptor activator of nuclear factor κβ ligand (RANKL) (Lian and Stein, 2003). The fundamental role of Runx2 in osteoblastogenesis is supported by *in vivo* experiments where mice without RUNX2 showed an almost complete absence of mineralized tissues (Takarada *et al.*, 2013).

The bone morphogenetic proteins BMPs, Hedgehog and Wnt are the major pathways known to regulate the commitment of MSCs to the osteoblast lineage. These pathways are activated by several factors such as parathyroid hormone (PTH), parathyroid hormone-related protein (PTHrP), fibroblast growth factors (FGF2 e 18), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), sex steroids, and other hormones (Buijs, J T, 2009).

Osteoclasts, derived from Human Stem Cells (HSC) monocyte/macrophage lineage, are specialized multinucleated cells with bone resorptive activity. Several factors are involved in osteoclastogenesis, some of them are produced by osteoblasts, like Macrophage colony stimulating factor (M-CSF), tumour necrosis factor alpha (TNFα) and RANKL (Boyle, Simonet and Lacey, 2003). In particular, RANKL binds its receptor on pre-OCs surface activating the differentiation. Osteoprotegerin (OPG) is another important factor responsible of osteoclastogenesis modulation: it is a decoy antibody against RANKL and works inhibiting OCs (Boyle, Simonet and Lacey, 2003).

Bone resorption occurs in so-called *Howship lacunae* where osteoclasts are anchored to bone matrix through integrin αvβ3. The side of the cell closest to the bone contains many small projections (microvilli) that form a brush, the cell's active region, where is an ATP-dependent pump. Osteoclasts produce a number of enzymes, active in presence of acid pH, chief among them acid phosphatase, that dissolve both the organic collagen and the inorganic calcium and phosphorus of the bone. Mineralized bone is first broken into fragments; the osteoclast then engulfs the fragments and digests them within cytoplasmic vacuoles. Calcium and phosphorus liberated by the breakdown of the mineralized bone are released into the bloodstream (Teitelbaum, 2000).

Osteocytes represent 95% of all bone cells in mature bone tissue and contribute to control the mineralization (Dallas, Prideaux and Bonewald, 2013) (Fig.3); in particular, osteocytes are becoming critical players in the regulation of osteoblasts and osteoclasts functions. In addition osteocytes act like mechanoreceptors integrating mechanical loading and hormonal signals to regulate bone mass in the skeleton (Bonewald and Johnson, 2008).

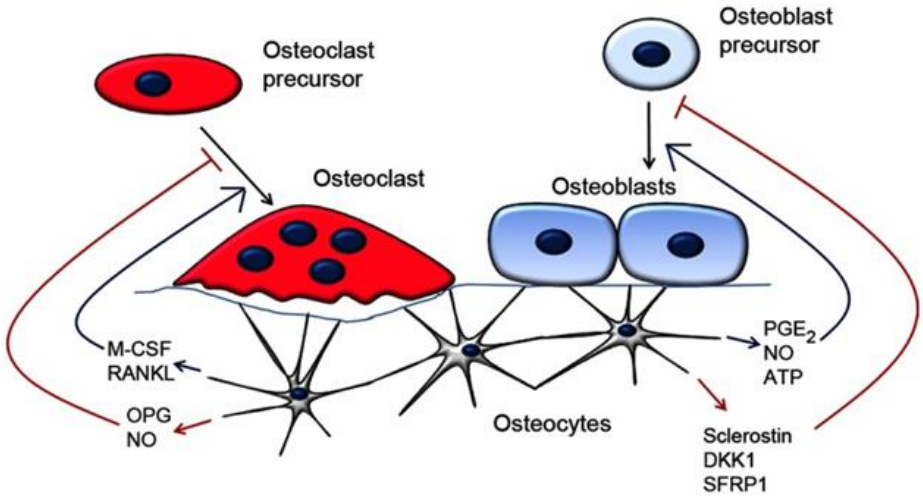

Figure 3. *Osteocyte funcions in bone turnover*
(Dallas, Prideaux and Bonewald, 2013)

Osteocytes express several modulators of Wnt signalling, as the sclerostin, leading to osteoblast activity stimulation. In addition to sclerostin, osteocytes express the LRP5/6 inhibitor DKK1, which inhibits osteoblast differentiation and bone formation. Moreover, they produce also secreted frizzled-related protein 1 (SFRP1), which is a competitive antagonist of Wnt ligand binding. Loss of SFPR1 expression *in vivo* results in increased bone mass and mineral density. SFRP1 overexpression is observed in the immature osteocyte and decreased in the mature cell, indicating a role for SFRP1 in the negative regulation of cell survival during matrix embedding (Dallas, Prideaux and Bonewald, 2013).

Intriguingly, osteocytes showed also a regulatory effect on osteoclast bone resorption through the modulation of RANKL/ OPG balance. In addition, osteocytes are also known to express another factor required for osteoclast activation, the macrophage-

colony stimulating factor (MCSF). Moreover, several signalling molecules such as nitric oxide (NO) and prostaglandin E2 (PGE2) are known to be rapidly released by osteocytes in response to external stimuli such as mechanical strain and both of them promote osteoblast activity and differentiation (Dallas, Prideaux and Bonewald, 2013). Despite the recent elucidation of osteocytes functions, their role in prostate cancer remains to be investigated.

In physiological condition, the number and activity of osteoblasts and osteoclasts are balanced resulting in an equal bone resorption and formation. The remodelling is realised in *basic multicellular units* (BMU) in which both osteoclasts and osteoblasts cooperate in the remodelling cycle (Katsimbri, 2017) (Fig.4).
In particular, the process requires the recruitment of monocytes (osteoclast precursors) to the bone surface. OBs secrete RANKL that binds its receptor RANK in monocyte membrane forming pre-OC that with macrophage colony stimulating factor 1 (CSF-1) and RANKL differentiates in multinuclear mature OC. In turn, mature OCs reabsorb mineralized matrix through a process of acidification and proteolytic digestion. Subsequently, pre-OBs migrate to the resorption site where they mature and produce *osteoid*, which is mineralized. Thus, a coordinating network of cells (OBs, OCs and osteocytes) regulates the mineralization process responding to different stimuli such as mechanical load, cytokines and hormonal signals. In this controlled system OPG/RANKL/RANK pathway is fundamental and acquires a central role in bone metastasis development (Mundy, 2002) (Dougall and Chaisson, 2006). Indeed, breast and lung tumours exert their osteolytic actions through the up- regulation of RANKL expression, whereas prostate cancer is characterized by an increase of OPG, thus promoting osteoblastic activity (Mountzios *et al.*, 2007) (Goldring and Goldring, 2007).

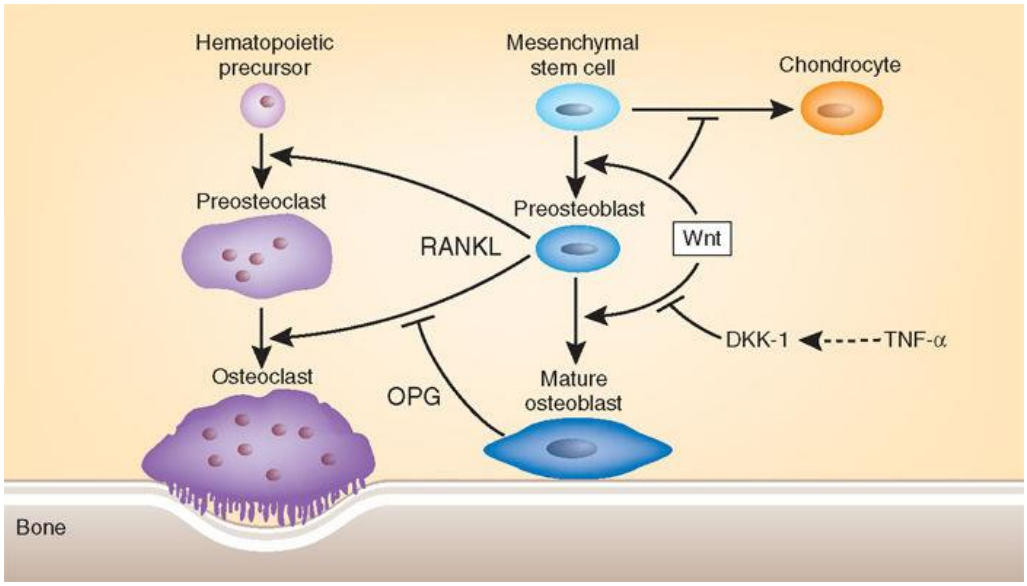

Figure 4. *Osteoblasts/ Osteoclasts interplay in bone turnover* (Goldring and Goldring, 2007)

3.4 Bone Metastasis Physiopathology

Bone metastasis development is determined by the crosstalk between tumour cells and bone microenvironment that interferes with physiological bone turnover, leading to abnormal bone formation.

3.4.1 The vicious cycle

Tumour cells and bone microenvironment interplay is the basis of so-called *vicious cycle* of cancer, described for the first time in 1997 by Mundy (Mundy, 1997) (Fig. 5). Cancer cells, migrated to the bone, release several factors known as OAF (Osteoclast Activating Factors) such as parathyroid hormone-related protein (PTHrP) and interleukine-6 (IL-6) that determine a switch in receptor activator of nuclear factor kappaB ligand/osteoprotegerin (RANKL/OPG) balance in favor of RANKL (Roodman *et al.*, 2004). RANKL overexpression stimulates osteoclastic bone resorption and then, the release of growth factors including bone morphogenetic proteins (BMPs), fibroblast growth factor (FGF), platelet derived growth factor

(PDGF), tumor necrosis factor β (TGF-β) that, in turn, promote cancer cell survival and proliferation. Recent evidences have shown that tumor cells release other factors like endothelin-1 (ET-1) and activate Wnt pathway, resulting in OPG secretion (Nelson , Bagnato and Battistini, 2003) (Takahashi *et al.*, 2011). OPG stimulates osteoblast differentiation and activity promoting the formation of new, but unstructured bone, prone to fracture (Dougall and Holen, 2014). RANKL production by activated osteoblast, promotes osteoclastic activity and thus the release of bone matrix-derived factors that, in turn, stimulate cancer cells closing the cycle (Roodman, 2004).

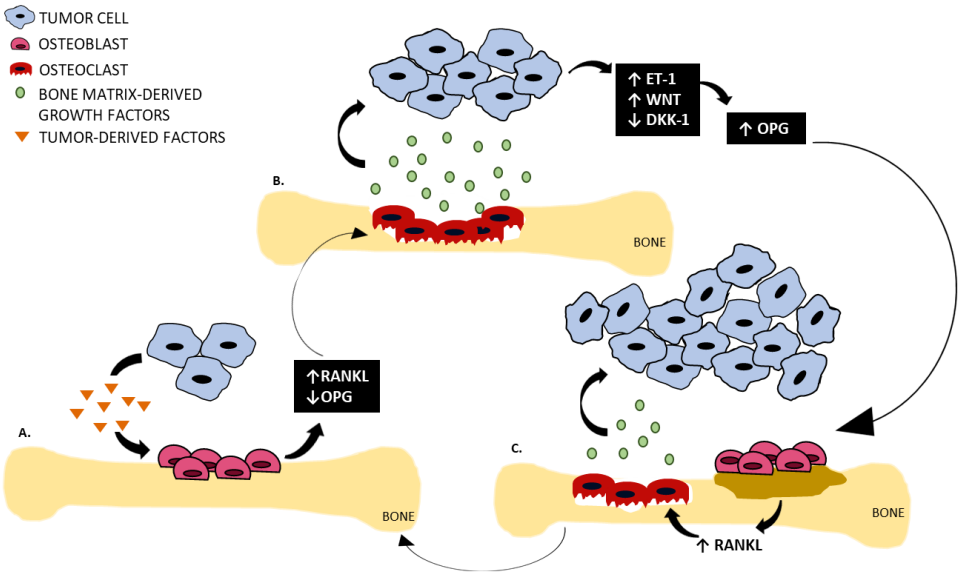

Figure 5. *The "Cancer Vicious Cycle"*

The mechanisms through which prostate cancer cells promote osteoblastic growth and bone mineralization remain poorly understood. However, a variety of bone-stimulating factors produced by CRPC cells, including PSA, endothelin-1 (ET-1), BMPs, IGF-1, and OPG have direct and indirect effects on bone stimulating abnormal mineralization (Autzen *et al.*, 1998)(Lee *et al.*, 2003)(Nelson *et al.*, 1995)(Guise, 2000)(Prisca *et al.*, 2000).

3.4.2 Bone metastasis regulator pathways

3.4.2.1 RANK/RANKL/OPG axis

RANK/RANKL/OPG axis play a crucial role in bone metastasis development. RANK expression has been founded in several tumor cell lines, including osteosarcomas, breast and prostate cancers (Danovi, 2010) (Van Poznak *et al.*, 2006). Moreover, RANK/RANKL expression has been reported in human tumor biopsies as well as breast, prostate cancers and hepatocellular carcinoma. Preclinical studies suggest that RANK expression in tumor cells facilities their migration to the bone, where RANKL is abundantly expressed. In particular, murine *in vivo* models showed RANKL as a potent chemoattractant in tumors and supported the pro-migratory activity of RANK-expressing breast and prostate cancer cell lines; moreover, in an *in vivo* melanoma model of bone metastases the inhibition of RANKL resulted in a reduction of bone lesions and tumor burden (Gonzalez-Suarez *et al.*, 2010). Finally, it has been demonstrated that RANK expression level in the primary tumor correlated with the occurrence of bone metastases, and RANK-expressing cancer could be found in up to 80% of bone metastases originated from solid tumor (Santini *et al.*, 2011). Recently, evidences suggest an important role for RANKL/ RANK in the immune system including in lymph node development, lymphocyte differentiation, dendritic cell survival, T-cell activation and tolerance induction. Detailed studies in mouse models have clearly demonstrated the involvement of RANKL signaling in the functions of immune regulatory cells, such as dendritic cells, M cells (specialized epithelial cells in mucosal tissues) and mTECs (epithelial cells localized in the thymic medulla) (Loser *et al.*, 2006). Notably, the functions of dendritic cells and the maintenance of M cell numbers were impaired by the inhibition of RANKL signaling in adult mice leading to T-regs lymphocytes expansion and subsequent local and systemic immunosuppression (Tan *et al.*, 2011). The result of these alterations was an increase in bone resorption, tumor invasiveness and cancer cells immune system evasion. The development and approval of denosumab, a fully monoclonal antibody against RANKL, has heralded a new era in the treatment of bone diseases by providing a potent, targeted and reversible inhibitor of bone resorption (Lacey *et al.*, 2012).

3.4.2.2 Endothelin-1 (ET-1)

Endothelin-1 (ET-1) is an important factor released by tumor cells with physiological and pathological functions that promotes bone metastasis development. ET-1 is responsible to induce the release of several pro-inflammatory molecules, such as IL-6, chemokine (C-C motif) ligand 2 (CCL2), monocyte chemoattractant protein 1 (MCP-1), cyclooxygenase (COX2) and MMPs that mediated tumor invasiveness and metastasis (Kandalaft *et al.*, 2009). It is the most common circulating ET produced in the endothelium and has a key role in vascular homeostasis binding the receptors ETA and ETB.

ET-1 promotes osteoblast proliferation and decreases osteoclast activity, leading to the formation of typical sclerotic lesions of metastatic prostate cancer (Nelson *et al.*, 2003). Indeed, elevated ET-1 plasma concentrations were observed in hormone refractory prostate cancer patients compared to healthy control. Moreover, immunohistochemistry of prostate cancer biopsies showed that ETA is present in prostatic gland and the expression is correlated with tumour grade (Nelson *et al.*, 1995). Activating this receptor, ET-1 promotes prostate cancer progression, enhancing proliferation, invasion and vascularization and inhibiting apoptosis (Zonnenberg and Voest, 2003). Altered mechanisms of ET-1 clearence are been observed in prostate cancer. In particular, endopeptidase-mediated ET-1 degradation is reduced and the bond with ETB is inhibited or abolished as a result of decreased expression of the receptor or/and alteration in ET-1 binding domain (Zonnenberg and Voest, 2003). ET-1expression could explain osteoblastic feature of prostate cancer bone metastases, indeed it has hypothesized that tumour released ET-1 promotes the release of interleuchin-1 (IL-1), Tumour Necrosis Factor α (TNF- α) and tumour growth factor β (TGFβ) that in turn stimulate osteoblasts decreasing bone resorption (Nelson *et al.*, 1999). Several preclinical data showed that there is an upregulation of ET-1 mRNA after tumour cells and bone fragments co-cultures, that osteoclastic inhibition is restored adding an ET-1 antibody and that tumour secreted ET-1 stimulates *in vitro* bone formation and *in vivo* bone metastasis development (Chiao *et al.*, 2000)(Yin *et al*, 2003).

Atrasentan is an inhibitor of the ETA receptor that has been showed to block formation of osteoblastic metastases in mice. Nevertheless, in a placebo-controlled phase III trial in men with metastatic prostate cancer, atrasentan failed to demonstrate a reduction of overall survival, risk of disease progression and cancer-induced bone pain *(Nelson et al.,* 2008).

Zibotentan (ZD4054) is an oral, specific ETA receptor antagonist extensively investigated in the ENTHUSE clinical development program. ENTHUSE M1 trial showed no significant improvement in OS with zibotentan monotherapy versus placebo in men with mildly symptomatic CRPC (24.5 versus 22.5 months respectively) (Nelson *et al.,* 2012). Moreover, the ENTHUSE M0 trial of zibotentan monotherapy in patients with non-metastatic CRPC has not demonstrated survival benefits (Miller *et al.,* 2013). Finally, in the ENTHUSE M1C randomized phase III trial zibotentan in combination with docetaxel has not showed improvement in OS compared to docetaxel alone in mCRPC patients (Fizazi *et al.,* 2013).

3.4.2.3 Integrin and Cadherin

Tumor metastases require the activity of several adhesion molecules including the superfamily of integrins and cadherins.

The heterodimeric (α and β monomers) transmembrane glycoproteins *integrins* have a cell type specificity and anchor cells to the extracellular matrix (ECM) binding their ligands. Stable adhesion to the ECM, is fundamental to cell survival, indeed detached cells undergo an apoptotic process, known as anoikis (Frisch, 2001). Metastatic cells elude this mechanism expressing aberrant integrins, activating different pathways like focal adhesion kinase (FAK) (Frisch *et al.,* 1996), epidermal growth factor receptor (EGFR) (Demers et *al.,* 2009) and Src (Windham *et al.,* 2002) and inhibiting apoptosis (Simpson *et al.,* 2008).

Several studies correlated integrin expression (αvβ3, αvβ5, α5β1, α6β4, α4β1, and αvβ6) with the progression of breast carcinoma, prostate, pancreatic and lung cancers and melanoma (Desgrosellier *et al.,* 2010). In addition, a correlation between integrins α2 e α6 - and also c-MET- expression and bone metastases development was found (Ricci *et al.,* 2013). Integrin β1 is another fundamental integrin in prostate cancer

progression that promotes bone and node metastasis formation through the activation of Akt pathway (Lee *et al.*, 2013).

Cadherins, calcium-dependent transmembrane proteins, regulate the formation of adherence junctions to bind cells with each other. Loss of function of cadherins has linked to bone metastasis development (Beavon, 2000). Depending on their form (E-cadherin or N-cadherin), these proteins can act as a suppressor or promotor of cancer invasion and metastases. Indeed, the switch from E-cadherin to N-cadherin is critical for epithelial to mesenchymal transition (EMT) and thus, for metastases onset. In particular, Gravdal and collegues demonstrated that this switch is associated with decreased OS and higher skeletal recurrence in patients with prostate cancer undergone radical prostatectomy (Gravdal *et al.*, 2007). Another group showed that in human samples E-cadherin is higher express in bone metastasis compared to primary tumor (Saha *et al.*, 2008). The overexpression of N-cadherin in prostate cancer cells (Gravdal et al., 2007) probably is due to a higher aggressiveness of the tumor and not by a bone tropic behavior of the cells, but nonetheless N-cadherin expression is a good marker of further skeletal recurrence.

Among all, cadherin 11 has demonstrated to promote bone metastases. In particular, it has observed that marrow stromal cells express cadherin-11 (OB-cadherin), that facilities the homing of breast cancer cells to the bone as well as stimulates osteoclastogenesis (Tamura *et al.*, 2008). Similarly, in preclinical models of prostate cancer, cadherin-11 enhances migration and invasiveness of tumor regulating also the expression of pro-invasive genes (Huang *et al.*, 2010).

3.4.2.4 WNT/ DKK-1 axis

Besides these pathways, Wnt/ β catenin system promotes bone neoformation as well. Wnt proteins represent a secreted group of glicoproteins that bind the 7-transmembrane domain receptors regulating several cellular functions (growth, differentiation and death). After the secretion, Wnt proteins undergo a post-translational modification, the palmitoylation necessary for their molecular proper functioning (Abrami *et al.*, 2008)

Wnt family include 19 glicoproteins (Wnt1, 2, 3a, 4 etc..) encoded by different genes, that activate different pathways. Among them, the most well known bone formation-stimulating pathway is Wnt/β catenin also called canonical pathway. This system promotes bone formation (Wang *et al.*, 2014):

- Stimulating osteoblast differentiation and activity and blocking mesenchymal differentiation into chondrocytes or adipocytes.
- Increasing osteoblast proliferation and inhibiting the apoptosis
- Inhibiting osteoclastogenesis

In the absence of Wnt or in case of missed bond with its receptor, β-catenin is destroy thanks to a destruction complex that include: Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GSK3) and casein kinase 1α (CK1α). This system targets β-catenin for ubiquitination that is recognised and digested by the proteasome. Instead, in presence of Wnt, the receptor binds the co-receptors Lrp5 and Lrp6 and inhibits GSK-3 activity, thanks to the formation of another complex made of Disheveled proteins (Dsh), Axin and Frat-1. This inhibition drives to β-catenin phosphorylation inhibition, stabilizing the protein that can translocate to the nucleus, where regulates gene transcription (Baron and Rawadi, 2007).

Several antagonists regulate the canonical pathway; for example, secreted frizzled-related protein) sFRP and Wnt inhibitory factor-1 (WIF-1) inhibit the bond between Wnt and its receptor. Sclerostin (SCL) and Dickkop-1 (DKK-1) inhibit Lrp5/6 bond with the receptor. In particular, Dkk-1 inhibits Wnt pathway through Kremen 1 and 2 (KRM1 and 2) receptors that bind and inactivate Lrp5/6 (Wang *et al.*, 2014).

The role of canonical Wnt signaling has been widely demonstrated in several tumor types (Duchartre, Kim and Kahn, 2016). The balance between Wnt and Dkk-1 activity determines the nature of bone metastasis in prostate cancer: several studies have showed in preclinical settings that Wnt activation or inhibition are, respectively, linked to sclerotic and lytic bone lesions (Hall *et al.*, 2005)(Thudi *et al.*, 2011). Indeed, prostate cancers usually express lower levels of Dkk-1 compared to normal prostate tissues, presenting mostly sclerotic metastases (Hall *et al.*, 2005).

Higher Dkk-1 serum levels are associated with poorer OS, as demonstrated by Rachner and colleagues (Rachner *et al.*, 2014). Prognostic value of Wnt/DKK1 axis was further investigated by Chen et al. who showed that high expression of miR34a in primary tumor, a negative regulator of the Wnt downstream effector TCF7, was found to be correlated to an improved OS in a retrospective analysis of 24 patients with metastatic prostate cancer (Chen *et al.*, 2015).

BHQ88O, a fully human anti-DKK1 neutralizing immunoglobulin G1 (IgG1) with high affinity for his target. The phase Ib trial showed that BHQ880 in combination with zoledronic acid and anti-myeloma therapy was well tolerated and demonstrated potential clinical activity in patients with relapsed/refractory multiple myeloma (Iyer *et al.*, 2014)

3.4.2.5 CXCR4/CXCL12

The chemokine CXCL12, called also SDF-1, is a chemoattracted cytokines that, binding its receptors, (CXCR4 and CXCR7) regulates cellular migration. Several studies have demonstrated the involvement of CXCL12/CXCR4/CXCR7 axis in the establishment of metastases from different tumors (Zlotnik, Burkhardt and Homey, 2011). Indeed, in prostate cancer high levels of CXCL12 regulates the metastatic spread in the bone marrow and the binding with its receptors, activates divergent cellular responses such as cell survival, proliferation and angiogenesis. Moreover, high levels of CXCR7 protein are associated to most aggressive tumors and promotes the release of proangiopoietic factors such as IL-8 and VEGF (Wang *et al.*, 2008).

3.4.2.6 TGF-β

TGF-β belongs to the TGF superfamily and has a central role in regulating cellular homeostasis. Indeed, TGF-β blocks cell cycle inducing differentiation and apoptosis preventing aberrant cellular proliferation (Derynck and Akhurst, 2007). Unfortunately, several tumors develop the resistance against this growth inhibition because of genetic loss of TGF-β signaling elements or downstream signaling perturbation. Moreover, TGF-β pathway is linked to bone metastasis onset in several tumor types. In particular, it has demonstrated that two TGF-β secreted proteins, bone

sialoprotein and osteopontin are highly expressed in prostate and breast cancer tissues, are associated with tumor grade and represent prognostic indicators for bone lesions. (Waltregny *et al.,* 2000)(Carlinfante *et al.,* 2003).

TGF-β exerts its pro-tumor action affecting directly bone microenvironment. Indeed, TGF-β secreted and activated from osteoclast bone resorption, promotes the release of PTHrP from tumor cells. PTHrP promotes osteoclastogenesis inhibiting at the same times osteoblastogenesis modulating RANKL OPG ratio (Yin *et al.*, 1999).

3.4.3 Osteotropism

The tendency of prostate cancer cells to metastasize to bone could be explained by Paget's "seed and soil" theory (Paget, 1989) according to which metastasis does not occur by chance, but depends on cross-talk between selected cancer cells (the 'seeds') and specific organ microenvironments (the 'soil'). In other words, tumour cells selectively colonize to distant organs because of the presence of a favourable microenvironment for their localization and growth.

This theory remains a basic principle in the field of cancer metastasis (Liotta and Kohn, 2001)(Langley and Fidler, 2007) and is particularly relevant to bone metastasis (Buijs, J T, 2009). Recent studies supported this theory showing that the acquisition of specific gene expression profiles (Kang *et al.*, 2003) or the activation of specific signalling pathways (Zhang *et al.*, 2009)(Zhang *et al.*, 2013) dictate the specificity of cancer cells growing in bone.

Bone represents an attractive "soil" for tumour cells thanks to the continuous and dynamic turnover of the bone matrix and bone marrow. Moreover, the osteoblasts, as well as the marrow cells, provide an environment rich in growth factors, cytokines and chemotactic factors that are crucial for metastatic cancer cell colonization and growth. Anyway, not all bone regions are same colonized; indeed there are some of them, like the metaphysis at the ends of the long bones, that are well vascularized and allow various cells to easily enter and exit the bone.

Analysis of bone extracts has revealed that a key factor that mediates prostate cancer cell invasion is the protein osteonectin or SPARC (Jacob *et al.*, 1999). SPARC

(secreted protein, acidic and rich in cysteine) is a bone matrix component that modulates cellular interactions with the extracellular matrix, exerting an anti-adhesive, promigratory effect on cells . SPARC is expressed by certain metastatic tumours, and its expression correlates with invasive activity. Despite the great importance of this bone matrix protein in the tumour development and metastasis, the mechanisms of its role in prostate cancer remain poorly understood.

3.4.4 EMT e MET

The metastatic process requires several long and coordinated steps including the degradation of the extracellular matrix, the detachment of tumour cells from the matrix and each other, migration through blood and lymphatic system to other organs (Hanahan and Weinberg, 2011)(Lamouille, Xu and Derynck, 2014). Each of these steps can failed stopping the entire process (Nieto, 2017).

In order to acquire a mesenchymal migratory phenotype, tumour cells must undergo the so-called *epithelial mesenchymal transition* (EMT) through which they lose their epithelial characteristics, detach from the epithelial layer and make a drastic alteration (Kalluri and Weinberg, 2009) (Thiery, 2002)(Thiery *et al.*, 2009).

The loss of cell-cell adhesion and apical-basal polarity and the cytoskeleton reorganization is induced by tumour infiltrating immune and stromal cells (Smith and Kang, 2013).

Following EMT process, tumour cells acquire motility and invasiveness capacity, changing the cytoskeletal proteins (expression of vimentin and α-SMA), adhesion receptor expression (switching from E-cadherin to N-cadherin (CDH2) or Cadherin-11 (Osteoblast-cadherin)) and secreting proteinase (especially metalloproteinases (MMPs) (Kalluri and Weinberg, 2009)(Thiery *et al.*, 2009)(Polyak, 2009). In particular, cadherin switch is typical of the more aggressive/castration resistant cell lines (Bussemakers *et al.*, 2000)(Jennbacken *et al.*, 2010) (Tran *et al.*, 1999).

The degradation of the basal membrane and the remodelling of extracellular matrix is mediated by protease, like metalloproteinase and cathepsins.

After EMT, migrating cancer cells have to undergone the *Mesenchymal to Epithelial Transision* (MET), the reverse process of EMT, that allows tumour cells to reacquire mesenchymal features, adhere to resident cells in bone, and survive and proliferate in the bone marrow. The key regulators of MET are cadherins, transcription factors (Pax, paraxis, Fox) and growth factors (Wnts, FGFs, Ephrins) (Sikes *et al.*, 2004).
Currently, the role of EMT and MET in bone metastasis is not fully understood, but it is known that these transitional stages are strongly affected by the bone microenvironment (Brabletz *et al.*, 2005).

3.4.5 Osteomimicry

It has known that prostate cancer cells migrated to the bone acquire bone-cell like properties. The ability of cancer cells to undergo phenotypic changes that allow them to form bone is termed "*osteomimicry*" and permits cancer cells to home, adhere, and migrate to bone, to invade bone space. Moreover, tumour cells utilize the rich bone microenvironment to proliferate and survive because of the abundance of growth factors and extracellular matrix molecules found in the bone niche (Bronner and Farach-Carson, 2009).
Prostate cancer cells, prostate stroma, endothelial, and inflammatory cells secrete a soluble factor, β2-microglobulin (β2-M) the major growth factor and signalling molecule that promotes cancer growth and progression in the bone environment (Huang *et al.*, 2006). In particular, β2-M regulates the signalling pathway that confers the expression of bone matrix proteins OCN and bone sialoproteins and induces growth of human prostate cancer cells through the activation of the angiogenesis factor and cell cycle regulatory cyclins. The blocking of β2-M downstream signalling is associated with an increase of caspase-cleaved cytokeratin 18 fragment (M30), activated caspases and PARP, markers known to be associated with programmed cell death (Huang *et al.*, 2006).

3.4.6 Bone metastatic niche

Primary tumour itself can influence and alter the environment of secondary organs by promoting the formation of supportive metastatic microenvironment, known as *premetastatic niche*, prior to tumour cell dissemination (Weilbaecher, Guise and McCauley 2011). Tumour-derived secreted factors and bone marrow derived cells are the principal actors in pre-metastatic niche formation. Indeed, tumour cells produce several factors that promote the mobilization and recruitment of bone marrow derived cells. These cells interact with the local stroma and extracellular matrix at secondary organs, to help create microenvironments suitable for colonization by metastasizing tumour cells.

Bone metastatic niche represents the ideal site for dormant tumour cells (DTCs) stabilization, where they can survive in a dormant state stopping to proliferate or proliferating at a reduced rate. DTCs are resistant to cancer therapies and can remain in quiescence for long time, even beyond 10 years (Mohme, Riethdorf and Pantel 2017) and then spread and colonize other organs.

The switch from dormant state to proliferative one is regulated by bone metastatic niche (Croucher, Mcdonald and Martin, 2016) (Fig. 6); in particular, it has known that factors including VEGF, fibronectin, and MMPs secreted from myeloid cells in the niche promote the angiogenic switch necessary to allow tumour cell escape from dormancy (Sceneay, Smyth and Möller, 2013). It has estimated that 72% of prostate cancer patients who have had a radical prostatectomy have DTCs in the bone marrow (Gallaher and Vessella, 2009).

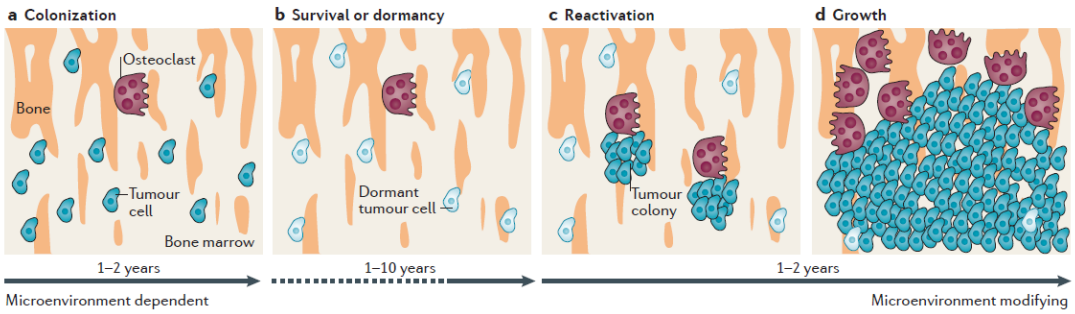

Figure 6. *The multi-step process of bone metastasis development*. (Croucher, Mcdonald and Martin, 2016)

3.4.7 Osteoimmunology in bone metastases

The immune system has long been known to have a central role in preventing tumor growth, but more recent evidence suggest the importance of the immune cell response in the tumor bone microenvironment as main regulator of cancer progression and metastases.

Once in the bone marrow, tumor cells can, directly or not, interact with different resident immune cells and modify the balance of immune effector and suppressor cells creating a microenvironment suitable for their growth (Dunn *et al.*, 2002)(Swann and Smyth, 2007).

In advanced bone metastatic cancers there is a prevalence of immunosuppressive cells, mainly myeloid-derived suppressors cells (MDSCs) and regulatory T-cells. Indeed, tumor cells secrete soluble factors such as IL-4, IL-13 VEGF, granulocyte–macrophage colony-stimulating factor (GM-CSF), granulocyte-colony-stimulating factor (G-CSF) and TGF-β that recruit and activate MDSCs. MDSCs stimulates osteoclast differentiation and activity and also support the polarization of macrophages into a tumor-promoting phenotype (Danilin *et al.*, 2012)(Gabrilovich *et al.*, 2012). Recent evidences support a role of osteoblasts in osteoimmunology mediated by the release of cytokines and growth factors in the microenvironment (Buenrostro, Park and Sterling, 2014). In particular, PTHrP, produced by tumor cells, stimulates osteoblasts to produce CCL2, IL-6 and VEGF (A) that recruit and stimulate MDSCs (Gratchev *et al.*, 2006).

Tumor–associated inflammation not always is a signal of immune system response to tumor cell growth, but sometimes creates a microenvironment that facilities neoplastic development. Indeed, CD68+ osteal macrophages, that have a pro-tumor phenotype, establish a complex crosstalk with cancer and bone cells leading to tumor progression in the skeleton, especially in breast and prostate cancers (Sinder, Pettit and McCauley, 2015).

Finally, different immune cell types are involved in the establishment of tumor cells in the metastatic niche, mainly in bone. Indeed, some inflammatory cells express

RANKL that mediates RANK+ tumor cells migration into the bone (Gonzalez-Suarez *et al.,* 2010)(Santini et al., 2011).

3.4.8 Markers of bone metastases

Bone metastatic cancers determine changes in bone metabolism and then in bone remodelling proteins whose serum levels could predict metastasis onset (Manca *et al.*, 2017). These proteins represent the bone turnover markers and include markers of bone formation and markers of bone resorption (Ferreira, Alho and Casimiro, 2015). Specifically, the bone formation markers include bone specific alkaline phosphatase (bALP), bone matrix proteins such as OCN and the procollagen extension peptides (P1NP and P1CP). bALP is an enzyme produced by osteoblasts that is released into circulation during the mineralization process (Jung and Lein, 2014).

OCN is a non-collagenous protein synthetized by osteoblasts that binds to hydroxyapatite and is involved in calcium binding (Seibel, 2005).

P1NP and P1CP are derived from the extracellular processing of the procollagen type I molecule, which contains amino- terminal and carboxy-terminal extensions that are enzymatically cleaved upon procollagen secretion (Hlaing and Compston, 2014).

In different stages of disease of prostate cancer, several bone turnover markers could predict the presence of bone metastasis on further radiologic imaging.

Jung et al.(Jung and Lein, 2014) found a correlation between the levels of several cone turnover markers and the disease state (bone metastatic vs non-metastatic) and they found that bALP, P1NP and -CTX predict overall survival. Moreover, de la Piedra et al. found that high levels of these proteins can predict SREs occurrence (de la Piedra *et al.,* 2003) .

Bone turnover markers might be a specific predictor of bone metastasis occurrence in a clinical setting since they could identify patients that are prone to bone metastasis formation due to comorbidities (i.e. osteoporosis), concomitant therapies (i.e. androgen deprivation therapy) or due to any metabolic condition that enhance bone remodelling.

Also PSA levels or PSA derivatives such as PSA velocity, PSA doubling time or PSA density can represent a marker of prostate cancer metastasis, but they can't differentiate patients who are going experience bone metastasis from patients who are going to experience visceral metastasis. Thus, PSA levels reflects tumour aggressiveness rather than bone metastasis onset.

Although bone-remodeling markers might be helpful in better defining the prognosis and the risk for bone complications in patients with bone metastatic disease, the level evidence is not yet sufficient to recommend them as part of the guidelines for clinical practice (Coleman *et al.*, 2011).

3.4.9 Treatment of bone metastases

Currently, several therapies are approved for the treatment of metastatic CRPC, based on their capacity to improve overall survival in randomized controlled trials. These include the cytotoxic drugs docetaxel and cabazitaxel, the immunotherapeutic vaccine sipuleucel-T, and the hormone-based therapies abiraterone and enzalutamide, and the bone-targeting radiopharmaceutical radium-223 dichloride (223Ra) (Santini *et al.*, 2016). Other bone-targeting agents are used in the supportive care of patients with metastatic CRPC to reduce pain and the incidence of SREs, including osteoclast inhibitors such as zoledronic acid or other bisphosphonates and denosumab. These agents have been shown to have the highest efficacy in delaying and reducing skeletal complications of metastatic CRPC in comparison with other drugs (Santini *et al.*, 2016). The first line-therapy for patients with metastatic CRPC is the chemotherapy with docetaxel, which demonstrated improved of overall survival (Tannock *et al.*, 2004), while cabazitaxel is administered as second line-therapy (de Bono *et al.*, 2010). Both of them are taxenes, a class of antimicrotubule agent. Interfering with microtubules, they block cell growth, stop mitosis, induce apoptosis and inhibit nuclear translocation of AR (Fitzpatrick and Wit, 2014).

Although chemotherapy has been shown to improve overall and progression free survival (de Bono *et al.*, 2010)(Fitzpatrick and Wit, 2014)(Harris *et al.*, 2011), it is not well tolerated by all CRPC patients because the majority are elderly men with limited bone marrow and several comorbidities (Paller and Antoranakis, 2011). In recent

years, wide varieties of novel therapeutic options have become available for patients with metastatic CRPC; among these, there are the so-called *targeted-therapies* that include abiraterone acetate (de Bono *et al.*, 2012) and enzalutamide (Scher *et al.*, 2012). Another approach to pain relief in patients with bone metastases is the use of radiopharmaceuticals.

3.4.9.1 Bone targeted agents

Bisphosphonates are analogues of pyrophosphate with a strong affinity for divalent metal ions, such as calcium ions and thus, for the skeleton. Bisphosphonates are the standard of care for the treatment of osteoporosis as well as bone metastases, thanks to their action against osteoclast bone resorption (Roelofs *et al.*, 2006). Indeed, binding hydroxyapatite crystals of bone matrix bisphosphonates form a barrier that prevents osteoclast activity and the subsequent osteoblast bone deposition. There are two classes of bisphosphonates, non-nitrogen-containing (alendronate, ibandronate, pamidronate, risedronate, and zoledronic acid) and nitrogen-containing (eg, clodronate, etidronate, and tiludronate), that inhibit differently osteoclasts. Particularly, nitrogen-containing bisphosphonates are more active than other in blocking osteoclasts (Luckman *et al.*, 1998), indeed, they inhibit farnesyl pyrophosphatase, the fundamental enzyme for osteoclast function, survive and morphology causing the accumulation of the cytotoxic nucleotide metabolite ApppI (Mönkkönen *et al.*, 2006). Moreover, several data demonstrated that bisphosphonates affect also immune cells (mainly macrophages and gamma delta T cells) and tumor cells through antitumor and/or antiangiogenic effects (Coleman *et al.*, 2012)
Zoledronic acid demonstrated beneficial effects in prostate cancer patients. Indeed, Zoledronic acid treatment increased bone density and significantly reduced bone fractures at 6, 12, 24 months in patients with non metastatic prostate cancer after Androgen Deprivation Therapy (Smith *et al.*, 2003). Zoledronic acid reduced SREs onset and pain also in patients that developed hormone-therapy resistance (Saad *et al.*, 2002) (Saad et al., 2004).

In a phase III clinical study (STAMPEDE) the addition of zoledronic acid to docetaxel showed no evidence of survival improvement or delay of SREs incidence in advanced metastatic prostate cancer patients (James *et al.*, 2016). Similar results were obtained from the CALGB/ALLIANCE 90202 study comparing early treated hormone-sensitive prostate cancer versus delayed treatment in Castration Resistant Prostate Cancer (CRPC) (Smith *et al.*, 2014).

· *Denosumab* is a monoclonal antibody against RANKL developed for the treatment of osteoporosis, skeletal pathologies and bone metastasis thanks to its inhibiting activity on osteoclasts (Lacey *et al.*, 2012). The superiority of denosumab compared to zoledronic acid in reducing SREs onset was demonstrated in a large randomized controlled trial (Stopeck *et al.*, 2010). Nevertheless, no differences in OS disease progression and rate of adverse events were observed (Martin *et al.*, 2012). In a castration-resistant prostate cancer patients population presenting bone metastases the median time-to-first on-study SRE for the denosumab arm was significantly prolonged (21 months) compared to the zoledronic acid ones (17 months) with no improvements in OS or progression of disease (Fizazi *et al.*, 2011). Another trial enrolled 1776 patients with myeloma induced osteolysis and solid tumors other than breast and prostate cancers (Henry *et al.*, 2011). The results showed a median time-to-first on-study SRE of 21 months in the denosumab group and 16 months in the arm receiving zoledronic acid demonstrating a non-inferiority for denosumab vs zoledronic acid, but neither a superiority after adjustment for multiple comparison nor an advantage in OS of denosumab over zoledronic acid (Henry *et al.*, 2011).
On the basis of these evidences the National Comprehensive Cancer Network the American Society of Clinical Oncology (ASCO), and the European Society of Medical Oncology (ESMO) recommend zoledronic acid or denosumab as the standard of care in bone metastatic patients (Coleman *et al.*, 2014)(Van Poznak *et al.*, 2011).

· *Radiopharmaceuticals* are a group of radioactive drugs that recognise reactive metastatic bone sites and emit radiations according to their nature (commonly beta emission). In patients who present metastases in different bone sites, the

radiopharmaceuticals are more effective than external local beam radiation, even if the combination of both is recommended for the most painful bone lesions (Agarawal *et al.*, 2006; Lutz and Chow, 2012).

Different types of radiopharmaceuticals are currently used for bone metastasis treatment. In bone metastatic prostate cancers stronzium-89 and samarium-153 represent useful palliation of bone pain. Therefore, in a randomised control trial, stronzium-89 improved progression free survival in mCRPC patients after six cycle of docetaxel (Sartor *et al.*, 2007; Silberstein , 1996).

Most recently, FDA approved the α-particle-emitting radiopharmaceutical, radium-223 as treatment for bone metastatic prostate cancer patients. As α-emitter, radium-223 delivers a highly localized radiation to bone surface than beta-emitters, causing DNA damages and the subsequent cell death giving less irradiation to healthy bone marrow (Allen, 2008). Radium-223 improved OS of bone metastatic CRPC patients previously treated with docetaxel or unfit to receive docetaxel (Parker *et al.*, 2013); moreover, it showed efficacy in all secondary end-points including time to the first symptomatic skeletal events (median, 15.6 months vs. 9.8 months respectively). Ongoing phase III trial are designed to evaluate the effect of a combined treatment of radium-223 and other new target therapies as abiraterone acetate in this group of patients (NCT01106352 and NCT02097303).

3.4.9.2 Anticancer agents with bone effect

Besides the traditional bone-targeted agents, others born as anticancer drugs (abiraterone and enzalutamide), showed at the same time, effects on bone microenvironment altering bone turnover.

- *Anti-androgen agents*

Abiraterone showed not only a significant survival advantage in metastatic prostate cancer patients (Fizazi *et al.*, 2012; Ryan *et al.*, 2013), but also a strong skeletal response. Indeed chemotherapy-treated patients treated with abiraterone showed better pain relief from skeletal metastases, a delay in time to development SREs (25 months vs 20.3 of placebo group) and in radiological skeletal progression

(Fizazi *et al.*, 2012). Abiraterone effects on metastatic bone disease may be not only secondary to a systemic control of the disease due to a direct antitumor effect but also due to a specific effect on bone microenvironment. Recently, our research team demonstrated the effect of abiraterone on bone microenvironment. In particular, abiraterone modulates bone cells leading to direct anabolic and anti-reabsorptive effects, suggesting a non-canonical mechanism of action (Iuliani *et al.*, 2015).

Enzalutamide have strong effects on bone responses, as demonstrated in two phase III trials. In particular, the AFFIRM study showed that mCRPC patients treated with docetaxel and then with enzalutamide had improvements in survival and skeletal responses compared to placebo group (Scher et al., 2012). In addition, the PREVAIL study demonstrated similar results in mCRPC patients treated with enzalutamide, who had not receive docetaxel compared to placebo. Indeed, it has observed improvements in primary endpoints (OS and radiographic progression) and also in the secondary endpoints, including delayed initiation of chemotherapy and reduction in risk of first SRE (Beer et al., 2014).

AIM OF THE STUDY

Androgen-deprivation therapy is the first-line therapy for advanced prostate cancer and is initially effective in the majority of patients. Despite the initial success of these treatments, castration-resistant prostate cancer (CRPC) inevitably occurs within few years and remains the primary unsolved challenge for clinicians (Harris WP, Mostaghel EA, Nelson PS, 2009). Multiple mechanisms of resistance contribute to the progression to castration resistant disease and the androgen receptor (AR) remains the most important driver in this progression (Chandrasekar *et al.*, 2015).

Moreover, about 90% of men with advanced prostate cancer develops bone metastases. As result, prostate cancer patients usually suffer SREs including pathological fractures, bone pain and spinal cord compression that severely affect the patients' quality of life (Coleman, 2006). Therefore, prostate cancer can be considered a microenvironment-driven disease, involving bi-directional biological interactions between tumour and bone microenvironment (Mundy, 2002).

This interplay between bone microenvironment and prostate cancer cells led to tumour vicious cycle theory revision (Ottewell, 2016). In particular, tumour cells, migrated to the bone, release several soluble factors such as PTHrP and IL-6 that determine a switch in RANKL/OPG balance in favour of RANKL. RANKL overexpression promotes osteoclastic bone resorption and then, the release of TGFβ and IGF-1 that stimulate tumour proliferation. Moreover, tumour cells release other factors like ET-1 and activate Wnt pathway resulting in OPG secretion. OPG stimulates osteoblast differentiation and activity promoting the formation of unstructured bone prone to fracture.

Based on these evidences, it is clear that osteoblasts play a central role, currently not completely understood, in the control of bone metastasis onset.

The aim of this study is to clarify if and how osteoblasts could influence CRPC progression. In particular, we investigated the possibility that human osteoblasts might

secrete factors that contribute to the emergence of androgen-independent prostate cancer.

In order to explore this point, we evaluated osteoblasts and castration resistant prostate cancer cells (C4-2B cells) interplay in *in vitro* human co-cultures, analysing the AR activity, tumour cell proliferation and cell cycle.

In detail, the specific aims of this research project are as follows:

- To analyse AR activity of C4-2B cells co-cultured with osteoblasts
- To evaluate osteoblastic effect on C4-2B cells proliferation and cell cycle
- To figure out the potential factor/ factors involved in osteoblast-mediated effects on C4-2B cells.

MATERIAL AND METHODS

1 Cell lines

1.1 Prostate Cancer Cell line

C4-2B cell line, that is a castration resistant prostate cancer (CRPC) cell line, was gently gifted by Thalman who isolated them in 1994 (Thalmann et al., 1994). Prostate cancer (PCa) cell line was stored in aliquots in liquid nitrogen and used within 6 months once resuscitated. C4-2B were grown in T-medium [80% Dulbecco's modified Eagle's medium, 20% F12K, 3 g/liter NaHCO3, 100 units/liter PenicillinG, 100 µg/mL Streptomycin, 5 µg/mL insulin, 13.6 pg/mL triiodothyronine, 5 µg/mL apo-transferrin, 0.25 µg/mL biotin, 25 µg/mL adenine] with 10% FBS. The cells were free of Mycoplasma.

To mimic androgen-deprivation therapy, typical feature of this stage of disease (CRPC) all experiments were performed using T-medium supplemented with 10% of charcoal-stripped serum (an androgen depleted serum).

1.1.1 GFP stable transfection

C4-2B cell line was transfected with MISSION® pLKO.1-puro-CMV-TurboGreenFluorescentProtein (GFP) ™ Positive Control Transduction Particles (Sigma Aldrich) to obtain GFP+ C4-2B cells. In particular, C4-2B cells were seeded in 96-multi well plate at 60-70 % of confluence. After 12 hours the culture media was removed and replaced with fresh culture media plus 8 mg/mL of Hexadimethrine bromide and lentiviral particles using a 0,5 of multiplicity of infection (MOI). 2 µg/mL of Puromycin was added after 48 hours in order to select the transfected GFP+ C4-2B cells.

1.1.2 AR-reporter stable transfection

To evaluate AR activation, C4-2B cells were transfected using Cignal AR luciferase reporter kit (QIagen). The cells were first transfected with lentiviral particles

expressing a transcription factor-responsive reporter gene (firefly luciferase) under the control of the AR promoter element. Then, the cells undergone to a second transfection with other lentiviral particles that expressed Renilla luciferase under the control of a housekeeping promoter. This second transfection was an internal control that could be helpful in overcoming technical variability and obtain more reliable data.
The transfection protocols were performed as previously described and the C4-2B Firefly/Renilla cells (C4-2B FR) were selected adding 100 µg/mL of Hygromycin and 2 µg/mL of Puromycin.

1.2 Primary Human Osteoblasts

Primary human OBs were differentiated from mesenchymal stem cells (MSCs). Pieces of spongy bone, which would otherwise have been discarded, were obtained from systemically healthy patients undergoing hip replacement at Policlinico Campus-Biomedico after approval by Ethical Committee of Campus Bio-Medico University of Rome and informed consent from patients in accordance with the Declaration of Helsinki principles (Prot 21/15 OSS). To obtain MSCs, bone fragments were digested with Collagenase II (1.5 mg/mL)/ Trypsin (1X) mix solution for 2 hours at 37°C and placed in a culture flask with alpha MEM (Euroclone) supplemented with 20% fetal bovine serum (Hyclone, Thermo Scientific), 100 units/ml penicillin, 100 mg/ml streptomycin and amphotericin B (250ng/mL). OB differentiation was achieved by culturing MSCs at an initial density of 5×10^4 in 24 well plate or at 3×10^6 in 75 cm^2 flask adding 10 mM beta-glycerophosphate (Sigma-Aldrich), 50 µM ascorbic acid (Sigma-Aldrich) and 100 nM dexamethasone to culture medium.

2 Cellular functional assays

2.1 Osteoblast functional assays

On day 21, OBs fixed with 4% formaldehyde for 5 minutes and stained with an alkaline phosphatase (ALP) kit (Sigma-Aldrich) according to the manufacturer's protocol. ALP positivity was quantified by ImageJ software. In order to detect bone matrix deposition, mature OBs were fixed with 4% formaldehyde for 20 minutes and

stained with alizarin red for 1 hour at room temperature (RT). Alizarin red fluorescence was detected at 470 nm and quantified by spectrofluorimeter (Tecan Infinite M200Pro).

2.2 C4-2B AR assay

To quantify AR activation in C4-2B FR cells, Dual-Luciferase Reporter Assay (Promega) was performed. In particular, C4-2B FR cells were seeded in 96-multi well plate at 70-80 % of confluence. After 24 hours, the activities of Firefly and Renilla luciferases were measured sequentially, following manufacturer's instructions, with spectrofluorimeter (Tecan Infinite M200Pro). 1 nM of R1881 (Molekula) was added as positive control, whether 35 μM of enzalutamide (AR inhibitor, (Selleckem)) was added as negative control.

3 Co-culture experiments

To study osteoblast/ prostate cancer cell crosstalk we performed *in-vitro* direct and indirect co-cultures experiments.

3.1 OBs-C4-2B cells direct co-culture

GFP+ C4-2B cells were plated to an initial density of 5×10^4 on treated or untreated OB layer in 24 well plate and GFP-fluorescent signal was measured every 24 hours until 120 hours with spectrofluorimeter (Tecan Infinite M200Pro). Images of each sample were acquired after 6 hours from the seeding (t0) in order to allow the cell attachment, and every 24 hours, using a Nikon Ti fluorescence microscope.

3.2 OBs-C4-2B cells indirect co-culture

Osteoblast conditionated-media (OCM) and C4-2B conditionated-media (C4-2B CM) were collected after culturing respectively treated or untreated OBs and C4-2B cells for 48 h in T-medium supplemented with 10% of charcoal-stripped serum.
C4-2B cells were seeded in an initial confluency of 10^4 in 96 well plates and after 24 hours, time necessary for their attachment, OCM and C4-2B CM were added. Time of

co-culture depended on the performed assay: 24 h for AR assay or 5 days for BrdU proliferation assay and cell cycle analysis.

4 Proliferation and Cell cycle analysis

C4-2B cell intracellular staining was performed by adding a panel of fluorochrome-conjugated monoclonal antibodies (mAbs) to single cell suspensions. The cells were fixed and permeabilized with Foxp3/Transcription Factor Staining Buffer Set (Termofisher eBioscience) for intracellular staining with anti-Ki67 antibody (clone 20Raj1 eBioscience) and a Propidium Iodure (PI) solution (50 µg/ml PI+ 40 ng/ml RNAseA+ 0.1% of Triton) (Sigma). Dead cell exclusion was performed with Viability Dye conjugated with eFluor780 fluorochrome (Affimetrix eBioscience). Samples were analysed by FACSCanto II instrument (BD Bioscience) and using FlowJo Software, v.10.0.7.

5 RNA extraction and gene expression analysis

Total RNA was extracted by Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was treated with DNase buffer and DNase (DNAse Turbo, Applied Biosystems) to avoid genomic DNA contamination. cDNA was produced using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. mRNA levels were measured by quantitative real-time polymerase chain reaction (qRT-PCR) using TaqMan Gene Expression Assays in 7900HT Real-Time PCR System (Applied Biosystems).

AR (Hs00171172_m1), IL-8 (Hs00174103_m1), IL-6 (Hs00985639_m1), WNT16 (Hs00365138_m1), DKK-1 (Hs00183740_m1) and CXCL-12 (Hs00171022_m1) expression levels were normalized to the endogenous housekeeping gene Glucuronidase Beta (GUSβ) (Hs99999908_m1) in both untreated and treated samples using the ΔCT calculation. Subsequently the relative expression levels in treated samples were normalized to the mRNA levels detected in control samples using the ΔΔCT calculation.

6 Western Blot Analysis

Cells were seeded in 6 well plates at $5x10^5$ concentration and after treatments lysed adding RIPA buffer (Sigma-Aldrich) and centrifuged (8000 g × 10 min). Protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Sigma-Aldrich) were added to the supernatant containing cellular proteins. Protein concentration was measured using a DC protein assay (Bio-Rad) following manufacture's instruction. Protein were dissolved in Laemli buffer (Bio-Rad) containing 5% of β-mercaptoethanol and warm at 95°C for 5 min. Equal amount of protein (20 μg) was load in each lane of gel (Bio-Rad Precasted gels) and after fractionation by SDS-PAGE on gels, proteins were electro-blotted onto nitrocellulose membrane (Bio-Rad) using Trans-blot turbo (Bio-Rad)

SDS-PAGE blots were blocked for 2 h in 10% defatted dry milk-TBS-0.1% Tween-20 and then incubated for 2 h at room temperature with AR primary antibodies (1:400) (D6F11XP Rabbit mAb, Cell Signaling), and then followed by incubation with secondary HRP-conjugated anti-Rabbit IgG Ab (1:10000, Cell Signaling).

Immunoreactive bands were visualized by ChemiDoc MTP Imaging System (Bio-Rad) using the Clarity™ Western ECL Substrate (Bio-Rad). Quantity One Software (Bio-Rad) was used to quantify bands signal intensity.

7 Cytokines detection in conditioned media

Cytokines quantification in conditioned media was evaluated by flow cytometry using BD Cytometric Bead Array (BD Biosciences). IL-12 p70, TNF, IL-10, IL-6, IL-1β and IL-8 were measured in pg/ml according to the manufacturer's instructions.

8 Statistical analysis

Data were analyzed using the Student t test and One-Way ANOVA test followed by Tukey's multiple comparison tests. The graphics processing and statistical tests were performed using the program GraphPad Prism (San Diego, CA).

RESULTS

1 OBs differentiation

Primary human OBs were differentiated from mesenchymal stem cells (MSCs) obtained from surgical bone pieces of healthy patients undergoing hip replacement. After 21-28 days of differentiation, we obtained mature ALP+ OBs (Fig.1A) and their activity (Fig.1B) was evaluated with Alizarin Red assay (for details see material and methods).

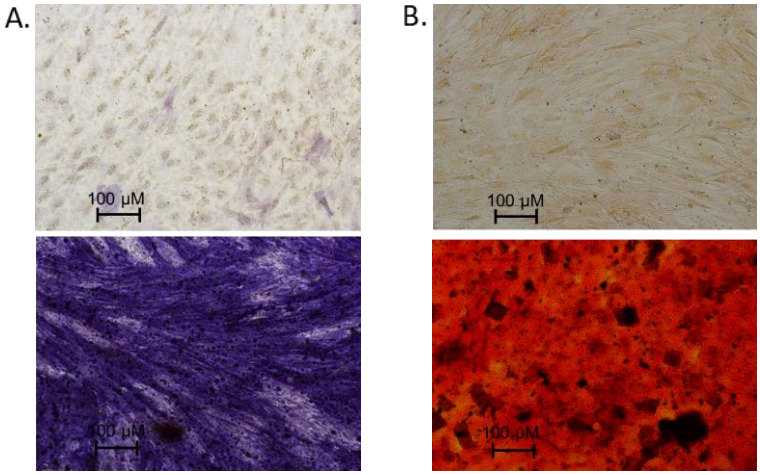

Figure 1. *Representative images of OBs differentiation and activity.* (A) Mesenchymal differentiation into OBs was evaluated using ALP assay: hMSCs are ALP^- (top), while mature OBs are ALP+ (bottom). (B) OBs mineralization was assessed through alizarin staining: hMSCs are unstained (top) while mature OBs produce calcium deposits stained by alizarin red (bottom)

2 OBs inhibit AR activity of C4-2B cells in direct co-culture system, stimulating their proliferation

To investigate the effect of OBs on C4-2B cells, we first established whether OBs modulated AR activity, which represents the major driving signalling for tumour cell proliferation. In particular, we performed OBs/ C4-2B FR cells direct co-cultures, seeding C4-2B FR cells on OBs monolayer. As control we used C4-2B FR cells cultured on C4-2B cells monolayer. As showed in Fig.2, AR activity of C4-2B cells was significantly inhibited (p= 0.0412) when they were cultured with OBs compared to control.

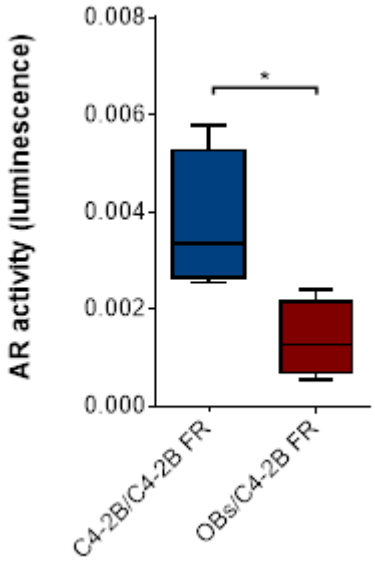

Figure 2. *AR activity of C4-2B cells cultured with OBs.* AR activity of C4-2B FR cells is measured as firefly-luciferase signal normalized with renilla-luciferase signal proportional to cell number (housekeeping control). *p<0.05

We also analysed C4-2B cells proliferation in the same experimental conditions, monitoring C4-2B GFP+ cell growth on OBs. Despite evidences support that AR inhibition is correlated to cell proliferation reduction, we observed a significant (p= 0.0413) increase of C4-2B cell growth (Fig.3).

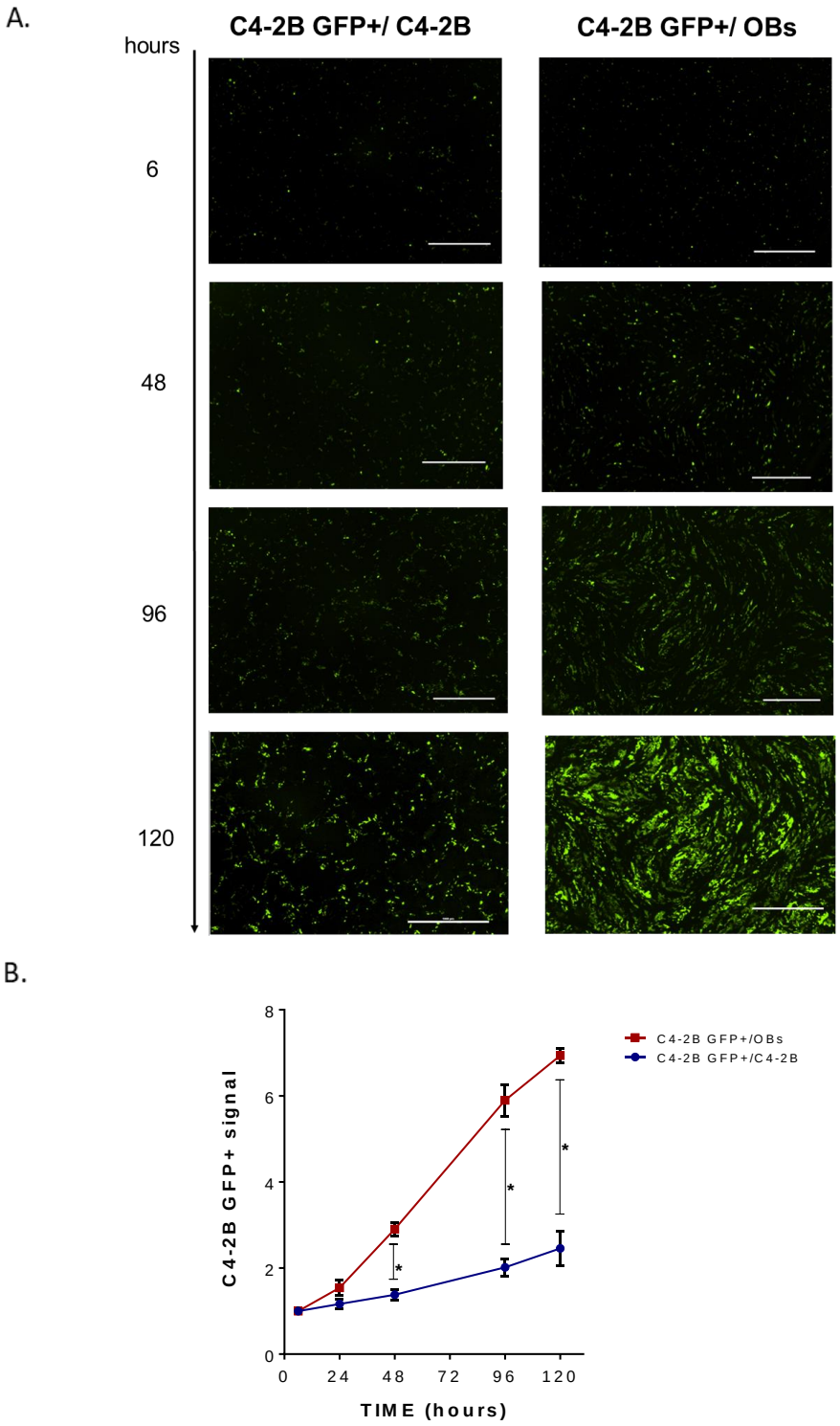

***Figure 3**. Proliferation of C4-2B cells co-cultured with OBs.* (A) Representative images of direct co-cultures captured at 6, 48, 96 and 120 hours after cell seeding. In green C4-2B GFP+ cells; scale bar= 100µm. (B) Schematic representation of proliferation analysis evaluated measuring GFP+ signal at different time points normalized with GFP signal at baseline (t= 6 hours). *p< 0.05

3 OBs inhibit AR activity of C4-2B cells in indirect co-culture

To evaluate if the OB-mediated AR inhibition needed a direct cell-to-cell contact between bone and tumour cells, we performed OB/PCa indirect co-cultures. In particular, we treated C4-2B FR cells with OB conditioned medium (OCM) or with C4-2B cells conditioned medium (C4-2B CM), that represents the control. Intriguingly, we observed that AR inhibition in C4-2B FR cells persisted also after OCM treatment ($p= 0.0029$) (Fig. 4A).

Further analyses of AR activity, showed that the OB-mediated inhibition of the receptor was only partially restored adding the exogenous androgen R1881 compared to the control ($p= 0.0033$), suggesting that this repression could be in part androgen independent (Fig. 4B).

In addition, treating C4-2B FR cells with an AR inhibitor (Enzalutamide) we found that the drug-mediated inhibition was significantly lower ($p= 0.0067$) in tumour cells treated with OCM compared to the control (Fig. 4C). This result led us to hypothesize that the reduced activity of Enzalutamide was due to a downregulation of its target, AR, following OCM treatment.

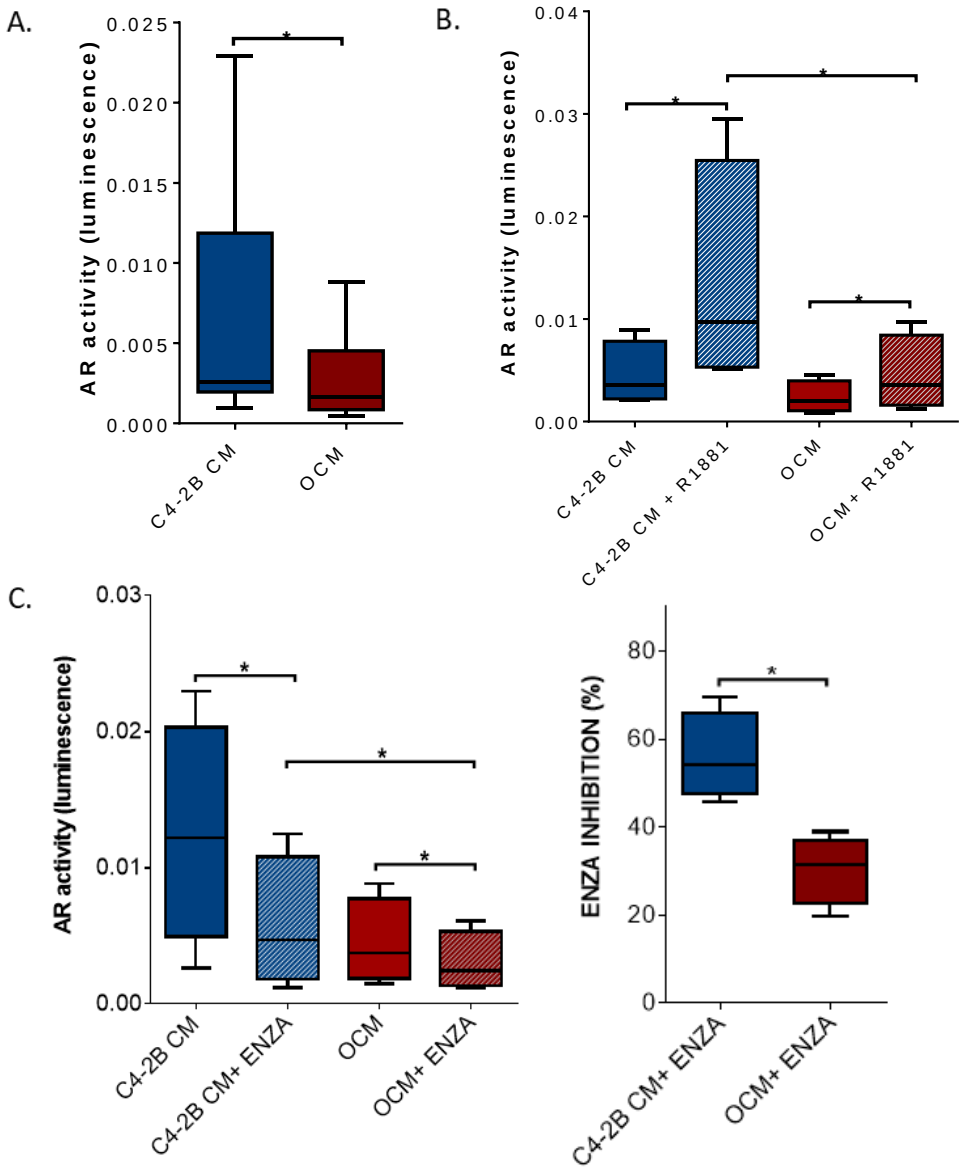

Figure 4. *AR activity in C4-2B cells after OCM treatment.* (A) AR activity is expressed as firefly-luciferase signal normalized with renilla-luciferase signal proportional to cell number (housekeeping control). (B) AR activity of C4-2B cells stimulated with C4-2B CM or OCM in presence of exogenous androgen R1881 (1nM). (C) AR activity of C4-2B cells after C4-2B CM or OCM treatment and with enzalutamide (ENZA 35 μM) (left). Enzalutamide (ENZA)-mediated AR inhibition in C4-2B cells treated with C4-2B CM or OCM is expressed as percentage normalized for the untreated samples (right). *$p < 0.05$

4 OB-mediated AR inhibition is due to AR mRNA and protein levels downregulation

To determine whether the AR was inhibited by OCM at the transcriptional or post-transcriptional level, we analysed AR mRNA and protein levels after OCM or C4-2B CM treatment. As showed in Fig. 5, we found that OCM treatment significantly downregulated AR mRNA (p= 0.0047) and, consequently, protein expression (p= 0.0247).

Thus, the AR inhibition mediated by OBs is due to a transcriptional regulation of the receptor that could be promoted by specific OB-secreted factors.

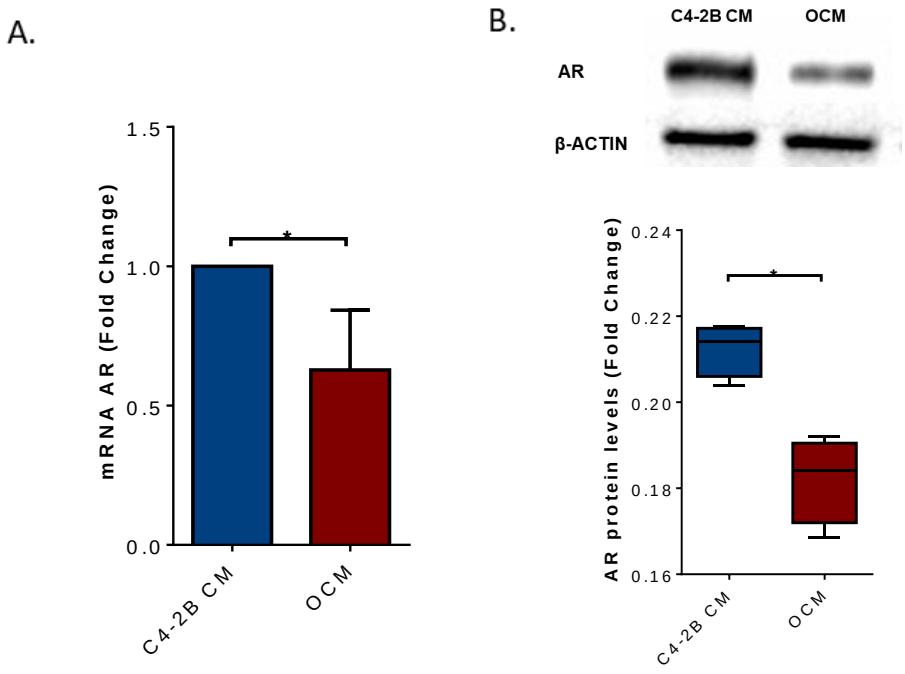

Figure 5. *AR mRNA and protein expression of OCM treated C4-2B cells.* (A) mRNA expression of AR is measured and normalized for the housekeeping β-glucoronidase (GUS-β). Values are expressed as fold change relative to the control. (B Top) Representative image of AR protein expression measured by western blotting. Equal amount of protein was loaded on each gel lane. (B Bottom) Schematic representation of AR protein expression obtained normalizing AR bands for the housekeeping β-actin. *p <0.05

5 OCM stimulates C4-2B cell proliferation

In order to evaluate the effect of OCM on cell proliferation, flow cytometry analyses were performed. In particular, the percentage of C4-2B proliferative cells was analysed using Ki-67/ PI staining (see Fig. 6 for our gating strategy, based on Forward and Side Scatter plots and exclusion of dead cells).

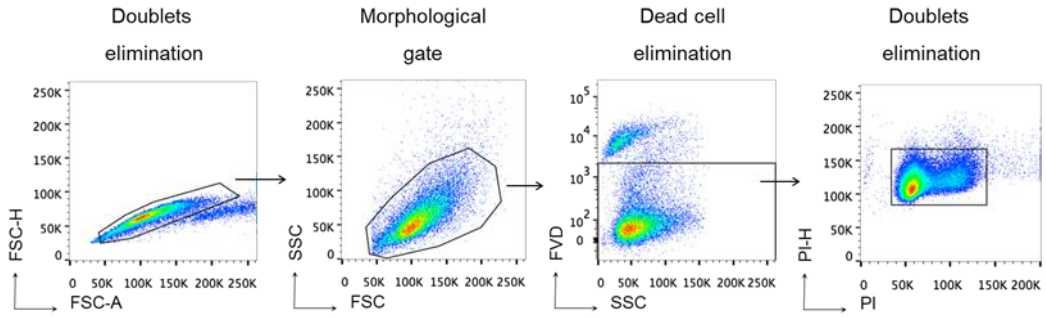

Figure 6. *Gating strategy for C4-2B cells proliferation and cell cycle.* Cells are stained with FVD to exclude death cells (FVD+). Gating strategy based on Forward and Side scatter is shown.

As showed in Fig. 7, we found that OCM treatment increased ($p= 0.0334$) the percentage of Ki-67^{+} C4-2B proliferative cells (in G1, S and G2-M phases), compared to the control.

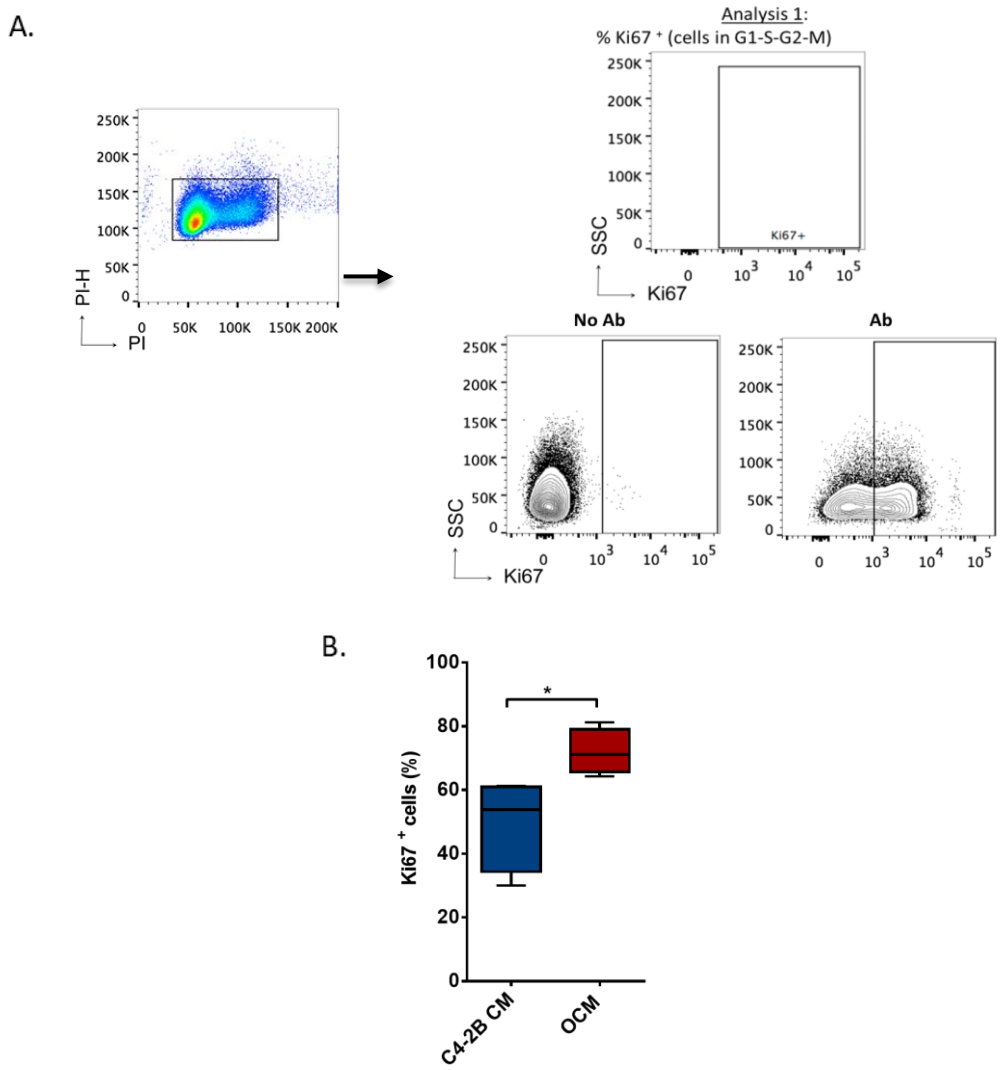

Figure 7. *C4-2B proliferation analysis after OCM treatment* (A) Representative dot plots showing Ki-67+ cell percentages among C4-2B cells. Ki-67 positivity is gated on the unstained control. (B) Schematic representation of Ki-67+ percentage obtained after C4-2B treatment with C4-2B CM or OCM with average value (bar). *p< 0.05

The combined analysis of Ki-67 and PI allowed us to identify all cell cycle phases (G0, G1, S and G2-M) as showed in Fig.8A. In particular, we found that OCM treatment increased the percentage of C4-2B in G1 (p= 0.0477), S (p= 0.0319) and G2-M (p= 0.0312) phases reducing the percentage of Ki-67$^-$ PI$^-$ resting cells in G0 phase (p= 0.0313) (Fig. 8B).

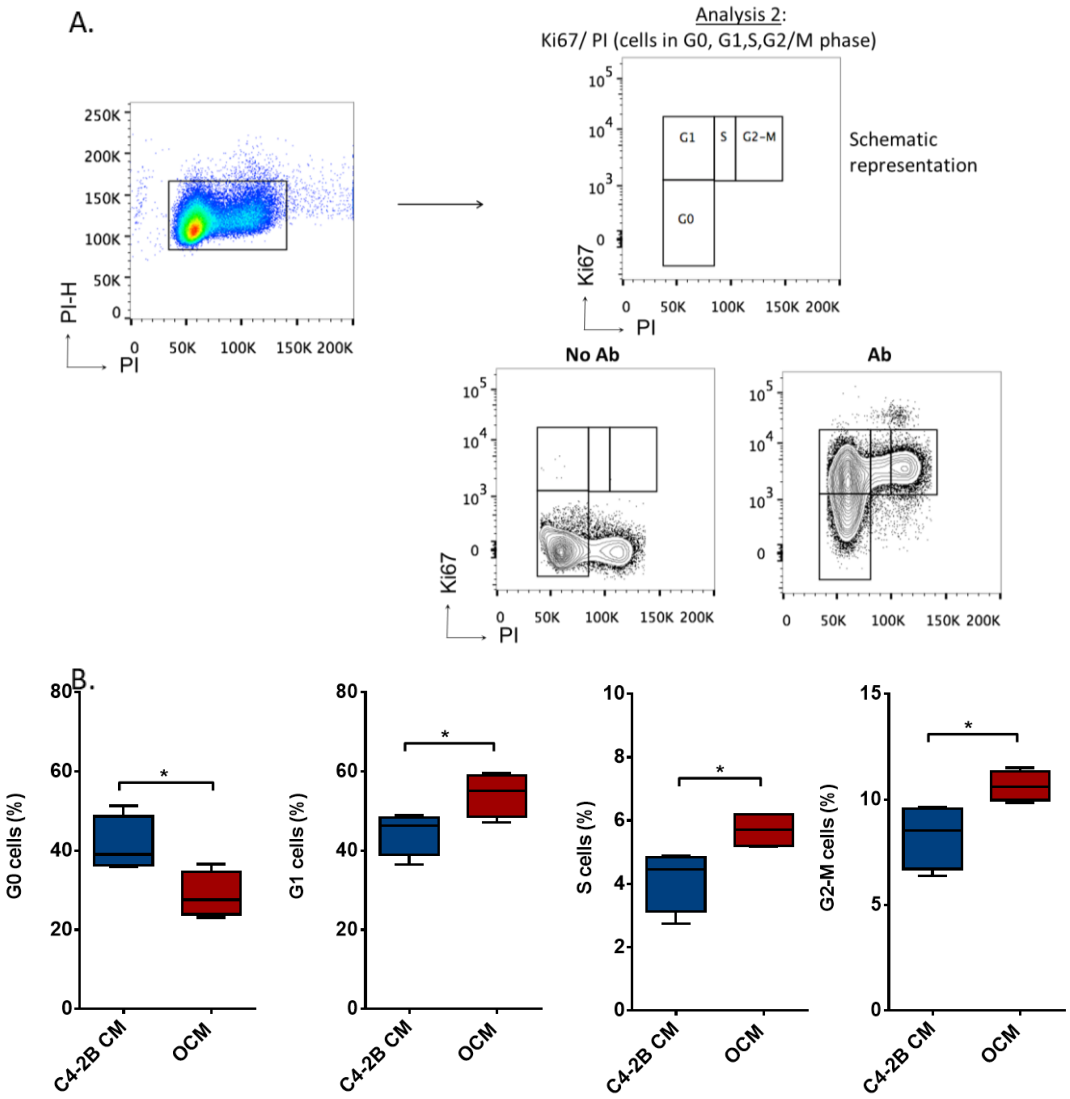

Figure 8. *Combination of PI and Ki-67 staining in C4-2B cells treating with OCM.* Ki-67 and PI stained C4-2B cells are analysed by flow cytometry (for gating strategy see Figure 4). (A) Typical dot plots showing Ki-67 and PI expression by C4-2B cells. Ki-67 and PI expression delineated 4 regions that correspond to different cell cycle phase: G0 (Ki-67-/ PI-), G1 (Ki-67+/ PI-), S and G2-M (both Ki-67+ with different PI intensity). (B) Schematic representation of C4-2B cells percentage in G0, G1, S and G2-M phases after OCM treatment with average value (bar). Cytometry profiles are representative of data from 4 different experiments. *p<0.05

6 IL-8, IL-6, WNT16, DKK1 and CXCL12 mRNA are highly express in OBs

Since the androgen-independent growth of C4-2B cells was sustained by OBs either directly or through conditioned medium, we looked for soluble factors potentially involved in this mechanism. For this purpose, we analyzed mRNA levels of IL-8, IL-6 WNT-16, DKK-1 and CXCL-12 in both OBs and C4-2B cells. Gene analyses showed that all these genes were expressed in OBs up to 30 times more compared to C4-2B cells (IL-8 p= 0.0026; IL-6 p= 0.0005; WNT-16 p= 0.0115; DKK-1 p= 0.0006; CXCL12 p= 0.0009) (Fig. 9).

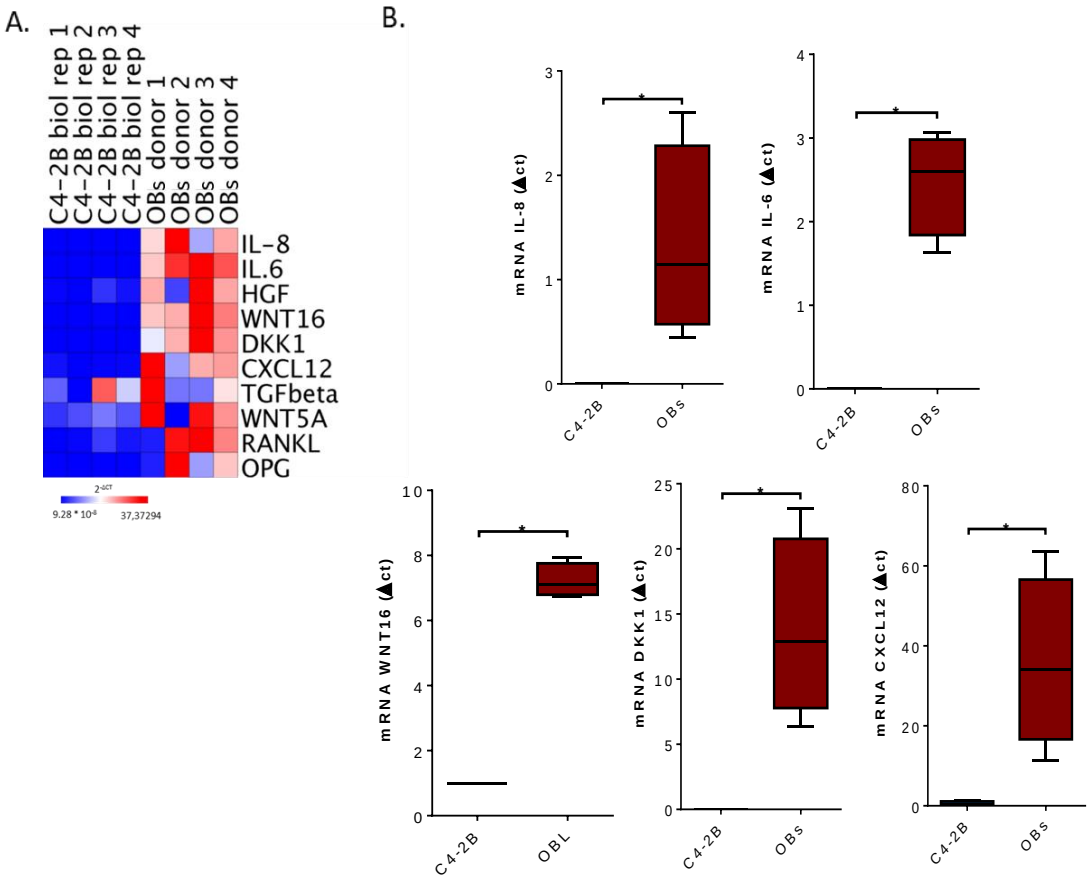

Figure 9. *IL-8, IL-6, WNT16, DKK1 and CXCL12 mRNA levels in OBs and C4-2B cells* (A) Heat-map of analysed genes. (B) IL-8, IL-6, WNT16, DKK1 and CXCL12 expression in OBs compared with C4-2B cells. mRNA expression of each gene is measured and normalized for the housekeeping β-glucoronidase (GUS-β). Values are expressed as fold change relative to the control. *p<0.05

7 IL-8 and IL-6 are present in OCM in higher concentration compared to C4-2B CM

IL-6 and IL-8 protein levels were subsequently quantified in C4-2B CM and OCM using flow cytometry analysis. As showed in Fig. 10, we found that IL-6 and IL-8 protein levels (pg/ml) were highly expressed (p= 0.0479 and p= 0.017 rispectively) in OCM, while they were almost absent in C4-2B CM.

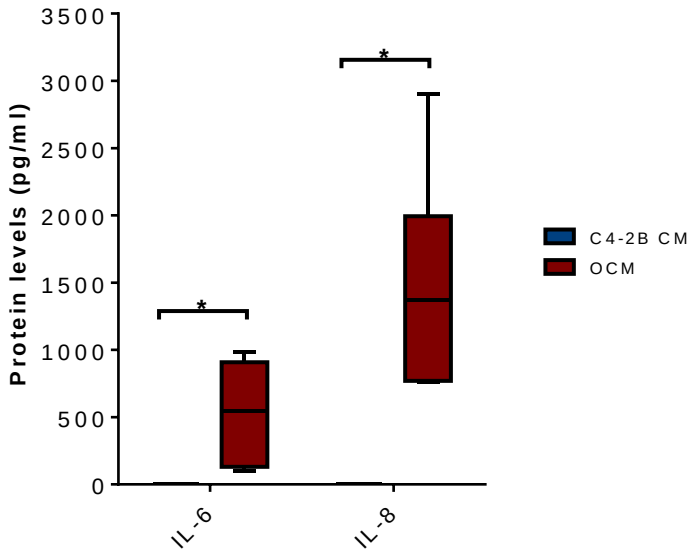

Figure 10. *IL-6 and IL-8 protein quantification in C4-2B CM and in OCM.* IL-8 and IL-6 are measured in OCM and C4-2B CM by flow cytometry and quantified as protein concentration (pg/ml). *p< 0.05

DISCUSSION

Prostate cancer has the propensity to metastasize to the bone forming mainly osteoblastic lesions (Coleman, 2001). Androgen ablation remains the standard of care for advanced metastatic disease leading to initial good responses, such as the decline in PSA levels. Nevertheless, an eventual tumour progression occurs after castration and androgen-independent disease develops (Harris WP, Mostaghel EA, Nelson PS, 2009).

Prostate cancer can be considered a microenvironment-driven disease. Indeed, it has known that bone and tumour cells interaction, through soluble factors release or a direct cell-to-cell contact, is crucial for tumour progression and bone metastasis onset (Mundy, 2002). Since this complex interplay is currently not completely elucidated, this research project was focused on osteoblast (OB) role in the progression of castration resistant prostate cancer.

To investigate this point, we first analysed androgen receptor (AR) activity in castration resistant prostate cancer cell line (C4-2B cells) cultured with OBs, demonstrating that OBs strongly inhibited the receptor. As control, we used C4-2B cells not cultured alone, but on C4-2B cells monolayer, in order to have the same cell number. Moreover, to mimic the typical feature of CRPC, in all experiments, the androgen deprivation was reproduced using a serum without androgens (charcoal stripped serum) in the culture media, replacing classical serum (FBS).

Since AR activation is the driving signal for prostate cancer cell growth, we investigated the impact of AR inhibition on tumour cell proliferation. Contrary to expectations, C4-2B GFP+ cells did proliferate significantly more when cultured with OBs, supporting the hypothesis that OBs stimulate an androgen-independent cancer growth.

Then, we wondered if the observed OBs-mediated effects were due to a direct cell-to-cell interaction between bone and cancer cells or were mediated by the release of specific soluble factors from OBs. In order to answer to this question, we treated C4-2B cells with OBs conditioned media (OCM) analysing AR activity.

Our data showed that the reduced activity of the receptor did persist also when prostate cancer cells were treated with OCM demonstrating that this effect was mediated by soluble factors released by OBs.

Further analyses of AR activity showed that the inhibition is only partially restored by the addition of the exogenous synthetic androgen, R1881 suggesting that this repression could be in part androgen independent.

In addition, the effect of Enzalutamide, a potent AR inhibitor was significantly lower when C4-2B were treated with OCM. These results could be explained by the fact that OCM treatment induced a downregulation of AR mRNA and protein levels in C4-2B cells. Collectively, the lower effect of AR agonist (R1881) and inhibitor (Enzalutamide) could be a consequence of the reduced expression of the receptor.

Once again, together with the repression of the receptor, we observed that OBs promoted tumour cell growth also in indirect co-culture. Indeed, the Ki-67 expression, a marker of proliferative cells (in G1, S and G2-M phases) increased after OCM treatment compared to the control. In addition, combining Ki-67 staining with PI, we demonstrated that OCM led to an increase of cell percentage in every single cell cycle phase (G1, S and G2-M), decreasing at the same times the percentage of quiescent cells in G0 phase.

Taking together, our data demonstrated that OBs play a crucial role in castration resistant prostate cancer promoting their androgen-independent proliferation. Moreover, we found that OBs exert their action on tumour cells through the secretion of soluble factors.

In order to identify the molecules involved in this process, we first analysed a panel of genes encoding for the principal soluble factors that are known to regulate prostate cancer proliferation and AR activity.

Beside these, we focused on IL-6, IL-8, WNT16, DKK-1 and CXCL12 expression. We found that OBs presented higher mRNA levels of all these factors compared with C4-2B cells. Thus, all these molecules could be potentially responsible of OB-mediated effects.

Several evidences from literature support our results. It has been demonstrated that IL-6 may contribute to prostate cancer progression: indeed, serum level correlated with

prostate tumor burden and patient morbidity. Moreover, IL-6 receptor is abundantly express by prostate cancer tissues (Adler *et al.*, 1999; Siegsmund *et al.*, 1994). In addition, Blaszezyk et al. observed that IL-6 was secreted by OBs and it promoted prostate cancer proliferation. Contrary to our data, the authors demonstrated that this proliferation was due to AR activation mediated by this cytokine. It must be noted, however, that they used LNCaP cell line that presents different molecular features compared to our C4-2B cells. In particular, LNCaP cell line mimics the sensitive disease and survive only in presence of androgens that activate AR, while C4-2B cells are a castration resistant cell line able to survive and proliferate also with a reduced activation of the receptor (Blaszczyk *et al.*, 2004). Other evidences from literature suggest that even IL-8 could mediate the observed OBs effect on tumour cells. In particular, it has been showed that this cytokine was undetectable in androgen-responsive prostate cancer cells (e.g., LNCaP), while it was highly expressed in androgen-independent metastatic cell lines (PC3). Moreover, continuous or transient exposure of LNCaP cells to IL-8 reduced their androgen-dependent growth, decreasing the sensitivity to anti-androgen drug. The authors concluded that IL-8 might help prostate cancer cells to become androgen independent and proliferate under androgen depletion (Araki *et al.*, 2007). These data are in accordance with our results suggesting that IL-8 could be the potential factor responsible of androgen independent prostate cancer growth.

Few evidences support the role of WNT16, DKK-1 and CXCL12 in prostate cancer progression. Sun et al. observed that WNT16b promoted prostate cancer cell resistance to chemotherapy, promoting their survival and proliferation (Sun *et al.*, 2012). Instead, DKK-1 overexpression was found in AR negative prostate cancer cell lines, suggesting that DKK-1 could stimulate tumour cells proliferation through androgen independent mechanism. (Rachner *et al.*, 2014).

Since IL-8 and IL-6 represent the best candidates to mediate OB effects, protein expression analysis on conditioned media was performed. As expected, we detected higher levels of these proteins in OCM compared with C4-2B CM.

These findings support the hypothesis that IL-8 and IL-6, secreted by OBs, could mediate AR inhibition and, at the same time, tumour cells proliferation.

However, further analyses are necessary to fully elucidate their role in the androgen-independent cancer cell growth. In particular, the next step will be to explore if the adding of IL-8/ IL-6 inhibitors in our co-culture models is able to prevent OBs mediated prostate cancer cells proliferation. In addition, we will perform an extensive transcriptomic analysis to completely clarify the activated molecular pathways in C4-2B cells following OCM treatment.

The identification of the targets responsible of OBs pro-tumour effects could represent a strong rationale for the development of new target therapies that could support the existing strategies of CRPC treatment.

CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, our study demonstrate that OBs establish a huge interplay with castration resistant prostate cancer cells sustaining their androgen-independent growth. This phenomenon is mediated by the secretion of osteoblastic soluble factor/ factors that inhibit AR and, at same time, promote alternative pathways of proliferation, not yet completely elucidated. Although we found some soluble factors (IL-8 and IL-6 *in primis*) that could be involved in the androgen-independent growth of tumour cells, further analyses are necessary to confirm our hypothesis.

BIBLIOGRAPHY

- Abrami L, Kunz B, Iacovache I, van der G. F. (2008) 'Palmitoylation and ubiquitination regulate exit of the Wnt signaling protein LRP6 from the endoplasmic reticulum', *Proc Natl Acad Sci*, 105(15), pp. 5384–9. doi: 10.1073/pnas.0710389105.
- Adler HL, McCurdy MA, Kattan MW, et al. Elevated levels of circulating interleukin-6 and transforming growth factor-beta1 in patients with metastatic prostatic carcinoma J Urol, 161 (1999), pp. 182-187
- Agarawal JP, Swangsilpa T, van der L. Y. et al. (2006) 'The role of external beam radiotherapy in the management of bone metastases.', *Clin Oncol (R Coll Radiol)*, 18, pp. 747–760.
- Allen BJ. (2008) 'Clinical trials of targeted alpha therapy for cancer.', *Rev Recent Clin Trials*, 3, pp. 185–91.
- Araki S, Omori Y, Lyn D, et al. Interleukin-8 is a molecular determinant of androgen independence and progression in prostate cancer. Cancer Res. 2007 Jul 15;67(14):6854-62. PubMed PMID: 17638896
- Attard G, Reid AHM, Auchus RJ, et al. (2015) 'Clinical and Biochemical Consequences of CYP17A1 Inhibition with Abiraterone Given with and without Exogenous Glucocorticoids in Castrate Men with Advanced Prostate Cancer', 97(February 2012), pp. 507–516. doi: 10.1210/jc.2011-2189.
- Attard G, Parker C, Eeles RA, et al. (2016) 'Prostate cancer', *Lancet*, 387(10013), pp. 70–82. doi: 10.1016/S0140-6736.
- Autzen P, Robson CN, Bjartell A, et al. (1998) 'Bone morphogenetic protein 6 in skeletal metastases from prostate cancer and other common human malignancies', 78(1 998), pp. 1219–1223.
- Baron R and Yale GR. (2007) 'Minireview: Targeting the Wnt/_-Catenin Pathway to Regulate Bone Formation in the Adult Skeleton', *Endocrinology*, 148(6), pp. 2635–2643. doi: 10.1210/en.2007-0270.
- Beavon IR. (2000) 'The E-cadherin-catenin complex in tumour metastasis: structure, function and regulation.', *Eur J Cancer.*, 36(13 Spec No), p. 1607–20.
- Beer TM, Armstrong AJ, Rathkopf DE, et al. (2014) 'Enzalutamide in metastatic prostate cancer before chemotherapy.', *N Engl J Med.*, 371, pp. 424–433. doi: 10.1056/NEJMoa1405095.
- Bianco P. (2015) 'Stem cells and bone : A historical perspective', *Bone*. Elsevier B.V., 70, pp. 2–9. doi: 10.1016/j.bone.2014.08.011.

- Blaszczyk N, Masri BA, Mawji NR, et al. (2004) 'Osteoblast-Derived Factors Induce Androgen-Independent Proliferation and Expression of Prostate-Specific Antigen in Human Prostate Cancer Cells', Clinical Cancer Research, 10(5), p. 1860 LP-1869.
- Bonewald LF and Johnson ML. (2008) 'Osteocytes, mechanosensing and Wnt signaling.', 42(4), pp. 606–615. doi: 10.1016/j.bone.2007.12.224.
- Bostwick DG, Burke HB, Djakiew D et al. (2004) 'Human prostate cancer risk factors', *Cancer*, 101(10 SUPPL.), pp. 2371–2490. doi: 10.1002/cncr.20408.
- Boyle WJ, Simonet WS and Lacey DL. (2003) 'Osteoclast differentiation and activation', *Nature*, 423(May), pp. 337–342.
- Brabletz T, Hlubek F, Spaderna S and Schmalhofer O. (2005) 'Invasion and Metastasis in Colorectal Cancer : Epithelial-Mesenchymal Transition , Stem Cells and β-Catenin', *Cells Tissues Organs*, pp. 56–65. doi: 10.1159/000084509.
- Bray F, Lortet-Tieulent J, Ferlay J et al. (2010) 'Prostate cancer incidence and mortality trends in 37 European countries: An overview', *European Journal of Cancer*. Elsevier Ltd, 46(17), pp. 3040–3052. doi: 10.1016/j.ejca.2010.09.013.
- Bronner F and Farach-Carson MC. (2009) *Bone and Cancer*. Edited by Bronner F and Farach-Carson MC. ISBN 9781848820180
- Buenrostro D, Park SI, and Storling JA. (2014) 'Sterling, Dissecting the role of bone marrow stromal cells on bone metastases.', *Biomed Res Int*, p. 875305.
- Buijs JT, Van der Pluijm G. (2009) 'Osteotropic cancers : From primary tumor to bone', *Cancer Letters*. Elsevier Ireland Ltd, 273(2), pp. 177–193. doi: 10.1016/j.canlet.2008.05.044.
- Bussemakers MJ, Van Bokhoven A, Tomita K, et al. (2000) 'Complex cadherin expression in human prostate cancer cells.', *Int. J. Cancer*, 450(June 1999), pp. 446–450.
- Carlinfante G, Vassiliou D, Svensson O, et al. (2003) 'Differential expression of osteopontin and bone sialoprotein in bone metastasis of breast and prostate carcinoma.', *Clin Exp Metastasis.*, 20(5), pp. 437–44.
- Cauchi C, Saracino B, Lopez M, Bonadonna G. (2011) *Carcinoma della Prostata. In: Lopez M. Oncologia medica pratica*. III. Edited by Società Editrice Universo.
- Chandrasekar T, Yang JC, Gao AC and Evans CP. (2015) 'Mechanisms of resistance in castration-resistant prostate cancer (CRPC).', *Translational andrology and urology*, 4(3), pp. 365–80. doi: 10.3978/j.issn.2223-4683.2015.05.02.
- Chang AJ, Autio KA, Roach M III and Scher HI (2014) 'High-risk prostate cancer—classification and therapy', *Nat. Rev. Clin. Oncol*, 11, pp. 308–323. doi: 10.1038/nrclinonc.2014.68.
- Chen WY, Liu SY, Chang YS, et al. (2015) 'MicroRNA-34a regulates WNT/TCF7 signaling and inhibits bone metastasis in Ras-activated prostate cancer.', *Oncotarget.*, 6(1), p. 441–457. doi: 10.18632/oncotarget.2690.

- Chiao JW, Moonga BS, Yang YM, et al. (2000) 'Endothelin-1 from prostate cancer cells is enhanced by bone contact which blocks osteoclastic bone resorption', *British journal of cancer*, 83(3), pp. 360–365.
- Coleman R, Costa L, Saad F, et al. (2011) 'Consensus on the utility of bone markers in the malignant bone disease setting', *Critical Reviews in Oncology / Hematology*. Elsevier Ireland Ltd, 80(3), pp. 411–432. doi: 10.1016/j.critrevonc.2011.02.005.
- Coleman RE. (2001) 'Metastatic bone disease : clinical features , pathophysiology and treatment strategies', *Cancer Treat Res*, 27, pp. 165–176. doi: 10.1053/ctr.
- Coleman, RE (2006) 'Clinical Features of Metastatic Bone Disease and Risk of Skeletal Morbidity', *Clin Cancer Res*, 12, p. 6243s–6249s. doi: 10.1158/1078-0432.CCR-06-0931.
- Coleman R, Body JJ, Aapro M, et al. (2014) 'Bone health in cancer patients: ESMO Clinical Practice Guidelines.', *Ann Oncol*, 25(Suppl 3), p. iii124-37. doi: 10.1093/annonc/mdu103.
- Coleman R, Gnant M , Morgan G and Clézardin P. (2012) 'Effects of Bone-Targeted Agents on Cancer Progression and Mortality', *JNCI: Journal of the National Cancer Institute*, 104(14), pp. 1059–1067. doi: doi.org/10.1093/jnci/djs263.
- Croucher PI, Mcdonald MM and Martin TJ. (2016) 'Bone metastasis : the importance of the neighbourhood', *Nat Rev Cancer*. Nature Publishing Group, 16(6), pp. 373–386. doi: 10.1038/nrc.2016.44.
- Dallas SL, Prideaux M and Bonewald L. (2013) 'The Osteocyte: An Endocrine Cell . . . and More', *Endocr Rev*, 34(5), pp. 658–690. doi: 10.1210/er.2012-1026.
- Danilin S, Merkel AR, Johnson JR, et al. (2012) 'Myeloid-derived suppressor cells expand during breast cancer progression and promote tumor-induced bone destruction.', *Oncoimmunology*, 1(9), p. 1484–1494.
- Danovi SA (2010) 'Tumorigenesis: Hormonally driven', *Nat Rev Cancer*, 10(7), p. 45.
- de Bono J., Oudard S, Ozguroglu M, et al. (2010) 'Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment : a randomised open-label trial', *The Lancet*. Elsevier Ltd, 376(9747), pp. 1147–1154. doi: 10.1016/S0140-6736(10)61389-X.
- de Bono JS, Logothetis CJ, Molina A, et al. (2012) 'Abiraterone and increased survival in metastatic prostate cancer.', *N Engl J Med*, 364(21), pp. 1995–2005. doi: 10.1056/NEJMoa1014618.Abiraterone.
- de la Piedra C, Castro-Errecaborde NA, Traba ML, et al. (2003) 'Bone remodeling markers in the detection of bone metastases in prostate cancer', *Clin Chim Acta*, 331(1–2), pp. 45–53. doi: 10.1016/S0009-8981(03)00081-0.
- Demers MJ, Thibodeau S, Noël D, et al. (2009) 'Intestinal epithelial cancer cell anoikis resistance: EGFR-mediated sustained activation of Src overrides Fak-dependent signaling to MEK/Erk and/or

PI3-K/Akt-1.', *J Cell Biochem.*, 107(4), p. 639–54. doi: doi: 10.1002/jcb.22131.

- Derynck R and Arkhurst R. (2007) 'Differentiation plasticity regulated by TGF-beta family proteins in development and disease.', *Nat Cell Biol.*, 9(9), pp. 1000–4.
- Desgrosellier JS, Cheresh DA. (2010) 'Integrins in cancer: biological implications and therapeutic opportunities.', *Nature reviews Cancer.*, 10(1), pp. 9–22. doi: doi:10.1038/nrc2748.
- Djulbegovic M, Beyth RJ, Neuberger MM et al. (2010) 'Screening for prostate cancer: systematic review and meta-analysis of randomised controlled trials', *Bmj*, 341(sep14 1), pp. c4543–c4543. doi: 10.1136/bmj.c4543.
- Dougall WC and Chaisson M. (2006) 'The RANK / RANKL / OPG triad in cancer-induced bone diseases', *Cancer Metastasis Rev*, pp. 541–549. doi: 10.1007/s10555-006-9021-3.
- Dougall WC, Holen I, González Suárez E. (2014) 'Targeting RANKL in metastasis.', *Bonekey Rep*, 3, p. 519. doi: 10.1038/bonekey.2014.14.
- Duchartre Y, Kim YM, Kahn M. (2016) 'The Wnt signaling pathway in cancer.', *Crit Rev Oncol Hematol.*, 99, pp. 141–9. doi: 10.1016/j.critrevonc.2015.12.005.
- Dunn GP, Bruce AT, Ikeda H, et al. (2002) 'Cancer immunoediting: from immunosurveillance to tumor escape.', *Nat Immunol.*, 3(11), pp. 991–8.
- Epstein JI, Egevad L, Amin MB, et al. (2015) 'The 2014 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma', *The American Journal of Surgical Pathology*, 29(9), p. 1. doi: 10.1097/PAS.0000000000000530.
- Ferreira A, Alho I and Casimiro S. (2015) 'Bone remodeling markers and bone metastases : From cancer research to clinical implications', *Bonekey*, 668(December 2014), pp. 1–9. doi: 10.1038/bonekey.2015.35.
- Fitzpatrick JM and de Wit R. (2014) 'Taxane Mechanisms of Action : Potential Implications for Treatment Sequencing in Metastatic Castration-resistant Prostate Cancer', *European Urology*, 65(6), pp. 1198–1204. doi: 10.1016/j.eururo.2013.07.022.
- Fizazi K, Carducci M, Smith M, et al. (2011) 'Denosumab versus zoledronic acid for treatment of bone metastases in men with castration-resistant prostate cancer: a randomised, double-blind study.', *Lancet*, 377(9768), pp. 813–22. doi: 10.1016/S0140-6736(10)62344-6.
- Fizazi K, Higano CS, Nelson JB, et al. (2013) 'Phase III, randomized, placebo-controlled study of docetaxel in combination with zibotentan in patients with metastatic castration-resistant prostate cancer.', *J Clin Oncol.*, 31(14), p. 1740–7. doi: doi: 10.1200/JCO.2012.46.4149.
- Fizazi K, Scher HI, Molina A, et al. (2012) 'Abiraterone acetate for treatment of metastatic castration-resistant prostate cancer: final overall survival analysis of the COU-AA-301 randomised, double-blind, placebo-controlled phase 3 study.', *Lancet Oncol*, 13, pp. 983–92.
- Frisch SM, Vuori K, Ruoslahti E, Chan-Hui PY. (1996) 'Control of adhesion-dependent cell survival by focal adhesion kinase. 8707856', *J Cell Biol.*, 134(3), pp. 793–9.

- Frisch SM, Screaton RA. (2001) 'Anoikis mechanisms.', *Curr Opin Cell Biol.*, 13(5), pp. 555–62.
- Furusato B, Tan S, Young D, et al. (2010) 'ERG oncoprotein expression in prostate cancer : clonal progression of ERG-positive tumor cells and potential for ERG-based stratification', *Prostate Cancer and Prostatic Diseases*. Nature Publishing Group, 13(3), pp. 228–237. doi: 10.1038/pcan.2010.23.
- Gabrilovich DI, Ostrand-Rosenberg S and Bronte V. (2012) 'Coordinated regulation of myeloid cells by tumours', *Nat Rev Immunol*, 12(4), p. 253–68.
- Gallaher IS and Vessella RL. (2009) 'Disseminated Tumor Cells in Prostate Cancer Patients after Radical Prostatectomy and without Evidence of Disease Predicts Biochemical Recurrence', 15(2), pp. 677–683. doi: 10.1158/1078-0432.CCR-08-1754.Disseminated.
- Goldring SR and Goldring MB. (2007) 'Eating bone or adding it : the Wnt pathway decides', *Nat Med*, 13(2), pp. 133–134.
- Gonzalez-Suarez E, Jacob AP, Jones J, et al. (2010) 'RANK ligand mediates progestin-induced mammary epithelial proliferation and carcinogenesis.', *Nature*, 468(7320), pp. 103–7. doi: 10.1038/nature09495.
- Gratchev A, Kzhyshkowska J, Köthe K, et al. (2006) 'Mphi1 and Mphi2 can be re-polarized by Th2 or Th1 cytokines, respectively, and respond to exogenous danger signals.', *Immunobiology*, 211(6–8), p. 473–86.
- Gravdal K, Halvorsen OJ, Haukaas SA, Akslen LA. (2007) 'A switch from E-cadherin to N-cadherin expression indicates epithelial to mesenchymal transition and is of strong and independent importance for the progress of prostate cancer.', *Clin Cancer Res.*, 13(23), p. 7003–11.
- Guise TA. (2000) 'Molecular Mechanisms of Osteolytic Bone Metastases', pp. 2892–2898.
- Hall CL, Bafico A, Dai J, et al. (2005) 'Prostate Cancer Cells Promote Osteoblastic Bone Metastases through Wnts', *Cancer research*, 65(17), pp. 7554–7561. doi: 10.1158/0008-5472.CAN-05-1317.
- Hanahan D and Weinberg RA. (2011) 'Hallmarks of cancer: The next generation', *Cell*. Elsevier Inc., 144(5), pp. 646–674. doi: 10.1016/j.cell.2011.02.013.
- Harris V, Lloyd K, Forsey S, et al. (2011) 'Short Report A Population-based Study of Prostate Cancer Chemotherapy', *Clinical Oncology*. Elsevier Ltd, 23(10), pp. 706–708. doi: 10.1016/j.clon.2011.04.014.
- Harris WP, Mostaghel EA, Nelson PS and Montgomery B. (2009) 'Androgen deprivation therapy: progress in understanding mechanisms of resistance and optimizing androgen depletion', *Nat Clin Pract Urol.*, 6(2), pp. 76–85. doi: 10.1038/ncpuro1296.Androgen.
- Heinlein CA, Chang C. (2004) 'Androgen Receptor in Prostate Cancer', *Endocrine Reviews*, 25(2), pp. 276–308. doi: 10.1210/er.2002-0032.
- Henry DH, Costa L, Goldwasser F, et al. (2011) 'Randomized, double-blind study of denosumab versus zoledronic acid in the treatment of bone metastases in patients with advanced cancer

(excluding breast and prostate cancer) or multiple myeloma.', *J Clin Oncol.*, 29(9), p. 1125–32. doi: 10.1200/JCO.2010.31.3304.

- Higano CS, Schellhammer PF, Small EJ et al. (2009) 'Integrated Data From 2 Randomized , Phase 3 Trials of Active Cellular Immunotherapy With Sipuleucel-T in Advanced Prostate Cancer', *Cancer*, 115(16):3670-9.doi: 10.1002/cncr.24429.
- Hlaing TT and Compston JE. (2014) 'Biochemical markers of bone turnover – uses and limitations Bone formation', *Annals of Clinical Biochemistry*, 51(2), pp. 189–202. doi: 10.1177/0004563213515190.
- Huang WC, Wu D, Xie Z et al. (2006) 'Beta2-Microglobulin Is a Signaling and Growth-Promoting Factor for Human Prostate Cancer Bone Metastasis.', *Cancer research*, 66(18), pp. 9108–9116. doi: 10.1158/0008-5472.CAN-06-1996.
- Huang CF, Lira C, Chu K, et al. (2010) 'Cadherin-11 increases migration and invasion of prostate cancer cells and enhances their interaction with osteoblasts.', *Cancer research.*, 70(11), pp. 4580–4589. doi: 10.1158/0008-5472.CAN-09-3016.
- Humphrey PA. (2004) 'Gleason grading and prognostic factors in carcinoma of the prostate', *Modern Pathology*, 17(3), pp. 292–306. doi: 10.1038/modpathol.3800054.
- Iuliani M, Pantano F, Buttigliero C et al. (2015) 'Biological and clinical effects of abiraterone on anti-resorptive and anabolic activity in bone microenvironment.', *Oncotarget*, 6(14), pp. 12520–8. doi: 10.18632/oncotarget.3724.
- Iyer SP, Beck JT, Stewart AK, et al. (2014) 'A Phase IB multicentre dose-determination study of BHQ880 in combination with anti-myeloma therapy and zoledronic acid in patients with relapsed or refractory multiple myeloma and prior skeletal-related events.', *Br J Haematol.*, 167(3), pp. 366–75. doi: doi: 10.1111/bjh.13056.
- James ND, Sydes MR, Clarke NW, et al. (2016) 'Addition of docetaxel, zoledronic acid, or both to first-line long-term hormone therapy in prostate cancer (STAMPEDE): survival results from an adaptive, multiarm, multistage, platform randomised controlled trial.', *Lancet*, 387(10024), pp. 1163–1177. doi: 10.1016/S0140-6736(15)01037-5.
- Jennbacken K, Tesan T, Wang W, et al. (2010) 'N-cadherin increases after androgen deprivation and is associated with metastasis in prostate cancer', *Endocrine-Related Cancer* (2010) 17 pp. 469–479. doi: 10.1677/ERC-10-0015.
- Jung K and Lein M. (2014) 'Biochimica et Biophysica Acta Bone turnover markers in serum and urine as diagnostic , prognostic and monitoring biomarkers of bone metastasis', *BBA - Reviews on Cancer*, 1846(2), pp. 425–438. doi: 10.1016/j.bbcan.2014.09.001.
- Kalluri R and Weinberg RA. (2009) 'The basics of epithelial-mesenchymal transition', *Journal of Clinical Investigation*, 119(6), pp. 1420–1428. doi: 10.1172/JCI39104.1420.
- Kandalaft LE, Facciabene A, Buckanovich RJ, Coukos G. (2009) 'Endothelin B receptor, a new target in cancer immune therapy', *Clin Cancer Res*, 15(14), pp. 4521–8. doi: 10.1158/1078-

0432.CCR-08-0543.

- Kang Y, Siegel PM, Shu W, et al. (2003) 'A multigenic program mediating breast cancer metastasis to bone', *Cancer Cell*, 3(6), pp. 537–549. doi: 10.1016/S1535-6108(03)00132-6.
- Karin J, Mukta W, Dafna B and Kleinman Hynda K (1999) 'Osteonectin Promotes Prostate Cancer Cell Migration and Invasion : A Possible Mechanism for Metastasis to Bone', *Cancer Research*, 59, pp. 4453–4457.
- Katsimbri P. (2017) 'The biology of normal bone remodelling', *Eur J Cancer Care*, pp. 1–5. doi: 10.1111/ecc.12740.
- Krieg M, Weisser H and Tunn S. (1995) 'Potential Activities of Androgen Metabolizing Enzymes in Human Prostate', *Chemistry, Clinical*, 53(1), pp. 395–400.
- Kwon ED, Drake CG, Scher HI et al. (2015) 'Ipilimumab versus placebo after radiotherapy in patients with metastatic castration-resistant prostate cancer that had progressed after docetaxel chemotherapy (CA184-043): a multicentre, randomised, double-blind, phase 3 trial', *Lancet Oncology*, 15(7), pp. 700–712. doi: 10.1016/S1470-2045(14)70189-5.
- Lacey DL, Boyle WJ, Simonet WS, et al (2012) 'Bench to bedside: elucidation of the OPG-RANK-RANKL pathway and the development of denosumab.', *Nat Rev Drug Discov*, 11(5), pp. 401–19. doi: 10.1038/nrd3705.
- Lamb AD, Massie CE and Neal DE. (2014) 'The transcriptional programme of the androgen receptor (AR) in prostate cancer', *BJU Int*, 113, pp. 358–366. doi: 10.1111/bju.12415.
- Lamouille S, Xu J and Derynck R. (2014) 'Molecular mechanisms of epithelial–mesenchymal transition', *Nature Reviews Molecular Cell Biology*, 15(3), pp. 178–196. doi: 10.1038/nrm3758.
- Langley RR and Fidler IJ. (2007) 'Tumor cell-organ microenvironment interactions in the pathogenesis of cancer metastasis', *Endocrine Reviews*, 28(3), pp. 297–321. doi: 10.1210/er.2006-0027.
- Lee H, Pienta KJ, Kim WJ and Cooper CR. (2003) 'The effect of bone-associated growth factors and cytokines on the growth of prostate cancer cells derived from soft tissue versus bone metastases in vitro', *International Journal of Oncology*, 22, pp. 921–926.
- Lee YC, Jin JK, Cheng CJ, et al. (2013) 'Targeting constitutively activated β1 integrins inhibits prostate cancer metastasis.', *Mol Cancer Res.*, 11(4), pp. 405–17. doi: doi: 10.1158/1541-7786.MCR-12-0551.
- Lian JB. and Stein GS. (2003) 'Runx2 / Cbfa1 : A Multifunctional Regulator of Bone Formation', *Current Pharmaceutical Design*, 9, pp. 2677–2685.
- Liotta LA and Kohn EC. (2001) 'The microenvironment of the tumour-host interface.', *Nature*, 411(6835), pp. 375–379. doi: 10.1038/35077241.
- Loser K, Mehling A, Loeser S, et al. (2006) 'Epidermal RANKL controls regulatory T-cell numbers via activation of dendritic cells.', *Nat Med*, 12(12), pp. 1372–9.

- Luckman SP, Hughes DE, Coxon FP, et al. (1998) 'Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras.', *J Bone Miner Res.*, 13(4), pp. 581–9.
- Lutz S, Chow E. (2012) 'A review of recently published radiotherapy treatment guidelines for bone metastases: contrasts or convergence?', *J Bone Oncol*, 1, pp. 18–23.
- Manca P, Pantano F, Iuliani M et al. (2017) 'Determinants of bone specific metastasis in prostate cancer', *Critical Reviews in Oncology / Hematology*. Elsevier Ireland Ltd, 112, pp. 59–66. doi: 10.1016/j.critrevonc.2017.02.013.
- Manni A, Pardridge WM, Cefalu W et al. (2015) 'Bioavailability of Albumin-Bound Testosterone *', *Journal of Clinical Endocrinology*, 61(4), p. 705-10
- Martin M, Bell R, Bourgeois H, et al. (2012) 'Bone-related complications and quality of life in advanced breast cancer: results from a randomized phase III trial of denosumab versus zoledronic acid.', *Clin Cancer Res.*, 18(17), p. 4841–9.
- McNeal JE, Redwine EA, Freiha FS, Stamey TA. (1988) 'Zonal Distribution of Prostatic Adenocarcinoma', *The American Journal of Surgical Pathology*, 12(12), pp. 897–906.
- Miller K, Moul JW, Gleave M et al. (2013) 'Phase III, randomized, placebo-controlled study of once-daily oral zibotentan (ZD4054) in patients with non-metastatic castration-resistant prostate cancer.', *Prostate Cancer Prostatic Dis.*, 16(2), p. 187–92. doi: doi: 10.1038/pcan.2013.2.
- Mohme M, Riethdorf S, Pantel K. (2017) 'Circulating and disseminated tumour cells — mechanisms of immune surveillance and escape', *Nat Rev Clin Oncol*, 14(3), pp. 155–167. doi: 10.1038/nrclinonc.2016.144.
- Mönkkönen H, Auriola S, Lehenkari P et al.(2006) 'A new endogenous ATP analog (ApppI) inhibits the mitochondrial adenine nucleotide translocase (ANT) and is responsible for the apoptosis induced by nitrogen-containing bisphosphonates.', *Br J Pharmacol.*, 147(4), p. 437–45.
- Mottet N, Bellmunt J, Bolla M et al. (2017) 'EAU-ESTRO-SIOG Guidelines on Prostate Cancer. Part 1: Screening, Diagnosis, and Local Treatment with Curative Intent', *European Urology*, 71(4), pp. 618–629. doi: 10.1016/j.eururo.2016.08.003.
- Mountzios G, Dimopoulos M, Bamias A et al. (2007) 'Abnormal bone remodeling process is due to an imbalance in the receptor activator of nuclear factor k B ligand (RANKL)/osteoprotegerin (OPG) axis in patients with solid tumors metastatic to the skeleton', *Acta Oncologica*, 46, p. 221-229. doi: 10.1080/02841860600635870.
- Mundy GR. (1997) 'Malignancy and the Skeleton', *Horm Metab Res*, 29(3), pp. 120–7.
- Mundy GR. (2002) 'Metastasis to bone : causes , consequences and therapeutic opportunities', *Nat Rev Cancer*, 2, pp. 584–593. doi: 10.1038/nrc867.
- Nelson J, Bagnato A, Battistini B, Nisen P. (2003) 'The endothelin axis: emerging role in cancer.', *Nat Rev Cancer*, 3(2), pp. 110–6.

- Nelson JB, Fizazi K, Miller K, et al. (2012) 'Phase 3, randomized, placebo-controlled study of zibotentan (ZD4054) in patients with castration-resistant prostate cancer metastatic to bone.', *Cancer*, 118(22), pp. 5709–18. doi: 10.1002/cncr.27674.
- Nelson JB, Hedican SP, George DJ et al. (1995) 'Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate.', *Nat Med*, 1(9), pp. 944–9.
- Nelson JB, Love W, Chin JL, et al. (2008) 'Phase 3, Randomized Controlled Trial of Atrasentan in Patients with Nonmetastatic Hormone-Refractory Prostate Cancer.', *Cancer*, 113(9), pp. 2478–2487. doi: 10.1002/cncr.23864.
- Nelson JB, Nguyen SH, Wu-Wong JR, et al. (1999) 'New bone formation in an osteoblastic tumor model is increased by endothelin-1 overexpression and decreased by endothelin a receptor blockade', *Urology*, 53(5), pp. 1063–9.
- Nieto MA. (2017) 'Context-specific roles of EMT programmes in cancer cell dissemination', *Nature Cell Biology*, 19(5), pp. 416–418. doi: 10.1038/ncb3520.
- O'Donnell A, Judson I, Dowsett M, et al. (2004) 'Hormonal impact of the 17alpha-hydroxylase/C(17,20)-lyase inhibitor abiraterone acetate (CB7630) in patients with prostate cancer.', *British journal of cancer*, 90(12), pp. 2317–25. doi: 10.1038/sj.bjc.6601879.
- Ottewell PD. (2016) 'The role of osteoblasts in bone metastasis', *Journal of Bone Oncology*, 5(3), pp. 124–127. doi: 10.1016/j.jbo.2016.03.007.
- Ozdamar O, Yildiz L and Atilla MK. (1996) 'Intraobserver and Interobserver Reproducibility of WHO and Gleason Histologic Grading Systems in Prostatic Adenocarcinomas', *International Urology and Nephrology*, 28(1), pp. 73–77.
- Paller CJ, Antonarakis ES. (2011) 'Cabazitaxel : a novel second-line treatment for metastatic castration-resistant prostate cancer', *Drug Design, Development and Therapy*, 5, pp. 117–124. doi: 10.2147/DDDT.S13029.
- Pantano F, Ribelli G, Iuliani M, et al. (2016) 'New Molecular Targets in Metastatic Prostate Cancer', *Journal of Cancer Therapy*, 7(6), p. 14. doi: 10.4236/jct.2016.76042.
- Pardoll DM. (2012) 'The blockade of immune checkpoints in cancer immunotherapy'. *Nat Rev Cancer*, 12, p. 252-64. doi: 10.1038/nrc3239.
- Parker C, Nilsson S, Heinrich D et al. (2013) 'Alpha Emitter Radium-223 and Survival in Metastatic Prostate Cancer', *N Engl J Med*, 369(3), pp. 213–223. doi: 10.1056/NEJMoa1213755.
- Pham H and Ziboh VA. (2002) '5 α-Reductase-catalyzed conversion of testosterone to dihydrotestosterone is increased in prostatic adenocarcinoma cells : suppression by 15-lipoxygenase metabolites of gamma-linolenic and eicosapentaenoic acids', *Journal of Steroid Biochemistry & Molecular Biology*, 82, pp. 393–400.
- Polyak K and Weinberg RA. (2009) 'Transitions between epithelial and mesenchymal states : acquisition of malignant and stem cell traits', *Nat Rev Cancer*, 9, p. 265-73. doi: 10.1038/nrc2620.

- Prisca H, Luger NM, Sabino MAC, et al. (2000) 'Osteoprotegerin blocks bone cancer-induced skeletal destruction , skeletal pain and pain-related neurochemical reorganization of the spinal cord', *Nature Medicine*, 6(5), p. 521-8.
- Rachner TD, Thiele S, Göbel A, et al. (2014) 'High serum levels of Dickkopf-1 are associated with a poor prognosis in prostate cancer patients', *BMC Cancer*, 2, p. 649. doi: 10.1186/1471-2407-14-649.
- Raisz LG. (1999) 'Physiology and Pathophysiology of Bone Remodeling', *Clinical Chemistry*, 45(8), pp. 1353–1358.
- Ricci E, Mattei E, Dumontet C et al. (2013) 'Increased expression of putative cancer stem cell markers in the bone marrow of prostate cancer patients is associated with bone metastasis progression.', *Prostate.*, 73(16), pp. 1738–46. doi: doi: 10.1002/pros.22689.
- Roelofs AJ, Thompson K, Gordon S, Rogers MJ. (2006) 'Molecular mechanisms of action of bisphosphonates: current status.', *Clin Cancer Res.*, 12(20 Pt 2), p. 6222s–6230s.
- Roodman GD. (2004) 'Mechanisms of Bone Metastasis', *N Engl J Med*, 350(16), pp. 1655–1664.
- Ryan CJ, Smith MR, de Bono JS, et al. (2013) 'Abiraterone in Metastatic Prostate Cancer without Previous Chemotherapy', *New Engl J Med*, 368, pp. 138–48. doi: 10.1056/NEJMoa1209096.
- Paget S. (1989) 'The distribution of secondary growths in cancer of the breast', *Cancer Metastasis Rev*, 8(2), pp. 98–101.
- Saad F, Gleason DM, Murray R et al. (2002) 'Zoledronic Acid Prostate Cancer Study Group. A randomized, placebo-controlled trial of zoledronic acid in patients with hormone-refractory metastatic prostate carcinoma', *J Natl Cancer Inst.*, 94(19), p. 1458–68.
- Saad F, Gleason DM, Murray R et al. (2004) 'Zoledronic Acid Prostate Cancer Study Group. Long-term efficacy of zoledronic acid for the prevention of skeletal complications in patients with metastatic hormone-refractory prostate cancer.', *J Natl Cancer Inst.*, 96(11), p. 879–82.
- Saha B, Arase A, Imam SS et al. (2008) 'Overexpression of E-cadherin and beta-catenin proteins in metastatic prostate cancer cells in bone.', *Prostate.*, 68(1), pp. 78–84.
- Santini D, Perrone G, Roato I et al. (2011) 'Expression pattern of receptor activator of NFκB (RANK) in a series of primary solid tumors and related bone metastases.', *J Cell Physiol*, 226(3), pp. 780–4. doi: 10.1002/jcp.22402.
- Santini D, Schiavon G, Vincenzi B, et al. (2011) 'Receptor Activator of NF-kB (RANK) Expression in Primary Tumors Associates with Bone Metastasis Occurrence in Breast Cancer Patients.', *PLoS ONE.*, 6(4), p. e19234. doi: 10.1371/journal.pone.0019234.
- Santini Daniele, Ripamonti Carla, Zoccoli Alice et al. (2016) 'Bone-Targeted Agents', in Bertoldo F., Boccardo F., Bombardieri E., Evangelista L., V. R. (ed.) *Bone Metastases from Prostate Cancer*, ISBN 3319423274 pp. 181–192. https://doi.org/10.1007/978-3-319-42327-2_15.
- Sartor O, Reid RH, Bushnell D. et al. (2007) 'Safety and efficacy of repeat administration of

samarium Sm-153 lexidronam to patients with metastatic bone pain.', *Cancer*, 109, pp. 637–43.

- Sceneay J, Smyth MJ and Möller A. (2013) 'The pre-metastatic niche: Finding common ground', *Cancer and Metastasis Reviews*, 32(3–4), pp. 449–464. doi: 10.1007/s10555-013-9420-1.
- Scher HI, Fizazi K, Saad F, et al. (2012) 'Increased Survival with Enzalutamide in Prostate Cancer after Chemotherapy', *N Engl J Med*, 367(13), pp. 1187–1197. doi: 10.1056/NEJMoa1207506.
- Seibel MJ. (2005) 'Biochemical Markers of Bone Turnover Part I : Biochemistry and Variability', *Clin Biochem Rev*, 26, pp. 97–122.
- Shafi AA, Yen AE and Weigel NL. (2013) 'Androgen receptors in hormone-dependent and castration-resistant prostate cancer', *Pharmacology & Therapeutics*, 140(3), pp. 223–238. doi: 10.1016/j.pharmthera.2013.07.003.
- Siegsmund MJ, Yamazaki H, Pastan I. Interleukin 6 receptor mRNA in prostate carcinomas and benign prostate hyperplasia J Urol, 151 (1994), pp. 1396-1399
- Sikes RA, Nicholson BE, Koeneman KS et al. (2004) 'Cellular interactions in the tropism of prostate cancer to Bone', *Int J Cancer*, 503, pp. 497–503. doi: 10.1002/ijc.20153.
- Silberstein EB. (1996) 'Dosage and response in radiopharmaceutical therapy of painful osseous metastases.', *J Nucl Med*, 37, pp. 249–52.
- Simpson CD, Anyiwe K, Schimmer AD. (2008) 'Anoikis resistance and tumor metastasis.', *Cancer Lett.*, 272(2), pp. 177–85. doi: doi: 10.1016/j.canlet.2008.05.029.
- Sinder BP, Pettit AR, McCauley LK. (2015) 'Macrophages: Their Emerging Roles in Bone', *Journal of bone and mineral research*, 30(12), p. 2140–2149. doi: doi:10.1002/jbmr.2735.
- Smith HA and Kang Y. (2013) 'The metastasis-promoting roles of tumor-associated immune cells', *Journal of Molecular Medicine*, 91(4), pp. 411–429. doi: 10.1007/s00109-013-1021-5.
- Smith MR, Eastham J, Gleason DM et al. (2003) 'Randomized controlled trial of zoledronic acid to prevent bone loss in men receiving androgen deprivation therapy for nonmetastatic prostate cancer.', *J Urol.*, 169(6), pp. 2008–12.
- Smith MR, Halabi S, Ryan CJ et al. (2014) 'Randomized controlled trial of early zoledronic acid in men with castration-sensitive prostate cancer and bone metastases: results of CALGB 90202 (alliance).', *J Clin Oncol.*, 32(11), p. 1143–50. doi: 10.1200/JCO.2013.51.6500.
- Sobin LH, Gospodariwicz M, Wittekind C. (2009) *TNM clasification of malignant tumours. UICC Internetional Union Against Cancer.* 7th edn. Edited by Wittekind C, Sobin LH, Gospodariwicz M. John Wiley & Sons.
- Stopeck AT, Lipton A, Body JJ et al. (2010) 'Denosumab compared with zoledronic acid for the treatment of bone metastases in patients with advanced breast cancer: a randomized, double-blind study.', *J Clin Oncol.*, 28(35), pp. 5132–9. doi: 10.1200/JCO.2010.29.7101.
- Studer UE, Hauri D, Hanselmann S et al. (2004) 'Immediate versus deferred hormonal treatment for patients with prostate cancer who are not suitable for curative local treatment: Results of the

randomized trial SAKK 08/88', *Journal of Clinical Oncology*, 22(20), pp. 4109–4118. doi: 10.1200/JCO.2004.11.514.

- Sun Y, Campisi J, Higano C, et al. Treatment-induced damage to the tumor microenvironment promotes prostate cancer therapy resistance through WNT16B. Nature medicine. 2012;18(9):1359-1368. doi:10.1038/nm.2890
- Swann JB, Smith M. (2007) 'Immune surveillance of tumors.', *J Clin Invest.*, 117(5), pp. 1137–46.
- Takahashi N, Maeda K, Ishihara A et al. (2011) 'Regulatory mechanism of osteoclastogenesis by RANKL and Wnt signals.', *Front Biosci*, 16, pp. 21–30.
- Takarada T, Hinoi E, Nakazato R et al. (2013) 'An analysis of skeletal development in osteoblast-specific and chondrocyte-specific runt-related transcription factor-2 (Runx2) knockout mice', *Journal of Bone and Mineral Research*, 28(10), pp. 2064–2069. doi: 10.1002/jbmr.1945.
- Tamura D, Hiraga T, Myoui A et al. (2008) 'Cadherin-11-mediated interactions with bone marrow stromal/osteoblastic cells support selective colonization of breast cancer cells in bone.', *Int J Oncol.*, 33(1), p. 17–24.
- Tan W, Zhang W, Strasner A et al. (2011) 'Tumour-infiltrating regulatory T cells stimulate mammary cancer metastasis through RANKL-RANK signalling', *Nature*, 470(7335), pp. 548–53. doi: 10.1038/nature09707.
- Tannock IF, de Wit R, Berry WR et al. (2004) 'Docetaxel plus Prednisone or Mitoxantrone plus Prednisone for Advanced Prostate Cancer', *New Engl J Med*, 351:15, pp. 1502–1512. doi: 10.1056/NEJMoa040720.
- Tannock IF, Osoba D, Stockler MR et al. (1996) 'Chemotherapy With Mitoxantrone Plus Prednisone or Prednisone Alone for Symptomatic Hormone-Resistant Prostate Cancer: A Canadian Randomized Trial With Palliative End Points', *New Engl J Med*, 14(6), pp. 1756–1764.
- Taplin ME. (2001) 'The Endocrinology of Prostate Cancer', *The Journal of Clinical Endocrinology & Metabolism*, 86(8), pp. 3467–3477.
- Teitelbaum SL. (2000) 'Bone Resorption by Osteoclasts', *Science*, 289, pp. 1504–1508.
- Thalmann N, Edmund E, Hopwood VL et al. (1994) 'Androgen-independent Cancer Progression and Bone Metastasis in the LNCaP Model of Human Prostate Cancer', *Cancer Res*, 54, pp. 2577–2582.
- Thiery JP. (2002) 'Epithelial–mesenchymal transitions in tumour progression', *Nature Reviews Cancer*, 2(6), pp. 442–454. doi: 10.1038/nrc822.
- Thiery JP, Acloque H, Huang RY et al. (2009) 'Epithelial-Mesenchymal Transitions in Development and Disease', *Cell*, 139(5), pp. 871–890. doi: 10.1016/j.cell.2009.11.007.
- Thompson IM Jr, Tangen CM, Paradelo J, et al. (2006) 'Adjuvant Radiotherapy for Pathologically Advanced Prostate Cancer: A Randomized Clinical Trial', *JAMA*, 76, pp. 180–182.
- Thompson IM. (2001) 'Flare Associated with LHRH-Agonist Therapy', *Rev Urol*, 3, pp. 10–14.

- Thudi NK, Martin CK, Murahari S et al. (2011) 'Dickkopf-1 (DKK-1) stimulated prostate cancer growth and metastasis and inhibited bone formation in osteoblastic bone metastases', *The Prostate*, 71(6), pp. 615–625. doi: 10.1002/pros.21277.
- Tollefson M, Karnes JR, Thompson HR et al. (2010) 'A randomized phase II study of Ipilimumab with androgen ablation compared with androgen ablation alone in patients with advanced prostate cancer', *The Journal of Urology*, 183(4), p. e261.
- Tomlins SA, Rhodes DR, Perner S et al. (2005) 'Recurrent Fusion of TMPRSS2 and ETS Transcription Factor Genes in Prostate Cancer', *Science*, 310(5748), pp. 644–649. doi: 10.1126/science.1117679.
- Tran NL, Nagle RB, Cress AE and Heimark RL. (1999) 'N-Cadherin Expression in Human Prostate Carcinoma Cell Lines', *American Journal of Pathology*, 155(3), pp. 787–798.
- Ung JO, Richie JP, Chen MH et al. (2002) 'Evolution of the presentation and pathologic and biochemical outcomes after radical prostatectomy for patients with clinically localized prostate cancer diagnosed during the PSA era', *Urology*, 60(3), pp. 458–463. doi: 10.1016/S0090-4295(02)01814-9.
- Van Poznak C, Cross SS, Saggese M, et al. (2006) 'Expression of osteoprotegerin (OPG), TNF related apoptosis inducing ligand (TRAIL), and receptor activator of nuclear factor k B ligand (RANKL) in human breast tumours', *J Clin Pathol*, 59(1), pp. 56–64. doi: doi: 10.1136/jcp.2005.026534.
- Van Poznak CH, Temin S, Yee GC, et al. (2011) 'American Society of Clinical Oncology executive summary of the clinical practice guideline update on the role of bone-modifying agents in metastatic breast cancer.', *J Clin Oncol*, 29(9), pp. 1221–7. doi: 10.1200/JCO.2010.32.5209.
- Vessella RL and Corey E. (2006) 'Targeting Factors Involved in Bone Remodeling as Treatment Strategies in Prostate Cancer Bone Metastasis', *Clin Cancer Res*, 12, pp. 6285–6291. doi: 10.1158/1078-0432.CCR-06-0813.
- Waltregny D, Bellahcène A, de Leval X et al. (2000) 'Increased expression of bone sialoprotein in bone metastases compared with visceral metastases in human breast and prostate cancers.', *J Bone Miner Res.*, 15(5), pp. 834–43.
- Wang J, Shiozawa Y, Wang J et al. (2008) 'The role of CXCR7/RDC1 as a chemokine receptor for CXCL12/SDF-1 in prostate cancer.', *J Biol Chem.*, 283(7), p. 4283–94.
- Wang Y, Li YP, Paulson C et al. (2014) 'Wnt and the Wnt signaling pathway in bone development and Disease', *Front Biosci*, 19, pp. 379–407.
- Weilbaecher KN, Guise TA, McCauley LK. (2011) 'Cancer to bone: a fatal attraction', *Nat Rev Cancer*, 11(6), pp. 411–425. doi: 10.1038/nrc3055.Cancer.
- Windham TC, Parikh NU, Siwak DR et al. (2002) 'Src activation regulates anoikis in human colon tumor cell lines.', *Oncogene.*, 21(51), pp. 7797–807.

· Yin JJ, Mohammad KS, Käkönen SM et al. (2003) 'A causal role for endothelin-1 in the pathogenesis of osteoblastic bone metastases', *Proc Natl Acad Sci*, 100(19), pp. 10954–9.

· Yin JJ, Selander K, Chirgwin JM et al. (1999) 'TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development.', *J Clin Invest.*, 103(2), pp. 197–206.

· Zeigler-Johnson CM, Spangler E, Jalloh M et al. (2008) 'Genetic susceptibility to prostate cancer in men of African descent: implications for global disparities in incidence and outcomes', *The Canadian journal of urology*, 15(1), pp. 3872–3882.

· Zhang XHF, Jin X, Malladi S et al. (2013) 'Selection of bone metastasis seeds by mesenchymal signals in the primary tumor stroma', *Cell*, 154(5), pp. 1060–1073. doi: 10.1016/j.cell.2013.07.036.

· Zhang XHF, Wang Q, Gerald W et al. (2009) 'Latent Bone Metastasis in Breast Cancer Tied to Src-Dependent Survival Signals', *Cancer Cell*, 16(1), pp. 67–78. doi: 10.1016/j.ccr.2009.05.017.

· Zlotnik A, Burkhardt AM, Homey B. (2011) 'Homeostatic chemokine receptors and organ-specific metastasis.', *Nat Rev Immunol.*, 11(9), pp. 597–606. doi: doi: 10.1038/nri3049.

· Zonnenberg, BA and Voest EE. (2003) 'The Role of Endothelin in Hormone-Refractory Prostate Cancer', *European Urology Supplements*, 2(3), pp. 9–14. doi: http://dx.doi.org/10.1016/S1569-9056(02)00204-X.