Experimental Models of Prostate Cancer Bone Metastasis

- Establishment, characterization and imaging of xenograft bone metastasis models -

PhD thesis presented by

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No entiendes realmente algo a menos que seas capaz
de explicárselo a tu abuela.

Albert Einstein

Summary

In industrialized countries, prostate cancer (PCa) is the most common malignancy in men, but mortality rates are much lower than those recorded in developing countries, reflecting benefits from advances in early diagnosis and effective treatment. However, the metastatic disease rather than the primary tumour is responsible for much of the resulting morbidity and mortality. Skeletal metastases occur in more than 70% of cases of late-stage of PCa and they confer a high level of morbidity, a 5-year survival rate of 25% and median survival of approximately 40 months. Though fractures and spinal cord compression are potential complications, the most common symptom of bone metastases is pain. Bone metastases from PCa lead to an accelerated bone turnover state that features pathological activation of both osteoblasts and osteoclasts. Raised activation of osteoclasts is directly correlated with an increased incidence of skeletal complications, cancer progression and death. Further, once tumour metastasizes to bone, the metastatic disease become incurable and current therapies are palliative and mostly target either tumour cells or osteoclasts.

Thus, to better understand the biology of PCa bone metastasis and to investigate new therapy options it is crucial to develop new animal models.

In this thesis, we have established new experimental models of PCa bone metastasis by intraosseous (i.o.), intracardiac (i.c.) or intratibial (i.t.) inoculation of human PCa cells in immunodeficient mice. Extensive bone metastasis were monitored by *in vivo* bioluminescence imaging. Different strategies were performed to describe new molecular targets involved in the mechanisms of PCa bone metastasis and to make a suitable model for evaluating novel compounds as future therapeutic approaches.

To conclude, these models provide a reliable reproduction of the clinical situation and allows characterization and design effective treatments by better understanding the molecular mechanisms of PCa bone metastasis.

Resum

En països industrialitzats, el càncer de pròstata (CP) és la neoplasia més comunment diagnosticada en homes i la segona causa de mort relacionada amb càncer, donat que els nivells de mortalitat en aquesta població són molt més baixos que els que es troben en els països en desenvolupament, es veu un clar benefici en els avenços tant en el diagnòstic precoç com el desenvolupament de teràpies eficients. No obstant, la disseminació metastàtica més que el tumour primari en sí és la responsable dels problemes de mortalitat i morbiditat associats al CP. Les metàstasis esquelètiques estan presents en més d'un 70% dels casos de CP avançat i confereixen alts nivells de morbiditat, un 25% de supervivència als 5 anys i una mitjana de supervivència de 40 mesos després de ser diagnosticats. Tot i que les fractures patològiques i la compressió de la columna vertebral són les complicacions més probables pel pacient metastàtic, el major símptoma és el dolor. Les metàstasis òssies del CP comporten un estat d'acceleració de la remodelació òssia que es caracteritza per una activació patològica tant dels osteoblasts com dels osteoclasts. Aquesta elevada activació dels osteoclasts està directament correlacionada amb un increment en la incidència de les complicacions òssies, de la progressió tumoural i la mort. A més, una vegada el tumour metastatitza a os, la malaltia esdevé incurable i les teràpies actuals són solament pal·liatives i principalment es dirigeixen a les cèl·lules tumourals o als osteoclasts.

Per tant, per entendre millor la biologia de les metàstasis òssies del PC i poder investigar noves teràpies és important desenvolupar nous models animals.

En aquesta tesi, s'han establert nous models experimentals de metàstasis del CP mitjançant la inoculació de cèl·lules humanes en ratolins immunodeficients per diferents vies, intraòssia, intracardíaca o intratibial. Finalment, diferents estratègies s'han dut a terme per descriure noves dianes moleculars involucrades en el mecanisme de les metàstasis i poder desenvolupar un model adequat per la evaluació de possibles compostos candidats a ser futures aproximacions terapèutiques.

Per concloure, aquests models proporcionen una fiable reproducció de la situació clínica i permeten tant la caracterització com el diseny de tractaments efectibles per compendre millor els mecanismes moleculars de les metàstasis òssies del CP.

Acknowledgements

"Començaré pels agraïments que segurament serà el més senzill" vaig pensar el dia que vaig decidir escriure la tesi...mentida! És igual o més complicat que la resta ja que no m'agradaria deixar-me a ningú per agrair la seva aportació ni tampoc vull agrair de més.

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tota la meva estada a la Vall Hebron i per la dedicació que han tingut amb mi. Un record especial també pel Servei de Protèomica. No em puc oblidar a la gent d'Anatomia Patològica que sempre han trobat un forat enmig de la seva feina i estrès per atendre els meus "pernilets" dels ratolins com solia dir en Pere Huguet.

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Definitions

Angiogenesis: The pathological growth of new blood vessels to support tumour growth.

Chemokine: A group of small signaling proteins (cytokines) that are usually secreted by immune cells.

Emboli: Clumped tumour cells that typically lodge in blood vessels.

Epithelial-mesenchymal transition: The conversion from an epithelial to a mesenchymal phenotype, which is a normal component of embryonic development. In carcinomas, this transformation results in altered cell morphology, the expression of mesenchymal proteins and increased invasiveness.

Extracellular matrix: A complex, three-dimensional network of very large macromolecules that provides contextual information and an architectural scaffold for cellular adhesion and migration.

Extravasation: Exit of tumour cells out of capillary beds into the parenchyma of an organ.

Gene ontology: is a major bioinformatics initiative to unify the representation of gene and gene product attributes across all species and databases.

Gleason grade: The 'gold standard' for grading prostate cancer, which is used by pathologists worldwide. This system involves assessing both the predominant and secondary pattern of gland formation within a prostate sample. The sample is scored to create a Gleason 'sum', ranging from 2 to 10, with the highest number indicating the most aggressive cancer. It is the most important prognostic marker.

Haematogenous dissemination: The spread of cancer cells through the bloodstream.

Histological grade: Morphologically identifiable steps of tumour progression that are used to classify disease stage.

Immunodeficient: A condition in which the body's immune response is damaged, weakened, or is not functioning properly.

Immunocompatible: Compatible with a targeted immune system.

Immunocompromised: Having the immune response attenuated by administration of immunosuppressive drugs, by irradiation, by malnutrition, or by certain disease processes (e.g., cancer).

Integrins: A family of more than 20 heterodimeric cell-surface extracellular-matrix receptors. They connect the structure of the extracellular matrix with the cytoskeleton and can transmit signaling information bidirectionally.

Intravasation: Entry of tumour cells into the bloodstream.

Melanoma: Skin cancer that is initiated by the transformation of melanocytes.

Metachronous seeding: multifocal development of cancers into other organs by cancer cell seeding.

Metastasis assays: In spontaneous metastasis assays, the tumour cells are inoculated either subcutaneously or orthotopically in animals, and spontaneous metastases from this primary site to distant locations are monitored. In experimental metastasis assays, tumour cells are injected into the bloodstream (for example, intravenously for lung metastasis, into the left heart ventricle for bone metastases and into the portal vein for liver metastases), thereby circumventing the first steps in the metastatic process.

Metastasis initiation genes: A gene that is engaged in the invasion and intravasation of metastatic cells.

Metastasis progression gene: A gene that has dual functions in mediating primary tumourigenesis and metastatic colonization.

Metastasis suppressor gene: A gene in which loss of function specifically enhances metastasis without affecting primary tumour growth.

Metastasis virulence gene: A gene that is exclusively involved in distant organ colonization.

Microarray: An array of polymerase chain reaction products (corresponding to either genomic or cDNA sequence) that is deposited onto solid glass slides.

Organ tropism: A predilection of a primary tumour to spread to specific secondary organs.

Orthotopic site: Transplant of tumour cells into the anatomical location of an animal that best recapitulates the original source of primary tumourigenesis.

Osteoclastogenesis: The differentiation and activation of osteoclasts that mediates bone resorption.

Paracrine factors: A form of bioregulation in which a secretion produced by one cell type in a tissue diffuses through the tissue and affects another cell type in the same tissue.

Parenchyma: The main functional portion of an organ.

Primary tumour: Cancer that arises from the malignant conversion of cells from an initial organ site.

Serial Analysis of Gene Expression (SAGE): A technique for the identification and quantitation of transcripts from two sources, including differentially expressed genes. SAGE is based on the isolation of short tags from a defined location within a transcript, which are sequenced as concademers and quantitated.

Subtractive hybridization: A technique that is used for identifying differentially expressed transcripts between two sources. cDNA from one source is hybridized to mRNA from another source to remove comparably expressed transcripts, and the resulting differentially expressed cDNAs are separated by chromatography.

Synchronous seeding: cancer lesions that have arisen from the same primary tumour

Syngeneic: means genetically identical, or sufficiently identical and immunologically compatible as to allow for transplantation.

Stromal activation: Stimulation and mobilization of host cells in the microenvironment that surrounds a tumour.

Xenogeneic: Originating outside the organism or from a foreign substance introduced into the organism.

Xenograft: Implantation of human tumour cells into an immunocompromize animal.

Abbreviations

ADT – Androgen-deprivation therapy

AS – Active surveillance

AP – Acid phosphatase

BC – Before Christ

BLI – Bioluminescence imaging

BMP – Bone morphogenetic protein

BMPR – Bone morphogenetic protein receptor

BPH – Benign prostatic hyperplasia

BSP – Bone sialoprotein

CRPC – Castration-resistant prostate cancer

COX – Cyclooxygenase

DKK – Dickkopf-related protein 1

DRE – Digital rectal examination

EBRT – External beam radiotherapy

ECM – Extracellular matrix

ET – Endothelin

FGF – Fibroblast growth factor

FFPE – Formalin-fixed paraffin-embedded

GO – Gene Ontology

HT – Hormonal therapy

i.c. – intracardiac

IGF – Insuline-like growth factor

IL – Interleukine

i.o. – intraosseous

i.p. – intraperitoneal

it. – intratibial

MBD – Metastatic bone disease

M-CSF – Macrophage colony-stimulating factor

MMP – Matrix metalloproteases

MS – Mass spectrometry

OPG – Osteoprotegerin

OPN – OSteopontin

PAGE – PolyAcrylamide Gel Electrophoresis

PAP – Prostatic acid phosphatase

PB – Prostate biopsy

PCa – Prostate cancer

PGE2 – Prostaglandina E2

PSA – Prostate specific antigen

PTHrP – Parathyroid hormone-related protein

RANK – Receptor Activator of nuclear factor Kappa-beta

RANKL – Receptor activator of nuclear factor kappa-beta ligand

RP – Radical prostatectomy

RT – Radiation therapy

SCID – Severe combined immunodeficiency

SDF-1 – Stromal-derived factor 1

SDS – sodium dodecyl sulfate

SILAC – Stable isotope labeling with amino acids in cell culture

TGFβ – Transforming growth factor beta

Abbreviations

uPA – Serine-type proteinase urokinase-type plasminogen activator

uPAR – Serine-type proteinase urokinase-type plasminogen activator receptor

VEGF – Vascular endothelial growth factor

WW – Watchful waiting

Introduction

I. CANCER AND METASTASES

a. Background

According to Medicine World (http://medicineworld.org), the first records of cancer appeared thousands of years ago. Cancer was documented during the ancient Egyptian period at a time when surgery was practiced, with an extremely radical treatment. Indeed, there was no anesthesia or antisepsis available. The first description of cancer takes place in approximately 1600 BC At this time, a papyrus described cases of tumours in the breast. The document comments about the disease: “There is no treatment”. Nowadays, despite the technological advances, cancer diseases still constitute a major public health problem.

Cancer is one of the most important health problems in our society, both in terms of morbidity and social impact. It affects the economy and quality of life of one in every four people throughout their lifetimes [1].

According to several agencies working in the cancer registries of Europe, such as EUROSTAT and WHO, there were an estimated 3.2 million new cases of cancer and 1.7 million deaths from cancer in 2008. Prostate cancer is the most numerous cancer diagnosed in men (382,000, 22.2% of the total), followed by lung (291,000, 17.0%), colorectal (231,000, 13.5%), bladder (110,000, 6.4%) and stomach (89,000, 5.2%) cancers [2].

Despite recent advances in cancer research the understanding of the molecular mechanisms that allow for the initiation and the progression of cancer represent a major objective in oncology research.

b. Cancer progression: general consensus

Genetic instability and/or genomic lesions in a plethora of genes characterize cancer disease. Activation of oncogenes (responsible for proteins who promote the

growth of the tumour), coupled with inhibition of tumour suppressor genes contribute to the transformation of healthy into malignant cancer cells. If these modifications allow for a proliferative advantage of the malignant cells over their normal counterpart, the result is a net abnormal cell growth and tumour formation (**Figure 1**). However, sustained growth is not the only hallmark that a tumour cell must possess in order to establish itself. Once the growth brakes have been removed, the tumour cell needs to change its environment such that the resources needed to fuel its development are readily supplied. Therefore, cancer occurs through the acquisition of several pro-cancer characteristics and can take several years before it develops. Accordingly, cancer progression is generally depicted as a multistep transformation, and a general consensus has been reached to describe the different steps that are required for a successful malignant transformation [3].

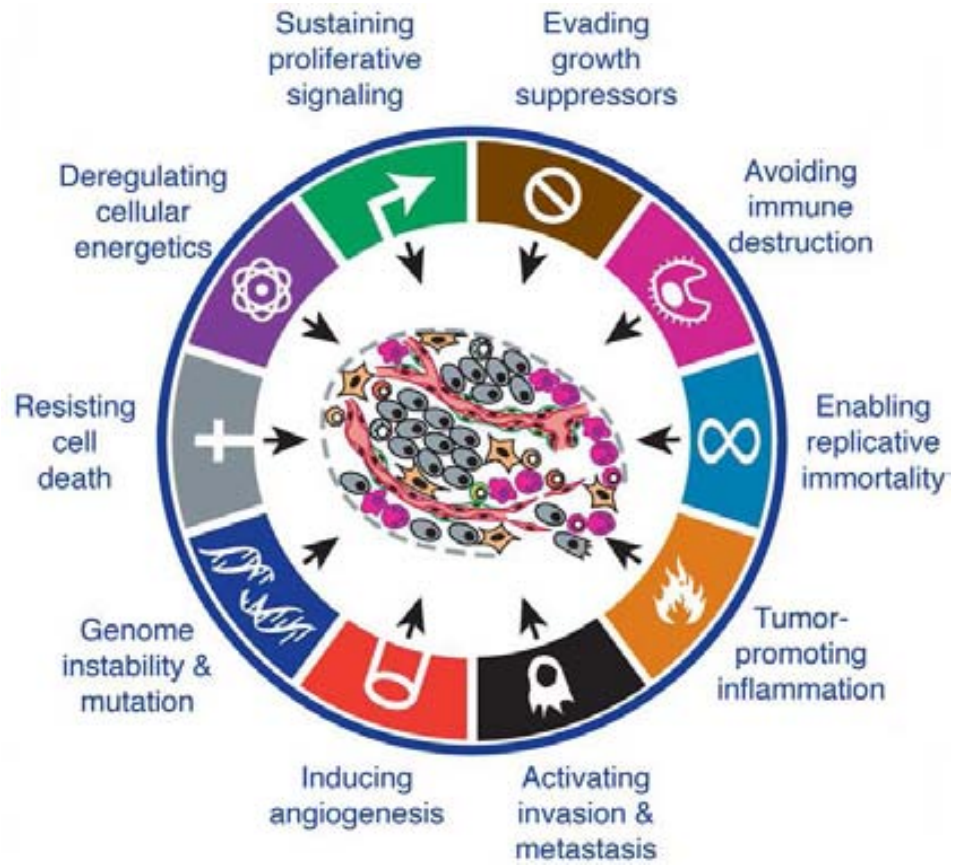

Figure 1. **The hallmarks of cancer: properties required for a successful tumour malignancy.** From REF [3].

These steps were defined to simplify the high complexity of a neoplastic disease. The steps include (i) the sustaining of the proliferative signaling, (ii) the evasion of growth suppressors, (iii) the resistance to the cell death, (iv) the enabling of replicative immortality, (v) the induction of the angiogenesis, (vi) the activation of invasion and metastasis, (vii) the reprogramming of the energy metabolism, and (viii) the escape of immune destruction [3].

In healthy cells, repair mechanisms exist to preserve the genomic integrity, keeping the probability that a critical mutation occurs during lifetime low. Even with these checks in place cancers are relatively frequent among the human population. This incidence can be explained by the emergence of a mutation in the DNA repair system itself (e.g. p53) that is responsible for the genome instability [4, 5]. However, genetic and epigenetic alterations in the cancer cells genome are not generally sufficient to allow the development of tumours and metastasis. Indeed, tumours are complex tissues composed by different cell types (cancerous but also surrounding normal cells) that interact with each other to promote tumour progression. Therefore, tumours cannot be simplified by the biology of cancer cells alone, but it is essential to consider the tumour as a whole [3, 4].

c. Metastatic cascade

An estimated 90% of cancer related deaths are attributed to the development of metastases, highlighting the urgent need for novel treatment strategies in this area [5, 6]. Metastases occur following the spread of cancer cells from a primary site resulting in the formation of secondary tumours in distant organs. Metastasis is common faith amongst all malignant tumours, but its progress is specific to the cancer type. Determinant for the secondary site of novel tumour growth is the origin of the cancer cells, their affinity to a specific tissue, and the ability of cancer cells to adapt to the novel environment. Interestingly, in 1889 Stephen Paget already hypothesized that different types of cancer can develop metastases in specific secondary sites and proposed the "dependence of the seed (= cancer cells) on the soil (= the distant organ)" concept [7]. This idea was contested in the 1920s by James Ewing, who proposed that adaptations of

the circulating cells between the primary tumour and the metastatic site were enough to form metastasis. In fact, these theories are not mutually exclusive, and both concepts can contribute to a successful metastatic process (**Figure 2**).

Figure 2. Founders who proposed the two major theories to explain organ selectivity of metastasis. Left, Stephen Paget (1889) with his "Seed and Soil" Hypothesis. Right, James Ewing (1929) with the "Mechanical-circulatory" Hypothesis

Nowadays, the development of a metastasis is typically described as a multistep process (**Figure 3**) [5, 6, 8]. The process starts with an initial tumourigenic mutation with potential to induce proliferative signals and/or the ability to perform indefinite cell division [9]. Secondly, to form distant tumours, cancer cells must adapt their phenotype to become motile in order to migrate through the extracellular space. This mobility can be explained by the loss of cell adhesion and the increase in factors that allow the degradation of the extracellular matrix. Furthermore, motility of cells facilitates the intravasation process (movement of the cancer cells through the endothelial cells layers) via the secretion of vascular destabilizing factor produced by the cancer cells. Subsequently, the vasculature is disrupted and cancer cells can move through the circulation, which is the major mode of cancer cell dissemination to distant organs. Once the metastatic cells reach the circulation, some properties of the vasculature system are

involved in the dissemination of metastatic cells. For example, metastatic cells can protect themselves from the immune system by interacting with the platelets. Moreover, the resulting aggregates can be responsible of the metastatic cells emboli playing a role during the extravasation [10, 11].

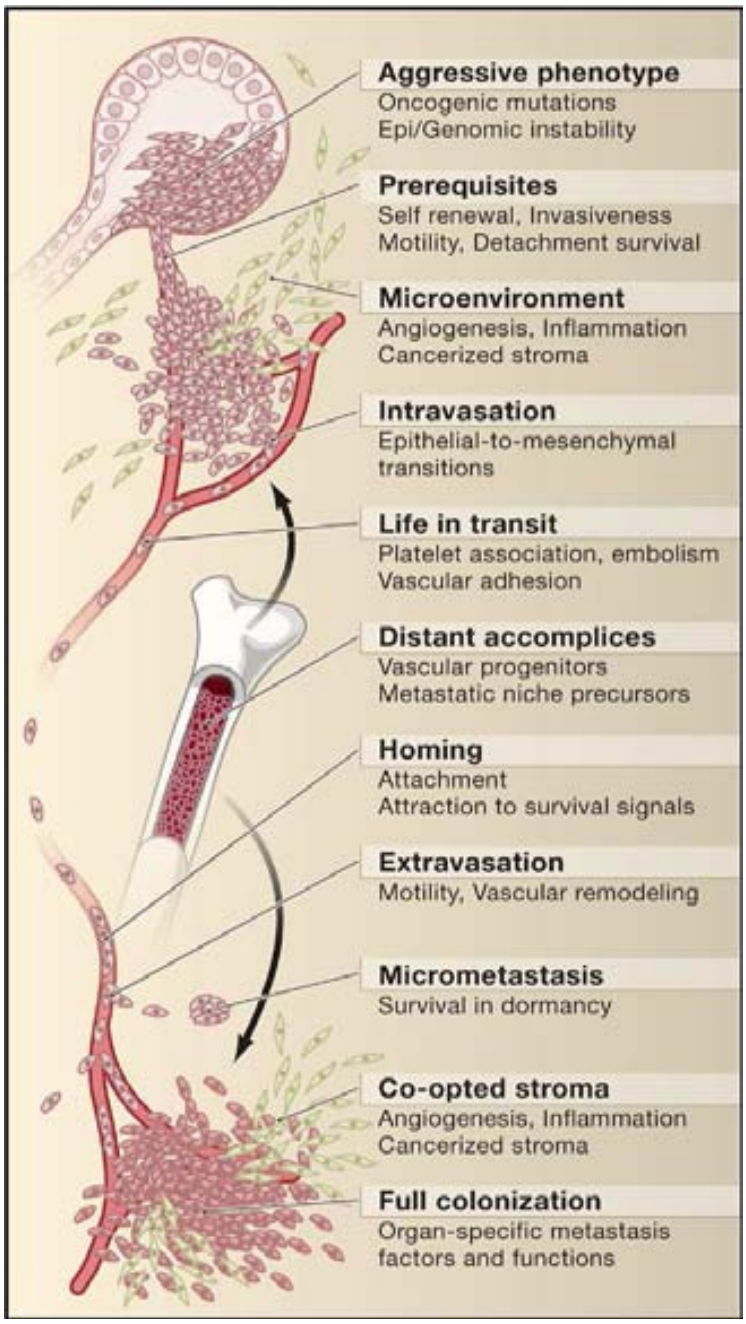

Figure 3. Different stages of a metastasis process. From REF [5]. A metastasis occurs through the multistep acquisition of capabilities that allow malignant cells of a primary tumour to disseminate and colonize a distant organ. The specific steps of this sequence may vary between the tumour types and the primary tumour site.

Although the migration of cancer cells is well-orchestrated and not a random process, the identification of the basic cellular and molecular processes that regulate their movement and subsequent arrival and survival at distant sites remain elusive [12]; however, little doubt exists that modulation of both the local host and tumour microenvironments is critical for the completion of the complex, multistep metastatic cascade (**Figure 4**).

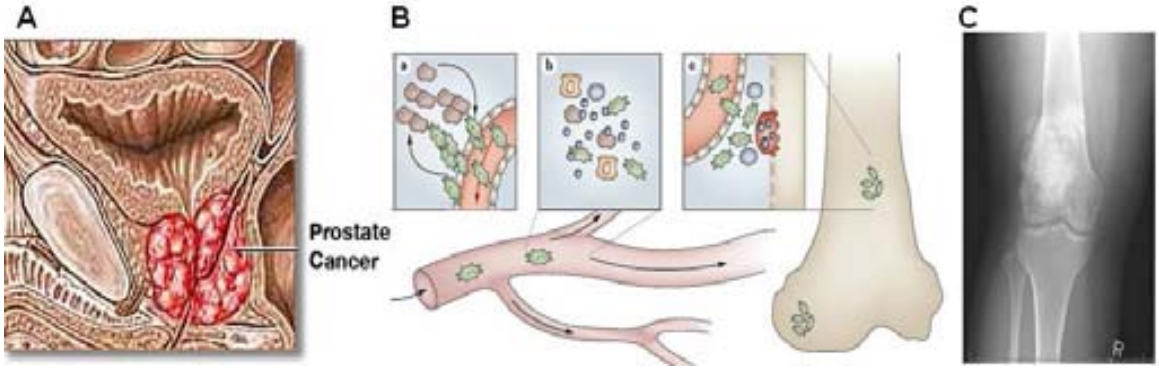

Figure 4. **Steps involved in tumour cell metastasis from a primary site to the skeleton**. Adapted from REF [13]. **A.** The primary tumour. **B.** Once in the vasculature, tumour cells (shown in green) interact with resident host blood-borne cells, such as erythrocytes, T cells and neutrophils and with platelets, which facilitate survival in the circulation. In the bone marrow, the tumour cells escape from the vasculature (extravasation) into the bone marrow where they interact with resident bone marrow cells for subsequent survival and eventual activation of resident bone cells, such as osteoclasts (shown in red). **C.** As a result, a bone metastatic foci is formed.

All the steps in the process of metastasis are sequential and depend both on the target organ as well as the primary tumour type. Therefore the process of metastatic tumour dissemination may take variable amounts of time and is usually characterized with a latency period during which no detection can be made in the clinics (**Figure 5**) [14]. For example, the 5-year recurrence-free rate in lung carcinoma patients is ~60 % in comparison with 98 % for breast cancer [15].

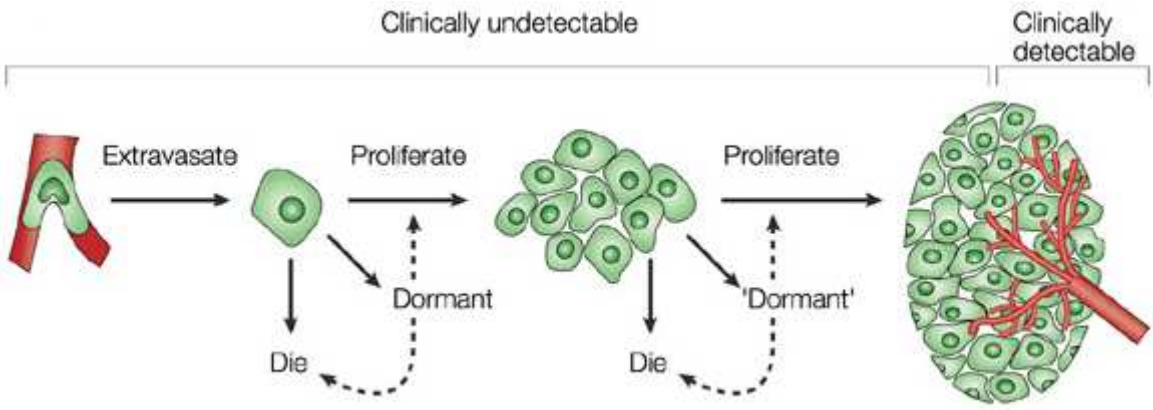

Figure 5. Hypothetic fates of cancer cells in the metastatic site, following the migration of circulating cancer cells in a distant organ. From REF [16].

Essentially it is still not know if the cancer cells are conditioned to metastasize in the primary tumour itself (deciding which tissue will/can be colonized) or they are first disseminated and then adapt their phenotype following the selective pressure of the novel microenvironment (**Figure 6**).

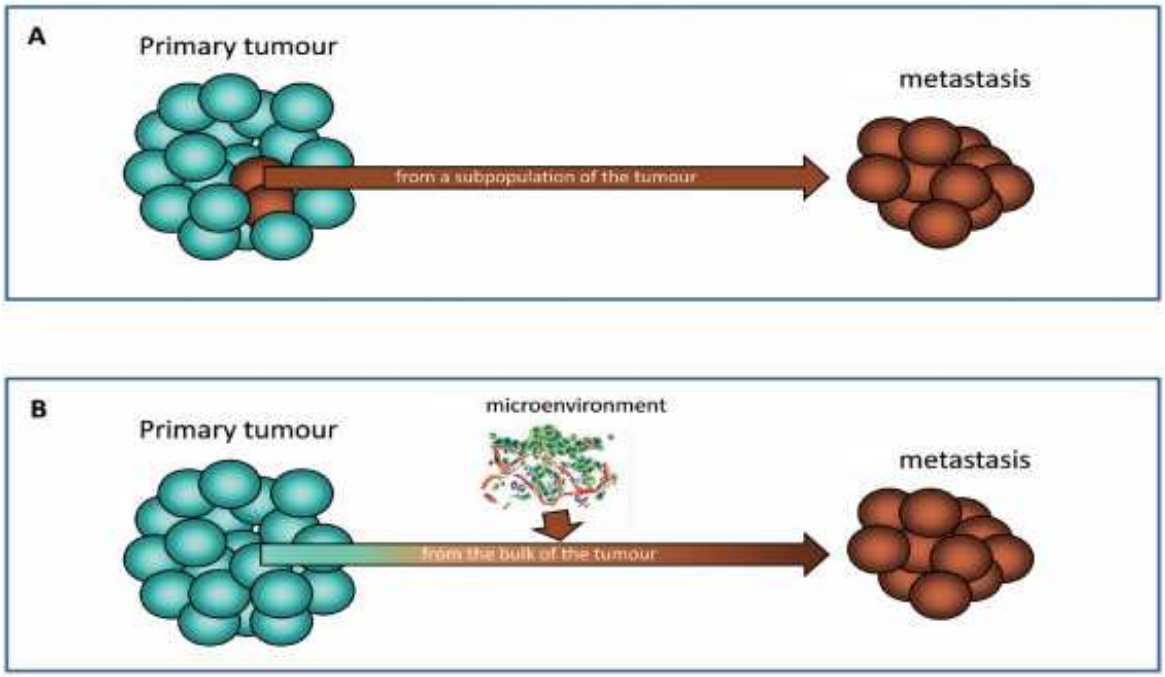

Figure 6. Possible mechanisms for the development of a metastasis. From REF [17]. A. Metastasis comes from a distinct group of cells present in the primary tumour. B. Metastasis comes from the tumour mass and changes its phenotype as a result of a selective adaptation to the novel microenvironment.

Box 1. **The metastasis process and its requirements.** Adapted from REF [18]

Prerequisites for metastasis

To metastasize, malignant cells must fulfill certain tumourigenic functions that become prerequisites for metastasis. These include: unlimited proliferation, evasion of cell-intrinsic and environmental constraints, attraction of a blood supply, and the capacity to detach and move away from the original location. Tumour-initiating mutations, alterations that are secondary to genomic instability and epigenetic changes, underlie the acquisition of these functions in developing tumours. As tumours grow, they must selectively conquer environmental pressures including cytotoxic immunity, low oxygen tension and an acidic environment, such functions must remain active throughout malignant progression, as they favour the emergence of metastasis-prone tumour cells in the context of local and distant microenvironments.

Metastatic initiation and dissemination

Dissemination starts in earnest when aggressive tumour cells become invasive and readily enter the bloodstream through the vasculature that they have attracted. Intravasation is also enhanced by an epithelial-to-mesenchymal transition that endows carcinoma cells with embryonic plasticity and added motility. The rate of malignant cell shedding generally increases with tumour size, but dissemination can occur in the early stages of the primary tumour, to the point that some metastases have no known primary source.

Metastatic colonization

The dissemination of tumour cells to various organs is influenced by circulation patterns and the mechanical lodging of tumour cells in capillary beds. Adhesion molecules and chemokine sources also have important roles. Metastatic cells enter the parenchyma of a target organ by breaching the capillaries in which they are embedded. Active colonization can be achieved by the co-option of organ-specific components of the tumour microenvironments, such as the activation of bone-resorbing osteoclasts by breast cancer cells during osteolytic metastasis. Full metastatic colonization can occur by immediate growth of tumour cells upon their extravasation, or after a prolonged period of micrometastatic dormancy. The survival of dormant lesions, their eventual activation and outgrowth requires additional tumour-intrinsic or tumour-extrinsic factors. As each organ presents a highly specialized microenvironment, distinct sets of functions are thought to be required for colonizing different tissues.

One of the most intriguing biological aspects of metastasis is the pattern of organ dissemination. The spread of tumour cells frequently includes invasion of local lymph nodes through the lymphatic system. However, aggressive tumour cells typically enter the bloodstream and reach distant tissues [18]. This dissemination has stereotypical patterns of organ tropism that reflects the heterogeneity of tumour cells and depends on the cancer type (**Table 1**).

Table 1. Stereotypical patterns of tumour metastasis to distant organs. From REF [18]

Cancer type	Site of metastasis
Breast cancers	Primarily bone and lung; less frequently liver and brain. ER-positive tumours preferentially spread to bone; ER-negative tumours metastasize more aggressively to visceral organs. This might be related to the cell of origin: ER-positive tumours can originate from luminal progenitor cells, whereas many ER-negative tumours emerge from cells in basal layers.
Lung cancers	The two most common types of lung cancer have different aetiologies. Small-cell lung carcinomas (SCLC) disseminate rapidly to many organs. Non-small-cell lung carcinomas (NSCLC) often spread to the contra-lateral lung, the brain and also to the adrenal glands, liver and bones.
Prostate cancer	Almost exclusively to bone, forming osteoblastic lesions and filling the marrow cavity with mineralized bone matrix, unlike the osteolytic metastasis that is caused by breast cancer.
Pancreatic cancer	Aggressive spread to the liver and the surrounding viscera.
Colon cancer	The mesenteric circulation pattern favours dissemination to the liver and peritoneal cavity, but metastasis also occurs in the lungs.
Ovarian cancer	Local spread in the peritoneal cavity; rarely affects other organs, as inadvertently demonstrated by the absence of distant metastases in patients who received palliative shunts to divert ascites to the circulation for abdominal pain relief [118].
Sarcomas	Various types of sarcoma (tumours of mesenchymal origin) mainly metastasize to the lungs.
Myeloma	Haematological malignancy of the bone marrow that causes osteolytic bone lesions, sometimes spreading to other organs.
Glioblastoma	These brain tumours show little propensity for distance organ metastasis, despite aggressively invading the CNS.
Neuroblastoma	Paediatric tumours that arise from nervous tissue of the adrenal gland. Forms bone, liver and lung metastases, which in some cases spontaneously regress.

ER – oestrogen receptor.

Patients can harbor simultaneous metastases in different organs, and for each patient it is unknown if such lesions have arisen from the same primary tumour (synchronous seeding), or whether they result from a sequence of seeding events in which some metastases themselves subsequently colonize other organs (metachronous seeding) [19]. Moreover, disseminated tumour cells could theoretically also return to their original site (self-seeding), thereby accounting for the progressive accumulation of aggressive cells in primary tumours and local recurrences. [20]

Along these lines several questions have to be answered. What are the phenotypic differences between primary tumour and corresponding metastasis? Do disseminated cancer cells develop the ability to colonize the host organ during the multistage process? Which genes/proteins enable metastatic cells dissemination, survival and growth?

i. Models of metastasis

The metastatic properties of tumour cells were extensively investigated in the late 1970s and early 1980s by means of 'experimental metastasis' assays. By studying the metastatic behaviour of cultured B16 melanoma cells that were injected intravenously into mice, researchers found that cells derived from outgrowths of these cells (metastases) have a higher metastatic potential than those derived from the original cell line [21]. These observations led to a metastasis model, which proposed that

most primary tumour cells have a low metastatic potential, and that during later stages of tumourigenesis rare cells acquire metastatic capacity through additional somatic mutations [22] (**Figure 7.A**).

A number of contrasting theories have re-emerged that provide possible explanations for the metastatic selectivity of cancer.

The **'traditional metastasis model'** suggests that select subpopulations of tumour cells acquire metastatic capacity during the late stages of tumourigenesis. This concept seems improbable, as numerous investigators have demonstrated that the vast majority of tumour cells, independent of tumour stage, have the potential to develop into a metastasis [23, 24] (**Figure 7.B**).

The **'dynamic heterogeneity theory'** suggests that the metastatic potential of tumour cells is determined by the rate at which tumour variants with increased metastatic potential occur within the primary tumour site [25] (**Figure 7.C**).

The **'clonal selection theory'** proposes that all primary tumours evolve from the same cell, and development of a primary tumour is the consequence of a series of multiple molecular changes resulting in clonal selection. This complex process specifically alters the phenotype of the tumour cell, allowing acquisition of different tumour-specific characteristics, such as the ability for site-specific metastasis [26] (**Figure 7.D**).

The **'stem cell theory'** of cancer proposes that the site selectivity of metastasis is the result of the activation of the so-called cancer stem cell compartment within a specific organ, such as the breast [26].

The **'genometastasis hypothesis'** proposes that metastasis occurs through transfection of susceptible cells in distant organs with dominant, plasma-circulating oncogenes that are derived from the primary tumour [27, 28] (**Figure 7.E**).

As these metastatic models are not mutually exclusive, it is reasonable to assume that a variety of as yet uncharacterized genetic, molecular, cellular and cell type-specific mechanisms regulate tumour initiation and metastasis to and survival within the skeleton, presumably involving features of all theories [26].

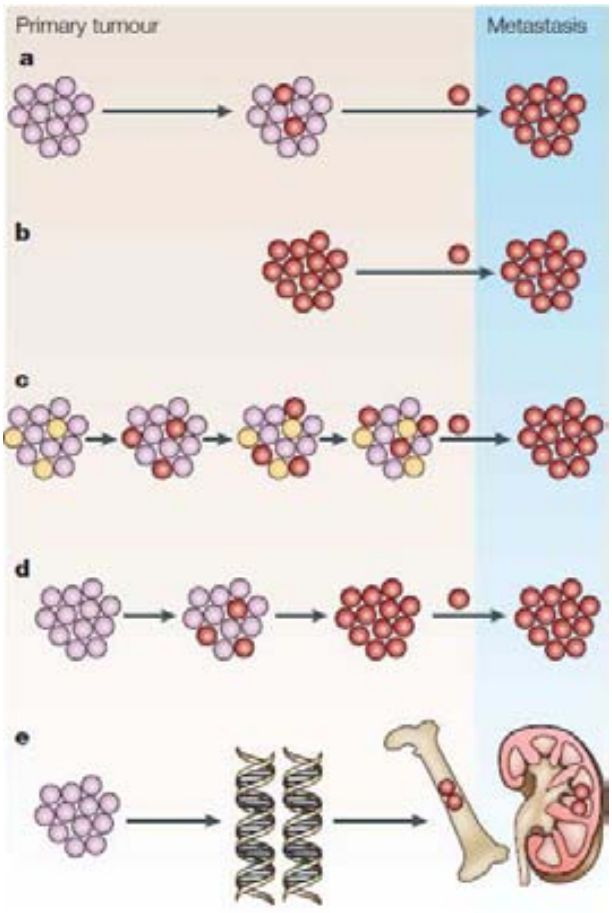

Figure 7. Models of the metastatic process. From REF [23]. **A.** The traditional model of metastasis suggests that only subpopulations of tumour cells (red) acquire metastatic capacity late in tumourigenesis. **B.** Spontaneous metastasis assays indicate that all tumour cells have the capability to develop a metastasis. **C.** The 'dynamic heterogeneity' model proposes that the frequency with which metastatic variants arise within the primary tumour determines its metastatic potential. **D.** The 'clonal dominance' theory proposes that metastatic subclones within a primary tumour can overgrow and dominate the tumour mass itself. **E.** The 'genometastasis hypothesis' proposes that metastasis occurs through transfection of susceptible cells in distant organs with circulating oncogenes.

ii. New models of metastatic process

Findings from DNA-microarray studies have revived the discussion about the metastatic process. A study by Ramaswamy and colleagues shows that different types of human primary adenocarcinoma harbor the same gene-expression signature that is associated with metastasis [29]. Furthermore, it was reported that pairs of human primary breast carcinomas and their distant metastases, which developed years later,

are highly similar at their transcriptome level, as are pre-malignant, pre-invasive, and invasive breast cancers [30, 31].

A variation to this model was proposed by Massagué and colleagues, in which a human breast cancer cell line was shown to harbor, besides a poor-prognosis signature, an additional gene set that mediated osteolytic bone metastasis [32]. These findings were interpreted to bridge the gap between the subpopulation metastasis model and the one based on the microarray data of human tumours (**Figure 8.A**) [22, 29, 33]. The authors propose the intriguing model that primary tumours with metastatic capacity possess the poor-prognosis signature and, in addition, subpopulations of cells also have a 'superimposed' tissue-specific gene-expression profile that predicts the site of metastasis (**Figure 8.B**)[32].

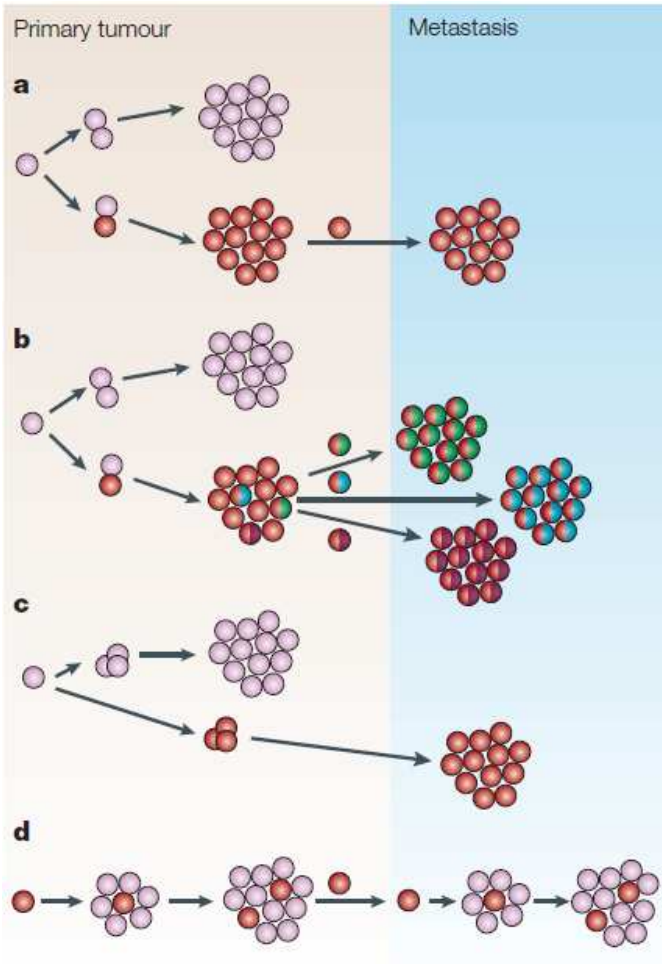

Figure 8. New models of the metastatic process in breast cancer. From REF [23]. **A.** Gene-expression profiling of human primary breast tumours can predict metastasis risk ('poor-prognosis' (red) versus 'good-prognosis' (pink) signature), which indicates that the capacity to metastasize might be acquired early during tumourigenesis. **B.** Primary tumours with metastasizing capacity display the poor-prognosis signature and an additional tissue-specific expression profile predicting the site of metastasis. **C.** The parallel evolution model proposes that the dissemination of metastatic cancer cells occurs early in oncogenesis and independently from tumour cells at the primary site. **D.** Only breast cancer stem cells, not the nontumourigenic bulk of the tumour, have the ability to metastasize and form new tumours.

The **'parallel evolution model'** proposes that the dissemination of metastatic cancer cells occurs early in oncogenesis and independently from tumour cells at the primary site (**Figure 8.C**) [34]. This theory clearly challenges the paradigm that tumour progression to metastasis occurs through clonal genomic evolution.

An alternative, attractive model of metastasis is based on the finding that tumours might contain **'cancer stem cells'** - rare cells with indefinite proliferative potential that drive the formation and growth of tumours [35]. Only breast cancer stem cells, not the non-tumourigenic bulk of the tumour, have the ability to metastasize and form new tumours (**Figure 8.D**) [36].

iii. Metastasis theories: seeds, soils and signatures

Already noted by the Egyptians of 1500 BC, malignant disease was described with remarkable coherence by Greek physicians in the time of Hippocrates (fifth century BC), who saw axillary swelling as a sign that breast tumours would be followed by secondary growths of fatal outcome. However, such lesions were considered as independent malignancies that arose from the spread of toxic humors. The humoral theory prevailed until the eighteenth century and beyond, when the discovery of the cell as the basic unit in live tissue stimulated new debate, with some viewing metastases as degenerate cell components arising from a primary tumour [18].

Congenial soils. Preference of various tumours to metastasize to certain organs must imply a hospitable environment in those tissues [7]. Building on this, Paget proposed that tumour cells ('seeds') must be predisposed to arrest and proliferate only in those anatomical sites ('soil') that provide a congenial ground - a basic tenet of his seed-and-soil hypothesis [7]. Ewing later postulated that the direction of circulation alone influences the distribution of metastasis. The importance of vasculature was further supported by mechanical distinctions between lymphatic and haematogenous dissemination [37]. Eventually, these disparate views were reconciled in the proposal that circulatory routes influence the distribution of cancer cells, but the eventual outgrowth of macrometastases depends on the seeding of compatible tissues [38].

Darwinian concepts. Inspired by evolutionary theory, neoplastic development started to be viewed as a process of natural selection, with genomic instability producing

variant cell populations from which aggressive clones emerge, suggesting that metastatic progression might require the acquisition of additional genetic alterations that confer a selective advantage to rare clones in the tumour cell population [22].

Pre-determining gene-expression signatures. Technological advances derived from the sequencing of the human genome have recently enabled the genome-wide analysis of tumours. Bioinformatic analysis from primary tumour samples has uncovered complex gene-expression patterns, or 'signatures' that can predict the risk of metastatic recurrence. According to this model, metastasis would be largely pre-determined by the combined action of mutations that give rise to a locally aggressive primary tumour. In this case, the acquisition of further pro-metastatic changes by rare variant clones would have only a complementary role [39].

Towards an integrative view. Because genetic instability enables clonal evolution, this suggests that some cells acquire mutations and disseminate early on, after which they continue to evolve independently of primary tumour cells. Whether overt metastasis eventually emerges from a progenitor pool that is present in such early seeding, or from the later dissemination of cells that acquired their aggressiveness in the primary tumour, remains an open question. These diverse lines of evidence can be assembled into an integrative model in which the proclivity to disseminate can be acquired as a developing tumour becomes locally aggressive, whereas the outgrowth of distal colonizing cells necessitates further selection from subsequent genetic heterogeneity. The cell type of origin and the circulation pattern represent other important factors [40].

iv. Classes of metastasis genes

The terms 'metastasis' or 'metastatic spread' have been rather liberally used to describe individual features of an aggressive tumour. Genes that mediate the initial formation and progression of tumours can confer tumourigenic properties, but might not specifically mediate the steps of metastasis. Genetic alterations that drive tumourigenic functions remain indispensable in metastatic cells, as shown by the clinical success of drugs [18]. Several classes of genes can be distinguished that fulfill these criteria (**Figure 9**).

Metastasis initiation genes. Includes genes that provide an advantage in a primary tumour and, in so doing, pave the way for tumour cells to escape into the circulation. Most genes that underlie tumour cell motility, invasion or angiogenesis would fall into this class. Invasiveness and local angiogenesis are therefore important events for metastasis, although they are not in themselves sufficient. Formation of distant metastases depends on whether some circulating tumour cells - usually a tiny minority of them - manage to enter and eventually overtake the parenchyma of a target organ. Genes that mediate the local progression of a primary tumour are not selected in response to the pressures that exist in distant organs, and so they might not be expected to satisfy demands that are unique to particular organ microenvironments [18].

Metastasis progression genes. Genes that fulfill certain rate-limiting functions in primary tumour growth and other specific functions in metastatic colonization. These genes are thus distinguished from oncogenes that carry out the same cell-autonomous transforming function throughout the course of a malignant disease. The specific advantage might be restricted to one particular target organ, in which case metastasis progression genes would mechanistically couple primary tumour progression and tissue-specific spread. As such, these genes can be found within gene-expression signatures that correlate certain primary tumours with risk of organ-specific dissemination [18].

'Metastasis-virulence' genes. Genes that provide a selective advantage in secondary sites but not in the primary tumour. These genes participate in metastatic colonization but not in primary tumour development. Consequently, metastasis virulence genes add to the aggressiveness of metastatic tumour cells in a secondary site. Such genes would rarely be present in primary tumour 'poor-prognosis' gene-expression signatures, as their function would not provide a selective advantage in primary tumours [18].

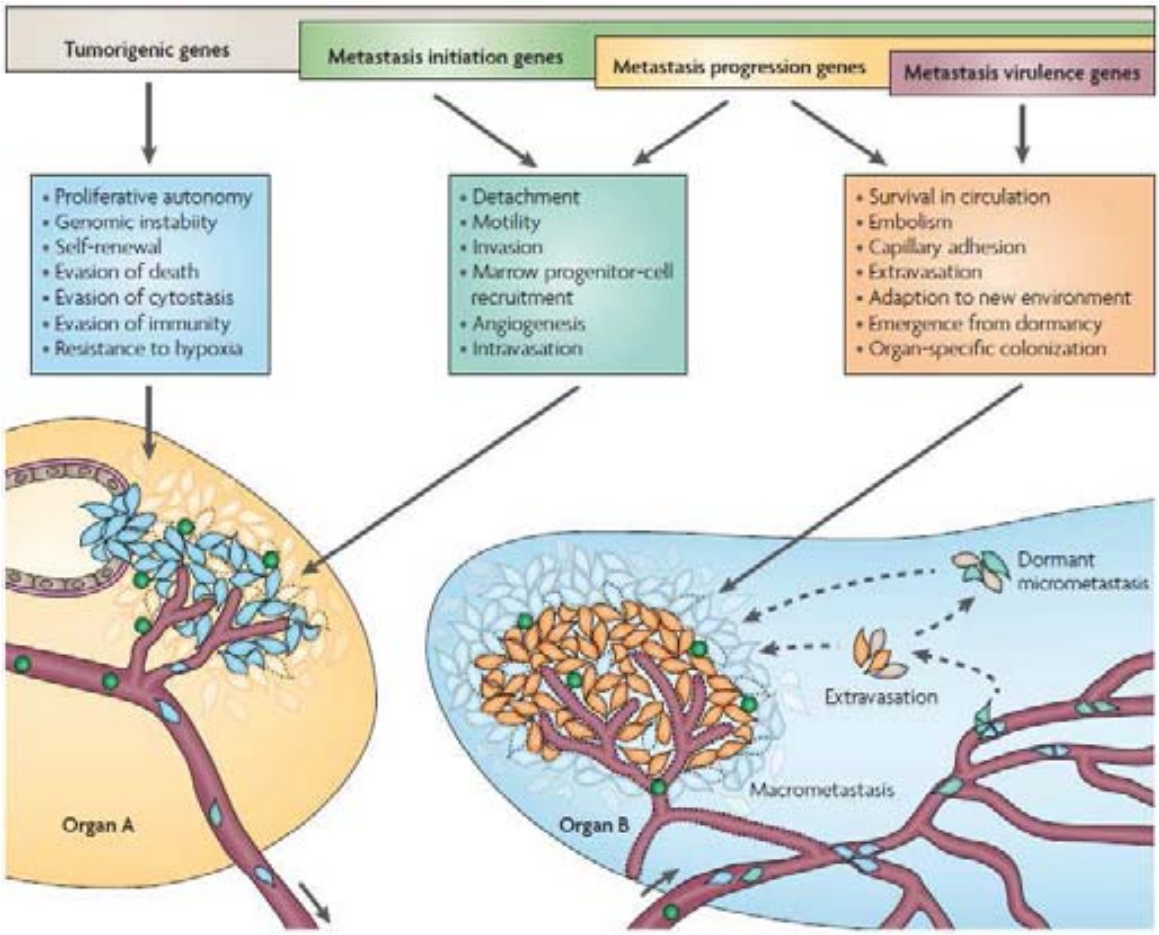

Figure 9. Classes of genes participating in the metastasis process. From REF [18]. '**Metastasis initiation**' genes are those that provide an advantage in primary tumours, paving the way for tumour cells to enter the circulation. '**Metastasis progression**' genes are those that fulfill certain rate-limiting functions in primary tumour growth, and other specific functions in metastatic colonization. '**Metastasis virulence**' genes are those that provide a selective advantage in secondary sites but not in the primary tumour, thus participating in metastatic colonization but not in primary tumour development. Metastasis genes might act from the tumour cells in the primary site (blue cells) or their metastatic derivatives in a distant organ (orange cells).

Box 2. Metastatic colonization. Adapted from REF [41]

Metastatic colonization is the outgrowth of tumour cells after they have arrived at a distant site. The contribution of angiogenesis to the outgrowth of micrometastases, so that they form tumours unlimited by the diffusion range of oxygen, is well described, and inhibitors are in clinical trial.

However, an incompletely understood component of metastatic colonization concerns the signal responsiveness of tumour cells in a distant location to the local microenvironment, paracrine signals and stress conditions. Devoid of the cell–cell, cell–extracellular-matrix and cell–local-growth factor interactions that are present at the primary tumour site, it can be hypothesized that the tumour cell that is resistant to apoptotic signals (or, conversely, sensitive to local survival factors), stimulated to proliferate by locally available cytokines and/or able to differentiate into a more independent and invasive form would have a survival advantage.

As an example, the ability of bone-metastasizing prostatic carcinoma cells to preferentially adhere, spread and proliferate on type I collagen,which is found in the bone-marrow extracellular matrix.

Data from this emerging field dictate that the responses of tumo cells that are grown as a primary tumour might be distinct from those at a metastatic site. As most primary tumours are removed by surgery and radiotherapy, the goal of most cancer chemotherapy is to eliminate metastatic cells or to halt metastatic colonization. Differences in signalling in tumour-cell colonization at a metastatic site might therefore affect the effectiveness of cancer therapies.

II. PROSTATE CANCER

a. The prostate gland

Researchers do not know all the functions of the prostate gland. However, it is known that the prostate gland plays an important role in both sexual and urinary function. It is common for the prostate gland to become enlarged as a man ages, and many men experience some type of prostate problem in their lifetime.

The prostate gland surrounds the neck of both bladder and urethra in front of the rectum. It is partly muscular and partly glandular, with ducts opening into the prostatic portion of the urethra. It is made up of three lobes: a center lobe with one lobe on each side (**Figure 10**).

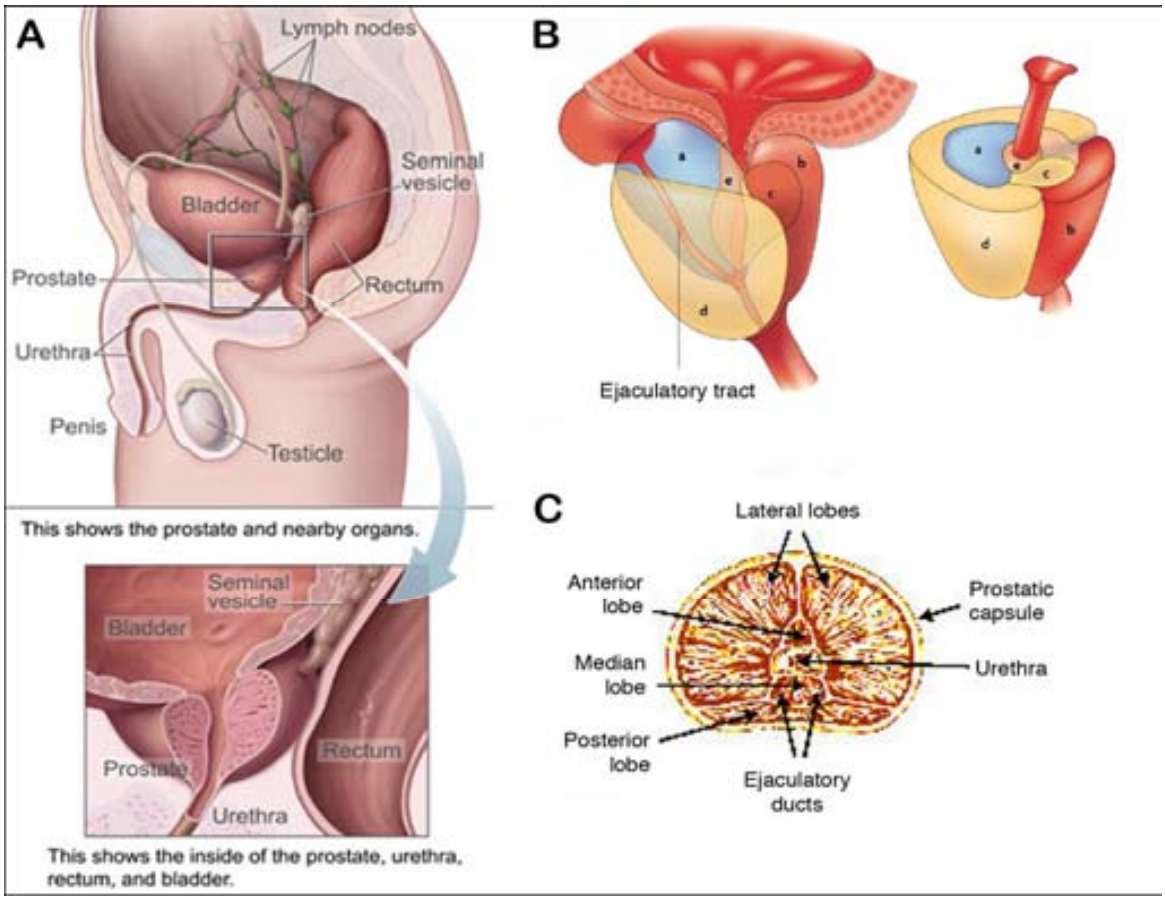

Figure 10. The human prostate. Adapted from http://en.wikipedia.org. **A.** Localization of the prostate in humans, adapted from http://en.wikipedia.org/. **B.** Prostate zones: a, central zone (CZ); b, fibromuscular zone (AFZ); c, transitional zone (TZ); d, peripheral zone (PZ); e, periurethral region, adapted from [42] **C.** Prostate lobes; lateral lobes, anterior lobes, median lobe and posterior lobe.

The prostate gland can suffer some of the following benign prostate problems:

Benign prostatic hyperplasia (BPH): an age-related condition of the prostate that is not malignant. Although it is not cancer, BPH symptoms are often similar to those of prostate cancer.

Prostatitis: inflammation or infection of the prostate gland characterized by discomfort, pain, frequent or infrequent urination, and sometimes fever.

Prostatism: the symptom of decreased urinary force due to obstruction of flow through the prostate gland. The most common cause of prostatism is BPH.

Prostatalgia: pain in the prostate gland, also called prostatodynia. It is frequently a symptom of prostatitis.

b. Epidemiology: incidence and mortality

Prostate cancer (PCa) is the most commonly diagnosed cancer among European and American men (24.1% of all cases) [43], and it is the second most common cause of cancer death among men [2] (**Figure 11**). In 2012 in the United States, PCa alone will account for 29% (241,740) of incident cases and it is estimated that 28,170 (9%) men will die as a result of this disease [1].

Even though the introduction of the PSA test in the late 1980s of the past century has led to a dramatic increase its detection [43], the risk of developing this type of cancer during a lifetime is estimated at 1 in 6 men in the US, and the risk of death due to this disease is 1 in 36 [44].

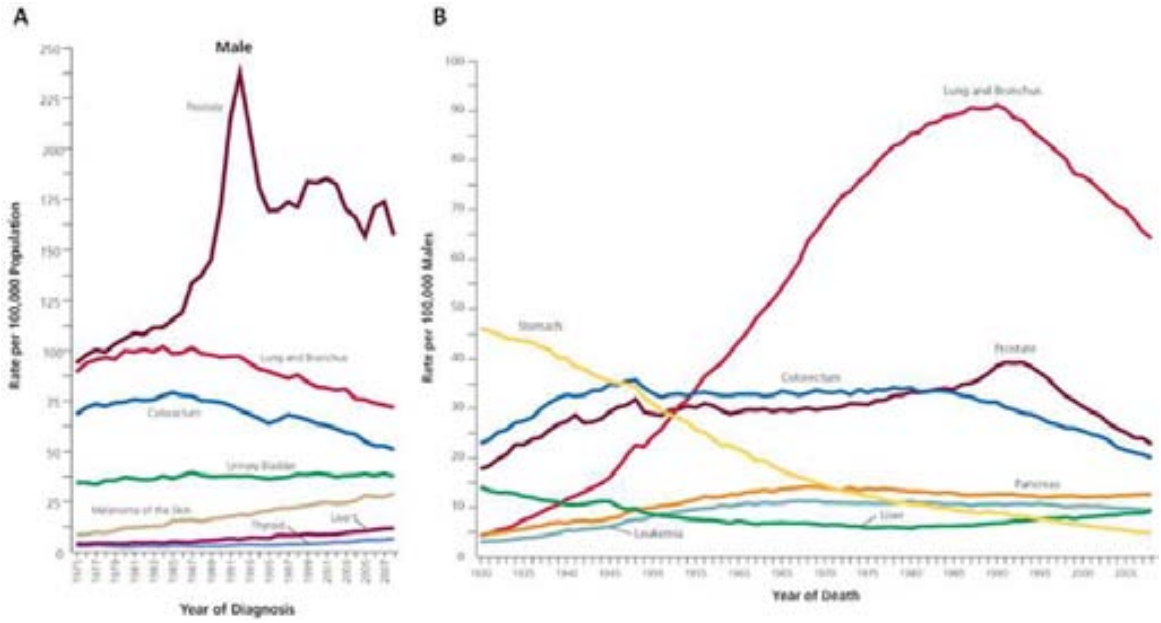

Figure 11. Cancer incidence. Adapted from REF [1]. **A.** Trends in incidence rates for selected cancers in males in United States from 1975 to 2008. **B.** Trends in death rates among males for selected Ccancers in United States from 1930 to 2008.

PCa is a chronic and progressive disease frequently accompanied by irreversible and lethal metastasis. PCa is notorious for its varied geographic distribution across the world [45]; a rare disease in Asia and Africa, whereas it is frequently diagnosed in other regions, especially in the West, where PCa is viewed as an ageing-related malignancy preferentially occurring in certain ethnic groups [46].

c. Etiology and pathogenesis

Defining the etiology, so as to provide measures of prediction and prevention, is the most priority of PCa research. During the last 20 years a large number on studies focusing on the influence of different risk factors on PCa incidence were published. The most important risk factors are age, race, family history, genetic and hormonal factors among other factors such as infective agents or diet. Age is an essential factor in PCa debut; while in males under 45 years PCa is unusual, as males get older, the incidence is progressively increasing, with a peak around 65 - 70 years. About genetic factors, current theories consider that PCa has a complex etiopathogeny, being caused by a multitude of factors. There are 2 general types of PCa, familial and sporadic. Familial prostatic disease is chiefly recognized because the cancer appears at early ages (< 55

years old) in the affected members of the same family. However, in sporadic PCa, the genetic material is damaged by external ambient exposure during the life of the individual. Both types of cancers have different incidence rates; in familial or hereditary cancer, the rates reach 15%, whereas in sporadic carcinoma, the rates approach values of 80 or 90% [47].

The vast majority (95%) of the malignant prostate tumours are adenocarcinomas (**Figure 12**), with origin in the epithelial cells covering the prostatic acini and glandular ducts. Over 50% of diagnosed prostate adenocarcinomas are already multifocal, but the volume of the isolated tumours is small (less than 0.5 mL) [48].

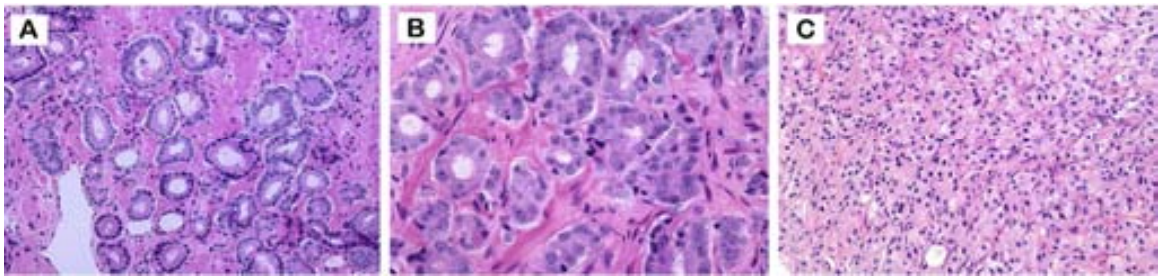

Figure 12. PCa features. From http://webpathology.com. **A.** Low grade adenocarcinoma where the majority of the glands are relatively uniform in size. **B.** Medium grade adenocarcinoma shows abundant amphophilic cytoplasm, enlarged nuclei with prominent nucleoli. **C.** High grade adenocarcinoma shows fused glands, no intervening stroma and disruption of the basal cell layer.

Histological grading of PCa is very important, allowing to evaluate the tumour aggressivity and, especially, to assess the prognosis of the patients. The essential criteria of the various grading systems are the modifications of glandular architecture and/or cellular anaplasy. The most widely accepted number of grading systems is the Gleason score (**Figure 13**). The Gleason score evaluates the architecture of the prostate glands, the pattern of tumoural growth and the relationship between the tumour cells and the surrounding stromal tissue. It has five levels of progressive tumour aggressivity, grade 1 being the least aggressive, while grade 5 is the most anaplasic.

Grade 1	Closely packed, uniform, well formed tumor glands, separated by a reduced quantity of stroma
Grade 2	Poorly circumcised nodules of less uniform glands, with more tissue between them
Grade 3	Marked variations of glands' size and architecture; some of the cells leave the glands and invade the surrounding tissue
Grade 4	Many fused glands, which have less recognizable structure; many cells invade the surrounding
Grade 5	Absence of cell differentiation, solid mass of cells or sheets of cells through the surrounding tissue

Figure 13. The five Gleason grades. Adapted from REF [48].

Because the majority of the prostate adenocarcinomas are not homogenous tumours, containing two or more different histological patterns in the tumour mass, Gleason score is established by the addition of the Gleason grades of the two most prominent tumour patterns. This allows for a more precise estimation of cancer prognosis. The assessment of Gleason score depends on pathologists' experience, having unfortunately a subjective component that gives a degree of inconsistency.

d. Tumour node metastasis classification

Two main classification systems are used to stage tumours: the Jewett system (stages A through D) described in 1975 [49] and the TNM system adopted in 1997 by the American Joint Committee on Cancer (AJCC) and the International Union against Cancer. In 2002, the TNM classification system was further revised by the AJCC [50]. These systems can reveal nonpalpable tumours by identifying an increase in serum prostate-specific antigen (PSA) level or an aberrant transrectal ultrasound image. These systems can also categorize patients based on tumour detection technique and distinguish nonpalpable PCa (those detected during transurethral resection) from palpable ones (those detected by digital rectal examination) [51].

The tumour node metastasis (TNM) classification is based on the status of the primary tumour, ranging from organ-confined to fully invasive (T1 to T4), with or without lymph node involvement (N0 or 1) and the presence and degree of distant metastasis (M0 and 1a-c) [52]. It corresponds to one of five stages of the traditional

staging system (a progression of the cancer from Stage 0 to Stage IV), but it has the advantage of revealing more detail by separating designations for the primary tumour, regional nodes, and distant metastases via more specific alpha-numeric subcategories [50]. An added number or letter is used to specify the size or extent of the tumour and the extent of spread. The staging system is important and essential, however insufficient it is by itself. Other significant variables that may contribute to the evaluation include the grade; PSA level; DNA ploidy; nuclear morphometry; and a number of cellular, molecular, genetic, and environmental factors [50].

The TNM staging system provides a basis for survival prediction, initial treatment selection, patient stratification in clinical trials, accurate communication among healthcare providers, and a uniform method for reporting the end result of cancer management [53]. The 2009 TNM classification for PCa [54] is shown in **Figure 14**.

The particular classification of bone metastases was described by Soloway *et al.* [55] in 1988, with a simple method to grade the extent of disease (EOD) observed on serial radionuclide bone scans. The study was done in patients with bone metastasis from PCa who received androgen deprivation therapy. They observed that EOD on the scan correlated with survival. On the basis of the number or extent of metastases the scans are divided into five EOD grades as shown in **Table 2**.

Table 2. The five EOD grades from Soloway Classification. Adapted from [55]

EOD Grade	Number or extent of metastases	2-year survival rates
0	normal or abnormal due to benign bone disease	
I	number of bony metastases less than 6, each of which is less than 50% the size of a vertebral body (one lesion about the size of a vertebral body would be counted as 2 lesions)	94%
II	number of bone metastases between 6 and 20, size of lesions as described above	74%
III	number of metastases more than 20 but less than a "super scan"	68%
IV	"superscan" or its equivalent, ***ie.,*** more than 75% of the ribs, vertebrae and pelvic bones.	40%

Accordingly with the EOD on the bone scan, men with metastatic PCa that enter into trials designed to evaluate the impact of treatment on survival should be stratified based

upon the EOD. This classification also indicates that patients in the EOD IV category have a particularly poor prognosis and may be candidates for alternative treatments [55].

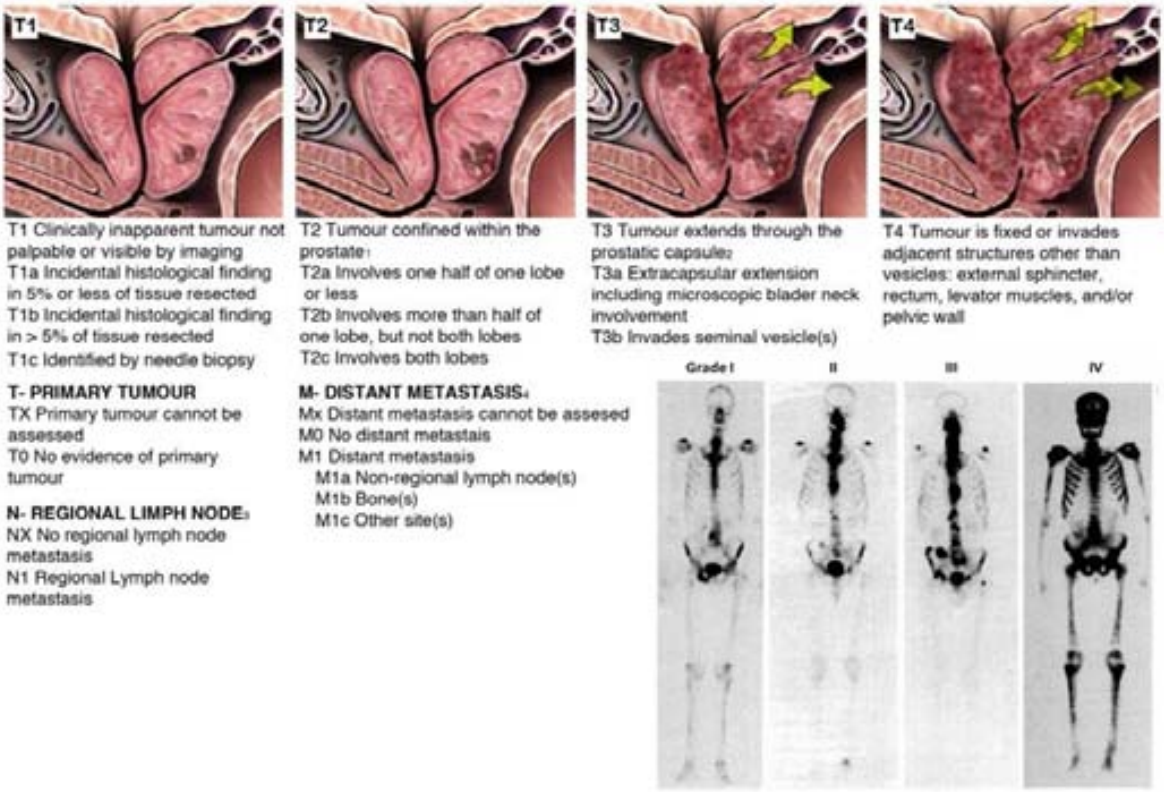

Figure 14. Tumour Node Metastasis (TNM) classification of PCa. Adapted from EAU guidelines 2010 edition [56] and [55]. Upper panel, TNM classification of PCa. Lower panel, EOD category of different bone scans showing a large number of bone lesions in each grade.

e. Therapeutic approaches in prostate cancer

The therapeutic management of PCa has become increasingly complex, due to the various therapeutic options available, even in cases of clinically localized disease, which have equal oncological efficacy but with different, treatment-related side effects. Treatment recommendations vary by disease severity and life expectancy, since the side effects of treatment may outweigh the potential benefits for men whose cancers are unlikely to progress in their lifetime (**Table 3**). Additionally, a multidisciplinary approach may be advisable from the beginning in patients with high risk PCa, because it is very likely that adjuvant treatment will be necessary for locally advanced disease.

The main treatments for PCa from the Clinical Practice Guidelines in Oncology 2009 [57] are summarized below:

Watchful waiting (WW): This term, which was coined in the pre-PSA screening era, refers to the conservative management of PCa until the development of local or systemic progression, at which point the patient is afforded palliative treatment. The rationale behind WW is the observation that PCa often progresses slowly and is often diagnosed in older men for whom there is a high incidence of death from other disease.

Active surveillance (AS): AS is now an accepted management strategy for men with low-risk PCa who previously faced radical whole gland treatment (surgery, external beam radiotheraoy (EBRT) or brachytherapy) [58]. AS involves monitoring the course of the disease with the expectation of intervening if and when the cancer progresses. It is often offered to men who have a limited life expectancy. Monitoring under AS involves PSA testing every 3 to 6 months, digital rectal examinations (DREs) every 6 to 12 months and possible, additional prostate biopsy (PBs).

Radical Prostatectomy (RP): This treatment involves the removal of the entire prostate gland between the urethra and the bladder and the resection of both seminal vesicles, along with sufficient surrounding tissue to obtain a negative margin. Regional lymph nodes may also be removed for examination to determine whether lymph node metastases are present.

Radiation therapy (RT): Radiation therapy normally consists of EBRT or brachytherapy for localized PCa. In EBRT the patient receives radiation treatment from an external source over an 8 to 9 week period. Brachytherapy involves placing small radioactive pellets, sometimes referred to as seeds, into the prostate tissue.

Hormonal therapy (HT): Androgen-deprivation therapy (ADT) alters the effects of male hormones on the prostate through medical or surgical castration (the elimination of the testicular function) and/or the administration of anti-androgen medications.

Table 3. PCa treatment recommendations, by disease characteristics and life expectancy. Adapted from REF [57].

Risk of progression and recurrence	Clinical characteristics of PCa	Life expectancy	Recommended initial treatment options
Low	T1-T2a and Gleason score 2-6, serum PSA levels <10 ng/mL	< 10 years	Active surveillance
		> 10 years	Active surveillance or radical prostatectomy or radiation therapy
Intermediate	T2b-T2c, or Gleason score 7 or serum PSA level 10-20 ng/mL	< 10 years	Active surveillance or radical prostatectomy or radiation therapy (EBRT+/brachytherapy-) +/-ADT
		> 10 years	Radical prostatectomy or radiation therapy (EBRT+/brachytherapy-) +/-ADT
High	T3a, or Gleason score 8-10 or serum PSA level >20 ng/mL	All	Radical prostatectomy (selected patients) or radiation therapy (EBRT) + long-term ADT

III. FROM PROSTATE CANCER TO BONE METASTASIS

a. Epidemiology

The incidence of bone metastasis is unknown [59] but of the estimated one million annual deaths associated with metastatic bone disease (MBD) in the USA, EU and Japan, approximately 20% are cases of advanced-stage PCa [59, 60]. The primary cancers that most frequently metastasize to bone are breast and prostate cancer, amongst many others (**Table 4**) [61]. Further, once tumour metastasizes to the bone, patients are incurable. For example, only 20% of patients with breast cancer is still alive 5 years after discovery of bone metastasis [62].

Table 4. Incidence of bone metastases at postmortem examination in different cancers. Adapted from REF [63]

Primary tumour	Incidence of bone metastases (%)
Breast	73
Prostate	68
Thyroid	42
Lung	36
Kidney	35
Gastrointestinal tract	5

Skeletal metastases occur in more than 70% of cases of advanced-stage PCa and they confer a high level of morbidity, a 5-year survival rate of 25% and median survival of approximately 40 months. Scintigraphic studies have shown that the areas most commonly affected are the axial skeleton (especially the lumbar spine) (60%) followed by the ribs (50%), appendicular skeleton (38%) and skull (14%) [64].

Bone metastases are responsible for tremendous morbidity in patients with cancer, including severe bone pain, pathologic fractures, spinal cord and nerve compression syndromes, life-threatening hypercalcemia, and increased mortality [65]. Further, bone metastasis results in impaired mobility, increased medical costs, a diminished quality of life, and as noted above, a negative impact on survival [66].

b. Mechanism of prostate cancer bone metastasis

The mode of dissemination to the skeleton is haematogenous and their distribution correlates closely wiht the distribution of the red marrow in bone [64]. Bone is a common site for metastasis owing to high blood flow in the red marrow; the presence of adhesive molecules on tumour cells that bind them to stromal cells in the bone marrow; and the production of angiogenic factors and bone-resorbing factors that enhance tumour growth, thereby providing access to the resorbed bone matrix for subsequent tumour adhesion and proliferation [13]. Cancer cells that survive the rigors of the systemic circulation invade sinusoids in the bone marrow cavity in preparation for progression to a bone metastasis. Thus, the affinity for the red marrow is the most likely factor determining the distribution of the metastatic spread correlating to the Batson's theory of "valveless" venous spread [64, 67]. However, the high incidence of metastases in the lumbar spine and pelvis is likely to be a consequence of the anatomical proximity and the pattern of venous drainage which dictates that this area of the skeleton has a higher exposure to cells released from the prostate and therefore has a higher number of epithelial cellular "hits" on the bone marrow endothelium itself [64].

Bone is a metabolic active tissue, it is a reservoir of growth factors, calcium and phosphor, which are liberated during the bone remodelling (**Figure 15**). The skeleton is continually remodelling, and the normal process of "bone turn-over" (resorption - formation) is extremely well equilibrated [68]. In normal conditions, micro-fractures, hormones, calcium levels and inflammation can initiate the remodelling of the bone. Bone turn-over is classically described by starting its cycle with bone degradation and ending with bone deposition [68]. However, the presence of cancer cells disturbs the equilibrium that exist between bone matrix depositing cells, osteoblasts, and bone degradating cells, osteoclasts.

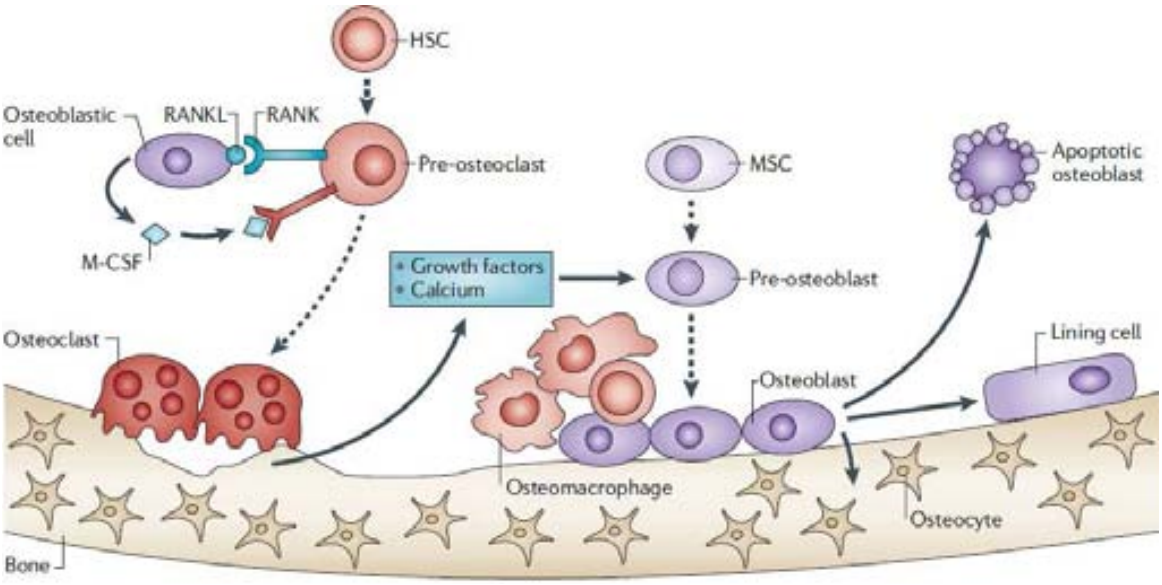

Figure 15. Bone remodelling. From REF [69].

Osteoblasts derive from mesenchymal stem cells in the bone marrow under the control of Runx2, a key osteoblastic transcription factor. Osteoclasts come from hematopoietic stem cells. Cells of this monocyte-macrophage lineage are stimulated to form by fusion the non-functional pre-osteoclasts (multinucleate cells). Osteoblasts produce macrophage colony stimulating factor (M-CSF) and receptor activator of NFkB ligand (RANKL), which both bind to pre-osteoclasts via their receptor c-fms and RANK, leading to their osteoclasts differentiation and activation. Osteoblasts also secrete osteoprotegrin (OPG), a trap receptor of RANKL that blocks its action. Therefore, balance between OPG and RANKL is crucial for the osteoclasts activity. Active osteoclasts bind the bone surface creating the 'sealed zone' where acid and proteolytic enzymes (e.g. cathepsin K) are secreted, resulting in bone matrix degradation [68]. Following the bone degradation, pre-osteoblasts are recruited from the mesenchymal stem cells and differentiated into active osteoblasts, which will repair the bone matrix. Once osteoblasts terminate bone deposition, they go through apoptosis, reside in the matrix or revert to mesenchymal cells [68].

The entry of cancer cells into the microenvironment of the bone will modify the cell-cell interaction (**Figure 16**). The initial site of tumour cell seeding is in areas of bone that are highly vascularized, and in agreement with this the majority of tumour models show that metastatic foci predominantly appear in areas of trabecular bone [70]. It has

been shown that tumour cells preferentially home to areas of high turn-over, attracted by the increased levels of bone-derived growth factors and cytokines generated by active bone resorption [70].

Following the arrival of tumour cells in bone, interactions with the bone microenvironment support their survival. Once localized in this niche, the tumour cells may be under the same microenvironmental control that ensures the quiescence of hematopoietic stem cell (HSC) in adult bone explaining the prolonged periods of tumour cell dormancy that often precedes the appearance of overt bone metastases [70].

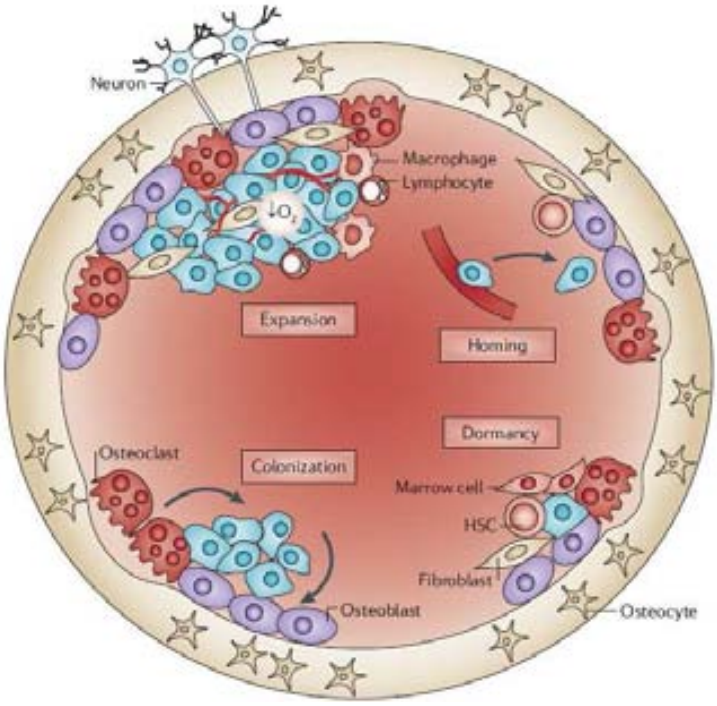

Figure 16. Cross-section of bone depicting stages of bone metastases. From REF [69]. Schematic representation of tumour cell interactions within the bone microenvironment during stages of tumour metastasis to bone: tumour cell homing, dormancy, colonization and expansion. Tumour cells home to and enter the bone marrow cavity and either remain quiescent or dormant or begin growth and colonization. Tumour-mediated recruitment and modulation of bone-residing cells and bone matrix modifications alter the bone environment thus favouring tumour growth and invasion and resulting in pain, fracture and further tumour dissemination.

It has been proposed that cancer cells metastasize into bone owing their gene expression that is considered to be “bone-related” [71]. With the expression of such proteins, cancer cells are able to colonize, survive and show persistent growth into the bone microenvironment [68]. Osteomimetic factors include osteopontin (OPN), bone sialoprotein (BSP) or PTHrP [72] and two chemo-attractants are particularly relevant: the expression of RANK by the metastatic cells that bind RANKL [73], and chemokine

receptor CXCR4 that bind stromal-derived factor 1 (SDF-1 or CXCL12) [74]. Both RANKL and SDF-1 are expressed by osteoblasts that are situated at the vascular surface of the bone, suggesting an important role of the osteoblast in guiding the metastatic cells to the bone.

Conventional wisdom has led many to propose that bone metastases are either osteolytic (bone destructive) or osteoblastic (bone forming, sclerotic) [59]. In this light, osteolytic bone metastases are presumed to be caused by the release of osteoclastogenic agents by tumour cells in the bone microenvironment [75, 76], whereas osteoblastic metastases are the result of the release of factors that stimulate osteoblast proliferation, differentiation and subsequently uncontrolled bone formation by metastatic cancer cells [77, 78]. Purely lytic or sclerotic bone lesions are, however, but two extremes of a spectrum of activity that drives tumour destruction of bone, and both processes are typically present in any skeletal site affected by metastases [13]. Accordingly, bone metastases are typically characterized as 'lytic', 'sclerotic' or 'mixed', according to the radiographic and/or pathologic appearance of the lesions [79].

In PCa, bone metastases are primarily osteoblastic [80]. In these metastatic sites, local stimulation of osteoblast activity results in bone formation directly adjacent to the metastatic tumour. Nevertheless, many patients with PCa will also exhibit osteolytic components in bone lesions [13]. Whereas in breast cancer, it has been described that either bone degradation or deposition occurs in the early metastatic process, but the great majority (~90%) ultimately cause bone loss [81]. It was originally noted that it was tumour cells that caused the bone degradation, but it is now widely accepted that healthy cell osteoclasts are largely responsible for the osteolysis of the bone metastatic lesions [68].

The presence of metastatic cells has been referred to as "vicious cycle of bone metastasis" (**Figure 17**) [82]. Tumours cells produce growth factors, such as parathyroid hormone-related protein (PTHrP), causing the production of RANKL and the down-regulation of OPG by the osteoblasts, and consequently activate the osteoclasts [83]. Bone degradation will release growth factors and others factors stored in the matrix such as transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), insulin-like growth factors (IGFs) and calcium. These factors can stimulate the tumour

cells, inducing their proliferation and their production of more growth factors and more PTHrP. Moreover, the cytokines (e.g. interleukin-6, -8, -11) secreted by metastatic cells also promote osteoclasts activation. Additionally, secretion of the cytokines is increased in the presence of TGF-β [32]. All these activations and secretions will supply and amplify the "vicious cycle".

Osteolytic metastasis of tumour cells involves a 'vicious cycle' between tumour cells and the skeleton that is propagated by tumour cells, bone-forming osteoblasts, bone resorbing osteoclasts and stored factors within bone matrix. Tumour cells release certain factors including interleukines, PTHrP and TNF that stimulate osteoclastic bone resorption. These factors enhance the expression of RANKL over OPG by osteoblasts, tipping the balance toward osteoclast activation thus causing bone resorption. This bone lysis stimulates the release of BMPs, TGFb, IGFs and FGFs for stimulating the growth of metastatic cancer cells to bone.

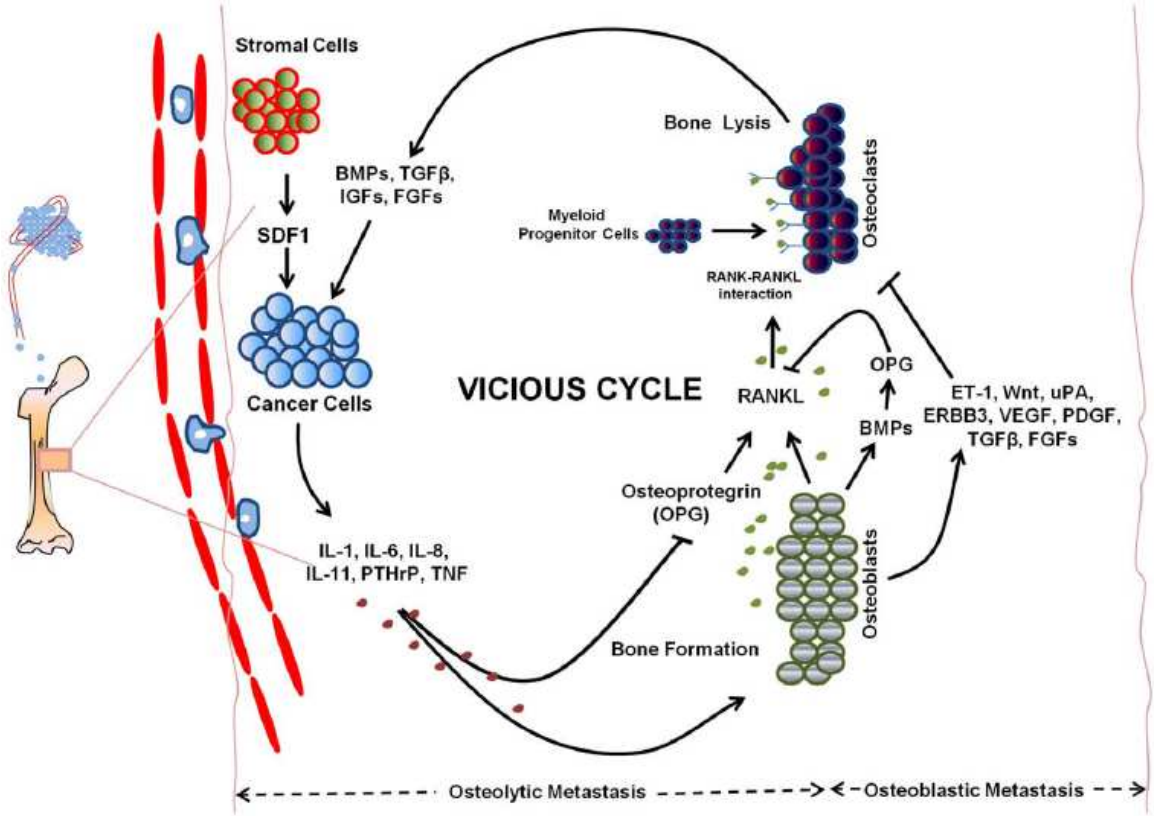

Figure 17. Regulatory mechanisms underlying metastasis to bone reflecting complex interplay of molecules. From REF [84]. Bone metastasis results from imbalance of normal bone remodelling process involving osteolytic (leading to bone destruction) and osteoblastic (leading to aberrant bone formation) mechanisms.

In **Osteoblastic metastasis**, factors released by osetoblastic cells, such as ET-1, Wnt, ERBB3, VEGF play an important role by increasing cancer cell proliferation and enhance the effect of other growth factors including PDGF, FGFs, IGF-1. Urokinase Plasminogen Activator (uPA), a protease, also acts as mediator for osteoblastic bone metastasis by cleaving osteoclast-mediated bone resorption factors responsible for regulation of osteoclast differentiation; thereby blocking the bone resorption.

In comparison the mechanisms of bone formation, the osteoblastic lesions are less understood [59]. One of the most well studied mediators is the ubiquitous growth factor endothelin-1 or TGF-β2, which stimulate osteoblasts formation and proliferation. Proteases can also playing a role in osteoblastic lesions by cleaving proteins such as PTHrP, reducing RANKL secretion, resulting in its inability to activate osteoclasts.

Still, little is known regarding the mechanism(s) of Pca metastasis to, and establishment of lesions in bone. Yet, how a tumour arrives at a metastatic site does not explain how a metastasis is established. Indeed, the favorable interaction of PCa cells with the bone microenvironment appears a critical determinant for the establishment and prevalence of PCa metastases in bone (**Figure 18**) [85].

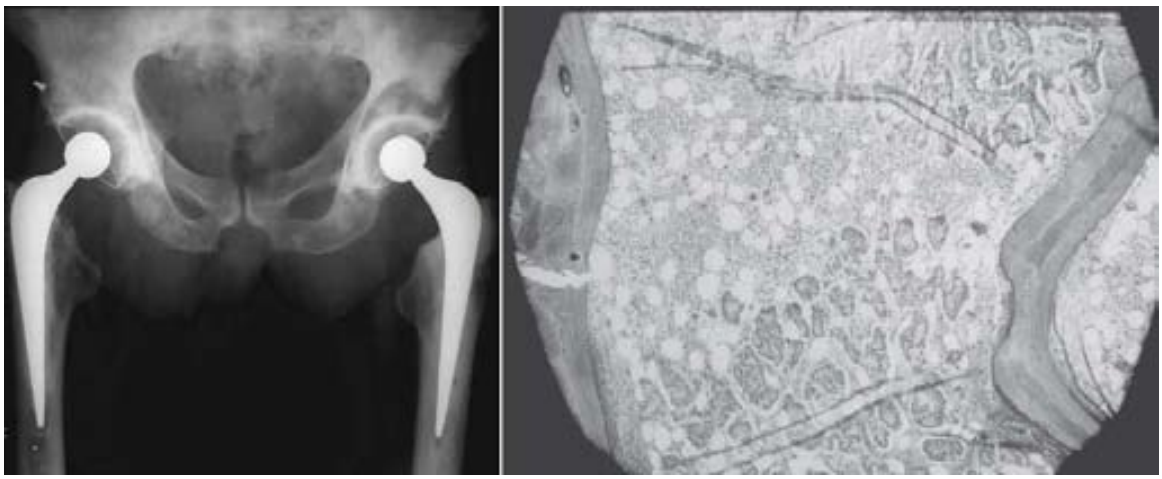

Figure 18. Radiograph of a 63 year old man and bone biopsy from a bone metastasis with hormone refractory PCa. Adapted from REF [64]. Left, the radiograph is illustrating the problem of long bone fracture. Right, in the bone biopsy, the trabeculae of the cancellous bone frame the marrow space containing normal red bone marrow (centre to upper left) and encroaching PCa cells (lower central and upper right). The PCa cells are displacing the red bone marrow progressively.

c. Molecular mediators of bone metastasis

Tumour cells disrupt a normal physiological process, redirecting the cells of the bone microenvironment towards increased activity to support tumour cell proliferation and expansion. This generates the physical space for tumour expansion as bone is resorbed, as well as increased supply of tumour growth factors and cytokines supporting further tumour growth [70].

i. Osteolytic mediators

Malignant cells secrete factors that stimulate osteoclastic activity both directly and indirectly.

Transforming growth factor beta

Once resident in bone, tumour cells, as well as factors released by the primary tumour, activate bone resorption and release transforming growth factor beta (TGF-β) from stores in the bone (**Figure 19**). TGF-β signaling occurs after specific ligand binding to the type II receptor serine kinase (TGFβRII) on diverse target cells [86]. TGFβRII activation results in phosphorylation of the type I receptor (TGFβRI) and signal transduction via phosphorylation of the downstream, regulatory Smad substrates Smad2 and Smad3 [86]. Subsequent binding of Smad2 or Smad3 to Smad4 results in nuclear translocation and increased transcription of target genes.

In addition TGF-β released from the mineralized bone matrix during osteoclastic bone resorption has direct effects on tumour progression in bone. Thus, both tumour cell-derived and bone-derived TGF-β stimulates local cell proliferation in the bone marrow microenvironment [78]. As many tumour types secrete TGF-β and respond to it by enhanced invasion and metastasis, targeting of TGF-β signaling pathways via direct antitumour actions or immunomodulation of the tumour microenvironment and/or effects on bone is a valid approach for the treatment of bone metastasis. In support of this concept, small molecule TGFβRI inhibitors have been shown to induce a variety of skeletal changes, including increased bone mass and improved bone material properties; results of current ongoing clinical trials are eagerly awaited [13].

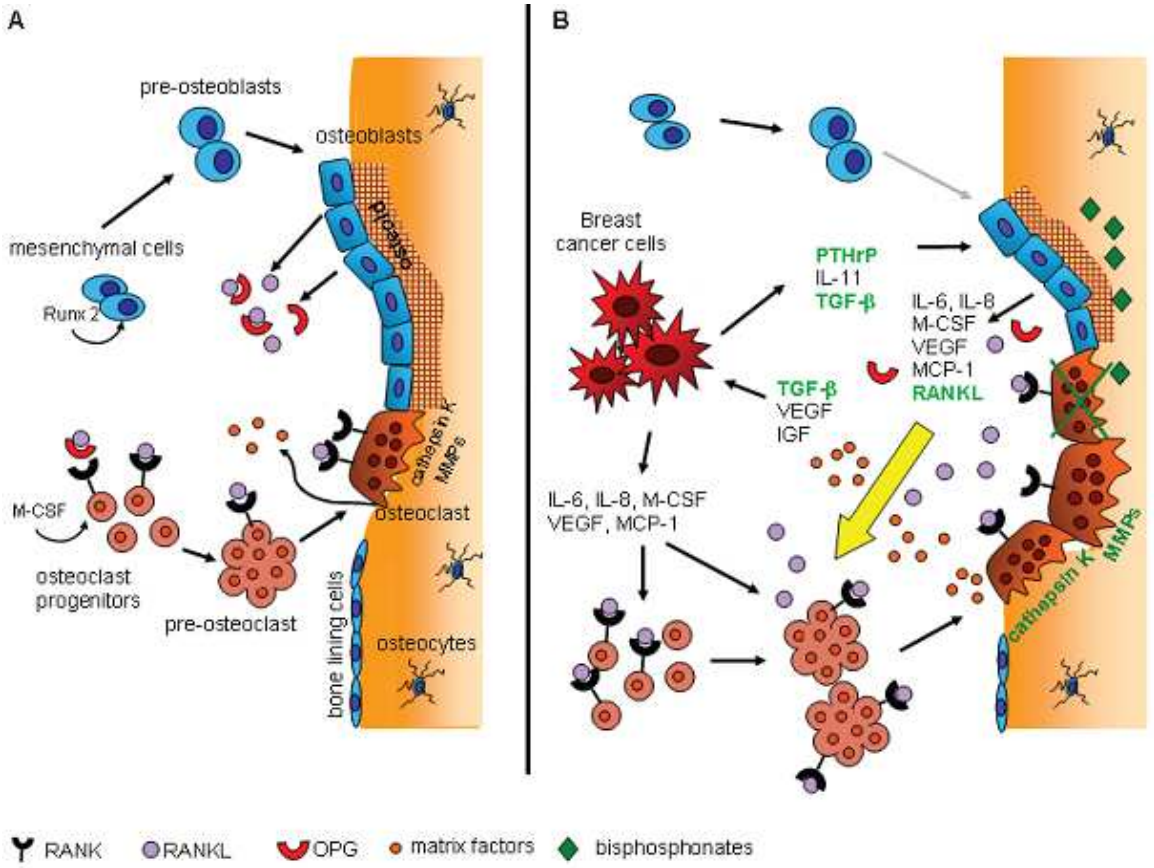

Figure 19. Bone turn-over [68]. **A.** In normal conditions. **B.** In osteolytic bone metastases.

In addition, the enhanced release of tumour-secreted factors such as parathyroid hormone related peptide (PTHrP) and IL-8 can activate T cells, thereby increasing the process of bone resorption, while suppressing T-cell function. As a result, antibody-producing B cells upregulate expression of CXC-chemokine receptor 4 (CXCR4) upon completion of differentiation. Both cancer and stromal cells commonly express the CXCR4 ligand stromal-derived factor-1 (SDF-1; also known as CXCL12). This ligand-receptor interaction has been suggested to facilitate cancer-cell migration throughout the bone microenvironment [13].

PTHrP and RANKL

PTHrP stimulates osteoclast activity via the cytokine RANKL, which causes osteoclast formation and activation by binding to its receptor RANK on osteoclasts and their precursors [87]. RANKL is the primary physiologic mediator of osteoclast formation,

function and survival, and the vast majority of pro-osteoclastogenic agents work via upregulation of RANKL [13, 88].

However, although strong evidence from numerous preclinical studies supports the concept that PTHrP is an important local mediator of osteolytic bone lesions [59, 89], clinical data from an antihuman PTHrP antibody trial in the treatment of bone metastases from breast cancer are currently lacking [13]. By contrast, a large prospective clinical trial demonstrated that the role of PTHrP is not in mediating metastasis but in other stages of cancer progression. PTHrP expression by primary breast cancers was associated with improved prognosis and decreased metastasis to all sites, including bone [90]. This result implicated an activity that remains unknown but is distinct from the well-characterized and widely accepted osteolytic action of tumour-derived PTHrP in bone [59]. In other words, the phenotype of metastatic breast cancer cells in bone, including the expression of PTHrP, is distinct from the phenotype of the tumour cells at the primary tumour site in the breast. Conceivably, the expression of PTHrP at sites of bone metastasis is the result of the tumour cells' successful completion of the metastatic cascade (**Figure 2**) and the influence of the bone microenvironment [13].

RANKL-independent mediators

Based on the complex interactions that mediate all aspects of tumour metastasis, the notion that RANKL activation and the TGF-β–PTHrP axis are the sole mediator of tumour osteolysis is naive. The most compelling evidence that supports RANKL-independent effects on tumour-induced osteoclastogenesis and osteolysis come from clinical trials of the fully human RANKL antibody (denosumab) in patients with bone metastases and elevated bone resorption [91]. Recent data [92-94] demonstrate the efficacy of the RANKL antibody for the inhibition of osteolysis in patients with cancer, but also reveal that in individuals with elevated bone resorption, tumour-derived factors other than RANKL probably contribute to the increased osteoclast activity [13].

Cyclooxygenase-2

Cyclooxygenase-2 (COX-2) expression by breast cancer cells is suggested to support development and progression of bone metastases through the generation of

the prostaglandin (PGE2), which in turn increases expression of RANKL in osteoblasts and stromal cells. Osteoclastic bone resorption releases TGFb from the bone matrix, leading to increased COX-2 expression by cancer cells and consequently PGE2 production, promoting further bone destrcution. COX-2 also induces production of IL-8 and IL-11, cytokines associated with bone metastases [95].

IL-8 and IL-11

Elevated IL-8 levels in the serum of breast cancer patients predict early metastatic spread, and human breast cancer cell lines expressing IL-8 induce higher levels of bone metastases compared to IL-8-negative cell lines. IL-8 increases bone resorption through binding to the CXCR1 receptor present on osteoclasts and their precursors, in a mechanism that is independent of RANKL pathway. In breast cancer cells, expression of IL-8 and IL-11 is induced by COX-2 in a PGE-depedent manner.

ii. Osteoblastic mediators

An accumulating data implicate a variety of factors in the stimulation of bone formation associated with metastatic PCa (**Figure 20**).

Growth factors

A major challenge in PCa biology is the lack of understanding of the mechanisms of PCa progression and the development of bone metastases. PCa cells express a large variety of growth factors capable of activating resident bone and bone marrow cells, such as acidic and basic fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) [96, 97].

Multiple BMPs and their cognate receptors (BMPRs) are expressed in normal prostate and PCa cells [98, 99]. Altered expression and function of BMPs and BMPRs has been reported during prostate development and PCa progression [100]. However, at present, the effects of specific BMPs on osteoblastic tumour progression are inconclusive, given the inconsistency of published results [97]. Expression of FGFs is elevated in PCa, and these factors can potentially act in either a paracrine or autocrine manner [101]. Similarly, FGFs also seem to regulate the osteoblastic response to bone

metastases from PCa. Both acidic and basic FGFs (FGF1 and FGF2) cause the profound stimulation of bone formation *in vivo* [102]. FGF2 was also shown to stimulate the proliferation and differentiation of osteoblasts via the concomitant upregulation of both RUNX2 and BMP2 [103]. Whether this regulation of bone formation via FGF2 also has a role in the formation of bone formation during bone metastasis from PCa remains undetermined [13].

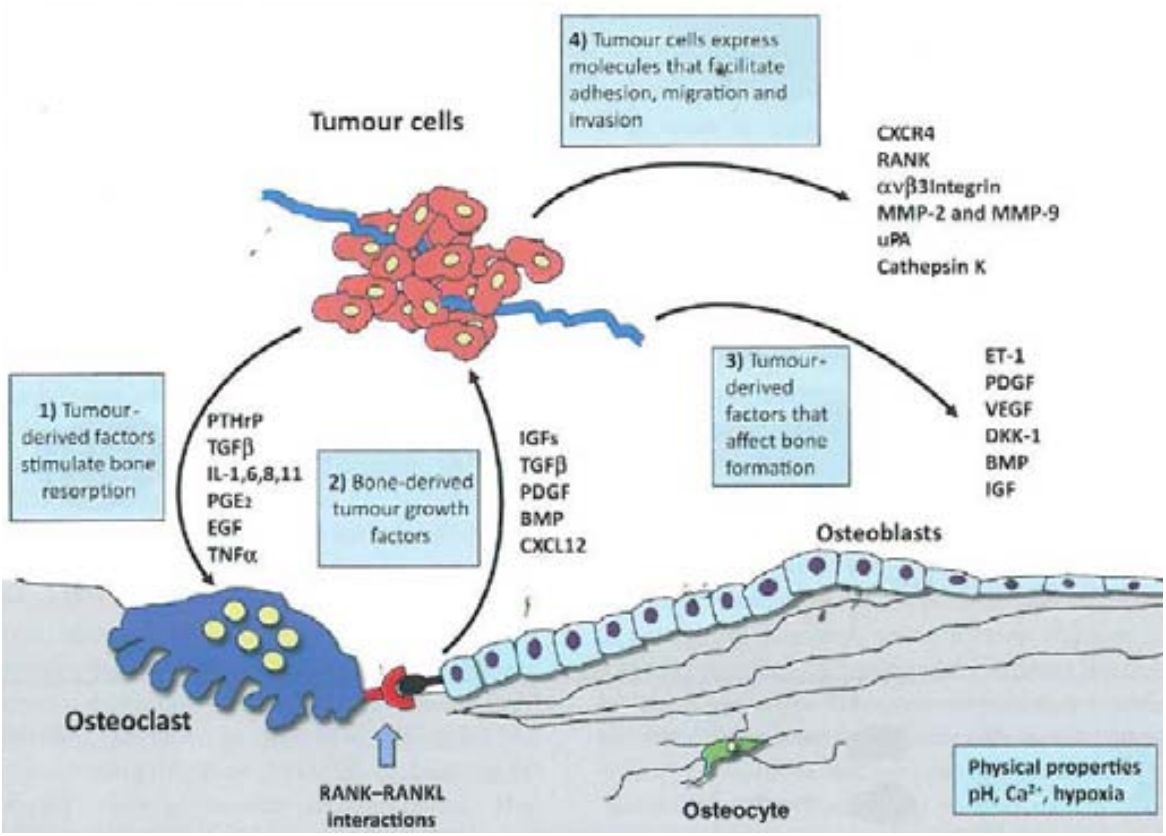

Figure 20. Molecular interactions in bone metastatic sites. From REF [70].

Endothelin-1

The potent vasoconstrictor endothelin-1 (ET-1) stimulates osteoblast and inhibits osteoclast activity, and has been demonstrated to mediate the formation of osteoblastic metastases. PCa cells express ET-1, which can potentiate the mitogenic effects of platelet-like growth factor (PDGF), EGF and FGF, thereby stimulating tumour cell proliferation and driving the development of osteoblastic bone metastases. High levels of plasma ET-1 correlate with the presence of bone metastases in PCa patients [70].

Breast cancer cells also express ET-1, potentially contributing to the development of the mixed bone lesions frequently observed in advanced disease [70].

Wnt signaling

In the past years, canonical Wnt signaling has been shown to play a central part in normal osteoblast development and bone formation [104]. This idea has been extended of late to suggest that Wnts also have a paracrine activity to regulate bone formation in bone metastasis from PCa [105]. PCa cells that metastasize to bone have been reported to secrete dickkopf-related protein 1 (DKK1), a secreted Wnt antagonist, early in the development of the skeletal metastasis. As the bone metastasis progresses, DKK1 expression decreases, leading to a Wnt-mediated increase in osteoblastic activity, which causes the well-described secondary osteoblastic lesions of PCa [106]. In addition, the effects of endothelin-1 to increase osteoblast proliferation and new bone formation also seem to be associated with activation of the Wnt signaling pathway via suppression of the Wnt pathway inhibitor DKK1 [107].

iii. Factors modulating the extracellular matrix

Matrix metalloproteinases (MMPs)

Proteolytic enzymes are implicated in processes involved in both bone metastasis and in normal bone turn-over, including matrix degradation, cell migration, angiogenesis, tumour promotion and growth factors. Secreted and cell surface-associated proteolytic enzymes are implicated in tumour induced bone disease, including MMP-2 and MMP-9, lysosomal cysteine proteinases and plasminogen activators, but their specific contribution to bone metastases has not been clarified due to lack of specificity of available inhibitors [70].

Cathepsin K

Cathepsin K is the major proteolytic enzyme secreted by osteoclasts contributing to degradation of extracellular matrix (ECM) proteins during bone resorption, responsible for breaking down type I collagen present in the bone matrix. Tumour-

derived cathepsin K has been suggested to play a role in cancer-induced bone disease in breast and prostate cancer [70].

uPA/uPAR

The serine-type proteinase urokinase-type plasminogen activator (uPA) and its receptor (uPAR) are involved in the conversion of plasminogen to plasmin, followed by a further cascade of proteolytic enzymes resulting in degradation of fibrin, vitronectin, proteoglycans and the major basement membrane components laminin and collagen type IV. A role for uPA in the mobilization of dormant tumour cells in bone marrow has been suggested, where uPA is proposed to regulate mitogenic signaling of the switching of tumour cells between active cell proliferation and dormancy [70].

d. Clinical outcome of metastatic bone disease

The growth of disseminated tumour metastases is a major cause of mortality in patients with cancer. In patients with aggressive tumour growth at the primary site, bone metastases are relatively uncommon. This finding does not mean that, in these particular instances, the tumour cells do not have the ability to grow avidly in bone, but that they may not have had the opportunity to do so. Invasion of the lymph nodes should be considered as an early step in the metastatic cascade and one that can lead to bone metastasis by drainage of tumour cells back into the systemic circulation [13]. PCa frequently metastasizes to bone, as approximately 90% of men with high-grade PCa show evidence of skeletal lesions. Even if localized to the prostate, a 15–20% incidence of subsequent metastatic disease has been reported [108].

e. Bone metastasis disease therapy

For patients with overt bone metastases, current treatment objectives are designed to decrease tumour burden, prevent further progression and metastasis and inhibit tumour-associated bone pathology, such as pathologic fracture, pain or hypercalcemia [76]. Several local bone metastasis treatment strategies are primarily palliative in nature; individual lesions are surgically excised and the tumour ‘bed’

irradiated, either before or after surgery. The decision for or against surgery and/or radiation, alone or in combination with select bone-targeted agents, is profoundly influenced by the extent of systemic disease at the time of treatment (**Table 5**) [76].

The concept that bone resorption and bone formation are critical for the progression of bone metastasis suggests that, if osteolysis is disrupted, then not only a decrease in bone resorption but also a decrease in tumour burden in bone, and thus potentially an increase in bone mass and strength, will occur. As the bone microenvironment is essential for the growth and aggressive behavior of metastatic cancers in the skeleton, the clinical rationale for the development of specific inhibitors of bone resorption and activators of osteoblastic activity is obvious. To date, other therapeutic regimens, which include but are not limited to bisphosphonates, are available for the treatment of cancer patients with bone metastases [92, 109].

Similarly, the increased understanding of the role of the bone-tumour microenvironment has been translated into the development of additional bone-targeted therapies, such as denosumab. This agent is FDA-approved for the prevention of fractures and skeletal problems in patients with bone metastases from solid tumours. Denosumab treatment (at higher and more frequent doses than those used in patients with osteoporosis) delayed the time to a first skeletal-related event compared with the bisphosphonate zoledronic acid in patients with bone metastases from breast or prostate cancer [13].

Treatment-naive metastatic PCa is largely sensitive to androgen-deprivation therapy (ADT) but progression to castration-resistant PCa (CRPC) occurs 18–20 months after starting treatment [110]. Metastatic bone diseases (MBD) causes some of the most distressing symptoms of advanced-stage cancer; estimates indicate that treatment of bone pain is required in approximately 30% of men with CRPC and associated MBD; with 22% requiring treatment for singular or multiple pathological skeletal fractures; 7% for spinal-cord compression; and 3-4% for hemiparesis or paresis [60]. Despite the complexities involved in the management of MBD, a number of guidelines for treatment have been recommended (**Figure 21**). At first diagnosis therapeutic intervention will usually involve systemic chemotherapy, hormonal therapy and bisphosphonates, which are mostly palliative options with the intention of reducing pain [111]. Once the disease

progresses and symptoms reoccur, or there is significant risk of skeletal fracture or spinal-cord compression, the use of localized radiotherapy, for solitary bone lesions, or radiopharmaceuticals, for widespread multiple lesions, will be considered [111]. Until recently, no drug had been shown to delay the development of bone metastases [70].

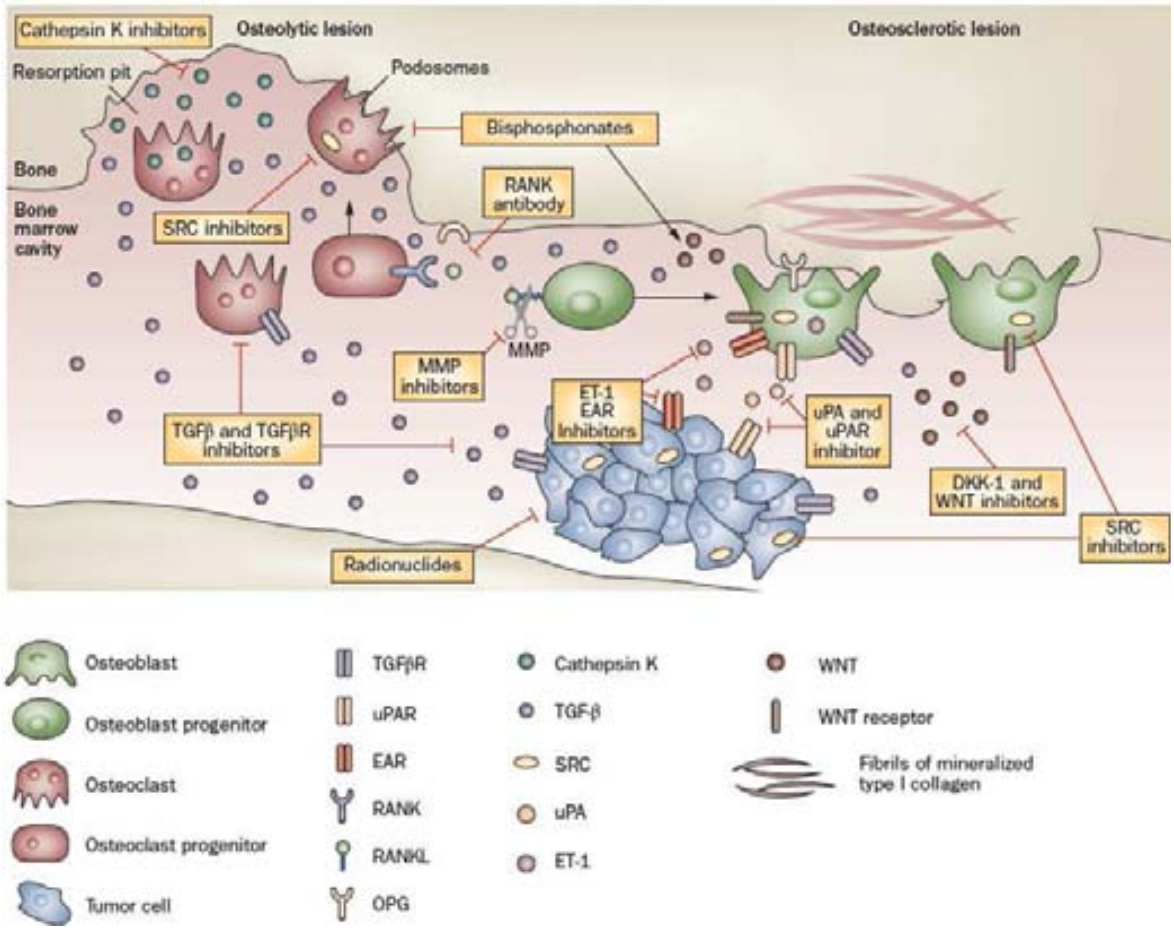

Figure 21. Bone-targeted therapy in metastatic lesions. From REF [60]. Therapeutic approaches to target the osteolytic and osteosclerotic microenvironment of metastatic bone disease in PCa include bisphosphonates, radionuclides and targeted inhibition of: RANKL, cathepsin K, SRC, EAR, TGF-β and uPA. Collagen remodelling is a proposed target. Abbreviations: ET-1, endothelin-1; EAR, ET-1 receptor; OPG, osteoprotegerin; RANK, receptor activator of nuclear factor-κB; RANKL, RANK ligand; TGF-β, transforming growth factor β; uPA urokinase-type plasminogen activator.

i. The current approach: bisphosphonates

Treatment with bisphosphonates has emerged as an effective measure for limiting osteolytic complications in patients with CRPC [112, 113]. Bisphosphonates are chemically stable derivatives of inorganic pyrophosphate that inhibit calcification by binding to hydroxyapatite (also known as bone mineral), preventing its breakdown by

osteoclasts. The second-generation of nitrogen-containing bisphosphonates have an added effect of inhibiting mevalonate pathway enzymes, which directly suppresses osteoclast function, and also have a direct effect on osteoblasts and tumour cells. These compounds can block apoptosis and promote differentiation in osteoblasts and can also promote apoptosis and inhibit growth-factor production, adhesion to bone matrix and the invasion of tumour cells [60].

ii. The new option: RANKL - RANK inhibition

Denosumab is a humanized RANKL monoclonal antibody that received FDA approval for subcutaneous administration for the treatment of osteoporosis. In November 2010, denosumab was also approved for the treatment of patients with MBD derived from solid tumours following the positive results of a randomized trial where its efficacy as an anti-osteolytic agent was compared with zoledronate in breast cancer patients with MBD [114]. An improved efficacy of denosumab compared with bisphosphonates was also reported in a phase II trial of patients with multiple tumour types-PCa (45%), breast cancer (40%) and other tumours (15%) [115].

iii. Some future approaches: SRC kinase inhibition

The tyrosine kinase SRC promotes cell proliferation and survival and has several pro-metastatic functions in PCa cells, including the promotion of cell adhesion, migration, invasion and dissemination to distant organs [116-118].

One clinical trial in a phase III will report on the impact of dasatinib, a SRC and BCR-ABL tyrosine kinase inhibitor, on the incidence of SREs and overall survival in men with CRPC and MBD. Saracatinib, another SRC and BCR-ABL tyrosine kinase inhibitor, which can limit RANKL-induced osteoclastogenesis and protect the bone architecture in the presence of PCa cells, is also under evaluation in a phase II trial for the treatment of men with CRPC and MBD.

iv. Inhibition of the TGF-β signaling axis

A critical role for TGF-β1 in normal bone homeostasis is to coordinate the temporal, spatial and quantitative coupling of new bone formation to sites where old bone degradation is occurring [119, 120] but it is also a critical regulator of several other key stages in bone formation [121]. Two critical findings have been that the TGF-β-signaling axis promotes the development of bone metastasis by regulating a gene-expression signature [32] and by promoting tumour cell homing to bone [122]. Studies have also confirmed the presence of high levels of TGF-β in osteolytic bone lesions, rendering TGF-β as a therapeutic target for limiting MBD progression [123].

Table 5. Novel targeted therapies against bone metastases. Adapted from REF [70]

MoA	NAME	TARGET	CLASS	ROUTE
RANKL	Denosumab	OC	mAB	SC
Cathepsin K	Odanacatib	OC	Inhibitor	PO
Src	Saracatinib Dasatinib	OC/OB	Inhibitor	PO
DKK-1	BHQ-880	OB	mAB	SC
ET-A receptor	Atrasentan Zibotentan	OB	Receptor anatgonist	PO
Activin receptor	ACE-011	OC/OB	Fusion protein	SC/IV

Abbreviations: IV, intravenous; mAB, monoclonal antibody; MoA, mode of action; OB, osteoblast; OC, osteoclast; PO, per os.

IV. ANIMAL MODELS OF PROSTATE CANCER BONE METASTASES

a. *In vitro* approaches

Considerable experimental work has been conducted on the standard PCa cell lines as an attempt to analyse metastasis. The PC3 cell line was originally isolated from a bone marrow metastasis [124], LNCaP is derived from a lymph node metastasis and DU145 from a brain metastasis (an extremely rare occurrence for PCa) [64]. Comparisons between these cell lines have been made, in attempts to define both site-specific changes, and androgen sensitivity of genes up-regulated in metastasis. After many years in culture, they have become grossly aneuploid and also heterogeneous. Unless cross-related to the tissue arrays (or similar), these simple models only offer a small fragment of the metastasis story [64]. A better approach would be to use malignant variants of the same cell type. Most cell lines do throw off variants in both culture and *in vivo* selection. Comparisons of non-malignant cells "progressed" by treatment with chemical and viral carcinogens offer the controlled baseline for comprehensive analysis of metastatic changes [64].

Certain fundamental properties of metastatic cells, including migration and invasiveness, have been the subject of many studies using a variety of *in vitro* model systems. Technological advances such as fluorescent or bioluminescent reporter molecules and sophisticated microscopy have allowed sensitive and accurate analysis of these processes and their molecular underpinnings at the single-cell or cell-cluster levels [125]. *In vitro* model systems have contributed to define the role of candidate metastasis genes in particular steps of the metastatic cascade [125, 126]. However, these models can provide surrogate systems for the analysis of only a limited set of events in the metastatic cascade, which *in vivo* involves multiple steps within specific tissue contexts.

b. Animal models

The ideal animal model for bone metastasis would be a reproduction of the genetic and phenotypic adaptations that occurs in human cancer. These include invasion, spread to the bone via the vasculature, and proliferation and survival in the bone microenvironment that lead to bone matrix modifications. PCa occurs naturally in dogs and in some strains of rats [127].

b.1. Dog model

The dog most closely resembles humans in terms of PCa characteristics [128]. Canine PCa is also age dependent, tumour cells metastasizes to bone in an osteoblastic manner in 24% of cases [129]. While dogs may seem an ideal model for studying PCa, there are limitations to their use. The instances of PCa do not diminish in castrated dogs indicates that tumour growth is not androgen dependent [129]. There is also a relatively long latency in dogs being an unrealistic experimental model [130].

b.2. Rat model

Several strains of rats, including the Dunning, Copenhagen, and Wistar rats, have been well characterized, and they also develop a wide range of cancer phenotypes in the prostate [131, 132]. However, due to the rarity of tumours, variability in phenotypes, long latency periods, and lack of metastases, the realistic probability of using them as models is low [130].

b.3. Mouse Prostate model

Naturally occurring PCa is uncommon in the mouse [133] and additionally, the histopathology and time-frame of prostatic disease development in these animals can be different [133]. Despite these several concerns, the mouse is still one of the best animals in which to model human cancer. First, mice are as susceptible to cancer as humans [134]. Second, the mouse and human genomes are approximately 95% identical, and mice have many structurally similar genes and genomic alterations that have been implicated in cancer [135]. Third, mice are relatively easy to genetically modify. And finally, because mice have a relatively short gestation time and are small,

they are reasonably easy and affordable to house and breed to generate large populations. The goal of every mouse model is to accurately imitate human disease so that molecular mechanisms can be found and new therapies can be tested [130].

b.4. Transgenic Mouse Models

To produce PCa in mice requires tissue specific expression of a strong oncogene from a tissue-specific promoter. Not all of the models however produce metastatic disease [64]. The TRAMP model (probasin promoter driven SV40 T antigen [136]) results in tumours in the dorsolateral lobe (murine equivalent of the peripheral zone) which metastasize to lymph node, lung and (in the correct genetic background) to bone [64]. However, the probasin promoter is active in luminal cells of the murine prostate, and most human PCa probably arise from the basal epithelium. The genetic changes observed are however similar if not identical to human disease, including the loss of E-cadherin expression.

b.5 Xenograft in nude/Scid Mice

Immunodeficient rodent models generally used for PCa studies are the nude mice model described in 1966 by Flanagan [137] and the Scid mice model (Severe Combined ImmunoDeficiency) described in 1983 by Bosma *et al.* [138]. The nude mutation results in thymus aplasy with quantitative and functional T-lymphocyte defects. The Scid mutation results in a lack of T- and B-lymphocyte function. However, normal NK cell and myeloid functions are present that may influence initial tumour growth and metastatic spread after implantation [139].

A new immunodeficient mouse model was described in 1995 by Shultz *et al.* [140] obtained by crossing the Scid and Nod mouse strains, the Nod strain (non obese diabetic) that is characterized by a functional deficit in NK cells, an absence of circulating complement and defects in the differentiation and function of antigen-presenting cells (APCs). The Nod Scid model combines multiple functional defects of adaptive and innate immunity. It is very suitable for xenografts of human tumoural lines [139].

However, it is rare to observe natural bone metastasis in these animals. This limitation has resulted in the use of a specific model representing specific stages of the

disease. Therefore, since animal models are still a representation of what occurs in patients, it was important to determine what can be considered as "bone metastasis". The general consensus defined that cancer lesions can be considered as bone metastasis once the tumour cell proliferation in bone modifies bone structure [127].

The use of immunodeficient and genetically engineered mouse models that mimic the human disease has been crucial in validating the importance of these genetic pathways in PCa. The production of metastases in immunodeficient mice is dependent on the site of implantation. Subcutaneous implantation rarely produces distant metastases, when orthotopic (intra-prostatic) implantation does allow metastatic spread. Orthotopic implantation in an immunodeficient rodent is a relevant model and the first description was made in 1992 by Stephenson et al. [141].

To better understand tumour development and progression *in vivo*, two general strategies have been pursued in mice: genetically engineered models (GEM) of cancer, and transplantable tumour model systems (**Figure 22**) [125]. In these models, cancer develops with high penetrance in a stepwise manner, enabling the study of tumour initiation and early steps of metastatic dissemination. Syngeneic and xenograft models in which mouse or human cancer cells are introduced into immunocompatible or immunocompromised mice provide at present methods of choice to experimentally address metastatic dissemination to, and colonization of relevant organs. Whereas syngeneic models the study of the complete microenvironmental interface in the mouse is limited to study the mouse cancer cell metastasis, xenograft models enable a superior alternative to the study of metastasis of human cancer cells *in vivo* [125].

However, both mouse model systems currently used for the study of metastasis have advantages and disadvantages (**Table 6**), but the combined use of these systems complemented with *in vitro* models is yielding an increasingly robust understanding of the multiple modes and steps of metastasis.

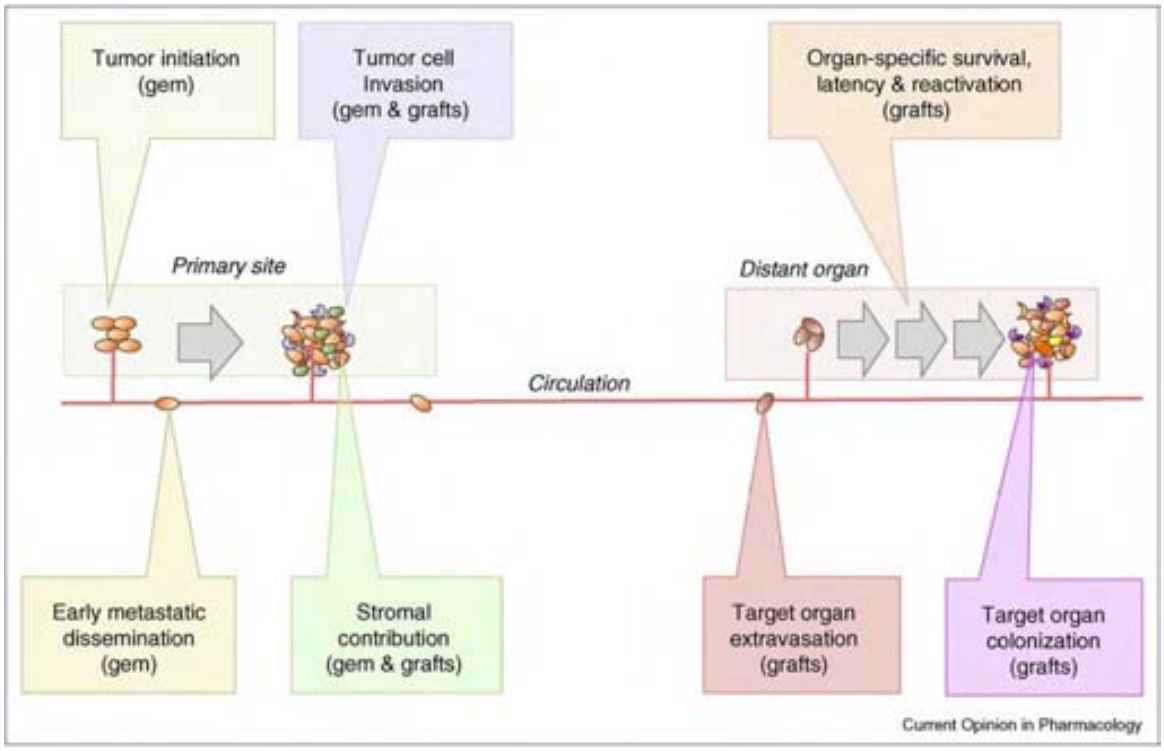

Figure 22. Contribution of different mouse models to the various steps of metastatic dissemination. From REF [125].
Abbreviations. GEM: genetically engineered mouse models, GRAFTS: xenograft o allograft transplantation.

Table 6. Advantages and disadvantages of genetically engineered and transplantable models for the study of metastasis. Adapted from REF [125].

Tumour model	Advantages	Disadvantages
GEM	Immunocompetent host Defined genetic background Tumours arising in tissue of origin, usually from clinical relevant mutations	Limited and/or atypical metastatic spread Laborious uncoupling of initiation from progression Long latency Requires validation in human
Xenografts	Wide range of human samples Range of orthologens metastatic sites Short latency or long latency	Lack of adaptative immune interactions Some species-specific incompatibility
Allografts	Immunocompetent host Wide range of metastatic sites Short latency	Limited range of useful mouse cell lines Requires validation in human samples

c. Metastatic dissemination and colonization

Animals used to study human PCa are immunodeficient. They allow tumour growth after implantation of human xenografts or cell lines in different locations [139]. Mice xenograft models provide an effective system to investigate secondary organ colonization of human cells, and remain the model of choice for preclinical studies of human tumour-derived cells.

Studies of the processes involved in metastasis of PCa cells to bone have been hampered by the limited availability of suitable *in vivo* models [142]. Compared to others metastasis, there is a very low incidence of bone metastasis development in transgenic model of prostate carcinoma in rodent. However, cancer cell lines can be selected *in vivo* to increase the frequency of bone metastasis development after orthotopic injection. Therefore the early steps in the metastatic process are often bypassed, and such models lack information on the complete metastatic process happening in humans. To get round these issues, models of prostate and breast cancer metastasis, in which both the cancer cells and the bone target of the osteotropic metastasis are of human origin, were developed [143]. In that model, orthotopic injection of human breast cancer cell lines later resulted in bone metastases, but only in the bones of human origin and not the mouse skeleton, indicating a "species-specific osteotropism" [142, 143].

Intracardiac inoculation of cancer cells (xenogeneic; derived from different species) into the arterial circulation of mice allows the systemic distribution of these cells to all organs [142, 144] for the analysis of metastatic functions including organ-specific extravasation, survival in the newly invaded parenchyma, retention of tumour-reinitiating capacity, and overt colonization [145]. The inoculation of human cancer cell lines into the left cardiac ventricle of immunodeficient mice has been a routinely used technique to induce bone metastasis *in vivo* [146, 147]. However, such models do not reflect the full process of metastasis occurring in patients and many of these metastases occur in the metaphyses of the long bones and it is the vascular arrangement that can favor the tumour cell arrest in young rodents and not the ability of the cancer cells themselves [148].

In contrast, tail-vein inoculation forces cancer cells to lodge in lung capillaries, which allows an assessment of lung extravasation and colonization functions [149]. Carotid artery inoculation likewise targets cancer cells to the brain [150].

Intratibial and intrafemoral injections have been used to model the invasion and growth of PCa cells in bone, providing a platform for studying bone microenvironment and bone-tumour crosstalk [142], which is essential to understanding why PCa tumours so frequently metastasize to the skeleton. The reason these models are so valuable is because there is no mouse model that spontaneously metastasizes to bone [151]. Intratibial injections were first described for PCa in 2002, when cells from three PCa cell lines were injected into the tibia of nude mice to compare their relative ability to invade and grow in bone [152]. Cancer cells may also be genetically altered to study the effect of specific genes on the ability of cells to grow in bone [106]. Intrafemoral injections can also be used in the same context as intratibial injections. Femurs are larger than tibia in overall size and cavity size, so it depends on the situation as to which bone is chosen for injection [151]. Intrabone injections represent an important model for the elucidation of the importance of genetic pathways and other factors in PCa metastasis to bone.

Although genetically engineered mouse models provide good systems for the preclinical evaluation of therapeutic agents [153], the response of human cancer cells to therapy *in vivo* requires the use of xenograft models. Of particular relevance is the xenografting of metastatic cell lines in orthotopic locations, followed by resection of the primary tumours and initiation of therapy. This setup approximates the situation observed in patients with advanced disease [154].

d. Visualizing metastasis

Tracking cancer cells in real time in whole animals has provided a tremendous advantage in the dynamic monitoring of metastatic development.

In order to improve the evaluation of experimental therapeutics in PCa metastasis, better models are needed to achieve two goals: (1) the ability to serially image tumour growth and colonization and (2) to produce pathologic features similar to those in patients. A new era of modeling cancer metastasis involves the use of optical

imaging technologies to assess tumour burden, and the effects of therapeutic interventions on tumour growth in bone [155-157] after introduction of cancer cells into the animal. The two predominant approaches involve using heterologous fluorescence- or bioluminescence-generating proteins expressed in cancer cells to monitor tumour growth [24, 25]. While fluorescent proteins are advantageous in that they do not require an exogenous substrate for signal emission, can be used for both *in vivo* and *ex vivo* microscopic analysis, and can be further analyzed by histological detection of the fluorescent molecule in frozen sections, or immunohistochemical detection of the reporter [158] this approach is generally 2-3 orders of magnitude less sensitive than bioluminescence imaging (BLI) [159]. This makes BLI a preferable optical imaging modality for monitoring small tumours and/or those growing deep beneath the surface of the animal [160].

i. Bioluminiescence imaging

Whole-body non-invasive BLI is applied for the detection of tumour growth and metastasis in small animals. The luciferase (*luc*) gene from the firefly *Photinus pyralis* is the most widely used bioluminescence reporter in life science research which can be inserted into tumour cells making the applicable for BLI. This technique is based on using a sensitive cooled charged coupled device camera (CCD) array to detect photons emitted from luciferase-expressing cells in tissues after conversion of the luciferin substrate in a reaction that requires ATP, O_2 and Mn^{2+} [161]. The D-luciferin is either injected i.v. or i.p., distributes rapidly throughout the body of the animal and is quickly taken up by the cells. Since BLI only measures viable cells, it can be more informative about cytotoxic effects than other modalities [160].

BLI has been used previously to study animal models of PCa metastasis (**Figure 23**) [127, 156, 162, 163]. Such models have aided in understanding the biology of cancer metastasis and evaluation of therapeutic interventions. However, BLI technology has limitations, such as the inability to absolutely define the size, shape and location of the signal source. Moreover, one must also consider how the pharmacodynamics, biodistribution and cellular uptake of luciferin as well as the disposition of other reaction components might affect light generation [160].

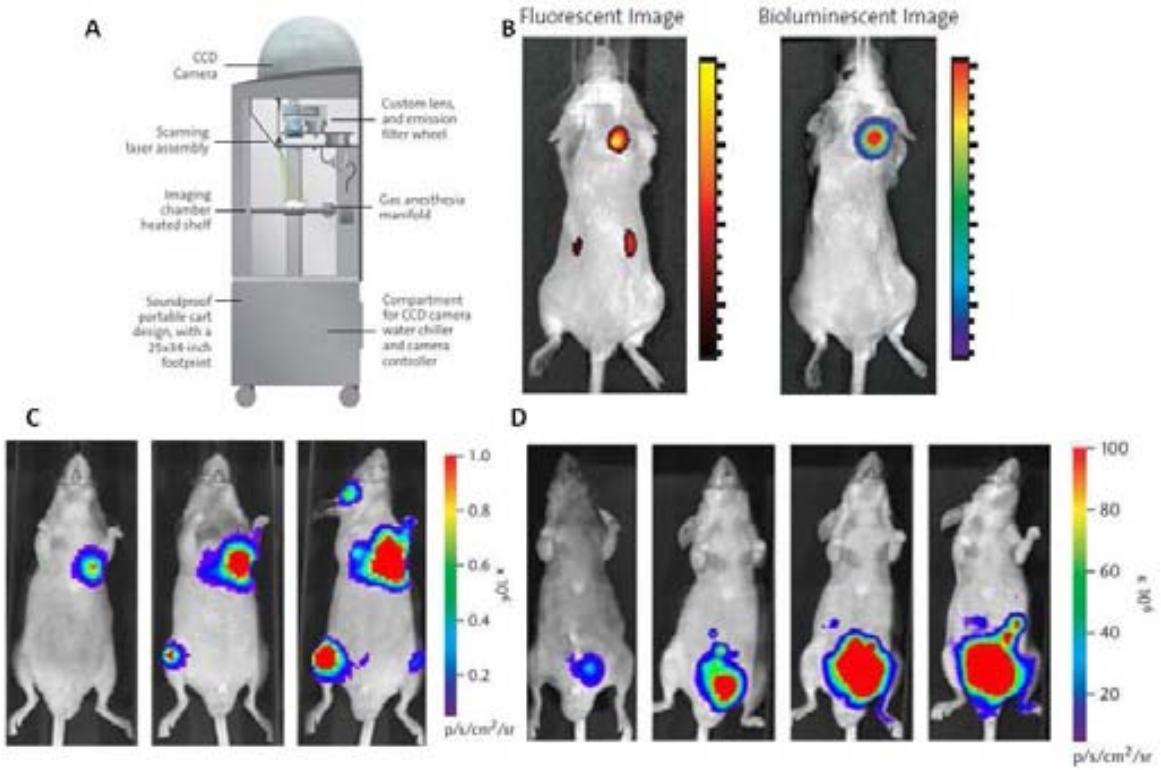

Figure 23. Optical imaging. All images are from Xenogen Corporation®. **A.** IVIS® Imaging System 200 Series components: thermoelectrically cooled CCD camera, imaging chamber, optics, single-view 3D reconstruction, acquisition computer, integrated anesthesia system, and Living Image® software. **B.** Example of *in vivo* detection of SKOV3-luc tumour with Her2-AF680 conjugate (Alexa Fluor® 680nm fluorescence). **C.** Example of PCa bone metastasis using the PC-3M-luc-C6 cell line injected intracardiacally into nude mice. **D.** Example of the orthotopic tumour growth of the PC-3M-luc-C6 cell line in nude mice.

ii. Others whole-body imaging technologies for bone metastasis

Computed tomography (CT) and radiography

Both plain radiography and CT are X-ray based methods and provide information about bone structure in a two- and three-dimensional manner respectively. However, micro-CT more accurately reflects the morphological changes in bone and allows an easier characterization of lesions as osteolytic, osteoblastic or mixed. Its resolution is about ten times greater than radiography and additionally offers the ability to obtain axial images. However, the quantitation of bone volume from micro-CT data is very time-consuming [164, 165].

Positron emission tomography (PET)

PET is a nuclear medicine imaging modality making it possible to visualize the uptake and accumulation of positron-emitting radiopharmaceuticals by tissues. A widely used PET-tracer is ^{18}F-fluorodeoxyglucose (^{18}F-FDG) which is taken up by the cells like glucose but is then metabolically trapped in the cell after phosphorylation by hexokinase and accumulates due to an enhanced glucose metabolism in tumour cells. Thus, PET imaging is especially useful to detect metastases in soft tissues or bone. However, the disadvantages of PET are high costs and long scanning times [166]. For skeletal metastases, 18F-fluoride, a bone-imaging agent, can be employed which uptake is not specific for tumoural bone involvement but deposits at sites of high bone turnover and remodelling [167].

Single-photon emission computed tomography (SPECT)

SPECT is a nuclear medicine modality used because of its ability to image in 3 dimensions. High energy emitting molecules are administered to the patient which differentially accumulate in tissue based on its cellular physiology. SPECT imaging is less sensitive than PET [168]. Respect to the detection of bone metastases, it could be shown that a ^{18}F-fluoride bone scan by PET is more sensitive than SPECT [169].

V. CANCER BIOMARKERS

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic or pathogenic processes, or pharmacologic responses to a therapeutic intervention [50]. In medicine, and according to the United States National Cancer Institute (U.S. NCI), a biomarker is "a biological molecule found in blood or another body fluid (urine, saliva, etc.) or tissue that signals a normal or abnormal process or of a condition or disease". In this context, biomarkers can help in early diagnosis, disease prevention, drug target identification, drug response and can further the development and evaluation of new therapies [50]. Biomarkers can be found using genomics, proteomics or imaging (**Table 7**) and are widely used as analytical tools to assess biological parameters for a rapid and comprehensive therapeutic analysis.

Table 7. Methods of detection of tumour markers. Adapted from REF [170]

Serology	**Enzyme assays**
Immunological	Immuno histo chemistry
	Radio immuno assay
	Enzyme-linked immuno sorbent assay
Flow cytometry	
Cytogenetic analysis	Fluorescent *in-situ* hybridization
	Spectral karyotyping
	Comparative genomic hybridization
Genetic analysis	Sequencing (automated)
	Reverse transcription
	Gel electrophoresis
	DNA micro-array analysis
Proteomics	Surface-enhanced laser desorption/Ionization

It facilitates screening and detecting the cancer, monitoring the progression of the disease, and predicting the prognosis and survival after clinical intervention. Generally, tumour markers include a variety of substances like cell surface antigens, cytoplasmic proteins, enzymes, hormones, oncofetal antigens, receptors, oncogenes and their products [170]. There have been numerous attempts to broaden the definition to accommodate the rapidly expanding set of identified tumour markers and include the following:

1. Substances present in, or produced by, a tumour itself or produced by host in response to a tumour that can be used to differentiate a tumour from normal tissue or to determine the presence of a tumour based on measurements in blood or secretions.
2. A molecule, a process or a substance that is altered quantitatively or qualitatively in precancerous or cancerous conditions, the alteration being detectable by an assay.
3. Biochemical indicators of the presence of a tumour. However, in common clinical practice, the term usually refers to a molecule that can be detected in plasma or other body fluids.

Box 3. Main types of cancer biomarkers. Adapted from REF [171]

Predisposition biomarkers: Identification of individuals at risk of developing cancer

Screening biomarkers: Early detection of cancer in the general population or at risk population

Diagnostic biomarkers: Definition of tumour type, stage and grade

Prognostic biomarkers: Identification of the likely clinical disease course (good or poor outcome) and hence appropriate therapeutic approach

Predictive biomarker: Patient enrichment to maximize likely benefit from individual therapies

Pharmacological biomarkers: Demonstration of potentially active drug concentrations (pharmacokinetics), drug target interaction (pharmacodynamics - proof of mechanism) and phenotypic effects (pharmacodynamics - proof of concept)

Surrogate biomarkers: Early prediction of ultimate clinical efficacy

Cancer biomarkers are usually classified into three categories: prognostic, predictive, and pharmacodynamic. **Prognostic biomarkers** predict the natural course of the cancer and to distinguish the tumour's outcome. They also help determine whom to treat, how aggressively to treat, and which candidates will likely respond to a given drug

and the most effective dose. **Predictive biomarkers** evaluate the probable benefit of a particular treatment. And **pharmacodynamic biomarkers** assess the imminent treatment effects of a drug on a tumour and can possibly determine the proper dosage in the early stages of clinical development of a new anticancer drug [170].

Conceptually, a single marker may be unable to contain sufficient information for clinical prognosis; alternatively, multiple markers may need to be measured simultaneously.

a. Ideal tumour marker

The three most important characteristics of an ideal tumour marker are: (a) it should be highly specific to a given tumour type, (b) it should provide a lead-time over clinical diagnosis and (c) it should be highly sensitive to avoid false positive results. Additionally, the levels of the marker should correlate reliably with the tumour burden, accurately reflecting any tumour progression or regression, along with a short half-life allowing frequent serial measurements. The test used for detection should be cheap for screening application at mass level and should be of such nature as to be acceptable to the target population (**Table 8**). In reality an ideal tumour marker does not exist (**Figure 24**) [170].

Table 8. Characteristics of an ideal tumour marker. Adapted from REF [170]

Characteristics	Remarks
Highly specific	Detectable only in one tumour type
Highly sensitive	Non-detectable in physiological or benign disease states
Long lead-time	Sufficient time for alteration of natural course of disease
Levels correlate with tumour burden	Prognostic and predictive utility of the tumour marker
Short half-life	Frequent serial monitoring of the marker levels after 5-6 half lives
Simple and cheap test	Applicability as screening test
Easily obtainable specimens	Acceptability by target population

Attempts to improve the sensitivity and/or specificity of tumour markers have led to combination of tumour markers with other procedures (e.g., combination of Carbohydrate antigen (CA) 125 with ultrasonography for early detection of ovarian malignancy) or to refining the evaluation criteria for tumour markers (e.g., PSA density or PSA velocity or age-specific PSA cut off ranges for early detection of PCa). However, these have either not stood the rigorous evaluation of randomized trials or have still not received widespread approval of professional clinical organizations [170].

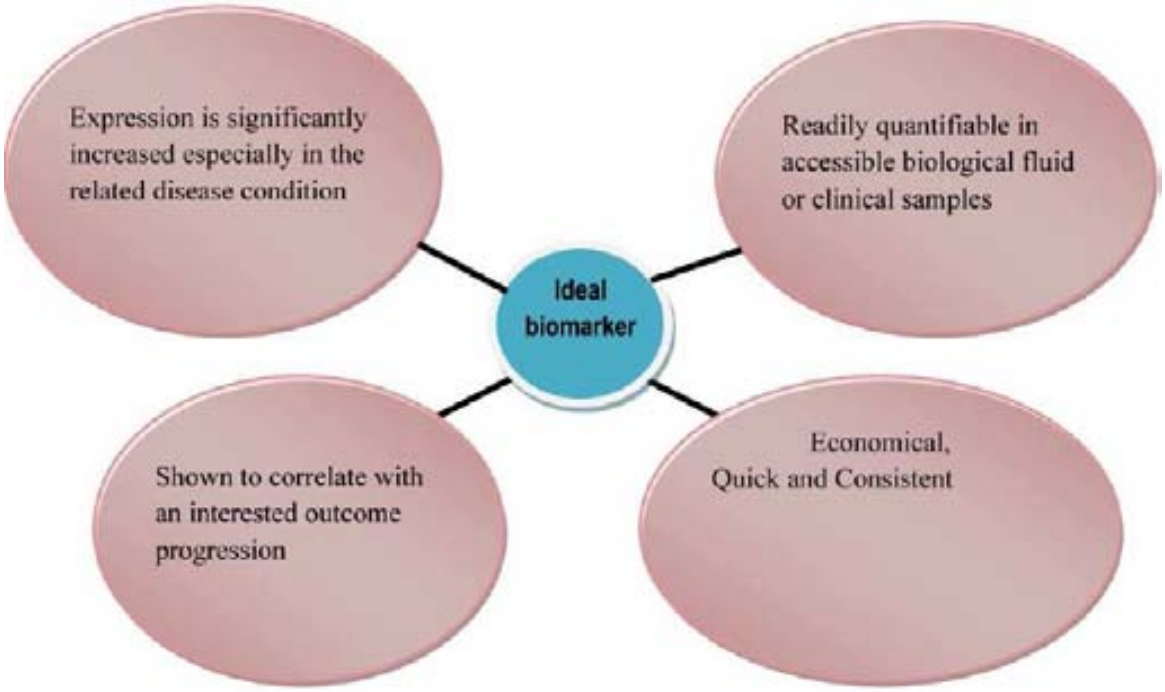

Figure 24. Characteristics of an Ideal Biomarker. From REF [50]

Monitoring disease, perhaps, constitutes the most common clinical use of serum tumour markers. Markers usually increase with progressive disease, decrease with remission and do not change significantly with stable disease. Tumour marker kinetics is generally more important than individual values [172]. Rising tumour marker levels may detect recurrence of disease well before any clinical or radiological evidence of disease is apparent, which it is called "biochemical recurrence" [170].

Of the numerous tumour markers identified, described and extensively researched upon, only a handful of them are used in routine clinical practice; and even of these, only a few have established consensus guidelines for use in day- to-day care of patients [170].

b. Prostate cancer biomarkers

The form of the PCa biomarkers can vary from metabolites and chemical products present in body fluid to genes and proteins in the prostate tissues [50]. Current advances in molecular techniques have provided new tools facilitating the discovery of new biomarkers for PCa. These emerging biomarkers will be beneficial and critical in developing new and clinically reliable indicators that will have a high specificity for the diagnosis and prognosis of PCa [50]. Both genes and proteins that reveal loss, mutation, or variation in expression between normal prostate and cancerous prostate tissues could be proposed as PCa candidate markers.

The management of PCa has undergone several dramatic changes as a result of the evolution of biomarkers used in screening, detecting, and predicting the disease [173, 174]. Human prostatic acid phosphatase (PAP) (or serum acid phosphatase (AP) was reportedly the first serum biomarker for PCa. Gutman and his colleagues [175] observed in the 1930s that patients with PCa metastasized to bone had elevated levels of PAP activity at the site of metastasis and high serum levels of the protein [176]. This finding effectively established the value of serum acid phosphatase activity as an aid in diagnosing metastatic prostate carcinoma and consequently as a biomarker for PCa progression and reaction to androgen deprivation therapy of PCa that had metastasized [50, 177]. Patients with localized cancer frequently display normal levels, and neither PAP nor AP show sufficient sensitivity to be used as a reliable biomarker for recurrence or response to systemic therapy. Furthermore, the use of AP has been reduced because of the development of PSA screening, which is a more sensitive and specific tumour marker [50].

PSA was later discovered as a biomarker for PCa following the discovery of serum PAP and was officially approved in 1994 for PCa screening by the FDA. The prostate gland produces PSA, and the test measures PSA levels in the blood (serum). Because PSA is from the body and can be used in disease detection, it is often referred to as a biological marker or a tumour marker. Both PCa and benign prostatic conditions (e.g., BPH) can increase PSA levels from a normally low level to an elevated state in the blood. PSA can be present in a free form or complexed with α1-antichymotrypsin or α2-macroglobulin in circulation. Patients with cancer can be distinguished from those with

BPH based on the percentage of free to total PSA in the serum. For those with an elevated level of PSA, particularly with a PSA range between 4 and 10 ng/ml, patients are more likely to have PCa when the free PSA is less than 20-25% of the total serum PSA level. ProPSA, the precursor form of PSA, may serve as an additional indicator in differentiating cancers from benign processes [50].

Serum PSA was initially used for screening men with an existing diagnosis of PCa and was regarded as an ideal marker for identifying recurring disease subsequent to treatment [178]. PSA gradually replaced serum PAP, which was considered inferior to PSA, for PCa screening, staging, and prognostication. However, PAP is once again attracting some attention because of the fact that several studies have shown that it is a good prognostic marker for patients with aggressive disease who went through local therapy and are at high risk for distant relapse [179]; however, PAP has no role as a diagnostic screening tool.

c. Candidate markers for prostate cancer

The need for effective PCa biomarkers is therefore urgent and great, and the search for them has been a priority of researchers for years. In the last decade, PSA has been widely used as a useful tool for screening PCa. However, PSA and other established biomarkers are still not ideal, as they lack diagnostic specificity and prognostic value and lead to a high rate of false-positives. Consequently, the lack of specific and sensitive biomarkers for early detection of PCa calls for investigating novel and existing biomarkers and developing new approaches such as current advancements in proteomics, tissue microarray, DNA microarray, immunohistochemical staining and other biotechnologies, to identify and validate more accurate diagnostic and prognostic biomarkers [50]. Using these methodologies, researchers have reported several biomarkers with great potential, and they are currently undergoing further investigation for validation. A few of the recent candidates that have generated some excitement for their potential as serum biomarkers for PCa are summarized in **Table 9**.

Table 9. Description of the Biological Function of Selected Serum Markers. Adapted from REF [50]

Serum Marker	Description/Type	Biological Function	Purpose
Chromogranin-A	Pro-hormone peptide released by neuroendocrine cells	Uncertain definite function. Possesses calcium-binding abilities and may act through paracrine and autocrine manners.	Prognosis
Neuron-specific enolase	Isomer of the glycolytic enzyme 2-phospho-D-glycerate hydrolase released by neuroendocrine cells	Uncertain definite function. Possibly serves as paracrine and autocrine factor.	Prognosis
Human kallikrein 2	Serine protease with trypsin-like substrate specificity	Splits pro-PSA to create PSA	Diagnosis
Urokinase-type plasminogen activator system	Serine protease and transmembrane receptors	Converts plasminogen to plasmin	Diagnosis (fragments) and prognosis
Interleukin-6	Cytokine	Implicated in hematopoiesis and the immune response through mediation of B-cell differentiation and the acute-phase inflammatory response	Prognosis
Transforming growth factor-β	Cytokine	Involved in cellular proliferation, cellular chemotaxis, cellular differentiation, angiogenesis, humoral immunity, cell-mediated immunity, and wound healing	Prognosis
Prostate membrane-specific antigen	Type II integral membrane glycoprotein with cell surface carboxypeptidase function	Possesses folate hydrolase function. Also is involved in the cell stress reaction, signal transduction, cell migration, and nutrient uptake. May possess questionable receptor function.	Diagnosis
Prostate-specific cell antigen	Glycosyl phosphatidylinositol-anchored cell surface glycoprotein	Known cell surface marker. Perhaps involved in several stem cell activities involving proliferation or signal transduction.	Prognosis
α-Methylacyl-CoA racemase (autoantibodies)	Peroxisomal and mitochondrial racemase	Engaged in bile acid synthesis, stereoisomerization, and β-oxidation of branched-chain fatty acids	Diagnosis
Early prostate cell antigen-1, -2	Nuclear matrix protein	May be involved in early prostate carcinogenesis; however, has uncertain contribution to nuclear morphology	Diagnosis
***GSTP1* hypermethylation**	CpG island hypermethylation of DNA encoding the protein, glutathione *S*-transferase π	Hypermethylation of GSTP1 inhibits transcription. GSTP1 usually acts by conjugation of oxidant and electrophilic carcinogens to glutathione to inactivate them	Diagnosis
Testosterone	Steroid hormone	Acts in the natural growth and support of the prostate gland and seminal vesicles. Many actions on sexual development and anabolism. Also involved in endocrine signal transduction.	Prognosis

Estrogen	Steroid hormone	Many effects on female sexual development. Also acts in the control of sperm development and in endocrine signal transduction.	Prognosis
Sex hormone-binding globulin	Serum glycoprotein-binding protein	Adheres to and carries testosterone and estradiol. Also involved in endocrine signal transduction.	Prognosis
Caveolin-1	Integral membrane protein	Works to regulate cholesterol metabolism and cellular transformation and is engaged in transducing cell-to-cell signals	Prognosis
E-cadherin	Calcium-dependent cell adhesion protein	Plays major role as a cellular adhesion molecule in cell-to-cell adhesion of secretory tissues	Prognosis
β-Catenin	Adhesion protein (80-kDa fragment isolated in prostate cancer)	Aggregates with cadherin to regulate the formation of adherent junctions between cells	Prognosis
MMP-9	Zinc-dependent endogenous protease	Acts in cell migration through and degradation of the ECM and in cell-cell adhesion.	Prognosis
Tissue inhibitor of MMPs (TIMP 1, 2)	Protease inhibitor	Prevents synthesis of ECM	Prognosis
Hepatocyte growth factor	Polypeptide growth factor (secretory protein of fibroblasts)	A cellular growth, motility, and morphogenic factor. Also, involved in cell scattering and angiogenesis.	Diagnosis/ prognosis
MIC-1	Cytokine (TGF-β superfamily)	Uncertain role, but may induce apoptosis	Diagnosis/ prognosis
Cytokine macrophage MIF	Cytokine (secreted by lymphocytes)	Modulates inflammation and the immune response. Activates cellular proliferation and angiogenesis, while inhibiting some tumour-suppressor genes.	Diagnosis
hK11	Serine protease (human kallikrein superfamily)	Has an uncertain function. Acts like trypsin but, depending on the tissue or body compartment in which it is present, may possibly have many different functions.	Diagnosis
Progastrin-releasing peptide (ProGRP 31-98)	Neuropeptide	Split to form GRP. GRP acts in the regulation of metabolism, behavior, smooth muscle activity, some exocrine and endocrine operations, and cellular chemotaxis.	Prognosis
Apolipoprotein A-II (8.9 kDa isoform)	Lipoprotein (abundant in HDL)	Effects plasma free fatty acid levels via operating in lipid metabolism and transport	Diagnosis
50.8-kDa protein	Unknown, identified by mass spectrometry	Uncertain function but possibly is parallel to the action of vitamin D-binding protein	Diagnosis
ILGF-1, -2	Growth hormone-dependent polypeptides	In the prostate gland, both modulate cellular proliferation, differentiation, and apoptosis. Also, acts in endocrine signal transduction.	Diagnosis
Leptin	Adipocyte-derived peptide	In metabolism, modulates hunger, energy use, and fat metabolism and is also known to induce angiogenesis	Diagnosis

Endoglin (CD105)	Homodimeric transmembrane glycoprotein	Controls TGF-β superfamily signaling pathway and therefore subsequently affects angiogenesis, cellular propagation, apoptosis, cell adhesion, and cell movement	Prognosis
EGFR family (c-erbB-1 (EGFR), c-erbB-2 (HER2/neu), c-erbB-3 (HER3) and c-erbB-4 (HER4))	Transmembrane glycoprotein receptors	Transduce signals for multiple growth factors	Diagnosis and prognosis
TSP-1	Homotrimeric extracellular matrix glycoprotein	Inhibits angiogenesis by inhibiting cell development, movement, and propagation and is also an effector molecule for the tumour suppressor gene p53	Diagnosis
VEGF	Dimeric, heparin-binding protein	An important endothelial cell growth factor that controls angiogenesis and augments vascular permeability	Prognosis
Huntingtin-interacting protein 1 (autoantibodies)	Cytoplasmic clathrin-binding protein	Acts in growth factor receptor transport. Also, transforms fibroblasts by lengthening the half-life of growth factor receptors.	Diagnosis
Prostasome (autoantibodies)	Prostatic secretory granules and vesicles composed of a lipid bilayer membrane and composite protein content	Consist of proteins that act in numerous enzymatic reactions, transport, structure, GTP activity, molecular chaperoning, and signal transduction	Diagnosis
ZAG	Glycoprotein	Induces lipid decline in adipocytes and therefore is implicated as possibly acting in cachexia	Diagnosis
CGRP	Neuropeptide	Vasodilatation and possibly regulation of protease secretion	Prognosis
PSP94	Nonglycosylated secretory peptide	In all probability acts as a growth and calcium regulator, apoptosis inducer, and an inhibitor of FSH.	Diagnosis
Other methylated genes including RASSF1α, APC, RARB2 and CDH1	Hypermethylated DNA encoding for various peptides	Hypermethylation predictably inactivates gene transcription	Diagnosis

d. Cancer biomarkers identification

Work over the past three decades has identified many genes for which gain or loss of function confers autonomous proliferative activity, resistance to cell death cues, angiogenesis, altered cell adhesion and motility. In other words, genetic alterations that mediate the initiation and local progression of tumours, and that collectively confer the

prerequisites for metastasis, have been identified. However, the society is only beginning to learn about the genetic determinants of metastasis proper (**Figure 9**); that is, those that mediate tumour cell invasion, intravasation, survival in circulation, scattering to distant tissues, extravasation into parenchyma, and colonization of vital organs [18]. Cellular functions reflect the state of the cell as a function of an intricate web of interactions among large number of genes, metabolites, proteins and RNA molecules [84].

By nature, PCa progresses slowly and can be treated effectively when it is detected early; however, the metastastatic disease presents a major challenge to improve survival rate and treatment efficacy. To overcome this problem, it is critical to identify predictors to distinguish those PCa that will progress and metastasize, from those that will not progress during the expected lifetime of the patient. Metastatic PCa proceeds through a series of distinct states such as transformation of normal prostatic epithelial cells to pre-invasive primary tumour, androgen-dependent invasive cancer, and androgen-independent metastatic disease these stages involve multiple molecular changes [180, 181] some of which can be implicated to alterations in gene expression [182].

Genomic technologies offer the promise of comprehensive understanding of cancer. These technologies are being used to characterize tumours at the molecular level, and several clinical successes have shown that such information can guide the design of drugs targeted to a relevant molecule. One of the main barriers to further progress is identifying the biological indicators or biomarkers of cancer that predict who will benefit from a particular targeted therapy [171].

Changes in gene expression that occur during the development of PCa have been extensively studied using DNA microarrays [183, 184] coupled with bioinformatics tools that can detect with remarkable resolution transcriptional signatures, gene copy number abnormalities, SNPs, epigenetic changes, microRNA levels, proteomic alterations and somatic mutations [18]. With an increasing appreciation for their potential clinical usefulness, these tools are regularly applied to interrogate tumour samples from large patient cohorts, and retrospectively derive indicators of disease outcome [18].

In many cases, however, gene expression levels do not accurately predict protein levels because additional control mechanisms exist, including post-transcriptional regulatory mechanisms [185]. Nevertheless, protein expression can only be determined by direct measurement of protein levels. Additionally, discovery of novel molecular markers, among which microRNAs (miRNAs), can be useful for improvement diagnosis, prognosis and classification of PCa [186]. miRNAs are attractive candidates as multifunctional regulators of metastatic progression because one miRNA can regulate an entire set of genes [187]. Such metastasis-associated miRNAs may serve as metastatic biomarkers and/or new targets for therapy of metastatic disease [188].

Box 4. Technologies for characterizing tumours. Adapted from REF [171]

Moleuclar alterations in tumours can be uncovered by using technologies that assess changes in the content or sequence of DNA, its transcription into messenger RNA or microRNA, the production of proteins or the synthesis of various metabolic products. Examples of technologies and information obtained about tumours are listed below:

DNA copy-number assessment
- Comparative genome hybridization to DNA microarrays

Mutation screening
- DNA screening
- Mass-spectrometry-based genotyping
- Mutation-specific PCR

Gene-expression profiling
- DNA microarrays
- Multiplex PCR

Proteomic profiling
- Mass spectrometry

Phosphoproteomic profiling
- Mass-spectrometry after immunoprecipitacion with phosphotyrosine-specific antibodies

Metabolomic profiling
- Mass spectrometry

e. Techniques to identify differentially expressed genes

The search for novel metastasis genes in PCa is more difficult. Many of the results are technology dependent, providing interesting new candidates from *in vitro* studies, which are infrequently confirmed in larger scale studies of human tumour material. The

ability to detect differences between populations of nucleic acids from metastatic and non-metastatic cellular populations has been exploited over many years. However many of the differences are subtle, and technology was unable to resolve these from background until the power of gene amplification was combined with the subtractive hybridization technologies. Also, most of the techniques require rather large starting quantities of RNA, which poses problems in heterogeneous metastatic lesions [64].

i. Subtractive Hybridization

The easiest way to compare two nucleic acid populations is to selectively hybridise them together, to leave an under and over represented population in an unpaired state, where imbalances have occurred. The enduring problem with a sound methodology has always been the yield of unpaired molecules, which restricted the changes detected to those of great magnitude, or aberrant hybrid formation. However, by combination with gene amplification, the technology is able to analyse much smaller differences in expression levels [64].

ii. DNA Microarray

A meta analysis of the major studies was published [184], but like most studies of this type in PCa, obtaining sufficient material from genuine metastatic lesions to carry our the analysis remains a problem. For a analysis of genes over-expressed in metastasis, the best measurement has currently been obtained from 64 primary and 12 metastatic adenocarcinomas originating from prostate, lung, colon, breast, ovary and uterus [29]. The initial screen produced a set of 128 genes, which could distinguish the metastatic lesions from primary tumours. The authors raised the possibility that these organ-confined tumours already contained cells pre-programmed to metastasize, particularly with lung tumours. This could equally apply in those “difficult” prostate tumours with a Gleason score of 5–7 where prognosis is a major diagnostic problem. Further refinement of the data set resulted in a minimal signature of genes over and under-expressed in metastases [64]. The power of microarray analysis may not be exploited to its fullest

extent, or in an extreme case, be providing misleading data. Most analyses reduce the differential expression to a ratio, relative to 'normal' tissues [64].

iii. Differential Display

One of the earliest methods of comparative gene expression, differential display (DD) has been used to analyse differences in gene expression between normal and tumour cell from prostate [189], but the required amounts of RNA for the analysis are relatively large, which precludes use with small metastatic lesions. By selection and cloning of individual products, the DD technology can isolate individual genes based on different sequence and biological criteria, but the procedure can be time consuming. To accelerate gene discovery, it can be combined with cDNA microarrays to reveal multiple expression alterations between metastatic and non-metastatic cell lines. These candidates remain to be confirmed on tissues however. A number of candidate genes have emerged from DD analysis [64].

iv. Serial Analysis of Gene Expression (SAGE)

This technology was devised to overcome the laborious nature of DD, by amplifying differentially expressed sequence tags of 10 base pairs as concatamers with defined ends. The small sequence tags are finally used to screen sequence databases to identify specific products, whose expression changes are confirmed by other technologies in the target tissue. With PCa, SAGE analysis has identified a number of expression changes [190] from a total of 156 detected changes. However, links to metastasis have still to be confirmed [64].

In summary therefore, the listing of the most common 'metastasis associated' genes, whose expression changes by the metastatic cell, is recorded in **Table 10** although is unlikely to be complete given the technique (and clinical material) dependency of the analyses carried out.

Table 10. Multiple gene expression changes implicated in PCa metastasis. Adapted from REF [64]

Gene	Function	Positive or negative effector	Reference(s)
Tazarotene-induced gene 1 (TIG1)	Retinoic acid responder gene	-	[191]
Hevin	Extracellular matrix, antiadhesive acidic cystein-rich glycoprotein	-	[192]
NF-kB	Transcription factor	+	[193]
VEGFC	Cytokine (angiogenesis)	+	[194]
Type XXIII collagen	Transmembrane (type 11 collagen)	+	[195]
Endothelin	Cytokine	+	[196]
Src-suppressed C Kinase substrate (SSeCKs/Gravin)	Tumour suppressor	-	[118]
Hepatocyte growth factor	Multiple growth factor-like activities	+	[197, 198]
CAT-like	Re-absorption of Ca++	+	[199]
c-erbB2/neu	cytokine	+	[200]
NKX3.1	Transcription factor	-	[201]
Elongin C	Multifunctional	+	[202]
Urokinase-type plasminogen activator	Protease	+	
Cutaneous fatty acid binding protein	Fatty acid binding	+	[203]
Osteoprotegerin	Cytokine (osteoblastic)	+	[204]
Prostate stem cell antigen	GPI anchored cell surface antigen	+	[205]
Annexin I	Calcium binding adhesion, membrane trafficking, cell signaling	-	[206]
Parathyroid hormone-related protein (PRHrP)	Peptide hormone	-	[207]
PHPrP receptor	Hormone receptor	+	
C13	Nuclear, glutamine and alpha helix rich	-	[208]
Autocrine motility factor (AMF)	Cytokine	+	[209]
Progastrin-releasing peptide (ProGRP)	Cytokine	+	[210]
Maspin	Serine protease inhibitor (adhesion to ECM)	-	[211]
TGF-b family	Cytokine	-	[212]
CLAR1	Proline-rich with SH3 binding domains	+	[213]
Bone morphogenetic proteins	Cytokine	+	[214, 215]
Matrix metalloproteinases	Proteases (tissue and vascular escape)	+	[216]
connexins	Intracellular communication	+/-	[217, 218]

VI. PROSTATE BONE METASTASIS SIGNATURE

a. Genomic biomarker discovery

Cancers are characterized by hallmark processes and shared mechanisms involved in expression of disease phenotype. It is a challenge to identify such genes involved in generic cancer mechanisms. Identification of such 'generic cancer genes' may help to focus on 'disease specific cancer genes' of potential therapeutic value. Due to complexity and subtle mechanisms involved in metastasis, it is difficult to identify their control mechanisms. Therefore it is important to have methods for identification of genes and regulatory mechanisms that are key to a complex pathogenic state such as secondary bone cancer [84].

Gene-expression profiling has been extensively used to classify cancers by gene-expression signatures [219-221]. It has also been used for predicting response to treatment and prognosis [222]. The hypothesis that screening of the gene expressed in the primary tumour from individual patients may be used to direct future therapy is very attractive, as currently a large number of patients receive unnecessary treatment [70]. In particular, identifying which patients would go on to develop bone metastases is of great interest, as this group may then be treated with organ-specific therapies like bisphosphonates [70].

In vivo selection of organ-specific metastatic variants from human malignant samples and cell lines, coupled with analysis of mRNA and microRNA expression patters has allowed the identification of organ-specific metastasis genes and functions. By comparing the results of this type of analysis with clinical gene expression data sets, it is possible to identify metastasis-associated genes of clinical relevance [125]. Several gene sets have been identified that are associated with organ-specific relapse in breast cancer patients [223, 224].

Another approach is based on interrogating clinical gene expression data sets for associations between specific pathways and particular disease outcomes [125]. By combining this information with functional assays it has been recently shown that a hyperactive Wnt pathway in lung adenocarcinoma tumours supports aggressive multi-

organ metastasis to brain and bones [225], whereas a high level of Src activity in breast tumours endows disseminating cancer cells with an enhanced capacity to survival in the bone marrow microenvironment and may contribute to late onset bone metastasis [226].

Gene signatures are useful tools to identify genes with functional roles in specific processes, as well to unravel new candidate prognostic or predictive genes, due to their specificity for a unique feature, like the target organ to metastasis [227]. In 2003, Kang et al. [32] demonstrated that only a fraction of the cells in a breast cancer population have the ability to exclusively spread into bone. Using the osteolytic breast cancer cell line MDA-MB-231 as a model, subpopulations with high metastatic ability either to bone or adrenal gland medulla were isolated and by transcriptomic profiling and comparison of these isolates, a gene set associated with the osteolytic bone metastatic ability was identified, denominated *Kang's BM signature*. This signature was retained after cell passage *in vitro* and *in vivo*, sustaining that the tissue-specific metastatic ability preexists in a certain parental population, and bone metastasis results from the selection and enrichment of these specific cells in the bone microenvironment [227].

In **PCa bone metastasis**, recent studies were performed to uncover differences in gene expression by using cDNA microarrays in comparison to liver and lymph node metastases, which could influence the pathologies associated with PCa metastasis at these sites [228]. Moreover, changes in integrin expression or function in malignant disease was found to be implicated in tumour growth, angiogenessis, and metastasis of human PCa, which make these receptors promising targets for novel anticancer therapies [229]. Therefore, an exhaustive analysis of gene expression in metastatic PCa cells is critically needed to identify new candidate genes that may play important roles in acquisition of the metastatic phenotype [182].

During the past year, three studies have been reported using genomic profiling to identify somatic copy number alterations specifically in clinical metastatic prostate tumours. In one study, a set of approximately 50 castration-resistant metastatic prostate tumours from 14 patients were analyzed using array-based comparative genomic hybridization (aCGH), gene expression profiling, and fluorescence in situ hybridization (FISH) [230]. This analysis implicated certain genes in metastatic PCa and associate

specific alterations with metastasis to specific distant sites [230]. In a more recent study, high-resolution single nucleotide polymorphism (SNP) arrays were used to assess copy number changes in a series of 94 metastatic tumours from 14 patients, where multiple lesions were studied from each patient [231]. They showed that most, if not all, lesions from each patient shared at least one somatic event, suggesting that metastatic tumours from any given patient were derived from a common progenitor tumour cell [231]. Furthermore, their data also suggested that there was no obvious relationship between specific lesions and specific systemic metastatic sites [231], contrary to results reported by Holcomb et al. (2009) [230]. More recently, a very comprehensive genomic study was published for 218 total primary and 5 metastatic tumours, including copy number analysis, gene expression, miRNA analysis, and mutational analysis of exons from more than 100 genes using Sanger sequencing methods [232]. The overall results of this study support a significant role for somatic alterations in the gene NCOA2 in PCa [232].

Together these studies have shed significant light on gross copy number changes, have implicated specific regions in metastatic disease, and in a few cases have implicated specific genes that might be involved in metastatic PCa. A number of candidate genes have been reported that harbor somatic mutations in localized PCa, including AR, TP53, KLF6, EPHB2, CHEK2, ZFHX3 (known as ATBF1), and NCOA2 [232-236]. However, there are limited reports of somatic coding mutations in metastatic tumours. One example is a study reported by Wong et al. (2007) [237], which describes mutations in the gene encoding plexin-B in metastatic PCa [237]; however, as of yet these results have not been replicated in the literature [238].

Numerous transcriptome studies have defined general PCa signatures (see **Table 9**), but, unlike breast cancer, these analyses have not identified robust subtypes of PCa with different prognoses [232].

b. Proteomic biomarker discovery

Study of disease protein interactomes offer a better understanding of disease-specific genes and processes involved and may offer better targets for drug development. It is increasingly evident that genes do not act as individual units but collaborate in a series of overlapping and interrelated networks, the deregulation of which is a classic hallmark of cancer [239]. These molecular interaction networks are characterized by the presence of a few highly connected nodes, often called 'hubs', suggesting a special role of these promiscuous interactors (**Figure 25**). Hubs of protein interactomes are more likely to be essential for the survival [240] and also reported to be important for cellular growth rate [241]. Proteins with high betweenness are reported to have much higher tendency to be essential genes [242]. Cancer proteins are reported to be more central in the protein interactome and are, on an average, involved in twice as many interactions as those of non-cancer proteins [84, 243].

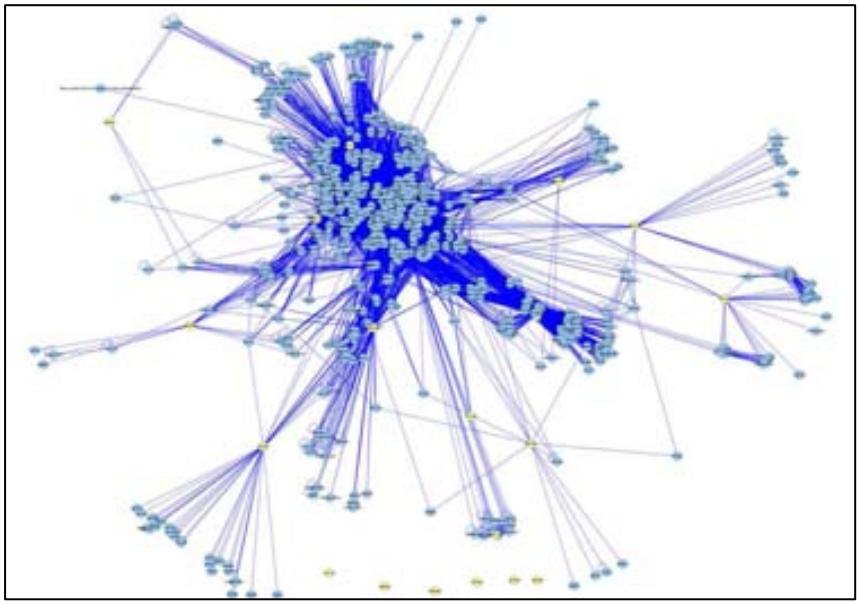

Figure 25. Example of organ-specific brain metastasis network as a model of breast cancer. From REF [244]. Proteins are represented by nodes and the edges between the nodes display interactions between the proteins.

Proteomics is more than the identification of proteins that are altered in expression as a consequence of pathophysiology, it also encompasses the search for novel biomarkers, a critical tool for the detection, treatment, and monitoring of disease [239]. The necessity for new methods to identify and validate biomarkers is underscored by the increased survival of patients diagnosed at early stages of cancer [239].

Proteomic biomarker discovery shares a lot of characteristics with genomic or transcriptomic profiling, including the analysis of biological samples within a complex matrix, and sophisticated statistical analysis, including large sets of variables. However, proteomic biomarker discovery is inherently more complex, mainly due to the vast range of analyte concentrations that must be detected and identified, as well as the fact that protein products cannot be amplified, since an equivalent PCR for proteins does not exist [245]. Innovative protein-based approaches to identify and quantify proteins in a high throughput manner have furthered our understanding of the molecular mechanisms involved in diseases [246]. Because of the inherent complexity of the proteome, all approaches to its examination are generally based on a separation step (gel-based or non-gel based) followed by ionization and a subsequent analysis by MS [247] (**Table 11**).

Table 11. Advantages and disadvantages of each mass spectrometry-based proteomics technique for use in clinical applications. Adapted from REF [248]

TECHNOLOGY	ADVANTAGES	DISADVANTAGES
2DE-MS	Detection of large molecules, enables estimation of their molecular weight. Sequencing of biomarkers easy to perform from 2D spots.	Not applicable to molecules < 10 KDa, no automation, time-consuming, quantification difficult, medium throughput, moderate comparability.
LC-MS	Automation, high sensitivity, used for detection of large molecules (> 20 Kda) after tryptic digest, sequence determination of biomarkers provided by MS/MS.	Time-consuming, relatively sensitive toward interfering compounds, restricted mass range, medium throughput.
SELDI-TOF*	Easy to use, high throughput, automation, low sample volume.	Restricted to selected proteins, low resolution MS, lack of comparability, sensitive toward interfering compounds, low information compound.
CE-MS#	Automation, high sensitivity, fast, low sample volume, multidimensional, low cost.	Generally not suited for larger molecules (> 20 kDa).

*Surface-enhanced Laser Desorption Ionization-Time-of-Flight (SELDI-TOF)
#Capillary electrophoresis coupled to mass spectometry (CE-MS)

The recent explosion in proteomic technologies centering on MS and protein microarrays has provided great opportunities for researchers to use these “bridging technologies” for the clinical, proteomic investigation of disease-relevant changes in tissues and biofluids [246]. Common proteomic methodologies are categorized into two classes: those for differential proteomics in the discovery phase and those for quantitative proteomics.

Differential proteomics is defined as the scientific principle that compares normal and diseased states for biomarker discovery without providing specific protein concentrations in the biological matrix.

Quantitative proteomics is defined as the absolute quantification of proteins used in targeted biomarker verification and quantification studies. Selected Reaction Monitoring (SRM) is introduced as one of the main multiplex quantitative methodologies in the biomarker pipeline for verification [246].

To date, mass spectrometry-derived protein signatures have been identified, characterized, modeled, and are now moving into validation in extensive patient cohorts [249, 250]. Further characterization and sequencing of these key features should provide new insights into disease etiology, and presumably, intervention [251]. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) or two-dimensional polyacrylamide gel electrophoresis (2D)-PAGE have emerged as the primary investigator-based modality for biomarker discovery of early stage cancers [252], although these approaches do have some limitations [239]. More recently, stable isotope labeling with amino acids in cell culture, or SILAC, has emerged as a valuable proteomic technique [253-255].

Two of the differential proteomic techniques for biomarker discovery used in this thesis are summarized below.

2D-DIGE technology

The term “proteomics” originated in the context of two-dimensional (2D) [256], a fundamental evolution in the field of separation technologies. 2D has proved to be a reliable and efficient method for the separation of proteins based on mass and charge. It can achieve the separation of thousands of different proteins on one gel [257]. In 2D

experiments, the staining pattern of proteins from two samples is compared, and the "up-regulated" and "down-regulated" proteins are identified. The main problem of 2D is its lack of reproducibility that difficult the comparison of different experiments.

To overcome 2D reproducibility, the simultaneous staining of two samples has been developed in recent years to allow a one-step analysis and a direct comparison of different mixtures of proteins [258]. The 2D-DIGE approach significantly improves the sensitivity and reproducibility of 2DE analyses because differently labelled protein samples are resolved on the same gel [259].This methodology is known as Differential In-Gel Electrophoresis (DIGE). The basis of the technique is the reaction of mass- and charge-matched N-hydroxy succinimidyl ester derivatives of the fluorescent cyanine dyes Cy3 and Cy5 with lysine residues of two different protein samples. This allows the co-detection of individual proteins originating from the different samples in a single spot and a direct comparison of the protein expression levels (**Figure 25**). Moreover, the application of a pooled standard protein sample labelled with a third fluorescent dye (Cy2) onto each gel allows the linking of gel images from individual gel runs. This not only avoids the complications of inter-gel comparison but also speeds up the analyses and reduces the number of gels that need to be run for a reliable comparison of protein patterns [259].

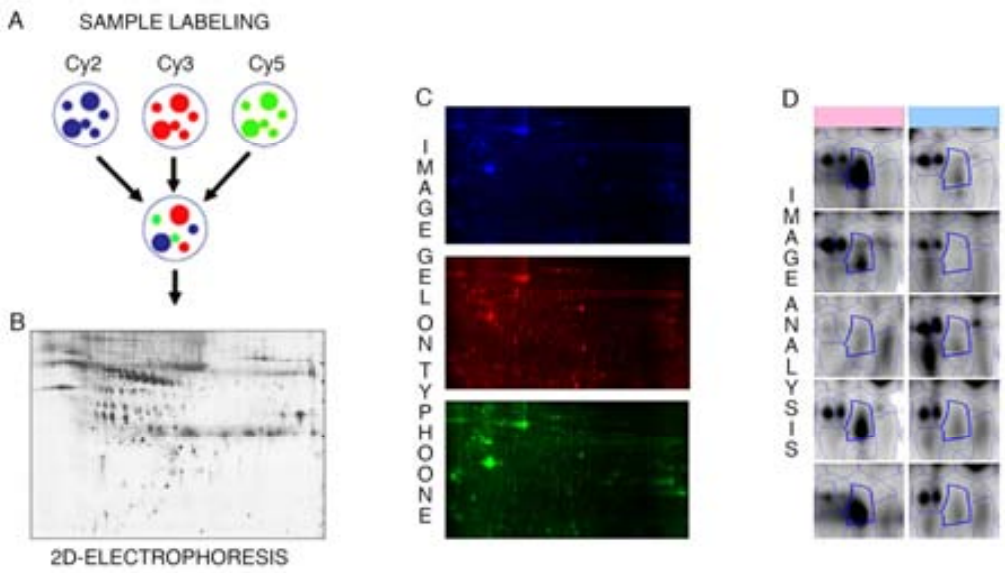

Figure 26. Differential In-Gel Electrophoresis (DIGE). A. Sample labeling with different dyes. **B.** 2DE of the different labelled samples in the same gel. **C.** Typhoone scanner using different wavelength to obtain the different images from the same gel. **D.** Image analysis using specific software (Progenesis Samespots v2.0 software (NonLinear Dynamics, Newcastle, U.K.)).

SILAC methodology

By SILAC, cells representing two biological conditions are cultured with 12C- or 13C-labelled amino acids and proteins effectively become isotopically labelled as "light" or "heavy." By adding stable, non-radioactive isotopic forms of amino acids to media when cells are growing cells, it is possible to achieve 100% amino acid incorporation. Upon isolation of proteins from these cells, samples can then be mixed in equal ratios and processed using conventional techniques for tandem mass spectrometry accurate protein identification [250]. Given that corresponding light and heavy peptides from the same protein will co-elute during chromatographic separation into the mass spectrometer, relative quantitative information can be gathered for each protein by calculating the ratio of intensities of the two peaks produced in the peptide mass spectrum (MS scan). Furthermore, sequence data can be acquired for these peptides by fragment analysis in the product ion mass spectrum (MS/MS scan) and used for accurate protein identification . Finally, when more than one peptide is identified from the same protein, the quantification is redundant, providing increased confidence in both the identification and quantification of the protein (**Figure 27**) [250].

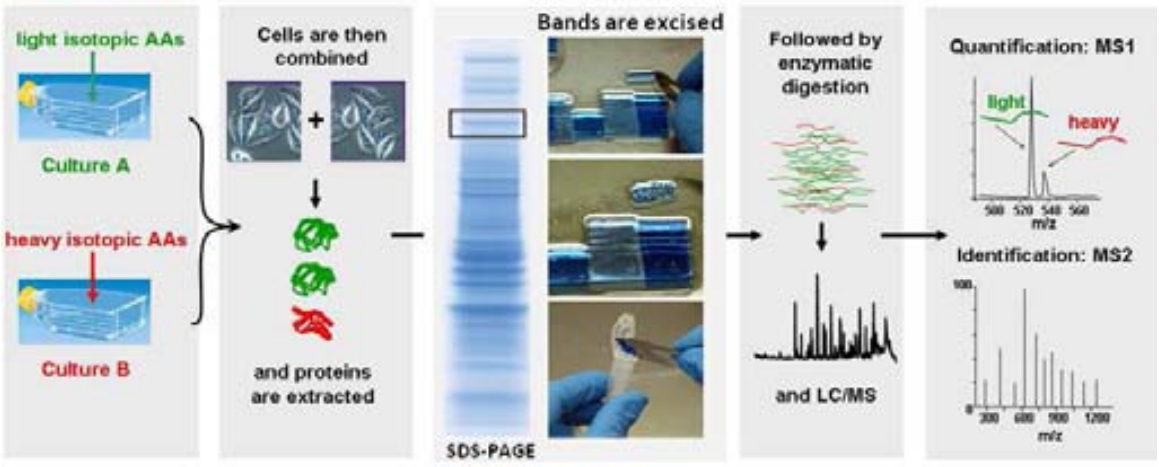

Figure 27. SILAC experiment workflow. Adapted from web page: http://www.grc.nia.nih.gov/branches/lci/muproteomics.htm

Because samples are mixed early on before processing and are subjected to the identical experimental protocol, experimental results achieve high fidelity with minimal bias, allowing relative quantitation of even small changes in protein abundance.

However, there is currently no way to reliably detect or predict which patients are at risk for metastatic cancer. Thus, the discovery of biomarkers that could distinguish patients with local disease from those with metastatic disease would be of great clinical value [239].

c. microRNA biomarker discovery

In recent years, evidence has accumulated that small non-coding RNAs are also used in a conserved manner to regulate key developmental events. At least four classes of regulatory small non-coding RNAs have been described, including microRNAs (miRNAs), short interfering RNAs (siRNA), repeat-associated small interfering RNAs (rasiRNAs) and piwi-interacting RNAs (piRNAs) [260]. Among these small RNAs, miRNAs (**Figure 28**) are the most phylogenetically conserved and function post-transcriptionally to regulate many physiologic processes, including embryonic development [261-263].

Therefore, investigations that are underway to find the molecular basis of metastatic PCa have focused on many novel molecules, among which microRNAs (miRNAs), that are becoming an attractive area of research.

The miRNAs are small, noncoding subset of RNAs which consist of about 18-22 nucleotides and bind to the 3’ untranslated region of messenger RNAs (mRNAs) [264]. By this action, they cause post-transcriptional inhibition or degradation of target mRNA, depending on the degree of complementary base pairing [186, 265]. During the past 12 years, significant advances have been made in miRNA research leading to the discovery of over 4,500 miRNAs in vertebrates, flies, worms, plants, and viruses out of which more than 1,000 miRNAs have been fully characterized and the number is expected to grow in the coming years [186].

The miRNAs are being implicated in the regulation of an increasing number of physiological processes. It is also believed now that they play an important role in the regulation of many cellular functions ranging from maintenance to differentiation and tissue development, from metabolism to cell cycle [186]. All of these facts leads to the conclusion that aberrant expression of miRNAs will have impact on various biological

processes where they are implicated, which will result in a variety of pathological events such as cancer [186, 266].

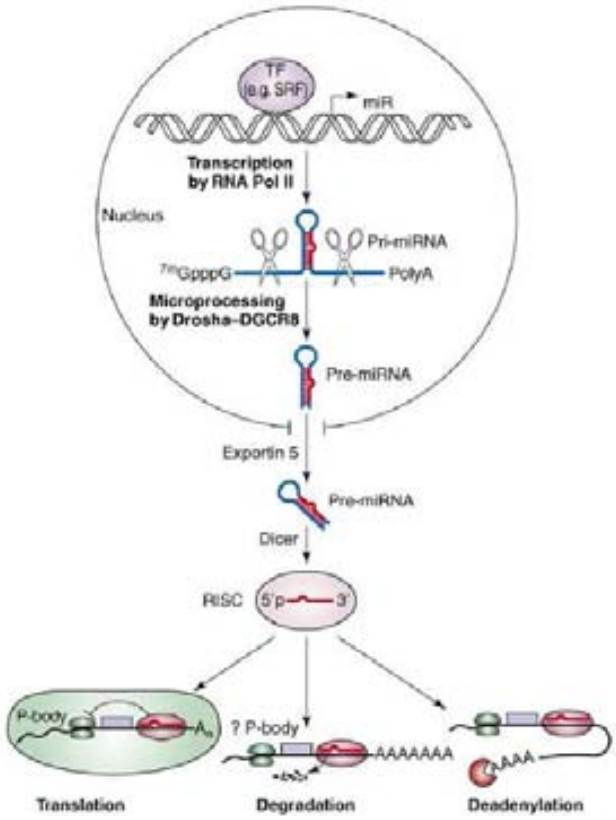

Figure 28. Current model of miRNA biogenesis and function. From REF [263]. Abbreviations: SRF, serum response factor; TF, transcription factor.

The role of miRNAs in cellular growth, differentiation and apoptosis of cancer cells through their interactions with their target mRNA has been studied [267, 268]. miRNAs may be oncogenic or tumour suppressors, with oncogenic being up-regulated and the tumour suppressors being down-regulated in cancers [186]. Generally, the importance of miRNAs in cancer is emphasized by the fact that around 50% of all miRNA genes are positioned in the so called 'fragile sites', the cancer associated genomic regions which are repeatedly changed in cancer [186]. Moreover, miRNAs are attractive candidates as multifunctional regulators of metastatic progression because one miRNA can regulate an entire set of genes [269].

The role of miRNAs in PCa is becoming clearer by understanding the interactions between miRNAs and their targets and the resulting impact on carcinogenesis of the prostate [186, 267, 270]. It is believed that several miRNAs and their targets are aberrantly expressed in PCa which, in turn, alter the cellular growth, invasion, and metastatic potential of PCa cells. The abnormal expressions of certain miRNAs are now

considered valuable biomarkers for diagnosis, prognosis and classification of PCa (**Table 12**) [271, 272]. All of the above information underscores the importance of the biology of miRNAs in PCa [186].

Table 12. miRNAs that influence PCa progression. Adapted from REF [186]

miRNA	Role in PCa	Function	Study
miR-15a and miR-16	Tumour suppressors	Inhibit cell proliferation, invasion and angiogenesis through regulation of multiple targets	Aqeilan 2010 [273], Musumeci 2011 [274]
miR-21	Onco-miRNA	Increases tumour growth, invasion and metastasis	Si 2007 [275], Selciklu 2009 [276], Li 2009 [277], Ribas 2009 [278]
miR-125b	Onco-miRNA	Increases cell proliferation and inhibits apoptosis	Lee 2005 [279], Shi 2007 [270]
miR-143	Tumour suppressor	Inhibits cell proliferation and migration by regulating KRAS, MAPK pathways and cell cycle. Also inhibits metastasis	Clape 2009 [280], Xu 2011 [281], Friedman 2009 [282]
miR-145	Tumour suppressor	Inhibits migration, invasion and metastasis	Friedman 2009 [282]
miR-200 s	Tumour suppressor	Inhibit cell migration and invasion by reversing EMT	Kong 2009 and 2010 [283, 284]
miR-221	Onco-miRNA	Stimulates cell growth and influences cell cycle progression	Zheng 2011 [285], Galardi 2007 [286], Sun 2009 [287], Pang 2010 [267]
miR-222	Onco-miRNA	Increased cell cycle progression	Galardi 2007 [286], Sun 2009 [287], Pang 2010 [267]
miR-488	Tumour suppressor	Inhibits Androgen Receptor-mediated cell growth	Sikand 2010 [288]

Thus far, only a small number of studies have investigated miRNA expression in PCa, and only a few have dealt with metastasis of this disease [188]. Differences in the expression profiles of miRNAs so far identified may have prognostic value for the various aspects of the disease and a better understanding of the role of miRNAs in the development and progression of PCa is needed [289]. Further research may also lead to identification of new miRNAs that are specifically related to PCa progression and

metastasis. Such metastasis-associated miRNAs may serve as metastatic biomarkers and/or new targets for therapy of metastatic disease [188].

miRNAs as biomarkers offer a number of advantages. First, compared with mRNAs, which are very sensitive to degradation, miRNAs are more stable in compromised human specimens (e.g., formalin-fixed paraffin-embedded; FFPE) [290, 291]. Second, their expression levels can be measured reliably in FFPE tissue samples. Third, minute amounts of RNA are needed to establish their expression using reliable, quantitative PCR amplification strategies, such as TaqManVR qRT-PCR. Finally, miRNA expression profiles are not dependent on the preservation of the specimen's architecture and cellular arrangement or the degree of cellular degeneration.

General format considerations

Due to the complexity of this study, this thesis has been structured in three chapters, each with its own Hypothesis, Objectives, Material & Methods and Results with Discussion, instead of the traditional format.

The main objective of this thesis is the study and characterization of prostate cancer bone metastases from different models to achieve an in-depth understanding of tumour cell dissemination and establishment of osseous metastases. The ultimate aim is to develop new metastasis-associated biomarkers that may be used as targets for effective therapies.

Chapter I.

Establishment and Characterization of Intraosseous Prostate Cancer Bone Metastasis Model

BACKGROUND

Bone is the most frequent location of metastases in patients with PCa. On postmortem examination, around 70% of patients that die of this cancer have evidence of metastatic bone disease [63]. For many patients, metastatic bone disease is a chronic condition with an increasing range of specific treatments that aim to slow the progression of the underlying disease [63]. In contrast, symptomatic PCa bone metastases are painful, debilitating, and generally fatal [292]. Metastatic bone disease results from the interaction between cancer cells and normal bone cells in the bone marrow microenvironment, where the stimulation of osteoclastic bone resorption triggers an uncoupled and unbalanced bone remodelling process [63].

The propensity of PCa to metastasize to bone is not well understood mechanistically. After PCa cells infiltrate the bone marrow, presumably via the venous plexus [293], two possible modes of engraftment have been postulated: the first is purely mechanical and involves trapping of PCa cells by filtration through the marrow; the second is biological and derives from the 'seed and soil' hypothesis of Paget, which holds that specific interactions between PCa cells and the bone marrow microenvironment are required for growth to occur [7]. In 1999, Koeneman *et al.* [294] focused on the interactions between PCa cells and the bone environment and hypothesised that prostate cells must acquire bone-like properties in order to grow and proliferate in the bone environment [292]. In this model, prostate cells become "osteoblast-like" and express proteins associated with osteoblast maturation, osteoblast differentiation and bone remodelling [292].

The generation of suitable *in vivo* models is critical for understanding the interactions between PCa cells and the bone microenvironment. The ideal animal model for PCa should mimic the clinical situation: the tumour should be of human origin, have a fast enough doubling time suitable for an experimental model, be androgen-dependent or androgen-sensitive, produce prostate specific antigen (PSA), create lymph node and bone metastases, and develop androgen-independent status after castration [139]. The lack of an ideal model for metastases hinders the study of the whole process and of the precise mechanisms in each step of this metastatic disease. The development of xenograft mice models has greatly contributed to this area [295] and facilitates the

research of new targets to improve current therapies. Currently, the best animal model of PCa metastasizing to bone is the Nod/Scid-humanized model, an immunodeficient mice grafted with a human bone, where both PCa cells and the bone target can be of human origin [295]. This *in vivo* model enables the analysis of the governing mechanisms of the interactions between human tumour cells and human organ environment [296] in the establishment of tissue-specific and species-specific metastases.

Proteomics is a useful tool for searching novel biomarkers for the detection, treatment and monitoring of diseases [252]. Thanks to the performance of mass spectrometry (MS), proteomic studies are able to identify a high number of proteins that are specific to a given malignancy.

Understanding the complex interactions that contribute to the metastatic behaviour of tumour cells is essential for developing biomarkers of disease progression, as well as for the development of more effective therapies.

To sum up, some molecular factors in human PCa cells promote metastases preferentially to the bone; there is a relationship between bone stromal components, metastasizing cells and bone marrow-derived cells that could explain the tissue-tropism observed in metastases. Finally, circulating tumour cells able to colonize secondary tissues may possess specific characteristics that differentiate them from other tumour cells.

HYPOTHESIS AND OBJECTIVES

Main hypothesis: It is possible to identify by differential proteomics molecules that are mediating bone metastases for PCa into the metastatic niche through an *in vivo* model of PCa cell dissemination.

Moreover, the use of those molecules can be used to identify those patients at risk of developing aggressive disease who may eventually benefit of a more personalized/targeted therapeutic approach.

General objectives

The main objective of Chapter I of this thesis was to identify differentially expressed proteins involved in the metastatic process of PCa to the bone to help clarify the complex nature of its development and its unusual mechanism(s) of disease progression.

To accomplish this aim, the research was focused on the following areas: **1)** to develop a humanized model of PCa bone metastases, **2)** to analyze the differential proteomic profiles of both bone stromal components and human metastatic PCa cells in the grafted metastatic niche. And finally, **3)** to further investigate the species-tropism of circulating human PCa cells to the human bone target in the same mice model.

Specific objectives

1. Development of a species- and tissue-specific metastases model of human PCa in immunodeficient and humanized mice engrafted with a human adult bone.

Specifically, the humanized and immunodeficient mice model of PCa bone metastases consisted in the following consecutive steps:

1a. Subcutaneous (s.c.) human adult bone implantation from non-cancerous patient into Nod/Scid immunodeficient mice;

1b. Neovascularization of the grafted human bone for a period of 4 weeks;

1c. Direct inoculation of PCa cells into the bone marrow of the implanted human bone;

1d. Tumour growth monitorization.

2. To analyze the differential proteomic profiling of the bone stromal component and human PCa cells in the grafted metastatic niche.

To further analyze changes in proteomic profiles for the PCa bone metastases dissemination, the 2D-DIGE approach was used to analyze those proteins whose expression was altered in bone metastases compared to normal bone.

Specifically, the following procedures took place:

2a. Protein extraction from bone implants, quantification and sample preparation;

2b. Performance of 2D-DIGE technique comparing bone with metastases, bone without metastases and the tumour cell component;

2c. Identification by MALDI-TOF-MS/MS of proteins differentially expressed and analysis of results.

3. Study of species-tropism of circulating human PCa cells to the human bone target.

Here, the preference of circulating human PCa cells introduced into humanized Nod/Scid mice to metastasize to the engrafted human bone or mouse bones was investigated.

The following steps took place:

3a. Nod/Scid mice were subcutaneously engrafted with human adult bone;

3b. After neovascularisation, human PCa cells were injected intracardiacally;

3c. Tumour progression and cell dissemination was monitored *in vivo*.

MATERIAL AND METHODS

1 Tumour cell lines

1.1 PCa cell lines

All the *in vivo* experiments shown in this thesis have been carried out with the human PCa cell line (PC-3), derived from a bone metastasis of a grade IV prostatic adenocarcinoma [124], and obtained from the LoGiCal Standards (LGC, UK). PC-3 cells were cultured in sterile 75-cm^2 tissue culture flasks filled with 15 mL complete media consisting of RPMI-1640 (Life Technologies, UK) supplemented with 10% foetal bovine serum (FBS; Gibco, Life Technologies, UK), 50 U/mL penicillin and streptomycin, 1X non-essential amino acids (MEM), 2 mM L-glutamine, 10 mM HEPES (all from Gibco, Life Technologies) and 1 mM sodium pyruvate (PAA Laboratories, UK) at 37ºC in a humidified atmosphere (5% CO_2/ 95% air). Cells were passaged and expanded every 6-7 days by trypsinization of cell monolayers. Culture media were changed every 3-4 days. The cells were harvested by trypsinization (0.25% trypsin, 0.02% EDTA) and washed three times by centrifugation in complete media. Cell concentration and viability were determined with 0.2% trypan blue solution using a Neubauer chamber.

1.2 Constructs and stable cell line generation

PC-3.pEGFPluc cells were generated by transfection of PC-3 cells with the expression vector pEGFPluc (a kind gift from Dr. Seoane), which uses the human cytomegalovirus (CMV) immediate early promoter to express the enhanced firefly Luc reporter gene upstream of the green fluorescent protein (GFP) gene. Plasmids (4 µg/well in a 6-well plate) were transfected into PC-3 cells using LipofectamineTM 2000 (Invitrogen, Life Technologies, UK), according to manufacturer's instructions. Twenty-four hours after transfection, transfected cells were selected with geneticin (Life Technologies, UK) at a concentration of 500 µg/mL. After expansion of the clones, isolated colonies were cloned and characterized. GFP-expressing cells were isolated by fluorescence-activated cell sorting using the FacsAria (BD Bioscience, USA).

2 Bioluminescence *in vitro* assay

To validate the bioluminescent light production (luciferase activity) of the clones, they were tested by *in vitro* assay using IVIS® Spectrum (Caliper Life Sciences, MA). Briefly, fifty microlitres of bioluminescent cells diluted from 200,000 to 196 cells were seeded into black, clear bottom 96-well plates. D-luciferin firefly (Promega Biotech Ibérica, Spain) at 300 µg/mL (50 µL) was added to each well just prior to imaging, and cells were incubated for less than 10 minutes at 37ºC. The production of light for each cell variant was linear, in proportion to the number of cells plated. All experiments were performed with pooled populations using the two highest luciferase expressing cell variants.

3 Animal care and human adult bone implant

Male Nod/Scid mice (NOD.C.B-17/IcrHsd-*Prkdc*^{scid}) were purchased from Harlan (Harlan Laboratories, Italy) at 4 weeks of age and maintained under specific pathogen-free conditions. Animals were kept for at least 1 week in the facility before experimental manipulation. Animals were kept in a sterile environment in cages with beds of sterilized soft wood granulate and fed irradiated rodent diet *ad libitum* with autoclaved tap water. An artificial cycle of 12h light/12h darkness was maintained in the room where the animals were kept. A maximum of five mice were kept in each box, and all manipulations were performed using sterile techniques within a laminar-flow hood at the animal facility. Experiments were performed on animals at 5 weeks of age. All the procedures associated with experimentation and animal care were performed according to the guidelines of the Spanish Council for Animal Care and the protocols of the Ethics Committee for Animal Experimentation at our institution.

After obtention of the informed consent, normal-appearing bone tissue (from the spinous process of a vertebra) was extracted from a non-cancerous patient (a 44-year-old man with hypertension and obesity) who underwent surgery in the Traumatology Department of the Hospital Vall d'Hebron in Barcelona. Implantation of human adult bone fragments into Nod/Scid mice was performed as described previously by Yonou *et. al* [296]. Briefly, after anesthetizing animals with 2% isofluorane, bone fragments were obtained and maintained under sterile conditions in RPMI 1640 at 4°C. Bone fragments of approximately 1 cm^3 were subcutaneously (s.c.) implanted into the left flank through

a small skin incision in Nod/Scid mice within 1 h after their procurement (**Figure 29**). The resulting grafted Nod/Scid mice were used for the metastasis assay at 4 weeks after implantation.

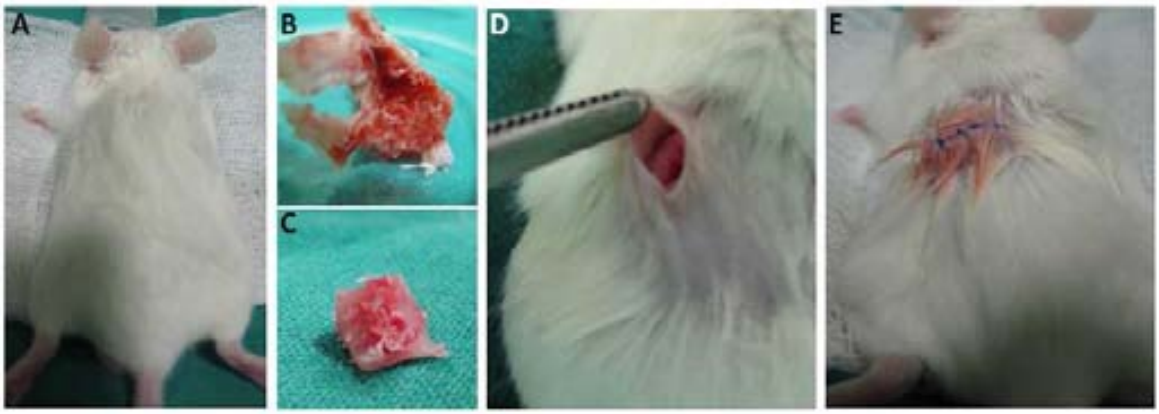

Figure 29. Human bone engraftment procedure. A. Male Nod/Scid mice were anesthetised with 2% isofluorane before surgery. **B-C.** Human adult bone, a spinous process from a vertebra of a non-cancerous patient, was maintained under pathogen-free conditions in RPMI media at 4ºC within 1 h after their procurement and cut into small pieces (**C**) of approximately 1 cm^3 before implantation. **D.** After wiping the area with 70% alcohol swabs, a skin incision to introduce the bone fragment was performed. **E.** The incision was closed by suture. The resulting grafted Nod/Scid mice were used for the metastasis assay 4 weeks after implantation.

4 Induction of bone tumours

Four weeks after bone implantation, a group of ten engrafted Nod/Scid mice (the bone with bone metastases group) were inoculated with single cell suspensions (1 x 10^6 cells/50 µL of sterile phosphate-buffered saline, PBS) of stable transfected PC-3 cells directly into the marrow spaces of the implanted human adult bone using a 27-gauge needle; another group of ten mice (the group of bone without metastases) with the same engraftment received a sterile solution instead of tumour cell suspension.

5 Xenograft by intracardiac inoculation of prostate cancer cell suspension

For the intracardiac (i.c.) injection of PC-3 luciferase-transfected cells, 5 week old male mice (n = 10 mice) were deeply anesthetized with ketamine (100mg/kg of body weight) and xylazine (10mg/kg of body weight) solution. Using a 25-gauge syringe, PCa cells (3 x 10^5 in 0.1 mL sterile PBS) were injected into the left cardiac ventricle of the heart. The presence of a rapid pulsatile flow of bright red arterial blood (as opposed to darker, burgundy coloured blood) into the syringe was indicative of correct needle placement

[297]. Mice were imaged for luciferase activity immediately after injection and continued to be monitored weekly using IVIS® imaging.

6 *In vivo* monitoring and *ex vivo* analysis

For the time-course of the *in vivo* bioluminescence imaging (BLI) of each group of mice throughout the study, the IVIS® Spectrum (Caliper Life Sciences, USA) was used as described by Drake *et al.* [160]. Before imaging, mice were given an intraperitoneal (i.p.) injection (150 mg/ kg body weight) of D-Luciferin substrate (Promega Biotech Ibérica, Spain) and were anesthetized with 2% isofluorane (ABBOT Laboratories, Spain) (**Figure 30**). Mice were imaged once weekly beginning on day 0 after tumour inoculation to evaluate and quantify bone tumour growth. Total image intensity was collected every 2 min until a high plateau was reached. Values were recorded and images obtained. BLI signal was quantified by measuring the amount of highlighted pixels in the regions of interest (ROIs) around the dorsal and ventral images of each mouse. Total photon flux was quantified using the Living Image Software (Xenogen, Caliper Life Sciences, USA) with photons/ second (ph/s) units. ROIs in all images were kept at a constant area.

In order to identify the location of tumours in these animals an *ex vivo* BLI was performed by injecting them with luciferin substrate followed by euthanasia after 5 minutes incubation. Individual organs were excised and examined using the Xenogen imaging system.

7 Histological analysis

Mice were sacrificed by cervical dislocation after sedation, and the tumour-induced legs were dissected. Limbs were immediately fixed in 4% formaldehyde for 24 h. Following fixation, bone samples were decalcified by a decalcification solution (Decalcifier II, Leica Microsystems, Spain) and paraffin-embedded for histological examination. Hematoxylin and eosin (H&E) staining was performed on paraffin-embedded 4 µm sections to assess tumour development.

Figure 30. *In vivo* BLI imaging using the IVIS System. A-C. Mice were anesthetized using 2% isofluorane into the induction chamber. **D-E.** Before imaging, mice were given an intraperitoneal (i.p.) injection (150 mg/ kg body weight) of D-Luciferin substrate, placed into the IVIS Instrument and maintained under anaesthesia. **F.** The IVIS® Spectrum was used for the time-course of the *in vivo* BLI of mice. Mice were imaged once weekly beginning on day 0 after tumour inoculation to evaluate and quantify the bone tumour growth until the end of the experiment 7 weeks after cell inoculation.

8 Protein separation by 2D-DIGE and gel imaging

After mice were sacrificed, human bone implants were excised and total protein extracted as described by Pastorelli *et al.* [298]. Briefly, bones were homogenized in 500 µL buffer solution containing 10mM K_2HPO_4, 10mM KH_2PO_4, 1mM EDTA, 10mM CHAPS (Sigma-Aldrich, UK) and a complete protease inhibitor cocktail tablet (Boehringer Mannheim, Germany). The homogenate was sonicated twice for 15 s and then centrifuged at 10, 000 x g for 10 min at 4ºC. The pellet was discarded and an aliquot of the supernatant was used to determine protein concentration. Protein extracts were kept at -20ºC.

8.1 Two dimensional differential in gel electrophoresis (2D-DIGE)

Following extraction, any interfering component was removed by a modified TCA-acetone precipitation (2D-CleanUp kit, Amersham Bioscience, USA) and the extracts were dissolved in DIGE lysis buffer (7M urea, 2M thiourea, 10mM Tris-HCl pH 8, 4% (w/v) CHAPS). Protein concentration was determined using the BioRad RC DC Protein Assay (BioRad, USA), according to the manufacturer's protocol. Lastly, the pH was adjusted to

8.5 for DIGE labelling. Labelling of samples: bone with PCa metastases, bone without metastases or tumour component were labelled with Cy3 or Cy5 cyanine dyes, while the internal standard pooled samples, a pool of equal amounts of each sample analyzed in the DIGE experiment, were labelled with Cy2 dye. After 30 min of incubation on ice in the dark, the reaction was quenched with 10mM lysine and additionally incubated for 10 min. According to the experimental design, the samples were finally combined at 50 ug of protein per Cy dye per gel, and diluted 2-fold with IEF sample buffer (7M urea, 2M thiourea, 4% (w/v) CHAPS, 2% 1.4-dithioerythritol (DTT), 2% pharmalyte pH 4-7 and 0.002% bromophenol blue). The 2D-DIGE was performed using GE-Healthcare reagents and equipment (GE Healthcare, UK). First-dimension IEF was performed on IPG strips (24cm; linear gradient pH 4-7) using an Ettan IPGphor system (GE Healthcare, UK). Prior to IEF, strips were incubated overnight in 450 µL of Rehydration buffer with 1% pharmalyte pH 3-7, 100mM DeStreak and 0.002% bromophenol blue. After focusing for a total of 67 kV x h, strips were equilibrated 15 min in reducing solution (6M urea, 100mM Tris-HCl pH 8, 30% (w/v) glycerol, 2% (w/v) SDS, 5mg/mL DTT and 0.002% bromophenol blue) and then 15 min on a rocking platform in alkylating solution (6M urea, 100mM Tris-HCl pH 8, 0% (w/v) glycerol, 2% (w/v) SDS, 22.55mg/mL iodoacetamide (IAA) and 0.002% bromophenol blue). Second dimension SDS-PAGE was run by overlaying the strips on 12-5% isocratic Laemmli gels, cast in low-fluorescence glass plates on an Ettan DALTsix system (GE Healthcare, UK). Gels were run at 20 ºC at a constant power of 2.5 W/gel for 30 min, followed by 17 W/gel until the bromophenol blue tracking front reached the end of the gel. Fluorescent images of the gels were acquired on a Typhoon 9400 scanner (GE Healthcare, UK). Image analysis and statistical quantification of relative protein abundance were performed using Progenesis SameSpots v2.0 software (NonLinear Dynamics, UK). The multivariate analysis tool of principal component analysis (PCA) was used as an explorative tool to visualize differences between datasets.

8.2 Protein identification

Protein spots of interest were excised from the gel using an automated Spot Picker (GE Healthcare, Sweden). Identification on spots selected was attempted after staining one

gel using Flamingo (BioRad, USA). Tryptic digests were purified using Zip Tip microtiter plates (Millipore, USA). MALDI-TOF-MS/MS analysis of tryptic peptides was performed on an Ultraflex TOF-TOF Instrument (Bruker, Germany). The spectra were processed using Flex Analysis 3.0 software (Bruker Daltonics, USA). Peak lists were generated using the signals in the m/z 800 - 4,000 region with a signal-to-noise threshold of greater than 3. After removing peaks corresponding to keratin and trypsin autolysis peptides the resulting final peak list was used for identification of the proteins by peptide mass fingerprint. The Mascot 2.2 program (Matrix Science, UK) was used to search the Swiss-Prot 55.4 database, limiting the search to human proteins. The criteria for positive identification were a significant Mascot probability score (score > 55, $p < 0.05$).

RESULTS AND DISCUSSION

Orthotopic Growth of PC-3 cells into Nod/Scid mice

In order to develop a xenograft model to characterize the molecules involved in the complex process of PCa bone metastases, 5-week old immunodeficient male mice were engrafted s.c. with a small piece of a human adult bone (**Figure 29**), approximately 1 cm^3 of a spinous process of a vertebra from a non-cancerous patient. After engraftment no signs of inflammation or granulation in the bone grafts or in the surrounding murine tissues were observed.

On day 0, single cell suspensions of the well-established and stable transfected human PCa cell line, PC-3 cells (1 x 106 cells/ 50 µL of sterile PBS), were inoculated directly into the human bone grafts of mice under anaesthesia with 2% isofluorane. Mice were imaged by BLI using the IVIS System (**Figure 30**) on day 0 after tumour inoculation and then weekly until the end of the experiment 7 weeks post-inoculation, when the tumour BLI signal had increased more than one order of magnitude without compromising the welfare of the mice, to evaluate and quantify tumour growth (**Figure 31**). At the time of anyeuthanasia, no severe signs of cachexia could be observed in any mice. PC-3 cells grew extensively after orthotopic implantation in the grafted adult human bone.

Intraosseous injection of PC-3 cells into human-engrafted Nod/Scid mice resulted in the development of tumours in bone grafts of 70% of mice. Only 3 out of 10 mice with a human bone xenograft did not develop bone tumour after cell inoculation. The control group (mice with the human bone graft but without tumour cell inoculation) received a PBS injection and was otherwise treated in the same conditions throughout the study. During 7 weeks of bone tumour expansion, *in vivo* monitoring showed an exponential growth of the BLI signal corresponding to the kinetics growth of tumour cells (**Figure 31.A-B**).

The *ex vivo* analysis demonstrated that the intraosseous BLI signals detected by the IVIS System were confined to the human bone graft (**Figure 30.C**). Histological analysis confirmed this result (Figure 31.D-E). Moreover, bone tumours showed an osteolytic phenotype derived from tumour proliferation of these human PCa cells (**Figure 31.D**).

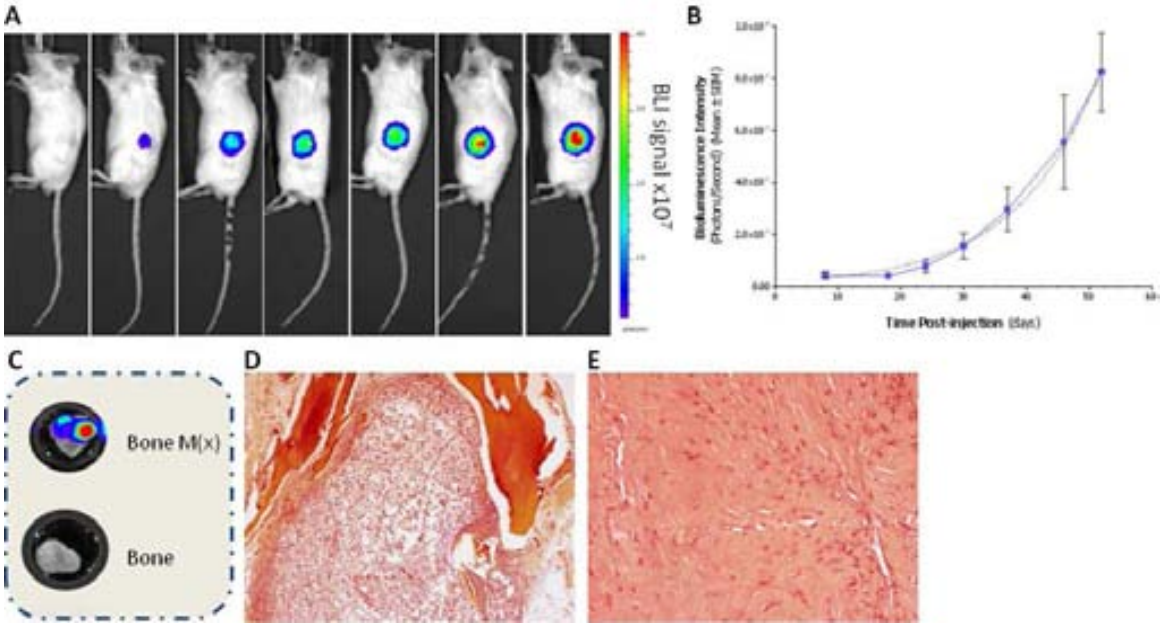

Figure 31. Tumour growth of human PC-3 into engrafted bone implants. A. A representative *in vivo* monitoring by BLI imaging in the IVIS System in Nod/Scid mouse engrafted with a bone implant after cell inoculation. Mice were imaged weekly from day 0 to day 52 post-injection. The colour scale on the right represents the BLI signal intensity (shown as radiance flux in photons per second) and corresponds to tumour burden. **B.** Histogram that represents the tumour growth of luciferase-expressing PC-3 cells into engrafted bone implants. Dots mean of BLI signal; bars, standard error of the mean (SEM). **C.** *Ex vivo* analysis of human bone implants grafted into Nod/Scid mice after 52 days of PCa tumour growth. *Ex vivo* BLI imaging of bone with metastasis confirmed that tumour cells were grown inside the bone implant. Control bones were excised from Nod/Scid mice with the engraftment but without cell inoculation. **D-E.** Histological examination by H&E staining of (**D**) bone implant with metastases (4x) and (**E**) without metastases (10x).
Abbreviations: Bone M(x): bone with metastases.

Engraftment was accompanied by a neovascularisation of bone tissue because bone tumours could not grow beyond an adequate blood supply within bone implants. However, no markers of vasculogenesis or angiogenesis, such as the vascular endothelial growth factor (VEGF), an important element in the creation of new blood vessels during embryonic development or after injury, were investigated.

Proteomic identification from intraosseous PCa tumours

After sacrificing the mice, the bone grafts were excised and proteins extracted, quantified and separated using 2D-DIGE technology (**Figure 32**). 2D-DIGE permitted the direct comparison of all components from all samples, since the protein gels were normalized with a pool of proteins from all of samples included in this study. The proteins were resolved on a 12.5% acrylamide gel after an IEF on non-linear gradient strips of pH 4-7. The strategy to achieve labelling efficiency was that half of the samples from each group were labelled with Cy3 dye and the other half with Cy5 dye; a third fluorescent dye, Cy2, was used to label the internal standard sample. Thus, alternative labelling was carried out for the three samples groups (bone with PCa metastases, bone without metastases and tumour cell component) (**Table 13**). Additionally, the internal standard pool was run on each gel, within a total number of six gels. Unfortunately, one out of six gels had to be discarded because of its different appearance from other gels. Spots differentially expressed in the 2D-DIGE analyses were picked and submitted to MALDI-TOF-MS/MS for identification. Statistical analysis was performed using the Progenesis SameSpots v2.0 software.

Table 13. Fluorescent labelling (Cy3 or Cy5) of human samples for the 2D-DIGE experiment

Gel	Cy3	Cy5
1	Bone M(x)_1	Bone_1
2	Bone M(x)_2	Bone_2
3	Bone M(x)_3	Tumour cells
4	Tumour cells	Bone M(x)_4
5	Bone_3	Bone M(x)_5
6	Bone_4	Bone M(x)_6

Abbreviations: Bone M(x): bone with metastases.

Comparing fluorescent gel images from bone with metastasis versus control bones, more than 30 proteins were found to be differentially expressed. Protein spots of interest were picked from the Flamingo staining gel and tryptic digested. From 31 spots of interest picked and analyzed using MALDI-TOF-MS/MS, only 17 proteins could be

identified by their peptide mass fingerprint and for subsequent search on the Swiss-Prot 55.4 database (**Table 14**). The search was limited to human proteins.

Additionally, keratin peptides were discarted from the final list. Principal Component Analysis (PCA) showed different behaviors between bones with PCa cells and control bones (**Figure 32**).

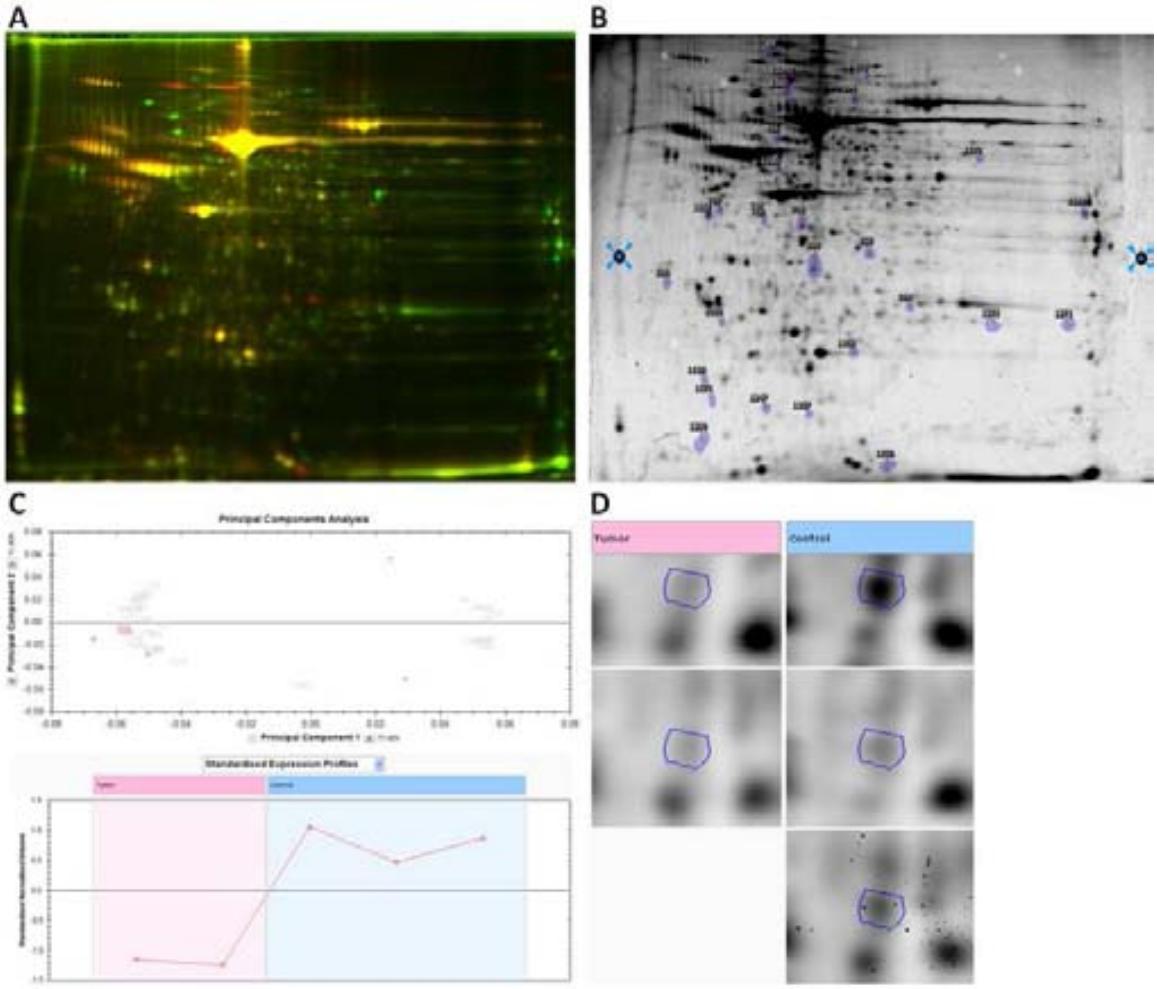

Figure 32. Proteomic experiment by 2D-DIGE technology. A. Example of a representative gel of a 2D-DIGE experiment on total protein from human bone implant samples. **B.** Silver staining gel where differentially expressed proteins are marked with blue circles to be picked and identified. **C.** Principal Component Analysis from DIGE experiment, where red dots correspond to bone with PCa metastases and blue dots to bone without metastases. **D.** Example of differential spots in PCa bone metastasis compared to control bones. Note: this specific spot is underexpressed in tumour samples compared to controls.

From the final list of proteins, none of them were identified solely as of human origin. All 17 proteins were either only from mouse origin or human, but with a high percentage of interaction with the protein from mouse origin.

Table 14. List of identified proteins from the DIGE experiment

SwissProt #	Name	Score	Pep match (%seq)	Error	% Inter
ALBU	Serum albumin	113	16 (32)	31	66.6
UAP1L	UDP-N-acetylhexosamine pyrophosphorylase-like protein 1	125	9 (23)	34	70.7
GALK1	Galactokinase	368	7 (18)	152	
ACTB	Actin, cytoplasmic 1	58	6 (19)	26	20.4
EIF3I	Eukaryotic translation initiation factor 3 subunit I	71	5 (19)	24	10.1
CAPZB	F-actin-capping protein subunit beta	91	12 (42)	29	32
EFHD2	EF-hand domain-containing protein D2	51	1 (5)	287	
FRIL1	Ferritin light chain 1	75	5 (34)	16	34.6
APT	Adenine phosphoribosyltransferase	64	4 (35)	26	16.8
VIME	Vimentin	171	20 (46)	36	90.4
KAP0	cAMP-dependent protein kinase type I-alpha regulatory subunit	66	5 (18)	26	53.2
GSTM1	Glutathione S-transferase Mu 1	88	8 (35)	22	30.5
ACTN4	Alpha-actinin-4	147	17 (22)	23	47.9
ACTN1	Alpha-actinin-1	267	7 (8)	98	
PSA	Puromycin-sensitive aminopeptidase	222	5 (5)	104	
ALDOA	Fructose-bisphosphate aldolase A	114	13 (37)	21	65.3
ACTA	Actin	116	3 (8)	58	

Circulating human tumour cells had a preference for targeting the human bone graft

To further study both the species- and tissue-tropism of circulating human PCa cells, an intracardiac inoculation of tumour cells was performed in immunodeficient mice engrafted with human adult bone, as previously described. Four weeks after bone implantation, ten Nod/Scid mice were inoculated with a tumour cell suspension (3 x 10^5 cells in 0.1 mL of sterile PBS) directly into the arterial circulation. Forty-eight hours after cell inoculation 2 mice out of 10 unexpectedly died presumably as a result of an inaccurate cell injection. Fortunately, two weeks post-inoculation 7 out of 8 mice showed a BLI signal in implanted human bones. No tumour signal was detected in mouse bones.

Animal models of cancer are important for identifying mechanisms of tumour growth and metastasis that replicate the clinical situation. Bone metastatic xenograft models involving the human PCa cell lines, PC3 [299-301], LNCaP [299, 300, 302, 303] or others [304, 305] have been developed using immunocompromised mice. These studies have documented new findings regarding bone remodelling and metastases. However, further studies are needed to clarify the pathogenesis of PCa metastases restricted to the microenvironment of the bone because the metastatic process is not only determined by the characteristics of the tumour cell itself but by its surrounding microenvironment [302]. Accordingly, the animal model presented in Chapter I of this thesis allowed to mimic PCa's bone metastases as accurately as possible by means of the subcutaneous implantation of human adult bone fragments followed by the intraosseous injection of human PCa cells, to assess the *in vivo* formation and progression of bone lesions.

In similar studies [296, 302] it has been observed that osteoblasts, osteoclasts and endothelial cells of implanted human bones were of human origin. Moreover, both human implanted cells and bone cells, including bone marrow stromal cells, survived and were functional for at least 16 weeks after subcutaneous implantation [302]. These findings confirmed that the human implanted bone is alive in this xenograft model. Although in this study no cytokeratin immunohistochemistry was carried out to assess the state of the implanted human bones into Nod/Scid mice, the *in vivo* tumour growth of PCa cells monitored by the IVIS System indicated that the environment allowed tumour growth and proliferation inside the human bone grafts. Moreover, the storage of growth factors in the bone matrix and their release into the intramedullary space may be necessary for PCa to remain viable and proliferate in bone metastases.

The humanized Nod/Scid model developed in this Chapter had been previously described; in most of these studies tested different therapies against tumour growth or osteolytic activity [296, 301, 303-309]. However, none of these reports included the differential proteomic profile of bone metastases.

In contrast to similar studies [300, 302-304], a bone fragment from a man without evidence of cancer nor bone metastases instead of bone from a cancer patient donor was used to be implanted subcutaneously. This model aimed to avoid the conditioning of the bone environment to establish bone metastases from other cancer types and the existence of undetectable micrometastases in the bone fragment previous cell inoculation.

Once the humanized mouse model had been established, proteomic techniques quantified the differential expression of protein profiles from bone with and without metastases, so that molecular changes could be attributed to PCa cells growing inside the implanted human bones. Using 2D-DIGE technology, several proteins were found to be differentially expressed in bone with metastases compared to control bones. Proteins of interest were picked, digested and analyzed by MS. However, only 17 proteins could be identified using the Swiss-Prot database. Even when the search was limited to human proteins none of them could be confirmed to be only of human origin. These findings suggest that in the Nod/Scid-human system the mouse stroma may play a role in facilitating tumour growth in metastatic lesions and that a mouse component that interacts with human bone and tumour cells may exist.

Regarding the final list of identified proteins, some were cytoskeletal proteins (ACTB, CAPZB, ACTN4, ACTN1 and ACTA) or enzymes for different metabolism compounds (UPAL1, GALK1, APT and KAP0). A comprehensive literature search to study the possible role of these proteins in the development of bone metastases did not yield any positive result. Consecuently, no candidate was used to be validated in human samples.

Concerning the results from the proteomic study, it is possible that the 2D-DIGE technology was not the best proteomic approach to perform this differential expression analysis. Indeed, 2D-DIGE has some limitations that could compromise the accuracy of the results [310]. Other proteomic approaches could be used to improve the identification of low abundant components with discriminatory expression in metastases, such as SELDI-TOF/MS (Surface-enhanced laser desorption/ionization time-

of-flight mass spectrometry), ICAT (Isotope-coded affinity tag) and iTRAQ (Isobaric tags for relative and absolute quantitation). Firstly, SELDI-TOF/MS has an affinity-based approach that allows sensitive and high-throughput protein profiling and screening of biological samples [311]. This proteomic technique was used for protein profiling of serum from PCa patients with and without bone metastases [311] and for biomarker discovery in these samples. Secondly, the ICAT method has the power to quantitatively identify proteins including membrane proteins, low copy number proteins and high molecular weight proteins [312]. It was developed to reduce sample complexity and identify low-abundance proteins in complex samples [313]. However, to increase the level of labelling the isotope-coded protein labelling (ICPL) was developed. And finally, iTRAQ is similar in concept to ICAT. The power of iTRAQ is that it allows the simultaneous analysis of 4, 6 or 8 biological samples, which makes the experimental work more cost-effective [312]. So far, few studies have used ICAT [314-319] or iTRAQ [320-327] in PCa and they are mainly applied to PCa cell lines. None has been applied to PCa bone metastases.

Regarding proteomic analysis, some studies have used 2D-PAGE coupled to MALDI-TOF or MS in metastatic PCa samples [328], PCa cell lines [329], breast cancer samples [330] or breast cancer cell lines [331-334]. Ronquist *et al*. [328] identified proteins derived from prostasomes that originated from cells of vertebral metastases of PCa patients but they did not study differences in protein profiles due to skeletal metastases. Although all these studies have initiated the characterization of molecular mechanisms of metastases suppressor molecules, further analyses are required to understand the tissue-tropism of PCa cells to the bone.

Despite all these promising proteomic techniques, the little amount of proteins obtained from the *in vivo* assay did not allow to try other proteomic approaches to increase the number of identified differentially expressed proteins in PCa bone metastases compared to healthy bones.

Ultimately, to further examine both the species- and tissue-tropism of circulating human PCa cells, an intracardiac tumour cell inoculation was performed into the Nod/Scid-human system. Two weeks after cell inoculation, circulating human PCa cells preferentially formed tumours in implanted human bone tissue in contrast to the host mouse tissue, thus corroborating the species-specificity observed by Yonou *et al.* [302]. These findings also indicate that human bone provides a favourable environment for the growth of PCa cells as suggested by Paget [7] in the "seed and soil" hypothesis. Additionally, similar studies [302] using LNCaP cells injected intravenously (i.v.) in mice in which adult human bone fragments were implanted s.c., demonstrated that two weeks after cell injection, LNCaP tumour foci were observed in human bone fragments, confirming that injecting human PCa cells into the circulation, either i.c. or i.v., can colonize the bone grafts whitin two weeks, as observed in this study.

This study also demonstrated that the human bone graft was vascularised and allowed the establishment of circulating tumour cells migrating through the arterial circulation and also tumour growth of PC-3 cells inside the bone grafted, as monitored by the IVIS System. Moreover, the study showed PC-3 cells species-tropism for human bone grafts when tumour cells were inoculated into the arterial circularion. In contrast, McCabe *et al.* [335] described a complete lack of bone metastases in human bone grafts after i.c. injection of murine prostate cells (RM1) regardless of the quantity of cells introduced into nude mice.

In addition, the colonization by human tumour cells of human bone grafts before any other tissue, such as lung or liver, suggests a tissue-tropism of PCa cells for their bone target, which correlates with the high incidence of skeletal metastases seen in advanced-stage PCa patients.

All these observations further indicate that the colonization of human bone grafts by PCa cells involves species- and tissue-specific mechanisms and is not attributable to the passive lodging of tumour cells in bone [302].

In conclusion, these data indicate that the Nod/Scid-humanized mice may provide a model to study species- and tissue-specific steps of the human metastatic process. Unfortunately, the differential proteomic approach of this study could not identify the molecular changes that attract human PCa cells to human bone implants.

Chapter II

Molecular characterization of highly bone metastatic prostate cancer cells obtained by *in vivo* selection in mice

BACKGROUND

Only a minute proportion of cancerous cells that enter the bloodstream manage to survive in distant organs [336, 337]. In xenograft models, the inoculation of thousands of tumour cells into mice at the corresponding (orthotopic) sites of the primary neoplasm or directly into the circulation results in a limited number of metastatic colonies [338]. The capacity of a few cells from a heterogeneous population to metastasize suggests that relevant metastatic cells can be separated from the rest by *in vivo* selection [21]. The selection imposed by this process incorporates multiple barriers of the metastatic cascade, especially when the xenograft is carried out in a non-orthotopic location; it also enables comparisons of phenotypically diverse cell populations that originate from the same tumour [18].

The distinction between tumorigenic events that constitute prior conditions for metastasizing such as transformation and immune evasion and events specifically aimed at metastatic invasiveness, intravasation, extravasation and colonization of distant organs must be emphasized, since the capacity of tumours to metastasize is mainly attributable to the second set of functions [18].

Bioinformatic analyses of data sets from primary tumour samples have uncovered complex gene-expression patterns, or 'signatures', able to predict the risk of metastatic recurrence [18]. It has also been reported that *in vivo* sorting of metastatic cell populations can isolate preexisting cell subpopulations with distinct metastatic organ tropism [339], which can be linked to the distinct proclivity for tumour colonization of gene-expression profiles. For example, in studies that used a cell line derived from a patient with advanced metastatic breast cancer, cell progenies showed preference for specific tissues were isolated following the injection into the arterial or venous circulation of immunodeficient animals [32]. In agreement with the selectivity of metastatic progression, these different cell variants were found in the parental, unselected population [32, 339]. Furthermore, subpopulations that metastasized to the bone, adrenal gland or lung tissue were associated with distinct gene-expression profiles [18]. These distinctive gene sets were therefore selected based on interactions with unique microenvironments, providing a genetic basis for metastatic tropism [18]. Thus, by combining bioinformatic analyses of transcriptional data sets with functional

validation in a xenograft mouse model, gene metastasis signature lists could be found differentially expressed in human primary tumours [223]. Consequently, the biological filter afforded by *in vivo* selection and the clinical filter provided by large gene-expression data sets can be exploited to identify genetic determinants of metastases relevant to human disease [18].

Metastatic genes should be defined as those with an activity that enables locally invasive tumour cells to enter the circulation, to home, to extravasate and to colonize distant tissues. These genes can be identified by combining functional testing in animal models with validation in clinical samples. To date, various candidate metastasis suppressor genes that impede metastatic progression without affecting the primary malignancy have been identified [41]. Metastasis suppressor genes seem to operate at several stages of the metastatic process, including the final step, i.e., metastatic colonization. Many of the known metastasis suppressor genes (**Table 15**) would not have been predicted *a priori* since they do not have a known biochemical function consistent with current knowledge on invasion and metastasis. Biochemical investigation of metastasis suppressors has led to the identification of new pathways and functions crucial to the metastatic process [41].

Table 15. Metastasis-suppressor genes. Adapted from REF [41]

Gene	Cancer cell type with suppressive activity	Function
NM23	Melanoma, breast, colon, oral squamous cell	Histidine kinase; phosphorylates KSR; which might reduce ERK 1/2 activation
MKK4	**Prostate**, ovarian	MAPKK; phosphorylates and activates p38 and JNK kinases
KAI1	**Prostate**, breast	Integrin interaction; EGFR desensitization
BRMS1	Breast, melanoma	Gap-junction communication
KiSS1	Melanoma, breast	G-protein-coupled-receptor ligand
RHOGDI2	Bladder	Regulates RHO and RAC function
CRSP3	Melanoma	Transcriptional co-activator
VDUP1	Melanoma	Thioredoxin inhibitor

EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; JNK, JUN-terminal kinase; KSR, kinase suppressor of RAS; MAPK, mitogen-activated protein kinase kinase.

By contrast, the identification of genetic alterations that would specifically mediate bone metastasis in PCa remains rather elusive.

HYPOTHESIS AND OBJECTIVES

PCa cells that invade sinusoids in the bone marrow and ultimately colonize bones may possess certain specific phenotypic characteristics.

Main hypothesis: molecular changes can be quantified by differential expression analysis. These changes may reflect differences in bone metastatic potential of tumour disseminated cells that would identify metastasis-associated biomarkers.

If any of these changes are already present in the primary tumour, it should be possible to detect them.

Therefore, the detection of those prognostic biomarkers in the primary tumour may be used to identify those patients prone to develop aggressive PCa disease and who would benefit from more aggressive therapies aim at abrogating the metastatic process.

General objectives

The main objective of the research described in Chapter II of this thesis was to identify molecules associated with PCa bone dissemination in order to describe a bone metastasis signature, and more specifically, the phenotypic characteristics of PCa cells that mediate tumour progression within the bone microenvironment.

The identification of biomarkers is essential for the characterization of PCa bone metastases, to better understand the complex process of this metastatic cascade from the primary tumour to distant organs and to design more effective therapies against tumour dissemination.

Consequently, this study has focused on two main areas: **1)** to generate a highly-specific bone metastatic PCa cell line using a dissemination mouse model; and **2)** to characterize PCa bone metastases by differential expression profiles.

Specific objectives

1. Generation of a bone metastatic-specific PCa cell line using a dissemination mice model

In this study, PC-3-luciferase expressing cells were introduced into nude mice through intracardiac injection (i.c.) and BLI images were performed serially to measure tumour growth over time. Intracardiac injection of tumour cells mimics hematogenous dissemination of cancer cells [340]. Even if this technique does not recapitulate every step of the metastatic cascade, it is useful to investigate metastatic colonization and tumour growth in sites relevant to clinical metastatic disease [160]. The aim of the serial intracardiac injections was to enrich subpopulations of PCa cells with a progressively increasing preference to metastasize to bone.

Specifically, the following steps took place:

1a. Three rounds of *in vivo* selection in immunodeficient mice until achievement of bone metastatic behaviour in human PCa cell subpopulations.

i. PCa cell inoculation by i.c. injection into immunodeficient mice

ii. *In vivo* monitoring of tumour colonization and tumour growth

iii. Isolation of metastatic cells in bone sites by flushing and expansion in culture

iv. Enrichment by cell sorting and reinjection of tumour cells into the next group of mice

1b. *In vitro* characterization of the PCa cell subpopulation with increased bone metastatic behaviour.

2. Identification of biomarkers of PCa bone metastasis by differential expression profiles

Metastatic variants were established from the inoculation of PC-3 human cancer cells that formed bone metastases. After 3 *in vivo* passages in the bone, the PC-3-BM

subclone was chosen for its high tendency to metastasize to the bone after intracardiac injection. Differential expression profiling allows the detection of signatures that may underlie prostate carcinogenesis.

The following steps took place:

2a. Performance of differential expression analysis by comparing cells with low (PC-3) and high (PC-3-BM) bone metastatic potential.

i. Analysis of differentially expressed miRNAs by Taqman Array Microfluidic cards
ii. Analysis of differentially expressed genes by Human Gene Array
iii. Analysis of differentially expressed proteins by SILAC technology

2b. Selection of significantly altered molecules to describe an integrated molecular bone metastasis signature for advanced-stage PCa dissemination.

Future objective

Validation of the descriptive bone metastasis signature: use of identified molecules as prognostic biomarkers in clinical samples

Understanding the pathophysiology that underlies bone metastases and identifying new therapeutic targets to prevent or treat them is critically important [66]. Moreover, the identification of novel targets or biomarkers to be validated in clinical samples and to provide new routes to regulate the mechanisms involved in PCa dissemination remains a priority.

The following ongoing experiments aim to accomplish the aforementioned objectives:

a. Validation of miRNA candidates by microdissection of tumour areas in formalin fixed paraffin-embedded tissues (FFPE) followed by RTqPCR detection.

b. Validation of protein candidates by immunohistochemical detection in formalin fixed paraffin-embedded tissues.

MATERIAL AND METHODS

1 Tumour cell lines and obtention of conditioned medium

The human PCa cell line (PC-3), the human foetal osteoblastic cell line (hFOB 1.19) and the mouse clonal osteogenic cell line (MC3T3-E1 subclone 4) were obtained from LoGiCal Standards (LGC, UK). **PC-3-BM** cells were clonally derived from the human PC-3 cell line and were isolated from bone metastases produced in immunodeficient mice after intracardiac injection of **PC-3** cells. Both PC-3 and PC-3-BM cells express the pEGFPluc expression vector and therefore, they carry the integrated firefly luciferase gene coding region cloned upstream of the green fluorescent protein (GFP) gene. PCa cells were cultured as previously described in Chapter I (Material and Methods).

The hFOB 1.19 (**hFOB**) are human foetal osteoblastic cells conditionally immortalized with a gene encoding for a temperature sensitive A58mutant of the SV40 large T antigen [341]. With an incubation of hFOB cells at a permissive temperature (33.5ºC), the temperature sensitive gene is expressed and these cells proliferate rapidly, whereas at a restrictive temperature (39ºC) the gene is not expressed and the cells proliferate less rapidly. hFOB cells were cultured at 33ºC in non-differentiating media for all experiments and therefore are considered premature osteoblastic cells. The hFOB 1.19 (hFOB) cell line was cultured in a 1:1 mixture of phenol-free DMEM/Ham's F12 medium containing 10% FBS and 1% gentamicin (Invitrogen, Life Technologies, UK) at the standard conditions (5% CO2) at the permissive temperature of 33ºC, which allows the expression of the large T antigen.

MC3T3-E1 (**MC3T3**) is a murine preosteoblast cell line [342] that has a well characterized osteoblastic phenotype and is frequently used as a model to study *in vitro* effects of proinflammatory cytokines [343]. These cells were cultured in α-minimal essential medium (α-MEM) (Sigma-Aldrich, UK) supplemented with 10% FBS, penicillin and streptomycin (50 U/mL).

To obtain conditioned medium (CM), PCa and osteoblast cells were seeded in 10 mm dishes at an equal number of cells (1 x 10^6) and incubated for 24 hours at 37ºC. The following day, the medium was replaced with fresh medium containing 1% FBS at a minimal volume to achieve concentration. Twenty-four hours later, the CM was

collected from the cultures, centrifuged to remove floating cells, filtered through a 0.2 µm polyethersulfone membrane (Millipore, USA) and stored at -80ºC.

2 *In vitro* Proliferation assay

PCa cells (2,000 cells/well) were seeded into 96-well tissue culture plates in sextuplicate. After 24 hours, cell proliferation was quantified using MTT CellTiter 96 Assay (Promega Biotech Ibérica, Spain), following manufacturer's instructions. Twenty microlitres of the One-Solution Reagent were added to each well, mixed by swirling the plates and returned to the incubator. After 2-3 hours, the absorbance was measured by dye solution using a BioTek ELx800 plate reader to directly test wavelengths of 490 nm.

For co-culture experiments, osteoblast cells were seeded (1.5 x 10^4/well) in 12-well plates for 24 hours in complete media. The following day, the old media was changed to a 1:1 mixture of PCa cells medium and cells were incubated for an additional 24 hours. Next, PCa cells (1.5 x 10^4 cells/ insert) were seeded onto polyethylene terephthalate (PET) membranes (containing 0.4 µm pores) of the Transwell inserts (BD Bioscience, USA) in the presence or absence of osteoblast-containing wells (**Figure 33**). After 2 days of co-culture, PCa cells from inserts were fixed with 4% paraformaldehyde (PFA) and stained with bisbenzimide (Hoescht, Sigma-Aldrich, UK) and 0.1% crystal violet (AppliChem, Germany). Cells were counted at x400 magnification in standard optical microscopy and the average number of cells per field in five random fields was recorded. Triplicate filters were used and the experiments were repeated three times.

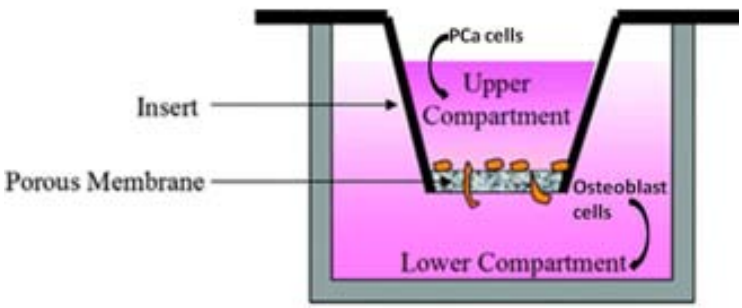

Figure 33. Boyden Chamber: cells are seeded on top of the insert in serum-free media, and chemoattractants are placed on the lower compartment. Migratory and invasive cells move through the pores toward the chemoattractant below.

3 *In vitro* migration assay

3.1 Wound healing assay

The PCa cells were allowed to reach confluent monolayers in 24-well plates and incubated overnight. A straight line (wound) was then gently performed at the bottom of the dish with a 0.5 mm plastic pipette tip. Next, cells were washed, incubated in medium with 2% FBS and kept in a computer controlled mini-incubator, which provided stable temperature of 37ºC with 95% humidity, 5% CO_2 and optical transparency for microscopic observations. The incubator was fastened to an inverted microscope (Live Cell Imaging CellR, Olympus, Japan) to monitor cell migration. Images were taken with the 4x objectives every 30 minutes at predetermined wound sites (3 sites per condition, performed in triplicate) and were analyzed using the ImageJ software (Wright Cell Imaging Facility, USA). Initial wound area (μm^2) and time needed to close the wound (hr) were the variables used to calculate the migration speed of the cells.

3.2 Transwell migration assay

For co-culture experiments, osteoblast cells (hFOB or MC3T3) were seeded (1.0×10^4/ well) at the bottom of a 24-well plate with complete media for 24h. The following day, the old media were replaced with a 1:1 mixture of PCa cells medium and osteoblasts were incubated for an additional 24 hours. Then, PCa cells were seeded (1.0×10^4 cells/ insert) onto Transwell inserts (BD Bioscience, USA) with 8 µm diameter pore membranes, in a medium supplemented with 2% FBS in the presence or absence of osteoblast-containing wells. After 24 hours, the number of migrating PCa cells was determined as described above. The migration experiments were performed at least three times.

4 *In vitro* Invasion assay

PCa cells were seeded (1.0×10^4/ insert) onto Matrigel Invasion Chamber inserts with 8 µm diameter pore membranes in triplicate, with the lower chamber containing medium supplemented with 10% FBS and incubated for 24 hours. Cells in the lower chamber determined the number of cells as previously described.

For co-culture experiments, osteoblast cells (hFOB or MC3T3) were seeded (1.0 x 10^4/ well) at the bottom of a 24-well plate with complete media for 24h. The following day, the old media were replaced with a 1:1 mixture of PCa cells medium and osteoblasts were incubated for an additional 24 hours. Next, PCa cells were seeded (1.0 x 10^4 cells/ insert) onto Matrigel Invasion Chamber inserts (BD Bioscience, USA) with 8 µm diameter pore membranes, in a medium supplemented with 2% FBS, in the presence or absence of osteoblast-containing wells. After 24 hours, the number of invading PCa cells was determined as previously described.

5 Animals and animal maintenance

Congenitally athymic male nude mice (Hsd.Athymic Nude-*Foxn1*^{nu}) and NOD-SCID mice (C.B-17/IcrHsd-*Prkdc*^{scid}) were purchased from Harlan (Harlan Laboratories, Italy) at 4 weeks of age and maintained under specific pathogen-free conditions. Animals were kept for at least 1 week in the facility before experimental manipulation. Animals were kept in a sterile environment in cages with beds of sterilized soft wood granulate and fed irradiated rodent diet *ad libitum* with autoclaved tap water. An artificial cycle of 12h light/12h darkness was maintained in the room where the animals were kept. A maximum of five mice were kept in each box, and all manipulations were performed using sterile techniques within a laminar-flow hood at the animal facility. Experiments were performed on animals at 5 weeks of age. All the procedures associated with experimentation and animal care were performed according to the guidelines of the Spanish Council for Animal Care and the protocols of the Ethics Committee for Animal Experimentation of our institution.

6 Xenograft by intracardiac inoculation of prostate cancer cell suspension and BLI imaging

The methodology for the intracardiac injection of PCa luciferase-transfected cells has been previously described in Chapter I (Material and Methods). Animals were sacrificed by cervical dislocation 5 or 6 weeks post-injection or earlier if there were signs of serious distress. For bioluminescence plots, the photon flux for each mouse was measured by the amount of highlighted pixels calculated by using a circular region of interest (ROI) in

a ventral or dorsal position and normalized to the value obtained immediately after xenografting the same area (day 0) of each mouse, so that all mice had an arbitrary starting BLI signal of 1.

7 Derivation of cell subclones from bone metastases

After sacrificing the mice, tumour cells from bone metastases were localized by *ex vivo* BLI imaging and freshly harvested under sterile conditions by flushing. Briefly, bones were dissected free of musculature and soft tissues using a scalpel and the upper and bottom part of the bone were cut. Bone marrow and tumour cells were rinsed twice with PBS using a 27-gauge syringe, centrifuged with additional fresh medium and put in cell culture dishes. The following day, the cells were washed twice with PBS to wash off mouse bone marrow cells that did not attach to the plate. Attached cells were maintained under geneticin resistance, expanded *in vitro* and selected by GFP fluorescence-activated cell sorting using the FacsAria (BD Bioscience, USA). These subpopulations were again inoculated intracardiacally in another group of animals. This process was repeated in a third group of mice.

8 Transcriptomic and miRNA expression analysis

8.1 Total RNA and miRNA isolation from PCa cells

Total RNA including miRNAs was extracted using the miRNeasy Mini Kit (Qiagen, Germany) following manufacturer's instructions. Total RNA concentration was assessed with a Spectrophotometer (NanoDrop 1000; Thermo Scientific, USA) and quality of RNA was ensured using the Agilent RNA 6000 Nano Kit in Bioanalyzer 2100 (Agilent Technologies, USA). Quality of miRNA was assessed by the amplification of small nuclear RNAs, RNU44 and U6 snRNA.

8.2 Reverse transcription and transcriptomic analysis

For microarray analysis, RNAs were amplified, labelled and hybridized on an Affymetrix GeneChip® Human Gene 1.0 ST Array (Affymetrix, UK). Images were processed using the Microarray Analysis Suite 5.0 (Affymetrix, UK).

8.3 Megaplex reverse transcription RT reactions and 384-well TaqMan MicroRNA Array

For each sample of PCa cell line, a multiplex RT reaction containing 2 different Megaplex RT primers (Applied Biosystems, USA) was performed. The Human Pool A v2.1 contains RT primers for 377 unique microRNAs and 5 controls, whereas the Human Pool B v2.0 contains RT primers for 290 unique microRNAs and the same endogenous controls. Three hundred nanograms of total RNA from PCa cells were used for a 7.5 µL reaction. Final Megaplex RT primers concentration was 1 nmol/L (0.05x). Reaction conditions: 40 cycles at 16ºC for 2 minutes, 42ºC for 60 seconds, 50ºC for 1 second, and finally 85ºC for 5 minutes. Next, 6 µL of the Megaplex™ RT product was mixed with 0.45 mL of TaqMan Universal PCR Master Mix (Applied Biosystems, USA) and 0.444 mL of nuclease-free water. Reactions were run and analyzed on an Applied Biosystems 7900 Real-Time PCR at default thermal-cycling conditions. Reaction conditions: 55ºC for 2 minutes and 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 15 seconds and 55ºC for 1 minute.

TaqMan Array Micro Fluidic cards profiled one sample for the expression level of 384 different miRNAs (including controls) using the comparative CT (ΔΔCT) method of relative quantitation. Each TaqMan Array Micro Fluidic card contains 5 types of endogenous controls: three replicates for U6 snRNA-001973, one replicate for RNU44-001094 and one for RNU48-001006. The stability of the five controls was computed with the Coefficient of Variation (CV).

8.4 Real Time Quantitative PCR (RTqPCR)

One microgram of total purified RNA was subjected to a reverse transcriptase reaction using Superscript III (Invitrogen, Life technologies, USA) using 1 µl of Random Primers (50 µM, Invitrogen), 1 µl of dNTPs mixture (10 mM, Promega) and sterile distilled water to 13 µl for each reaction. Reaction conditions: 5 min at 65 ºC, followed by an incubation of 1 minute on ice; addition of 4 µl of 5X First-Strand Buffer, 1 µl of DTT (0.1 M, Invitrogen) and 1 µl of SuperScript III (Invitrogen); and incubation for 5 min at 25 ºC, 60 min at 50 ºC and 10 min at 70 ºC, with a final step at 4 ºC.

Next cDNA, corresponding to approximately 1 µg of starting RNA, was used in three replicates for quantitative PCR (Taqman, Applied Biosystems, USA).

For the confirmation of the microarray analysis the CAV-1-Hs00971716_m1, ANXA-10-Hs00200464_m1 and ITGA2-Hs00158127_m1 probes were used. The GAPDH-Hs9999905_m1 probe was used for normalization (Applied Biosystems).

8.5 Signalling pathway analysis

The analysis of biological significance was based on the enrichment analysis of the Gene Ontology (GO) and the Kyoto Encyclopaedia of Genes and Genomes (KEGG), whereas the functional pathway and network analyses were performed through the Ingenuity Pathway Analysis (version 9.0, Ingenuity® Systems, USA).

9 Protein expression analysis

9.1 Western Blot of EMT molecules

PCa cells growing in culture were scrapped, centrifuged and cleaned with 1x PBS. Whole cell extracts were prepared using Laemmli Buffer (100 mM Tris-HCl pH 6.8, 4% SDS and 20% glycerol) and pellets and lysis buffer were incubated at 90 ºC for 5 min. Cell lysates were centrifuged at maximum speed for 45 min at 4ºC. After centrifugation, the supernatants of the total protein extraction were stored at -20 ºC. Protein concentration was determined with a BioRad RCDC Protein Assay kit (BioRad, Spain). Equal amounts of protein (50 µg/lane) were then resolved on an 8% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, USA). Membranes were blocked in 5% milk solution (Tris buffered saline (TBS)–0.1% Tween) for 1 hr at room temperature and then incubated with the appropriate primary antibody in 5% milk solution overnight at 4ºC. Membranes were washed three times in TBS-0.1%Tween at room temperature and incubated for 1 hour with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (Dako, Denmark). Final detection was obtained through the enhanced chemiluminiscence system (Amersham Pharmacia Biotech Inc., USA), following manufacturer's instructions. Primary antibodies used for Western blot were: anti-integrin β1 (1/1000; 610467, BD Transduction Laboratories, USA), anti-integrin α2

(1/200; sc-74466, Santa Cruz Biotechnologies, USA), anti-Protein_B (1/100), anti-Protein_A (1/200) and anti-α-tubulin (1/1000; 2125, Cell signalling). Densitometric analysis of the protein bands was performed with ImageJ software (Wright Cell Imaging Facility, USA) [344].

9.2 Cell culture and protein preparation for SILAC

PCa cells, PC-3 and PC-3-BM, were grown in parallel at 37°C in L-lysine- and L-arginine-depleted RPMI-1640 medium (Thermo Scientific, USA) supplemented with antibiotics, 10% dialyzed foetal bovine serum (Sigma-Aldrich, UK) and either L-lysine monohydrochloride (100 mg/L; Sigma-Aldrich, UK) plus L-Arginine (100 mg/L; Arg-SILAC™, Invitrogen, Life technologies, USA) (**LIGHT** medium) or Lysine HCl ^{13}C-labelled (100 mg/L; Silantes GmbH, Germany) plus $^{13}C_6$-L-Arginine HCl (50 mg/L; Silantes GmbH, Germany) (**HEAVY** medium). Fresh medium was replaced every 2 days and PCa cells were cultured for approximately 7 doublings to achieve complete labelling of cellular proteins with heavy and light labelled amino acids. Next, cells were washed three times with ice-cold PBS, and cell lysis and protein extraction were carried out.

The cells were lysed in lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, 1 mmol/L EDTA, 0.25% DOC (deoxycholic acid) and 2% SDS (pH 7.6), in addition to a mixture of protease inhibitor cocktail (Boehringer Mannheim, Germany). Lysates were clarified by centrifugation at 12,000 x g for 20 min at 12ºC. Relative protein concentrations were measured using the BioRad RCDC Protein Assay kit (BioRad, Spain).

9.3 Protein Separation and Trypsin In-gel Digestion

Equal amounts of protein (100 µg) from each sample were mixed at a 1:1 ratio, and mixed with 6M urea (GndCl 6M) and pH was adjusted to pH 8. Next, a reducing buffer containing 700 mM Dithiothreitol (DTT; GE Healthcare, UK) and 50 mM Ammonium bicarbonate (AB; Sigma-Aldrich, UK) was added and samples were incubated for 1 h. For protein alkylation 700 mM Iodoacetamide (IAA; GE Healthcare, UK) containing 50 mM AB was added and incubated for 30 minutes in the dark. Mixed samples were run on a 12% Acrylamide gel in SDS-PAGE, fixed 1 h with a fix solution (45% ethanol, 1% acetic acid) and lightly stained using Coomassie Blue overnight. Gel lanes were destained with

water, excised and cut horizontally into 20 equal sections. Excised sections were cut into ≈1 mm^3 pieces and destained using 50% acetonitrile/50% 25 mM AB solution, followed by dehydration in 100% acetonitrile for 10 min. Acetonitrile was discarded, and gel pieces were placed under vacuum centrifugation until completely dry. Each sample was then incubated overnight at 30ºC in a 0.2 µg/µL trypsin solution (in 25 mM AB). Peptides were extracted in 100% acetonitrile for 15 min at 37ºC, and then an extraction solution using 0.2% trifluoroacetic acid (TFA; Sigma-Aldrich, UK) was added for 30 min at room temperature. After a centrifuge spin, peptides were placed into PCR tubes, dried using vacuum centrifugation and stored at -20°C until analysis by mass spectrometry.

9.4 Protein Identification and Quantification

The digested proteins were analysed on an Esquire HCT Ultra Ion Trap mass spectrometer (Bruker, Germany), coupled to a nano-HPLC system (Proxeon, Thermo Scientific, USA). Peptide mixtures were first concentrated on a 300 mm id, 1 mm PepMap nanotrapping column and then loaded onto a 75 mm id, 15 cm PepMap nanoseparation column (LC Packings). Peptides were then eluted by a 0.1% formic acid - acetonitrile gradient (0%-40% in 100 min; flow rate ca. 300 nL/min) through a nano-flow ESI Sprayer (Bruker, Germany) onto the nanospray ionization source of the Ion Trap mass spectrometer. MS/MS fragmentation (3 x 0.3 s, 100–2800 m/z) was performed on three of the most intense ions, as determined from a 0.8 s MS survey scan (310–1500 m/z), using a dynamic exclusion time of 1.2 min for precursor selection and excluding single-charged ions. An automated optimization of MS/MS fragmentation amplitude was used, starting from 0.60 V. Proteins were identified using Mascot (Matrix Science, UK) to search the SwissProt database. Searches were restricted to human (20407 sequences) taxon. MS/MS spectra were searched with a precursor mass tolerance of 1.5 Da, fragment tolerance 0.5 Da, trypsin specificity with a maximum of two missed cleavages; cystein carbamidomethylation was set as fixed modification, with methionine oxidation as well as the corresponding Lys and Arg SILAC labels as variable modifications.

Data processing for protein identification and quantification was performed using WARP-LC 1.3 (Bruker, Germany), a software platform integrating liquid chromatography-MS run data processing, protein identification through a database search of MS/MS

spectra and protein quantification based on the integration of the chromatographic peaks of MS-extracted ion chromatograms for each precursor. For protein quantification, both H/L and L/H ratios were calculated averaging the measured H/L and L/H ratios for the observed peptides after discarding outliers.

10 Statistical analysis

Student's *t*-test was used to compare means in proliferation, migration (wound healing and transwell), invasion assays and also in miRNA expression levels (RTqPCR).

Taqman MicroRNA Array cards were normalized by deltaCt method [345] and filtered out to increase statistical power and reduce unnecessary noise. Non-specific filters were applied to remove endogenous controls, undetermined categories and features whose standard deviation was below the 20th percentile of all standard deviations. The analysis to select differentially expressed miRNAs was based on adjusting a linear model with empirical Bayes moderation of the variance (*p*-value). The cut off for statistical significance was a *p*-value under 0.05. The *t*-test was used to compare expression levels between groups. *In silico* analysis of putative miRNA targets was performed using annotation methods from Bioconductor and based on miRBase (http://www.mirbase.org/) and TargetScanS (http://genes.mit.edu/tscan/). However, for experimentally verified miRNA targets two databases were used: the TarBase v6.0 [346] and the mirTarBase v3.5 [347].

Microarray data were normalized using the RMA method described in [348, 349]. The analysis to select differentially expressed genes were based on adjusting a linear model with empirical Bayes moderation of the variance, a technique similar to ANOVA specifically developed for microarray data analysis by Smyth GK. [350]. The selection of differentially expressed genes was based on adjusted *p*-values below 0.01 and a absolute value of LogFC above 2. Statistical analyses were performed using the Statistical Package for Social Science version 15.0 (SPSS, IBM, USA).

RESULTS AND DISCUSSION

In vivo selection of PC-3 cell subpopulations with a preference to metastasize to bone

Tumour cell populations are heterogeneous and have different abilities to metastasize to secondary organs such as the bone. In order to isolate highly metastatic subpopulations that home preferably to the bone, the *in vivo* selection approach was used. This methodology consisted of injecting PCa cells into the left ventricle of immunodeficient mice to recapitulate the natural spreading of tumour cells through arterial circulation, resulting in metastasis formation. To this end, 3 x 10^5 PC-3 luciferase-expressing cells were injected intracardiacally into immunodeficient mice in two different mouse strains, nude and Nod/Scid mice (n = 5 mice/strain), in order to generate a subpopulation of PC-3 with a strain-independent growth in bone sites. Mice developed bone lesions 2 weeks after cell inoculation and animals were monitored until the end of the study (week 9). Mice were sacrificed immediately if they presented any symptom of paraplegia or cachexia. BLI imaging was used to monitor tumour burden and tumour cell localization, whereas tumour-induced bone destruction was assessed using micro-computed tomography. After the sacrifice, tumour cells were isolated from bone metastases and reinoculated after expansion in culture (**Figure 34**).

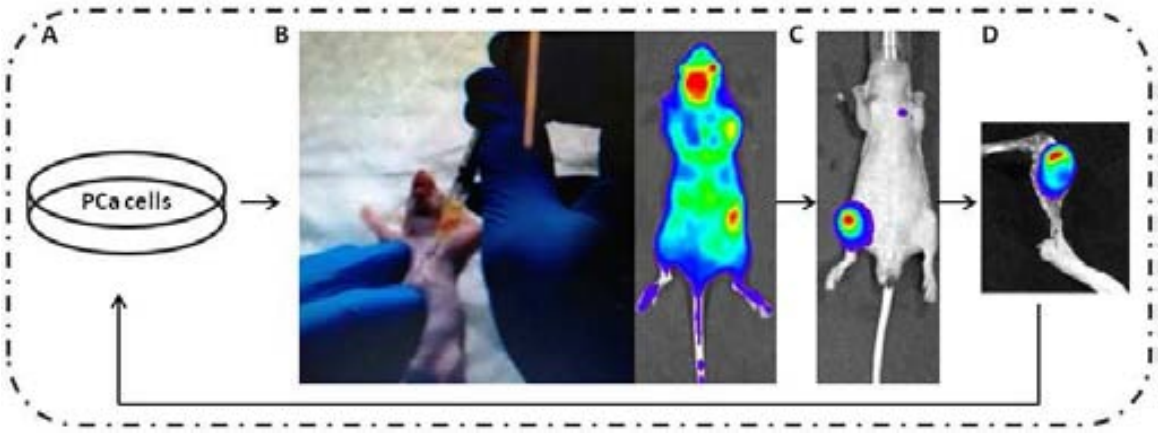

Figure 34. View of the sequential method for the *in vivo* selection of PCa cells with a preference to metastasize to bone. **A.** Following PCa cell culture of luciferase-expressing cells, the first step consists of **B** intracardiac inoculation of cells into immunodeficient mice and *in vivo* monitoring of tumour growth by BLI imaging using the IVIS System. In the second step, circulating tumour cells target multiple organs, including bones. **C.** The final step collects tumour cells from bone metastatic sites by flushing and puts them in culture to be injected again.

PC-3-BM, a metastatic subclone of PC-3 cells with a preference to home to bone was isolated after three rounds of this process of *in vivo* selection. Although the incidence of bone metastases increased concomitantly with each round of selection (**Figure 35**), the development of osteolytic lesions did not grow faster when compared to the parental cell line. Moreover, the distribution and number of bone metastases generated by PC-3-BM cells was higher in leg, spinal cord and scapula areas. Non-bone metastases were less frequent and involved primarily lung and soft tissues such as the adipose tissue or the musculature. PC-3, the parental cell line with a poor metastatic phenotype, has an incidence of metastasizing to long bones of only 20% compared to the 100% of the PC-3-BM subclone.

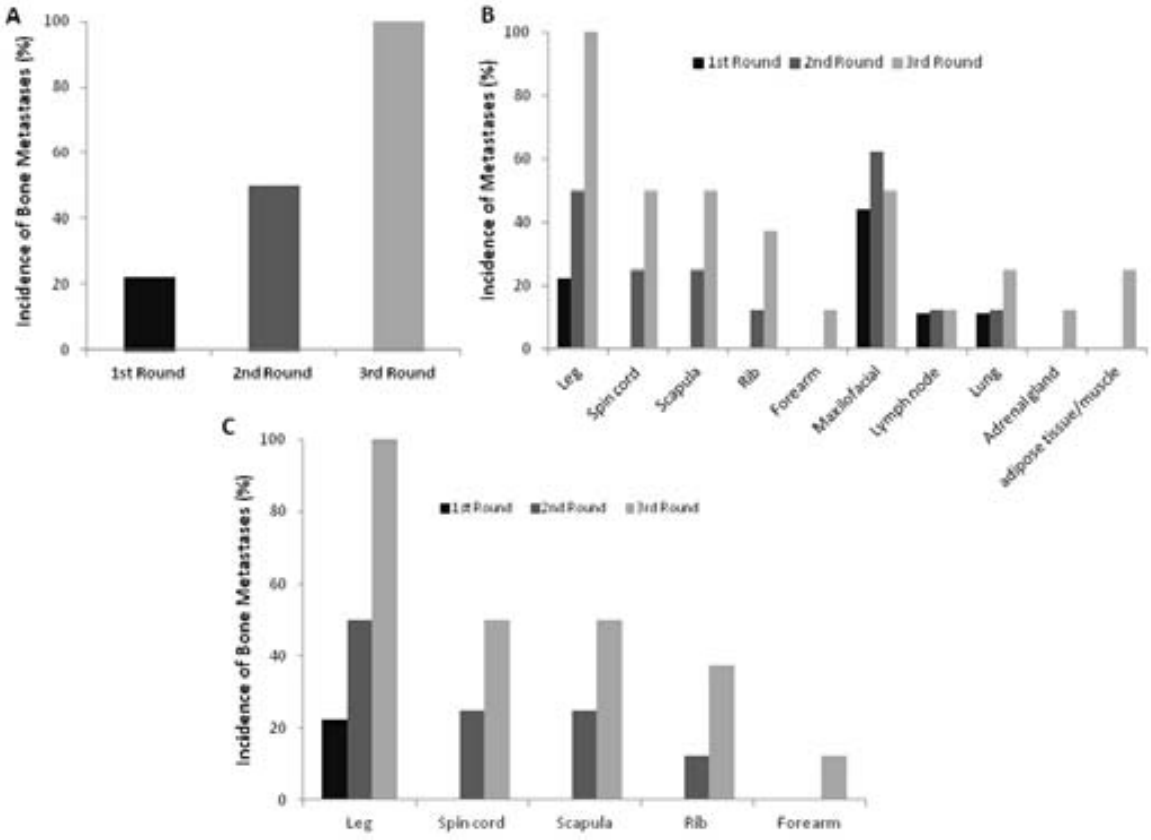

Figure 35. Incidence of metastases after three rounds of *in vivo* selection in immunodeficient mice. **A.** Incidence of bone metastases in long bones over the whole experiment. **B.** Distribution and incidence of metastases in bones and soft tissues. **C.** Distribution and incidence of metastases only in bones. Results are expressed as percentage of metastases per total number of animals well injected in each round.

Figure 36 shows the time course of bone lesions development as monitored weekly by the IVIS System, from day 0 until day 63 after cell inoculation, comparing the parental PC-3 to the PC-3-BM subclone.

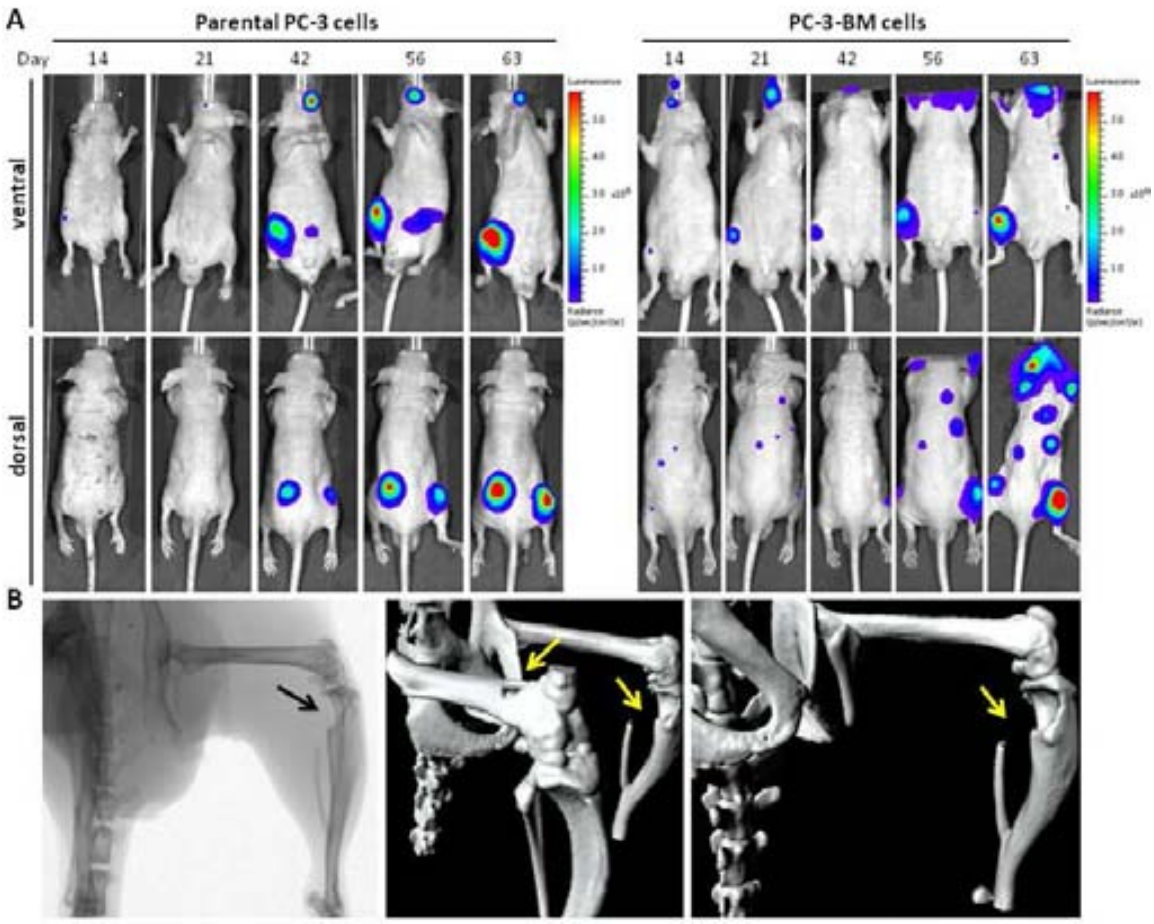

Figure 36. Bone metastases development of PCa cells injected intracardiacally into nude mice. To select subpopulations of PC-3 cells which home preferably to bone, these tumour cells were inoculated directly through the arterial circulation and then isolated from bone metastases in long bones. **A.** Representative BLI imaging of the metastatic distribution pattern of parental PC-3 cells compared to PC-3-BM cells, both injected intracardiacally into nude mice over time. The ventral and dorsal positions from each mouse were recorded weekly from day 0 until the end of the experiment. **B.** Representative micro-CT images showing osteolytic lesions caused by PCa cells from different mice before sacrifice. Black arrows show the metastatic lesions.

In vitro characterization of the PC-3-BM subpopulation

To further study the *in vitro* metastatic activity of two different human PCa subpopulations, which present high and low *in vivo* metastatic potential to bone, PCa cells were co-cultured with bone cells and multiple *in vitro* assays were performed.

Subsequently, tumour cells were cultured at 1:1 ratio with either bone mouse (MC-3T3) or human (hFOB1.19) osteoblast cells in co-culture systems using Boyden chambers as described in the Material and Methods section of Chapter II. Dissimilarities on tumour proliferation, migration and invasion rates were assessed in highly metastatic PC-3-BM cells compared to parental PC-3 cells (**Figure 37**).

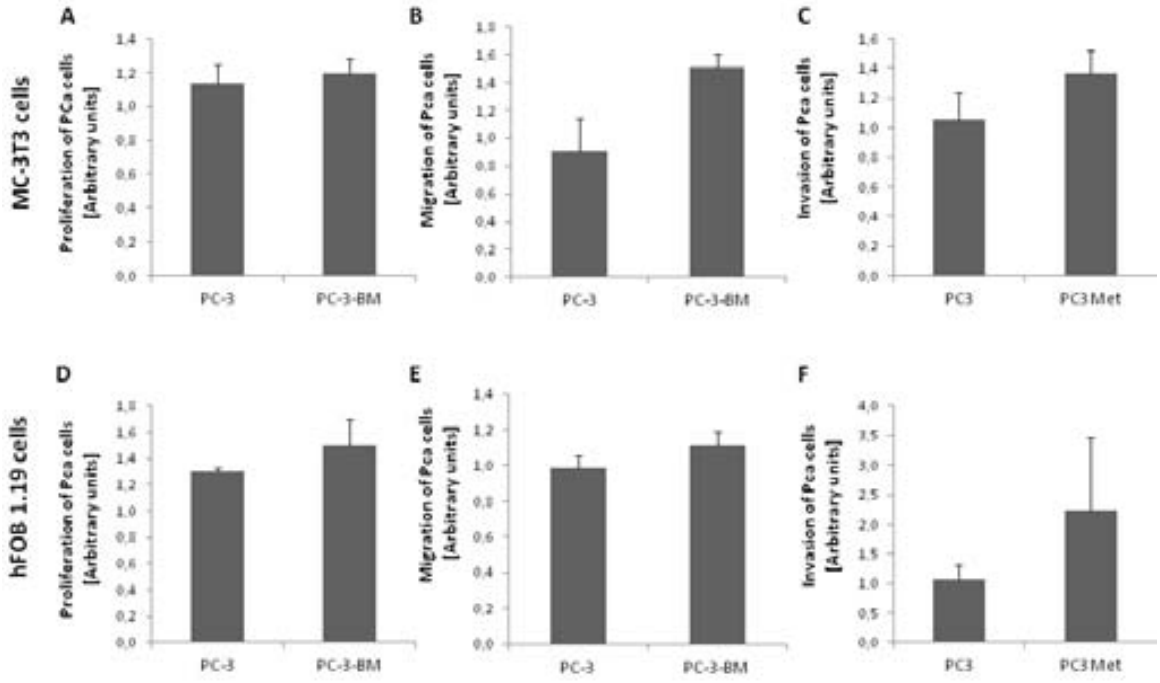

Figure 37. *In vitro* characterization of the *in vivo* selected clone PC-3-BM compared to the parental PC-3 cell line. To assess the metastatic activity from PC-3 and PC-3-BM cells, both cell populations were characterized by (**A-D**) proliferation, (**B-E**) migration and (**C-F**) invasion assays using Boyden chambers to establish co-culture systems. The PC-3-BM subpopulation showed induction of invasiveness and migration compared to parental PC-3 cells.

Although the bone-metastatic subclone PC-3-BM obtained in a third round of selection had a growth rate comparable to that of parental PC-3 cells (**Figure 37.A-D**), they generated a higher number of bone lesions in immunodeficient mice and migrated and invaded more easily the transwell inserts *in vitro* in the presence of bone cells (**Figure 37**). In terms of invasion, this induction of the metastatic potential was slightly stronger when human PCa cells were placed at the upper compartment of the insert and human bone cells were at the bottom site (**Figure 37.F**), compared to results from mouse bone cells (**Figure 37.C**).

To elucidate if the *in vivo* changes observed in the phenotype of PC-3 subclones resulted from an inherent cell proliferation and migration trait or from the specificity of tumour cells to metastasize to bone, two types of tests were performed after each round of *in vivo* selection. Consequently, PC-3 subclones that differed from the parental cells on cell proliferation (by MTT assay) and migration (by Wound Healing assay) were discarded.

To further evaluate changes in cell proliferation and cell communication between PCa and bone cells, additional *in vitro* studies using conditioned media (CM) were performed. Osteoblast cells were grown in fresh medium containing 1% FBS at minimal volume for 24 hours. Next, the CM was collected, centrifuged, filtered and placed in each well of 96-well plate where PCa cells, PC-3 or PC-3-BM had been seeded 24 hours before. Cell proliferation assays demonstrated that PCa cells did not proliferate more when CM from osteoblast cells were added, corroborating the results observed in the co-culture system (**Figure 38**).

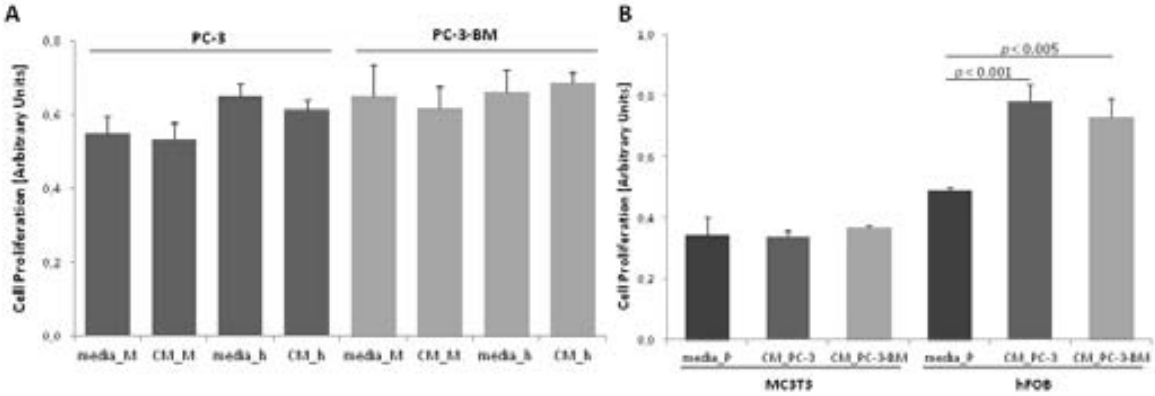

Figure 38. *In vitro* cell proliferation assay using conditioned media (CM) from PCa and bone cells. To assess changes in cell proliferation on both PCa and bone cells, the CM from another cell type was used. **A.** After 24 h of bone cell growth, CM were collected, centrifuged and added to each well where PCa cells had been seeded 24 h before. Cell proliferation was quantified using MTT CellTiter Assay. No changes were observed in PCa cells with mouse (MC3T3) or human (hFOB) bone cells CM. **B.** A significant increase in cell proliferation was observed in hFOB cells when CM from both PC-3 ($p < 0.001$) and PC-3-BM ($p < 0.005$) cells were added to the plate.
Abbreviations: CM, conditioned media; M, MC3T3 cells; h, hFOB cells; P, PCa cells.

Remarkably, when CM from PCa cells were added to human osteoblast cells, there was a significant increase in cell proliferation (CM from PC-3 cells, $p < 0.001$; PC-3-BM cells, $p < 0.05$) using the Student's *t*-test.

miRNAs expression profiling in PCa bone metastases

The main goal of this discovery phase to identify metastasis-associated biomarkers was to investigate the differentially expressed miRNAs from two types of PCa cell lines, PC-3-BM with high and PC-3 with low *in vivo* metastatic potential to bone, by collecting their expression data using TaqMan Array Micro Fluidic cards (Applied Biosystems).

Following array processing, U6-snRNA, RNU44 and RNU48 normalization and filtering of the raw array data, the pairwise comparison of PC-3-BM and PC-3 cells identified 87 miRNAs dysregulated in metastases, with an absolute log fold change value above 1.5. The analysis to select differentially expressed miRNAs was based on adjusting a linear model with empirical Bayes moderation of the variance. Of the 88 dysregulated miRNAs, 20 miRNAs were consistently upregulated (**Table 16**) and 67 miRNAs were down-regulated in the metastatic cell line compared to the parental one (**Table 17**). Those miRNAs can be regarded as biomarker candidates with a potential biological relevance for PCa development.

Table 16. Upregulated miRNAs (Log FC > 1.5) in PC-3-BM cells relative to PC-3 cells.

miRNA name	Log FC	miRNA name	Log FC
miR-582-3p	4.38	**miR-34a-3p**	2.31
miR-155	3.94	**miR-202**	2.24
miR-628-5p	3.51	**miR-582-5p**	2.13
miR-150	3.33	**miR-33a**	2.03
miR-615-3p	3.18	**miR-580**	1.94
let-7b-3p	2.98	**miR-548b-5p**	1.88
miR-639	2.80	**miR-142-3p**	1.82
miR-489	2.54	**miR-1225-3p**	1.81
miR-650	2.50	**let-7d-3p**	1.56
miR-105	2.33	**miR-30a-3p**	1.50

Abbreviations: LogFC, log2-fold change.

Table 17. Downregulated miRNAs (Log FC < -1.5) in PC-3-BM cells relative to PC-3 cells.

miRNA name	Log FC	miRNA name	Log FC
miRNA_A	-7.34	**miRNA_F**	-2.02
miRNA_B	-6.69	**miR-192**	-2.01
miRNA_C	-6.65	**miR-545-5p**	-1.95
miR-630	-6.41	**miR-18b**	-1.93
miR-16-2-3p	-5.10	**miRNA_G**	-1.90
miR-135b	-4.62	**miR-134**	-1.89
miR-194	-4.29	**miR-875-5p**	-1.87
miR-199a-3p	-4.02	**miRNA_H**	-1.86
miR-376a	-3.97	**miR-423-5p**	-1.83
miR-382	-3.90	**miR-323-3p**	-1.81
miR-148a	-3.60	**miR-126-5p**	-1.80
miR-190b	-3.31	**miR-122**	-1.80
miR-495-3p	-3.27	**miR-374a-3p**	-1.78
miR-376a-5p	-3.08	**miR-766**	-1.78
miR-331-5p	-3.04	**miR-450b-5p**	-1.77
miR-409-3p	-3.01	**miR-411**	-1.76
let-7a-3p	-2.93	**miR-376c**	-1.75
miR-34b	-2.90	**miR-151-5p**	-1.70
miR-502	-2.87	**miR-182-3p**	-1.70
miR-181c-3p	-2.82	**miR-1285**	-1.68
miR-20b	-2.76	**miR-571**	-1.68
miRNA_D	-2.75	**miR-195**	-1.67
miR-550	-2.73	**miR-664**	-1.67
miR-363	-2.58	**miR-888**	-1.65
miR-9	-2.53	**miR-425-5p**	-1.64
miR-181c	-2.53	**miR-191-3p**	-1.62
miR-7-5p	-2.52	**miR-125**	-1.61
miR-432-3p	-2.52	**miR-182**	-1.59
miR-378	-2.41	**miR-422a**	-1.55
miR-30d	-2.39	**miR-1296**	-1.53
miRNA_E	-2.32	**miR-137**	-1.52
let-7f-2-3p	-2.18	**miR-9-3p**	-1.51
miR-133a	-2.15	**miRNA_I**	-1.51
hsa-miR-1238	-2.03		

Abbreviations: Log FC, log2-fold change.
Note: Some miRNA identifications are coded due to intellectual property reasons

To verify the accuracy of the array data, RTqPCR experiments were performed for the most dysregulated miRNAs in the ranking of the candidate list and several additional miRNAs with potential involvement in metastatic PCa disease found in the literature. As a result, 20 miRNAs of interest plus the normalizer RNU44 were analyzed by RTqPCR. The association between array data and RTqPCR was good for all candidates except for miR-191, -195, -100, -21 and -210, and particularly strong for candidates with significant fold changes, such as miR-105, miRNA_A, miRNA_B and -135b.

Table 18. Comparison of Log FC between Microfluidic Array and RTqPCR data.

miRNA name	Microfluidic card (Log FC)	RTqPCR (Log FC)
miR-105	2.33	1.60
miR-146a	0.71	2.56
miRNA_A	-7.34	-6.31
miRNA_B	-6.69	-5.69
miR-194	-4.29	-1.52
miRNA_G	-1.90	-1.56
miR-135b	-4.62	-6.39
miR-9	-2.53	-1.74
miR-191	-0.80	0.25
miR-195	-1.67	0.82
miR-100	-0.76	0.99
miR-21	-0.77	0.64
miR-210	1.11	-0.23
miRNA_J	-1.33	-
miR-141	0.35	-
miR-218	-	-1.15
miR-96	-	-1.02
miR-34c	-	-

Abbreviations: Log FC, log2-fold change.
Note: Some miRNA identifications are coded due to intellectual property reasons

Differentially expressed genes involved in PCa bone metastases

In order to investigate which were the most relevant differentially expressed genes, but also to further associate the dysregulation of miRNAs with their target genes at mRNA level, the two subpopulations of PCa with different *in vivo* metastatic potential were examined for differential mRNA expression using Human Gene Array (Affymetrix). As

shown in **Tables 19** and **20**, 32 mRNAs were upregulated and 13 downregulated in the more metastatic cell line with an absolute log fold change value above 2.5.

Table 19. Upregulated mRNA (Log FC > 2.5) in PC-3-BM cells relative to PC-3 cells.

Entrez ID	GeneSymbols	Name	log FC	adj. *p*-value
285	ANGPT2	angiopoietin 2	**4.67**	3.77E-07
1134	CHRNA1	cholinergic receptor, nicotinic, alpha 1 (muscle)	**4.06**	4.48E-06
255324	EPGN	epithelial mitogen homolog (mouse)	**3.86**	3.77E-07
8787	RGS9	regulator of G-protein signaling 9	**3.30**	3.77E-07
158511	CSAG1	chondrosarcoma associated gene 1	**3.19**	7.34E-07
28984	C13orf15	chromosome 13 open reading frame 15	**3.12**	7.34E-07
728461	CSAG2// CSAG3	CSAG family, member 2// CSAG family, member 3	**3.04**	7.34E-07
84189	SLITRK6	SLIT and NTRK-like family, member 6	**3.00**	1.21E-06
9966	TNFSF15	tumor necrosis factor (ligand) superfamily, member 15	**2.99**	1.47E-06
	Gene_A		**2.99**	1.47E-06
344901	OSTN	osteocrin	**2.97**	6.73E-06
167681	PRSS35	protease, serine, 35	**2.88**	2.08E-06
245972	ATP6V0D2	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d2	**2.85**	5.20E-06
	Gene_B		**2.84**	4.23E-06
2591	GALNT3	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GalNAc-T3)	**2.80**	7.34E-07
4113	MAGEB2	melanoma antigen family B, 2	**2.78**	1.27E-06
80763	C12orf39	chromosome 12 open reading frame 39	**2.74**	2.54E-05
2895	GRID2	glutamate receptor, ionotropic, delta 2	**2.74**	3.86E-06
23224	SYNE2	spectrin repeat containing, nuclear envelope 2	**2.73**	7.34E-07
80017	C14orf159	chromosome 14 open reading frame 159	**2.72**	1.41E-06
26002	MOXD1	monooxygenase, DBH-like 1	**2.72**	7.34E-07
27063	ANKRD1	ankyrin repeat domain 1 (cardiac muscle)	**2.67**	1.64E-06
25891	PAMR1	peptidase domain containing associated with muscle regeneration 1	**2.65**	7.34E-07
	Gene_C		**2.65**	1.79E-06
81832	NETO1	neuropilin (NRP) and tolloid (TLL)-like 1	**2.63**	1.36E-06
731220	RFX8	regulatory factor X, 8	**2.63**	1.27E-06
57717	PCDHB16	protocadherin beta 16	**2.57**	7.34E-07
80177	MYCT1	myc target 1	**2.57**	1.36E-06
6347	CCL2	chemokine (C-C motif) ligand 2	**2.56**	8.84E-06
8515	ITGA10	integrin, alpha 10	**2.52**	7.93E-07
401024	FSIP2	fibrous sheath interacting protein 2	**2.52**	1.53E-06
1293	COL6A3	collagen, type VI, alpha 3	**2.50**	7.34E-07

Abbreviations: Log FC, log2-fold change; adj. *p*-value, adjusted *p*-value.
Note: Some dysregulated mRNA are coded due to intellectual property reasons.

Table 20. Downregulated mRNA (Log FC < -2.5) in PC-3-BM cells relative to PC-3 cells.

Entrez ID	Gene Symbol	Name	Log FC	adj. *p*-value
100128252	LOC100128252// LOC100288114	Uncharacterized LOC100128252// uncharacterized LOC100288114	**-5.82**	7.34E-07
9674	KIAA0040	KIAA0040	**-4.14**	3.77E-07
100129543	ZNF730	zinc finger protein 730	**-4.01**	7.06E-06
3294	HSD17B2	hydroxysteroid (17-beta) dehydrogenase 2	**-3.38**	3.77E-07
9119	KRT75	keratin 75	**-3.27**	3.77E-07
55612	FERMT1	fermitin family member 1	**-3.21**	7.93E-07
1803	DPP4	dipeptidyl-peptidase 4	**-3.12**	5.28E-07
100033432	SNORD116-21// SNORD116	small nucleolar RNA, C/D box 116-21	**-2.98**	8.33E-06
440519	ZNF724P	zinc finger protein 724, pseudogene	**-2.90**	4.79E-06
6507	SLC1A3	solute carrier family 1 (glial high affinity glutamate transporter), member 3	**-2.86**	7.34E-07
2239	GPC4	glypican 4	**-2.83**	1.61E-06
112770	C1orf85	chromosome 1 open reading frame 85	**-2.79**	1.10E-06
139886	SPIN4	spindlin family, member 4	**-2.63**	1.21E-06

Abbreviations: Log FC, log2-fold change; adj. *p*-value, adjusted *p*-value.

To verify the accuracy of the microarray data, RTqPCR experiments were performed for some of the dysregulated genes, such as integrin alpha 2 (*ITGA2*), caveolin (*CAV1*) and annexin alpha 10 (*ANXA10*), among others. Those selected genes were found dysregulated at mRNA and also at protein level and were previously reported to have a role in metastases. The association between the microarray data and RTqPCR was good for all genes (**Figure 39**).

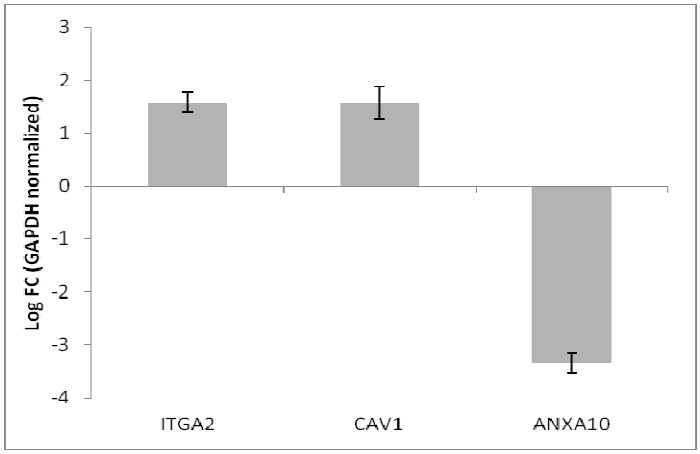

Figure 39. *ITGA2*, *CAV1* and *ANXA10* are dysregulated in PC-3-BM cells compared to PC-3 cells. *ITGA2* and *CAV1* are upregulated in the bone metastatic cell line compared to the parental one, whereas ANXA10 is downregulated. *GAPDH*, a housekeeping gene, was used to normalize mRNA levels in the analysis of those genes by RTqPCR.

Differentially expressed proteins involved in PCa bone metastasis

Similarly to Chapter I, one of the main objectives of Chapter II was to investigate proteins associated to PCa's metastatic process. In order to identify differentially expressed proteins, changes in PC-3 and PC-3-BM tumour cell lines were evaluated using the SILAC method.

PCa cells were grown in culture under light and heavy isotopic conditions for 7 generations. A crossed-labelling experiment was performed where PC-3 and PC-3-BM were labelled with light and heavy isotopic conditions, respectively, but also inversely, with heavy and light labelling, to rule out chance changes in protein expression levels. After cell lysis and protein extraction, equal amounts of extracted proteins were mixed and run on a 12% acrylamide gel in SDS-PAGE for protein separation based on their molecular weight. After gel fixation and staining, gel lanes were excised and cut horizontally into 20 equal sections. Next, each section was cut again into ≈1 mm^3 pieces. After trypsin digestion, peptides were analysed by MS through a nano-flow ESI Sprayer. Proteins were identified using Mascot searching into the SwissProt database, whereas protein quantification was calculated averaging the measured Heavy/Light ratio (PC-3-BM/PC-3 cells, SILAC experiment 1) and Light/Heavy ratio (PC-3-BM/PC-3, SILAC experiment 2) of quantified peptides (**Figure 40**).

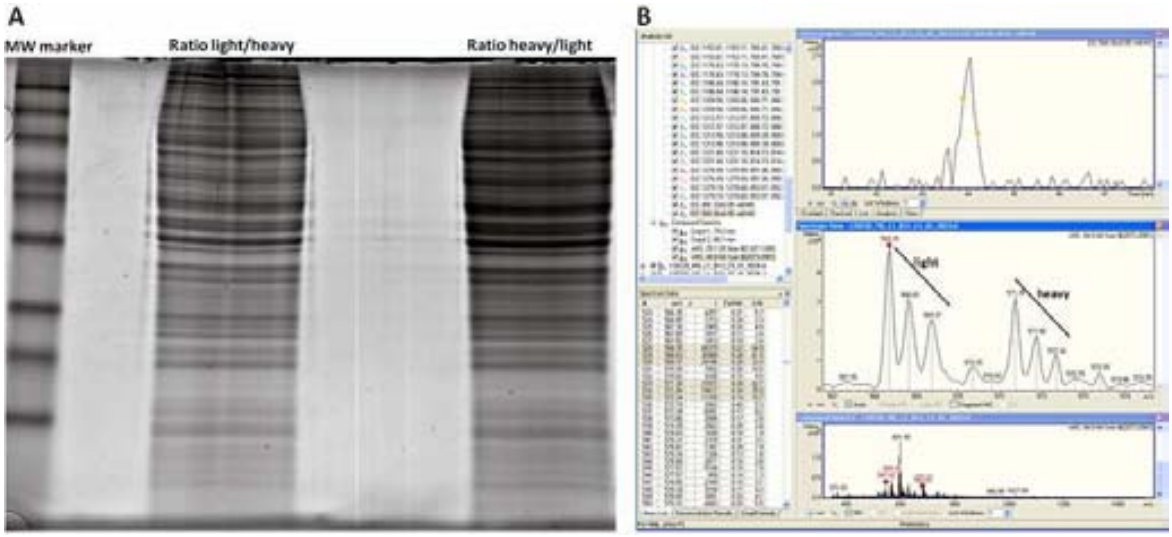

Figure 40. SILAC method. **A.** Gel after Coomassie Blue staining. Left, molecular weight marker for proteins, the middle lane corresponds to the light (PC-3-BM) and heavy (PC-3) labelling and the right lane corresponds to the heavy (PC-3-BM) and light (PC-3) labelling. **B.** Example of MS spectrum from one peptide after MS/MS fragmentation. Notice in the middle panel the ratio determination between the light (non-labelled) and heavy (labelled) peaks.

Almost 992 proteins were identified in one experiment (SILAC L1) and 1,078 proteins in the other (SILAC L2). 749 proteins were shared by the two lists (**Figure 41.A**). In this study, nearly 18% of proteins were identified by a single peptide and were consequently discarded from the differential expression analysis since confidence in their identification and quantification was poor. In addition, proteins identified in the SwissProt database as keratins were also discarded from the analysis as possible sources of contamination. Furthermore, the MS/MS spectra for all peptides used for quantification were manually inspected and verified.

To perform the relative protein quantification, a protein was considered as differentially expressed when it (i) was identified in the SwissProt database of human origin, (ii) was identified for more than one peptide, and (iii) was found dysregulated with a fold change at least 1.5 times higher or lower in relation to the parental cell line. Consequently, after filtering the final differentially expressed list, 14 upregulated proteins in the highly bone metastatic PC-3-BM cell line (**Table 21** and **Figure 41.B**) and 17 down-regulated proteins (**Table 22** and **Figure 41.C**) were obtained. Finally, two additional restrictive criteria were applied in order to include candidate proteins into the further integrative analysis when: (i) the percentage of quantified peptides from the total identified peptides for each protein was higher than 50%, and (ii) the coefficient of variation (CV) was lower than 30%. The CV is a normalized measure of dispersion of a probability distribution. After filtering with all the aforementioned criteria, a final list of 21 proteins were differentially expressed and were found in both SILAC experiments, of which 9 were upregulated and 12 downregulated in the PC-3-BM cell line.

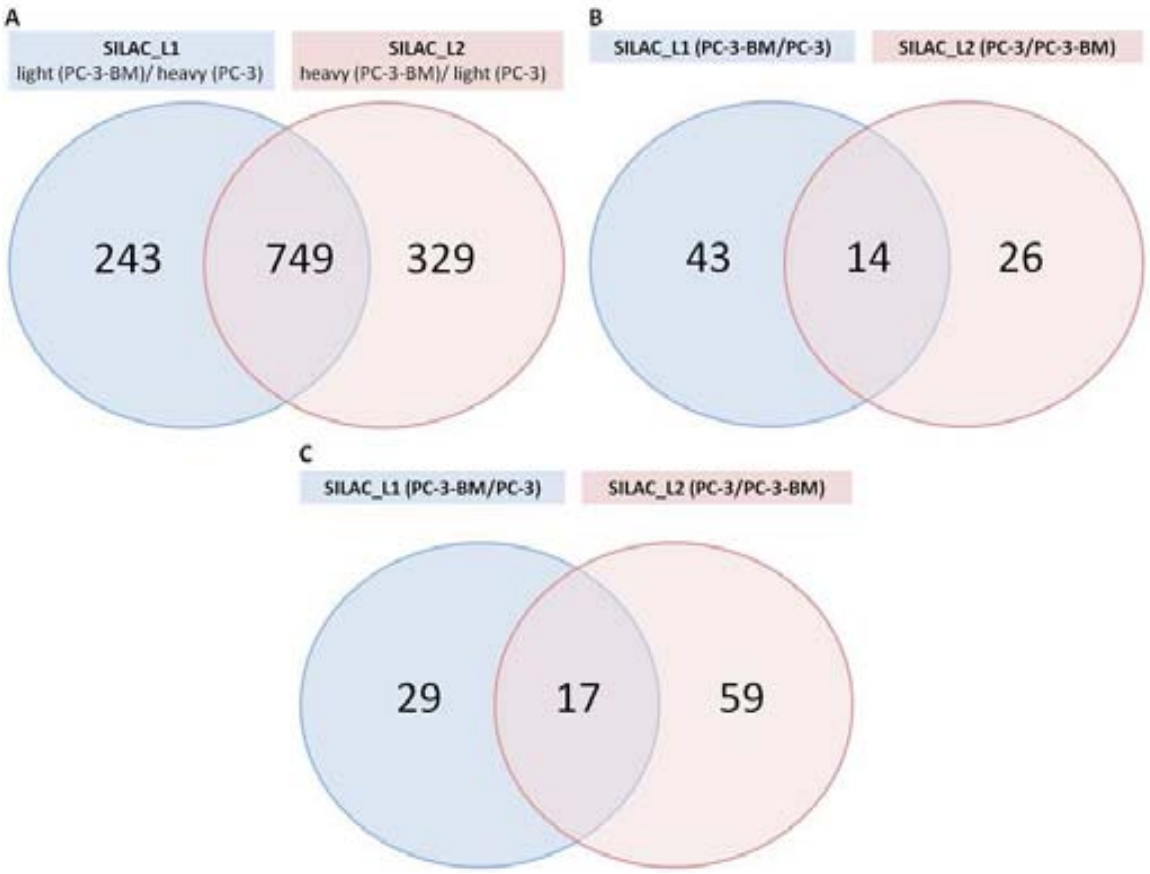

Figure 41. Venn diagrams overlapping proteins between the highly (PC-3-BM) and lower (PC-3) metastatic PCa cell lines analyzed by the SILAC method. In experiment L1 (blue circles), PC-3-BM cells were labelled with light and PC-3 cells with heavy isotopic conditions, and inversely, in experiment L2 (red circles) PC-3-BM cells were labelled with heavy and PC-3 cells with light isotopic conditions. **A.** The diagram shows the total number of identified proteins. **B.** The diagram shows the number of upregulated proteins and **C** the number of downregulated proteins after filtering.

Table 21. Upregulated proteins (FC > 1.5) in PC-3-BM cells relative to PC-3 cells.

Entrez ID	Symbol	Name	FC	% ID peptides	CV [%]
	Protein_A		**6.60**	72	28.53
	Protein_B		**2.39**	100	27.71
3673	**ITGA2**	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	**2.26**	100	24.16
10226	**M6PRBP1**	Mannose-6-phosphate receptor-binding protein 1 - Homo sapiens (Human)	**2.11**	67	18.6
493869	**GPX8**	glutathione peroxidase 8 (putative)	**2.08**	67	25.43
7167	**TPI1**	triosephosphate isomerase 1	**1.95**	48	8.04
2597	**GAPDH**	glyceraldehyde-3-phosphate dehydrogenase	**1.90**	78	15.97
	Protein_C		**1.84**	100	24.13
3688	**ITGB1**	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	**1.77**	70	25.23
301	**ANXA1**	Annexin A1	**1.74**	68	20.78
6888	**TALDO**	Transaldolase	**1.74**	67	22.99
4082	**MARCKS**	myristoylated alanine-rich protein kinase C substrate	**1.62**	100	26.64
	Protein_D		**1.53**	100	18.15
2023	**ENOA**	Alpha-enolase - Homo sapiens (Human)	**1.52**	62	18.85

Abbreviations: FC, fold change; % ID peptides, percentage of identified peptides; CV, coefficient of variation.

Note: Some proteins are coded due to intellectual property reasons.

Table 22. Downregulated proteins (FC < -1.5) in PC-3-BM cells relative to PC-3 cells.

Entrez ID	Symbol	Name	FC	% ID peptides	CV [%]
	Protein_E		**-4.88**	100	22.23
10105	**PPIF**	peptidylprolyl isomerase F	**-2.61**	60	14.35
9144	**SYNGR2**	synaptogyrin 2	**-2.39**	67	10.56
94081	**SFXN1**	sideroflexin-1	**-2.29**	71	20.41
1327	**COX4I1**	cytochrome c oxidase subunit IV isoform 1	**-2.16**	100	32.31
373156	**GSTK1**	glutathione S-transferase kappa 1	**-2.08**	100	12.82
1666	**DECR1**	2,4-dienoyl CoA reductase 1, mitochondrial	**-2.04**	100	17.23
9377	**COX5A**	cytochrome c oxidase subunit Va	**-2.03**	60	18.46
	Protein_F		**-1.97**	100	16.37
3192	**HNRPU**	heterogeneous nuclear ribonucleoprotein U	**-1.97**	80	23.94
5250	**SLC25A3**	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	**-1.93**	60	12.65
1537	**CYC1**	cytochrome c-1	**-1.90**	67	7.09
7791	**ZYX**	zyxin	**-1.78**	75	15.49
4191	**MDHM**	malate dehydrogenase 2, NAD	**-1.75**	74	15.57
515	**ATP5F1**	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit B1	**-1.70**	80	21.23
498	**ATP5A1**	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1	**-1.70**	71	16.25
84908	**F136A**	family with sequence similarity 136, member A	**-1.70**	100	29.59

Abbreviations: FC, fold change; % ID peptides, percentage of identified peptides; CV, coefficient of variation.
Note: Some proteins are coded due to intellectual property reasons.

Figure 42 shows the SILAC ratio distribution for all quantified proteins following a Gaussian distribution of the data, where the maximum number of proteins indicated an abundance ratio of 1.0.

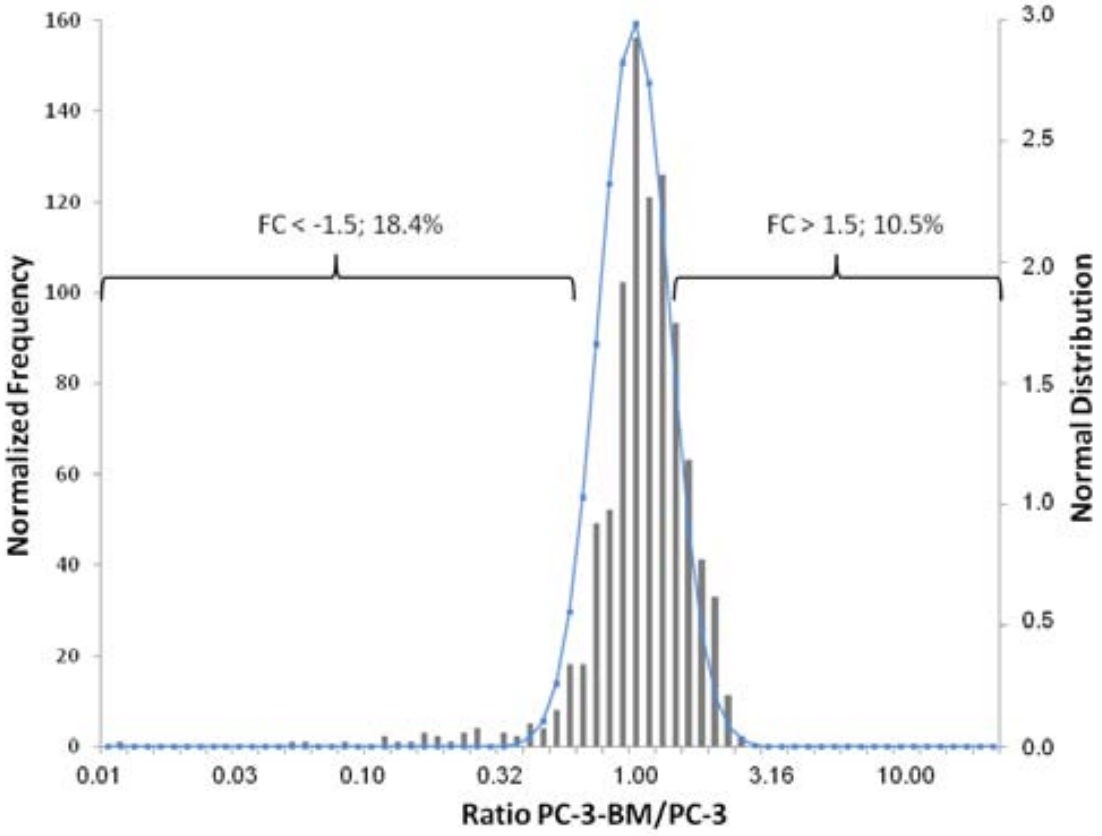

Figure 42. Distribution of protein expression ratio as determined by SILAC. The SILAC ratio for each protein represents the relative expression difference between high (PC-3-BM) and low (PC-3) metastatic PCa cells. The percentages indicate the respective portions of quantified proteins that were up- or downregulated in the metastatic cell line with an absolute fold change of 1.5.

To further confirm the SILAC results, four proteins for which antibodies were readily available were tested by Western blot. The level of integrin alpha-2 (ITGA2), integrin beta-1 (ITGB1), Protein_A and Protein_B, all proteins related to the EMT process, were assessed by immunoblot (**Figure 43**). Alpha-tubulin was used as a loading control because relatively no change was observed by SILAC. The expression level of these proteins had a good association with that observed by the SILAC experiment except for Protein_A, whose expression level did not change in PC-3-BM compared to PC-3 cells.

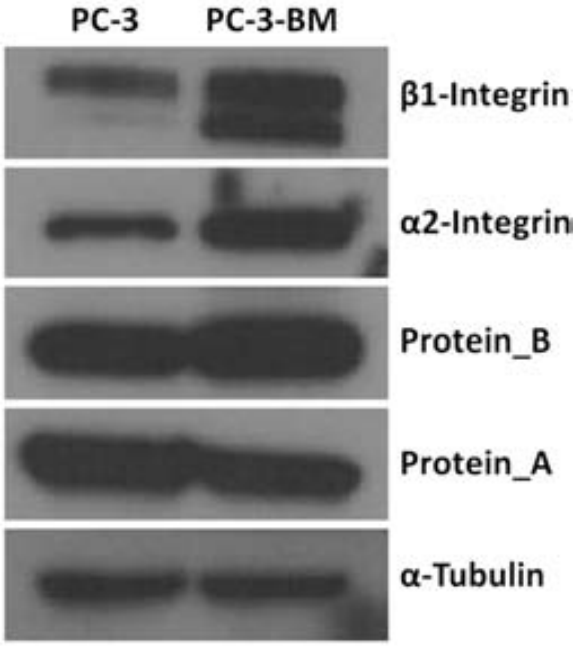

Figure 43. Western blot of EMT differentially expressed proteins in parental PC-3 and metastatic PC-3-BM cells. Proteins from PCa cells grown in culture were extracted and resolved on 8% SDS-PAGE gel. Primary antibodies of anti-integrin β1 (1/1000; 610467), anti-integrin α2 (1/200; sc-74466), anti-Protein_A (1/100), anti-Protein_B (1/200) and anti-α-tubulin (1/1000; 2125) were used for the detection of EMT associated proteins. Alpha-tubulin was used as loading control.

Integrative view of differentially expressed molecules responsible for prostate cancer bone metastases

The overview of the integration method to describe a PCa bone metastasis signature, essentially composed of 3 step levels, is presented in **Figure 44**. The screening method was based on three differential expression analysis by high-throughput, which compared two human PCa cell populations with high (PC-3-BM) and low (PC-3) *in vivo* metastatic potential to bone. The first level led to the identification of the differentially expressed miRNAs as determined by Array Microfluidic Cards. The second level was the differentially expressed genes highly associated with dysregulated miRNAs as assessed by Gene expression Array. And finally, the third step corresponded to differentially expressed proteins found by the SILAC method. Since miRNAs and proteins can be potentially used as biomarkers, they may provide a suitable list of candidates for the detection of prognostic biomarkers for PCa metastases.

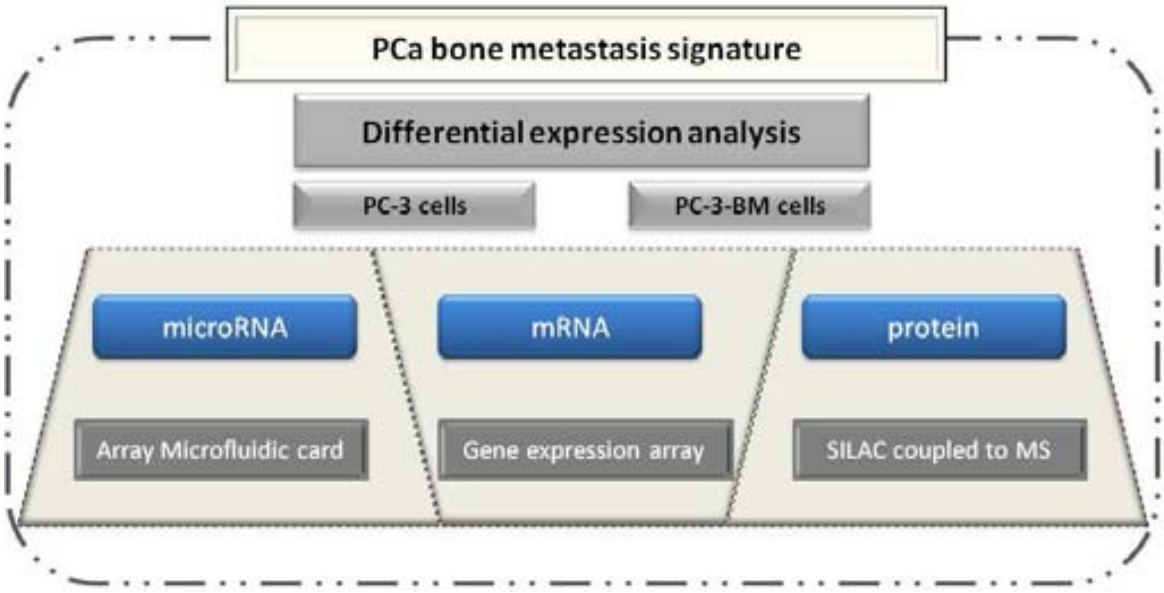

Figure 44. Overview of the three differential expression analyses carried out in this study to describe a PCa bone metastasis signature.

In order to select the candidate miRNAs and proteins, two individual approaches were performed with all data collected in the three screening methods. Firstly, a comparative analysis between miRNAs and their target genes was carried out to select which of the miRNA-target gene association was represented in the metastatic model. Secondly, the analysis compared the differentially expressed genes and proteins. Finally, it was considered that proteins and miRNAs highly related in the differential expression analysis play a role in the metastatic development of PCa.

As a first step in the identification of miRNAs with potential significance in the PCa metastatic process, putative target genes for each of the differentially expressed miRNAs were identified using annotation methods from Bioconductor and based on the miRBase and the Gene Ontology enRIchment anaLysis and visuaLizAtion (GOrilla) tool. Overlapping the results extracted from the microRNA array to the gene microarray data, an mRNA enrichment analysis was obtained with a list of the biological processes most altered in metastases. The most altered were response to chemical stimulus, such as cell surface receptor signalling pathways or cell-to-cell signalling, and cell motility or cell adhesion functions. However, to further identify which biological processes regulated by the differentially expressed miRNAs were the most affected, a biologically-guided approach based on a miRNA GO enrichment analysis was performed using a GO

enrichment of the targeted genes according to their miRNA expression levels. In this case, two experimentally verified miRNA target databases were used, the TarBase v6.0 and the mirTarBase v3.5. The miRNA GO enrichment analysis (**Figure 45**) showed that the generation of precursor metabolites and energy, the small molecule metabolism, oxidation-reduction process and gene expression among other processes were affected, offering an overview of the main functions altered due to metastases.

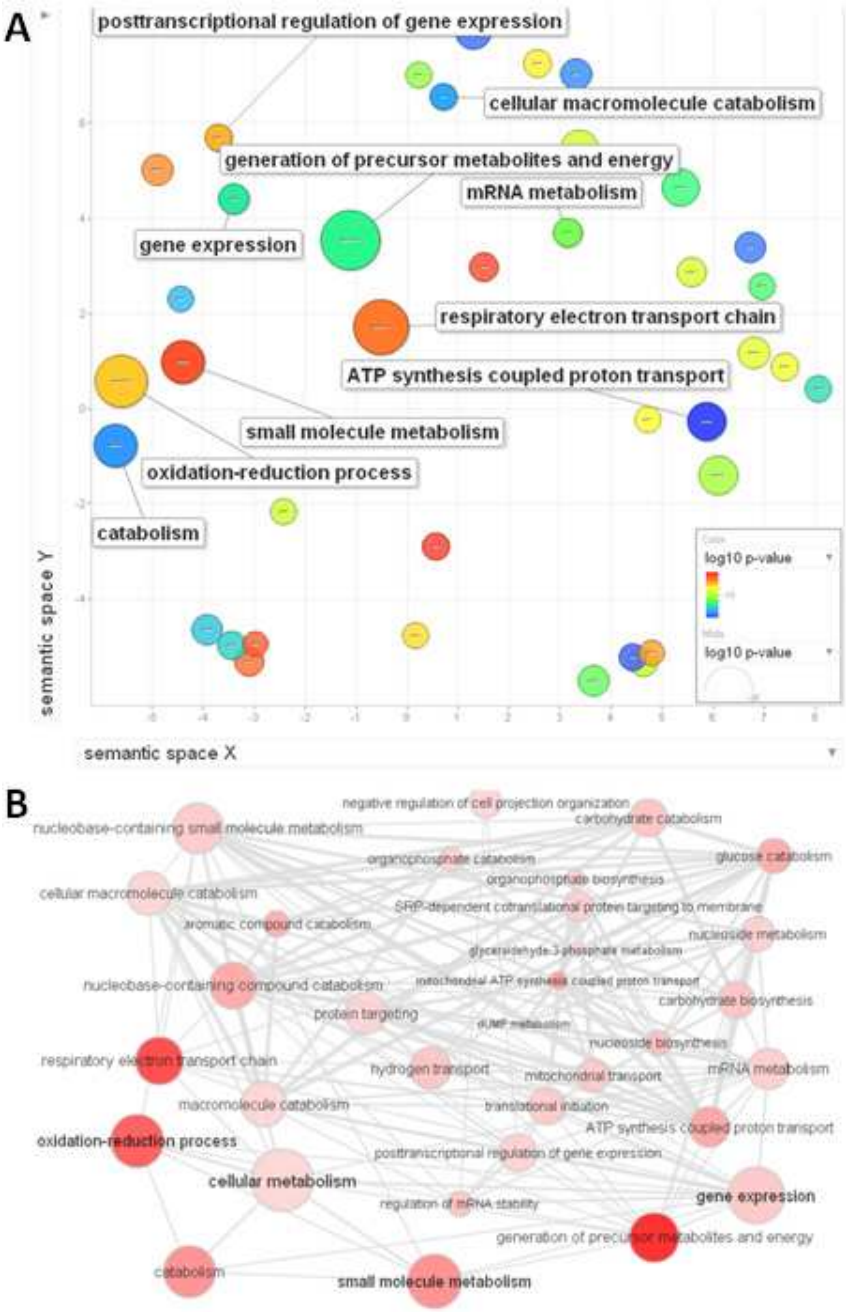

Figure 45. Main GO terms altered in the study after GO enrichment analysis. **A.** Scatter plot where some of the main GO terms altered are identified. The significance of the log *p*-value for each GO term is represented by the colour and size (*see legend*). **B.** Interactive graph of the same GO terms altered.

On the other hand, with the standardization of gene and protein codes, the comparative analysis identified 80 differentially expressed molecules present in both microarray and SILAC data. This association between differentially expressed genes and proteins is represented in **Figure 46**. Focusing on the 21 differentially expressed proteins obtained after the most restrictive filter, an association between gene and protein expression levels was found for all molecules except for 2 where gene-protein expression was inversely correlated (*Gene_D* and *RL27A*) and a further 2 molecules that were not expressed in the microarray data (*Protein_C* and *Protein_D*).

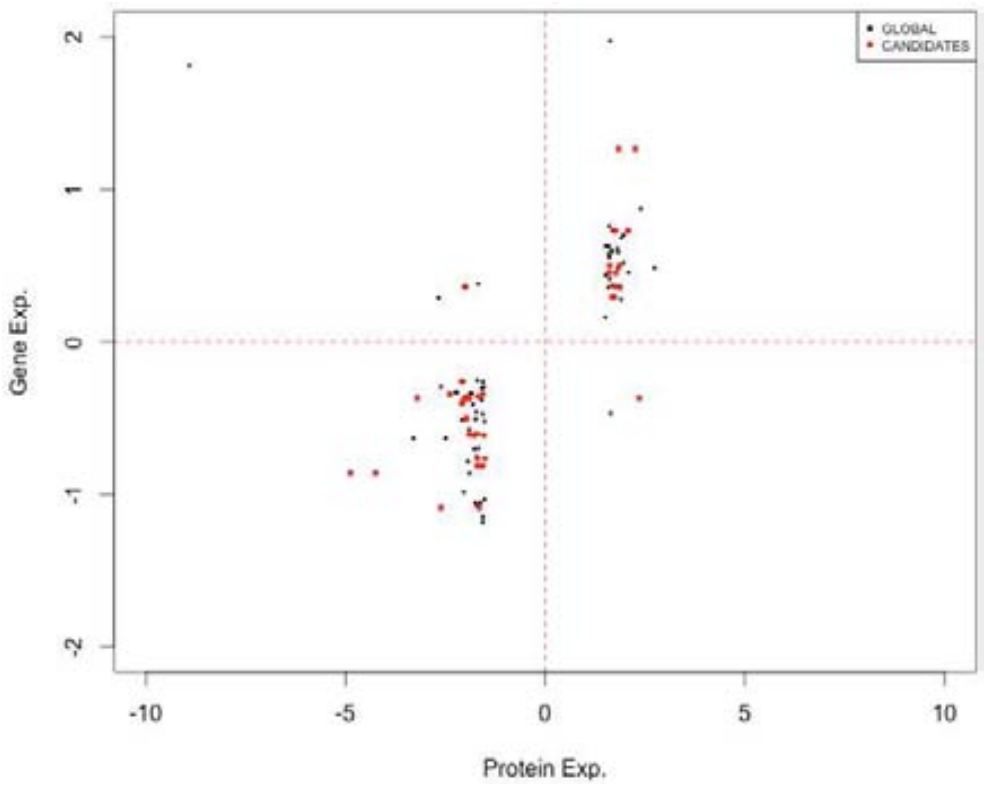

Figure 46. Distribution of genes and proteins differentially expressed depending on their expression levels. Most molecules have well-correlated expression levels. Each dot represents a molecule differentially expressed in the microarray and SILAC experiments. Black dots are proteins found only in one SILAC experiment; red dots represent proteins in the two SILAC experiments.

In order to identify which miRNAs and proteins were associated through their target genes and were differentially expressed in the PC-3-BM compared to the parental PC-3 cell line, an accurate analysis using experimentally verified miRNA target databases was carried out. This analysis generated a ranking of potential miRNA-protein candidates to be further validated (**Table 23**). From this list, proteins differentially expressed could be related to miRNA, also found differentially expressed in the Microfluidic Taqman Array,

and vice versa, a ranking list of differentially expressed miRNAs could be related to their final protein product (**Table 24**).

Table 23. Protein and miRNA candidates found in the integrative analysis

Entrez Id	Symbol	Name	Protein FC	miRNA name	miRNA FC	Source
3688	**ITGB1**	integrin, beta 1	1.77	**miR-183-5p**	-2.53	tarbase6
				miR-192-5p	-4.03	tarbase6
				miRNA_*a*	1.26	tarbase6
				miRNA_*b*	-6.71	mirTarBase
3673	**ITGA2**	integrin, alpha 2	2.26	**miRNA_*c***	-3.62	tarbase6
				miR-30a-5p	-2.05	tarbase6
				miRNA_*d*	-3.72	tarbase6
4082	**MARCKS**	myristoylated alanine-rich protein kinase C substrate	1.85	**miR-122-5p**	-3.47	tarbase6
				miR-155-5p	15.39	tarbase6
				miR-21-5p	-1.70	mirTarBase
	Protein_B		2.39	**miR-7-5p**	-5.75	tarbase6
				miR-155-5p	15.39	tarbase6
				miR-30a-5p	-2.05	tarbase6
				miR-34a-5p	4.85	tarbase6
				miRNA_*a*	1.26	tarbase6

Abbreviations: FC, fold change.
Note: most miRNAs are inversely expressed to their protein expression level and are regulating more than one protein. Some names are coded due to intellectual property reasons.

Table 24. Ranking list of the top ten differentially expressed miRNAs with their protein targets.

miRNA name	Number of target proteins
miRNA_*b*	22
miR-30a-5p	10
miRNA_*d*	10
miR-21-5p	9
miRNA_*a*	9
miR-34a-5p	9
miR-7-5p	8
miR-192-5p	8
let-7b-5p	6
miR-155-5p	5

Note: Some names are coded due to intellectual property reasons.

To summarize, this study shows that intracardiac injection of human PCa cells in immunodeficient mice is a useful experimental model to investigate the pathophysiology of bone metastases *in vivo*. Furthermore, the bone metastases model described in this Chapter developed by intracardiac injection of the PC-3 cell line followed by subsequent selection of a sub-line with increased *in vivo* propensity for bone metastases is a promising alternative to spontaneous metastatic models such as the TRAMP mice, since it offers a high experimental bone-metastatic tumour incidence; it frequently metastasizes to specific bone sites such as the femur and tibia; and it recapitulates the early stages of the metastatic process from tumour cells entering into the blood to disseminate to distant organs.

After three rounds of *in vivo* selection, the PC-3-BM subpopulation of PC-3 showed an increased preference to metastasize to bone, with up to 100% of bone lesions (**Figure 36**) when tumour cells were injected intracardiacally into immunodeficient mice, compared to the 20% incidence of PC-3 cells. The most affected bones were leg, spinal cord and scapula (**Figure 36**).

When located in the bone marrow, prostate tumour cells with the potential to metastasize to bone would come in contact with bone lining cells. Bone lining cells are quiescent or premature osteoblasts and therefore immature hFOB and MC3T3 cells are particularly good models for bone cells and those PCa cells that would come in contact with the bone microenvironment after tumour colonization [351]. In addition to the results observed *in vivo*, the PC-3-BM cells also showed an increase migration and invasiveness capacity when they were grown in co-culture with osteoblast cells compared to parental PC-3 cells. In particular, a even more increase was observed when the co-culture was composed of human PCa (PC-3) and human osteoblast cells (hFOB) instead of murine bone cells (**Figure 37**). The intrinsic species-specificity of human PCa cells for human bone cells is an interesting feature that corroborates the species-tropism observed by Yonou *et al.* [302] and also the findings previously described and discussed in Chapter I.

Moreover, cell proliferation assays demonstrated that PCa cells did not proliferate faster when they were directly co-cultured or when CM from osteoblast cells were added to PCa cells (**Figure 37**-**38**). However, a significant increase in bone cell proliferation was observed when CM from PC-3 or PC-3-BM was added to those cells. In accordance with these results, studies with paired clinical samples [352] in breast, colon and prostate cancer have shown that the metastatic growth rate correlated with the corresponding rates for the primary tumour, i.e., tumour cells in metastatic sites do not proliferate quicker than cells in the primary tumour. The same pattern of cell proliferation has been observed in this model (**Figure 37**-**38**). These data suggest that factors released from bone cells do not stimulate tumour cells to grow faster, but PCa cells may acquire characteristics that facilitate their establishment in the bone marrow microenvironment during the metastatic process. Bidirectional paracrine interactions between PCa cells and osteoblasts may enhance PCa cell survival and proliferation of osteoblasts and are believed to be responsible for the resistance of prostatic metastatic cancer to treatment [353]. However, many factors and molecules released from numerous cells, such as bone stromal components and bone lining cells, enhance tumour growth and survival of metastatic cancer cells within the bone microenvironment [69]. Similarly, primary tumours may condition the bone marrow by means of the production of circulating factors that target cells in the bone microenvironment and thus render it conducive to tumour localization and colonization [69]. Examples include osteopontin (OPN), secreted by tumour cells promoting bone marrow cell recruitment or tumour formation [354], MMP production from osteoclasts supporting PCa skeletal metastasis [355] and PTHrP, produced by various tumours and able to promote bone resorption [78] and to enhance the production of local factors in the bone marrow [69]. All these data suggest that bone cells together with bone marrow stromal cells may attract tumour cells to the bone and may further provide a favourable niche through the modulation of a large number of genes and proteins in the bone microenvironment.

In order to identify bone metastasis-associated biomarkers in PCa, three strategies were carried out by differential expression analysis (**Figure 44**). Firstly, a Taqman Array of microRNA was carried out to detect those miRNAs differentially expressed in the PC-3-

BM cell line compared to the parental one. From this analysis, a final list gathered 20 upregulated miRNAs which may function as oncogenes (**Table 16**), and 68 downregulated miRNAs in the metastatic cell line, acting as tumour suppressors (**Table 17**). In general, multiple miRNAs known as oncomiRs have been shown to have oncogenic properties or act like tumour suppressor genes. An alteration in oncomiR expression is causatively linked to cancer development [356].

Some of the differentially expressed miRNAs found in this study have been previously associated with PCa (miR-21 [277], miR-126* [357], miR-34 cluster [358]), PCa metastases (miRNA_J) or metastases of other types of cancer (miR-582 [359], miR-155 [360], miRNA_G). The miR-200 family is comprised of five miRNAs that are encoded within two clusters. Each cluster encodes a polycistronic gene. One cluster resides on the human chromosome 1 and encodes miR-200b, miR-200a, and miR-429, while the other cluster is located on human chromosome 12, and encodes miR-200c and miR-141 [267, 361]. In this study, some miRNAs from the miR-200 family were found dysregulated in the metastatic cell line. Vrba *et al.* [361] determined that the miR-200 family participates in the maintenance of an epithelial phenotype and loss of its expression can result in epithelial to mesenchymal transition (EMT). Furthermore, the loss of expression of miR-200 family members is linked to an aggressive cancer phenotype in prostate [283, 361] and in breast cancer [362].

In addition to the miRNA differential expression profile, the second study was performed at mRNA level using Human Gene Array to further correlate the miRNA expression profile with the one found at mRNA level. It is known that regulatory effect on miRNAs is mediated by the interaction between miRNAs and their target mRNAs and nearly 30% of gene expression is probably regulated by miRNAs via this interaction (**Figure 47**) [267]. Individual miRNA may regulate around 200 targets by partial base pairing to mRNAs, whereas a particular target is probably modulated by few miRNAs via different number and types of binding sites in the 3' UTR of the targets, suggesting that one miRNA may control numerous biological or pathological signalling pathways by affecting the expression and function of their targets [267].

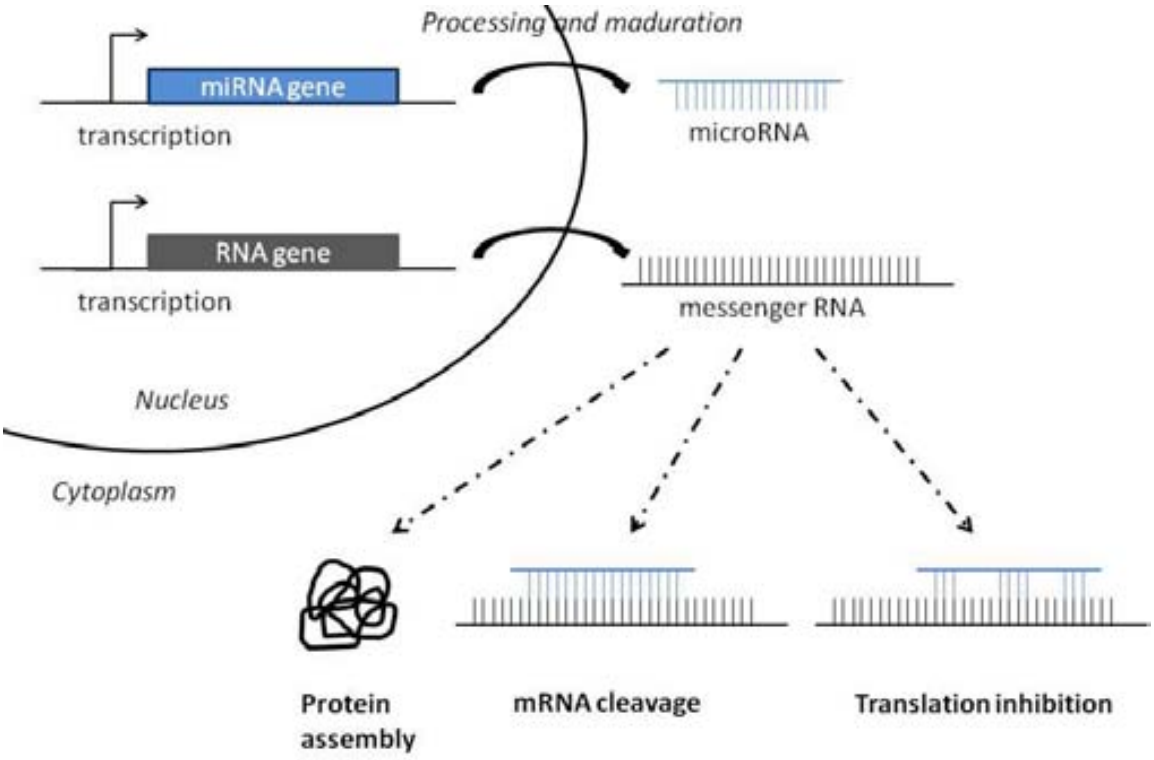

Figure 47. A diagram of genes and microRNA pathways in the cell. Within the nucleus, the initial expression of genes and miRNA genes are transcribed. Both the messenger RNA (mRNA) and the precursor miRNA (pre-miRNA) are exported to the cytoplasm where the pre-miRNA is processed into mature miRNA. Next, the following three events may can occur: (i) the direct protein assembly; (ii) the degradation of the mRNA when the miRNA binds complementarily to the mRNA; (iii) the expression inhibition of the mRNA when a total complementary binding between miRNA and mRNA does not exist and consequently the protein assembly is blocked.

Some of the genes identified by differential mRNA expression were potentially targeted by both up- and downregulated miRNAs. In view of these results, further analysis was restricted to target genes whose dysregulation was potentially solely associated either with upregulated or downregulated miRNAs.

Among the mRNAs upregulated in the most metastatic cell line, some such as the *ADAMTS1* [363] and *Gene_D* have been reported to have a role in PCa metastases. Similarly, mRNAs downregulated in the PC-3-BM cell subpopulation, such as *SERPINB5*, have been found to be a tumor suppressor gene in mammary and prostate [364] tumours, which further confirms the reliability of this analysis.

Finally, the third differential expression analysis was based on proteomics in order to associate the major changes observed in protein abundance between the two cell populations with their differences in bone metastatic *in vivo* potential.

In contrast to RNA analysis, proteomics addresses the relative abundance of the protein product, post-translational modifications, subcellular localization, turnover, interaction with other proteins and functional aspects. Additionally, in terms of gene expression levels, a correlation of less than 0.5 has been determined between mRNA and protein levels, probably due to differences in the rates of degradation of individual mRNAs and proteins and post-translational modifications. Moreover, one gene can have various isoforms of mRNA giving rise to more than one protein. Therefore, in humans could be at least three times more proteins than genes [312]. For this reason, verification of a gene product by proteomic methods is an important step in providing key information about levels of expression, post-translational modifications, protein-protein interactions and intracellular localization of gene products [312]. In PCa research, large-scale proteomic approaches have only been applied to date to the discovery of new biomarkers in body fluids or to characterize changes in the proteome of prostate cells. However, none of them have been applied to PCa metastases.

In the SILAC method, a crossed-labelling condition was performed to increase the analytical stringency and to rule out protein changes observed by chance. A total of 749 different proteins were common to the two SILAC experiments (**Figure 41.A**). A high percentage of these proteins (71%) were found in an abundance ratio of 1 (**Figure 42**). However, after filtering, 14 proteins were found upregulated (**Table 21**) and 17 were downregulated (**Table 22**) in the PC-3-BM metastatic cell line, with a dysregulation at least 1.5 times different from the parental PC-3 cell line (**Figure 41**). This number changed to 9 and 12, respectively, when two additional criteria for a more stringent filter were applied.

Among the most upregulated proteins in the metastatic PCa cell line, some had already been associated with PCa, such as Protein_D, or PCa metastases, such as integrin alpha 2

[365, 366] and beta 1 [367, 368]. A recent study [365] demonstrated that the integrin heterodimer α2β1 protein, a receptor for collagen and other matrix molecules, was elevated in PCa skeletal metastases compared to PCa primary lesions and soft tissue metastases, thus contributing to the selective metastasization to the bone. Hall *et al.* [369] also suggested that its ligation by collagen I activates RhoC guanosine triphosphatase, which mediates PCa invasion, a mechanism for the preferential metastasization of PCa cells within the bone. The activation of integrin β1 with β3 has been seen to contribute to the migration [370] and invasion [371] of PCa cells. Interestingly, Virtakoivu *et al.* [368] defined that down-regulation of AKT1 and AKT2 induced activation of β1 integrins and enhanced adhesion, migration and invasion of PCa cells. Moreover, the dysregulation of the miR-200 family induced integrin activity and cell migration in PC-3 cells [368], corroborating the miRNA and proteomic results described above.

Annexin A1 (ANXA1) has been implicated in metastases of lung [372] and breast [373-376] cancer, but no reports suggest a role for metastases of PCa. ANXA1 promotes metastases formation by enhancing TGFβ/Smad signalling and actin reorganization, which facilitates an EMT-like switch, thereby allowing efficient cell migration and invasion of metastatic breast cancer cells [375, 376], or through specific activation of the NF-κB signalling pathway [377]. Paradoxically, ANXA1 expression, which inversely correlates with the increasing histological grade of PCa [378-381], was found to be upregulated in the SILAC experiment.

Protein_D has exhibited an inverse correlation with the metastatic potential of breast cancer cells and poor prognosis of patients. This protein has been reported to be upregulated in adenocarcinoma of the prostate and is associated with disease progression and adverse patient prognosis. Its expression was found to be higher in prostasomes from cancer cells than those of normal cells.

Other molecules such as Protein_C could be a good candidate to identify potential biomarkers associated with skeletal metastases because it has been described to be

upregulated in hepatocellular carcinomas with invasive behaviour. On the other hand, the myristoylated alanine-rich protein kinase C substrate (MARCKS) has a role in adherens junction formation and tumorigenesis in PC-3 cells [382] and it plays key roles in cell motility and invasion, partly because it is a target of the miR-21 [277]. Also, Protein_B could be a novel marker for PCa metastasis as it has been described as a prognostic and therapeutic target marker in colorectal cancer. Moreover, Protein_B, an oncogenic gene directly regulated by the tumour suppressive miR-1285 showed a significant induction of cell proliferation and invasion in renal cell carcinoma. In this work, a marked down-regulation of the miR-1285 and an increased up-regulation of the Protein_B were found in the metastatic cell line compared to the parental PCa cell line, confirming the results previously published on PCa.

Similarly, downregulated proteins in the PC-3-BM cell subpopulation such as zyxin (ZYX) could behave as a tumour suppressor gene through its direct binding to myopodin, a protein frequently deleted in aggressive PCa [383].

Some proteins related with the EMT process such as integrin alpha 2 (ITA2), integrin beta 1 (ITB1), Protein_B and Protein_A were found to be differentially expressed in the PC-3-BM subpopulation. They were confirmed by Western Blot (**Figure 43**) to be upregulated in PC-3-BM compared to PC-3 cells, except for Protein_A, the expression of which did not change. Preliminary observations of these EMT proteins have shown substantial implication in PCa progression and dissemination. However, further studies are required to determine whether some of these identified molecules have a prognostic significance in PCa patients.

Future aspects:

The validation phase of these and other differentially expressed proteins in high versus low bone metastatic PCa cell capability is ongoing in order to investigate their role as potential candidates for PCa biomarkers.

The identification of specific protein changes served as a starting point for further studies which will advance the understanding of the molecular mechanisms underlying metastases and provide potential prognostic markers for disseminated PCa.

Chapter III

Generation of a bone metastasis mice model for therapeutic approaches.

The selective cyclooxygenase-2 inhibitor suppresses tumour progression in prostate cancer bone metastasis

BACKGROUND

Many studies investigating agents to halt bone metastatic disease progression have found that the inhibition of cyclooxygenase-2 (COX-2) can be an effective therapeutic option [384, 385].

COX-2, one of the two main inducible isoforms of COX [386], is an enzyme that converts arachidonic acid (AA) to prostaglandins (PGD2, PGE2, PGF2α), prostacyclin (PGI2) or thromboxane A2 through tissue-specific isomerases [387]. It is associated with inflammation, carcinogenesis [388] and has also been implicated in cell growth promotion and apoptosis inhibition. More importantly, it has been shown to be over-expressed in PCa [389-393]. The contribution of COX-2 to tumour progression could be partly mediated by the vascular endothelial growth factor (VEGF) [394].

Celecoxib is a selective COX-2 inhibitor commonly used in the management of osteoarthritis that has also shown anti-neoplastic properties [395, 396]. Moreover, clinical assays have also suggested that celecoxib reduces the risk of breast, lung and colon cancer [397-400]. Celecoxib can also exert its anti-cancer effect via COX-2-independent mechanisms, which include interference with Akt (signal transduction), NF-κB (inflammatory mediator of tumorigenesis) and other mediators in cancer development and progression [401, 402]. It seems that celecoxib can inhibit cancer progression at different stages through COX-2-dependent and COX-2-independent actions.

PCa prevention with celecoxib has been proved in many preclinical and clinical studies [403-405]. Also, selective inhibition of COX-2 has specifically suppressed the progression of PCa cell lines [406]. Xenograft models provide an effective system to investigate secondary organ colonization of human cells and remain the model of choice for preclinical studies of human tumour-derived cells [125]. In a preclinical breast cancer bone metastasis model, a high dose of intraperitoneal celecoxib plus minocycline hydrochloride had an inhibitory effect [407].

There are no current preclinical or clinical studies on the effect of oral celecoxib at standard human doses on established bone metastatic disease in PCa.

HYPOTHESIS AND OBJECTIVES

Main hypothesis: celecoxib can be used as a preventive or therapeutic drug in PCa bone metastases thanks to its anticancer properties.

General objectives

The main objectives of this Chapter were 1) to develop PCa bone metastases models in immunodeficient mice through two different strategies to evaluate the efficacy of celecoxib as a preventive or 2) as a suppressive agent for bone metastatic disease.

Specific objectives

1. Development of tumour cell dissemination animal model to evaluate the efficacy of celecoxib as a preventive agent for bone metastatic disease.

PCa cells were introduced into the blood of nude mice through intracardiac inoculation modelling the hematogenous dissemination of cancer cells and enabling the study of metastatic colonization and tumour growth in bone sites.

Specifically, the following steps took place:

1a. Intracardiac inoculation of PC-3-BM luciferase-expressing cells into immunodeficient mice to develop a tumour cell dissemination animal model.

1b. Bone metastases were quantified in order to examine whether celecoxib was suitable to be used as a preventive agent for bone metastatic disease.

2. Development of an established bone metastasis animal model to assess the efficacy of celecoxib as a suppressive agent for bone metastatic disease.

Through intratibial (i.t.) inoculation, bone lesions of PCa cells were formed directly in the bones of immunodeficient mice to assess the efficacy of celecoxib in the development of established bone metastases.

In particular:

2a. Intratibial inoculation of PC-3 luciferase-expressing cells into immunodeficient mice to generate bone lesions directly into the tibias of nude mice.

2b. Tumour burden was quantified weekly in order to assess whether the suppressive drug celecoxib was able to block tumour growth of implanted bone metastasis.

Future objective

Both bone metastases models would be used to investigate the use of other therapeutic approaches or target strategies in the implantation and establishment of PCa bone lesions.

MATERIAL AND METHODS

1 Materials and reagents

Celecoxib for *in vitro* studies was purchased from Sigma (PZ0008, Sigma Aldrich, UK) and was dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma Aldrich, UK) as stock solution. It was then diluted further in PBS solution as assay media for cell culture experiments. The final concentration of DMSO for all experiments was maintained at 0.1%. For *in vivo* studies, celecoxib (Celebrex, Pfizer Pharmaceuticals Group, USA) was added to the AIN-76A semi-purified rodent diet at 15 ppm. The commercially available kit for MTT CellTiter 96 Assay (Promega Biotech Ibérica, Spain) was used.

2 Cell viability assay in monolayer cell cultures

The number of viable cells after treatment was determined using a Neubauer chamber under a light microscope. PC-3 cells (2.5 x 10^5) were plated in triplicate on 100-mm tissue culture dishes in complete medium (10% FBS). The following day, the medium was replaced by medium with celecoxib (25, 35, 50 and 100 µM) or without (DMSO) and allowed to grow for 72 hours. Cell viability was determined by the trypan blue exclusion assay, which was done by mixing 80 µL of cell suspension and 20 µL of 0.4% trypan blue solution for 2 min. Blue cells were counted as dead cells, and the cells that did not absorb dye were counted as live cells. The results are presented as percentages relative to the value determined with solvent-treated control cultures.

3 Cell viability assay in anchorage-independent cell cultures

Human PC-3 cell aggregates were generated using a liquid overlay technique, as previously described [408]. Briefly, 24-well plates were coated with 0.5% agarose (SeaKemVRLE agarose, Lonza, Switzerland) in serum-free medium. Cells were released from the monolayer cultures and resuspended in complete medium. PC-3 cells (100,000 cells/ mL) were deposited in each well and maintained at 37ºC for 48 hours. Celecoxib was added (25, 50 and 100 µM) or not (DMSO) and maintained for 72 hours. Cell death was determined by trypan blue staining of the cell suspension. The results are presented as percentages relative to the value determined with solvent-treated control cultures.

4 Anchorage-dependent clonogenic assay

To determine the involvement of COX-2 in the ability of cancer cells to form colonies, a clonogenic assay was performed. PC-3 cells (200 cells/ mL) were plated in RPMI-1640 medium with FBS in a six-well plate. After treatment for 14 days with celecoxib (10, 25, 50 and 100 µM) or without it (DMSO), cell colonies were rinsed with PBS and stained with 0.05% crystal violet in PBS for 20 minutes after fixation with 4% paraformaldehyde (PFA). Plates were rinsed in water three times and then a 10% acetic acid solution in PBS was added to each well and incubated more than 30 minutes. After swirling and pipetting up and down, 0.2 mL of stain were placed in a 96-well plate and the absorbance was measured at 590 nm using the BioTek ELx800 plate reader (BioTek Instruments Inc., UK). The results are presented as percentages relative to the value determined with solvent-treated control cultures.

5 Immunofluorescence of COX-2

PC-3 cells were placed onto glass coverslips in 12-well plates and incubated for 24 hr. Then, old medium was removed and fresh medium was added with celecoxib (25, 50 and 100 µM) or without (DMSO), and cells were cultured for 48 hours more. Cells were fixed with 4% PFA for 10 min, treated with 50 mM NH_4Cl for 30 min to prevent autofluorescence and permeabilized with 4% Triton X-100 for 10 min. Cells were incubated with primary antibody anti-COX-2 (M19 clone, Santa Cruz Biotechnology Inc., USA) followed by an 647-Alexa Fluor (Molecular Probes, Invitrogen, USA) red-conjugated secondary antibody for 1h at RT in dark. For detection of nuclei, bisbenzimide (Hoescht, Sigma Aldrich, UK) was mixed at 10 µg/ml with the secondary Ab solution. Coverslips were mounted using the Aqua/Poly Mount Medium (Polysciences Europe GmbH, Germany). Fluorescence images were captured using DM-IRBE inverted fluorescence microscope (Leica, Germany) coupled to a TCS-NT argon/krypton confocal laser (Leica). Z-stacks were collected and averaged projected and the fluorescence intensity was quantified with the Leica Confocal Software.

6 Animals and animal maintenance

Congenitally athymic male nude mice (Hsd.Athymic Nude-*Foxn1*^{nu}) were purchased from Harlan (Harlan Laboratories, Italy) at 4 weeks of age and maintained under specific pathogen-free conditions. Animals were kept in same conditions as previously described in Chapter I (Material and Methods).

7 Xenograft by intracardiac inoculation of prostate cancer cell suspension

Five-week old athymic male nude mice were used for the intracardiac (i.c.) inoculation of human PC-3-BM cells. Prior to the inoculation, mice were randomized into 2 groups after the quarantine (n = 10 control group, n = 10 prevention group). The prevention group began the 15 ppm COX inhibitor-supplemented diet seven days before the cell inoculation, while the control group was fed the standard diet without the COX inhibitor. Both groups continued their diets until the end of the experiment. The methodology for the i.c. injection of luciferase-transfected PC-3-BM cells (3 x 10^5) has been previously described in Chapter I (Material and Methods).

8 Xenograft by intratibial implantation of prostate cancer cell suspension

Prior to inoculation, the cells were harvested at near confluence and suspended at a concentration of 2 x 10^5 in 10 µL in a sterile PBS solution. Cells were kept on ice until being used for mouse inoculation (0-2 h). Cell viability was 97% or above at the time of inoculation, as determined by trypan blue staining of the cell suspension. Five-week old nude mice were used for the intratibial (i.t.) inoculation of cancer cells. After one week of quarantine, mice were randomized into 2 groups (n = 15 control group, n = 15 treated group), and 0.01 mL of luciferase-transfected PC-3 cells were inoculated into the right tibia, similar to described by Berlin *et al.* [409], under appropriate anesthetics and analgesics. Briefly, right legs were cleaned with 70% ethanol, and a 26-gauge needle was inserted ~5 mm deep into the diaphysis of the tibia through the knee joint using a drilling motion. When the needle was well inserted into the tibia, the needle was removed, and a 27G insulin syringe (Myjector® 1mL, Terumo Corporation, USA) was inserted. Next, 10 µL of cell suspension was slowly inoculated into the tibia (**Figure 48**).

At day 0, the success of the inoculation was verified by bioluminescence imaging (BLI) of the mice in the IVIS® System.

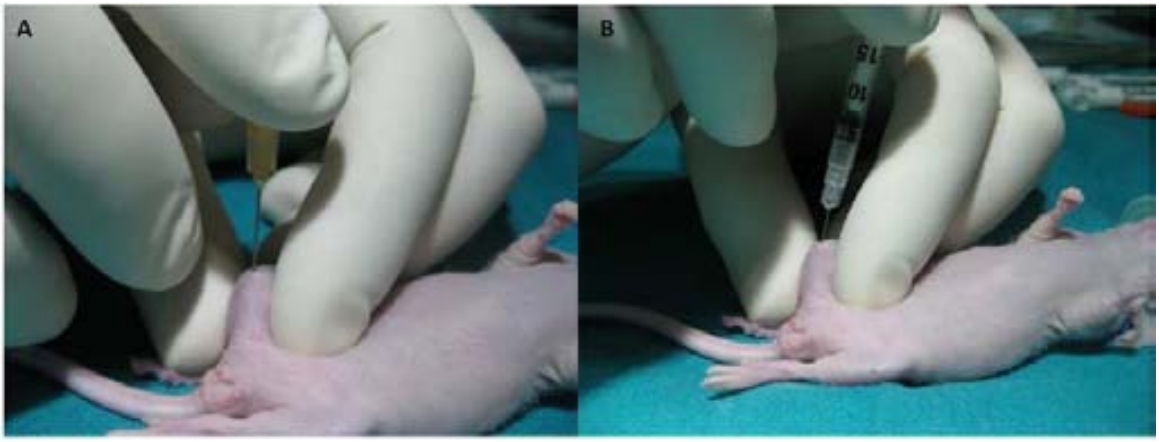

Figure 48. Schematic representation of the intratibial inoculation procedure. Male nude mice were anesthesized with 2% isofluorane before surgery and right legs were cleaned with 70% ethanol. **A.** A 26-gauge needle was used to drill into the diaphysis of the tibia through the knee joint using a drilling motion. **B.** Next, a 27-gauge needle with the tumour cell suspension was inserted and cells were administrated slowly into the medullary space of the tibia.

9 Study design and celecoxib supplementation diet

Treated mice from both experimental groups, metastases prevention and therapeutic, were fed with a special diet formulated by Research Diets, Inc. (New Brunswick, USA) based on the AIN-76A semi-purified rodent diet. Celecoxib (Celebrex, Pfizer Pharmaceuticals Group, USA) was added to the diet prior to pelleting at a final concentration of 15 ppm and stored in a cold room. Mice were fed either the AIN-76A control diet or the COX inhibitor-supplemented AIN-76A diet. Throughout the study, mice were permitted free access to the diet and drinking water. Dietary administration of celecoxib in the prevention study started 7 days before the tumour cell inoculation and continued until the end of the experiment. Mice in the therapeutic study group began to receive the COX inhibitor diet 7 days after the tumour cell tibial injection and continued this diet until the end of the study (**Figure 49**). Body weights were recorded weekly, and animals were monitored daily for their general health. At the end of the study, mice were sacrificed by cervical dislocation after sedation. Bones and intratibial prostate tumours were stored frozen at -80ºC and paraffin-embedded for further biochemical and histological analysis.

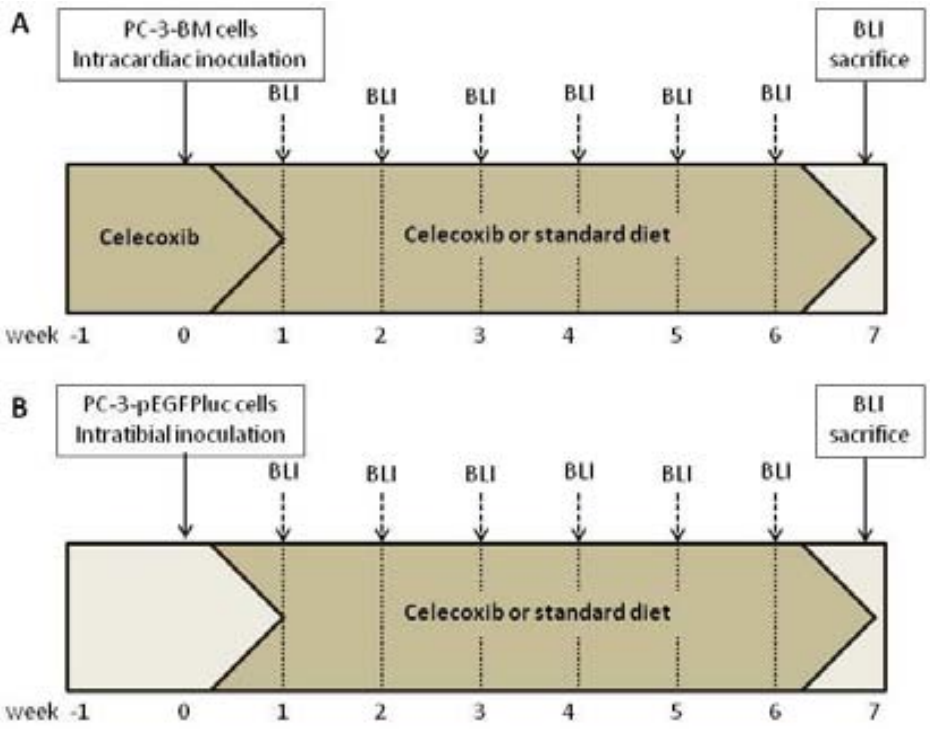

Figure 49. Experimental procedure diagram. Animals were randomized into two independent studies to test the potential effects of celecoxib as a bone metastasis preventive agent (**A**) starting one week before intracardiac cell inoculation or as a therapeutic agent, and (**B**) starting one week after intratibial cell injection. To evaluate the incidence of bone metastasis and quantify the tumour growth inside bone, mice were monitored by BLI in the IVIS System

10 Western blot analysis for the detection of COX-2

Total protein from frozen intratibial prostate tumour tissues from celecoxib-treated and control groups were lysed by the addition of 0.25 mL of lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, 1 mmol/L EDTA, 0.25% DOC (deoxycholic acid) and 2% SDS (pH 7.6), in addition to a mixture of protease inhibitor cocktail (Boehringer Mannheim, Germany). Mixtures were homogenized using a syringe to break up the cell aggregates and then cleared by centrifugation at 12,000xg for 20 min at 4ºC. The supernatant (total lysate) was stored at -80ºC for further analysis. Protein concentration and gel preparation were performed as previously described in Chapter II (Material and Methods). The primary antibodies for the Western blot were anti–COX-2 primary antibody (M19 clone, Santa Cruz Biotechnology) at final concentration of 200 µg/ mL and anti-alpha-tubulin (2125, Cell Signaling Technology Inc., USA). Densitometric analysis of the protein bands was performed with ImageJ software (Wright Cell Imaging Facility, USA) [344].

11 Bone Histology

Mice were sacrificed by cervical dislocation after sedation, and the tumour-induced legs were dissected. Limbs were immediately fixed in 4% formaldehyde for 24 h. Following fixation, bone samples were decalcified by a decalcification solution (Decalcifier II, Leica Microsystems, Spain) and paraffin-embedded for histological and immunohistochemical examination. Hematoxylin and eosin (H&E) staining was performed on paraffin-embedded 4 µm sections to assess tumour development in these limbs, including mitotic count in 10-fields (40x). Histological examination was performed by an experienced pathologist.

12 Immunohistochemical analysis

To determine the effect of celecoxib on the human prostate PC-3 cells injected into the tibia of mice, immunohistochemical (IHC) analysis was performed to detect the tissue level expression of COX-2 and cleaved caspase-3. Paraffin sections of 4 µm were deparaffinized using xylen and rehydrated using descending concentrations of ethanol according to standard protocols. Goat monoclonal antibody for COX-2 (sc-1747, Santa Cruz Biotechnologies Inc., USA) and rabbit monoclonal antibody for cleaved caspase-3 (#9664P, Cell Signaling Technology Inc., USA) were incubated at dilution 1:100 overnight at 4ºC. For primary antibody detection, incubation with secondary biotinylated antibody (LSAB kit, Dako, Denmark) was achieved at room temperature for 30 minutes. The visualization of antigen-antibody reaction was accomplished through incubation with streptavidin-HRP for 15-30 minutes, and further incubation with DAB chromogen substrate (Dako, Denmark) was performed for 5 minutes. All cases were counterstained with hematoxylin.

13 Statistical analysis

All *in vitro* experiments were repeated a minimum of three times. Statistical analyses using Statistical Package for Social Science software (SPSS Inc., V16, USA) were carried out, and results were represented using the GraphPad 5 Prism software. The one-way analysis of variance (ANOVA) and Tukey post-hoc analysis were used for the viability, clonogenicity and proliferation assays, whereas two-sided unpaired Student's *t*-test was

used for the number of mitotic cells and body weights among the different groups. The mean BLI intensity and corresponding standard errors of the mean (SEM) were determined for each experiment. The comparison analysis of mean BLI was done using Student's *t*-test with Welch's correction. Nonlinear regression plots were used to describe the relationship between BLI intensity and time after cell injection and R^2 values were reported to assess the quality of the nonlinear regression model. *p*-values of 0.05 or less were considered significant.

RESULTS AND DISCUSSION

Effect of celecoxib on cell viability and proliferation in monolayer cultures of human PC-3 cells

In this study, the effect of various concentrations of celecoxib on the growth and death of PC-3 cells cultured in monolayer was assessed. In these experiments, PC-3 cells were treated with indicated concentrations of celecoxib (5 to 100 µmol/L) for 48 or 72 hours. The number of viable and dead cells was determined by a trypan blue exclusion assay and MTT CellTiter 96 Assay. The treatment of PC-3 cells with celecoxib inhibited cell growth and caused cell death in a concentration-dependent manner (**Figure 50**).

Treatment of PC-3 cells cultured in monolayer with 50 and 100 µmol/L of celecoxib for 72 h resulted in a decrease in the number of viable cells to 14% and 73%, respectively (**Figure 50.A**), when compared to control cells treated only with DMSO solvent. Statistical analysis using the One-Way ANOVA with a Tukey's multiple comparison test showed that the difference for the number of viable cells between the control and 50-100 µmol/L celecoxib-treated cells was statistically significant ($p < 0.05$ and $p < 0.001$, respectively).

The effects of celecoxib on the growth of PC-3 cells were also studied. In these experiments, PC-3 cells were treated with celecoxib (5 to 50 µmol/L) for 48 hours. At lower doses, treatment with 10 µmol/L celecoxib caused a small 37% ($p < 0.05$) decrease in the number of viable cells (**Figure 50.B**) compared to the solvent-treated control, whereas a higher concentration of celecoxib (50 µmol/L) caused stronger growth inhibition, an 80% ($p < 0.001$) decrease, compared to the control.

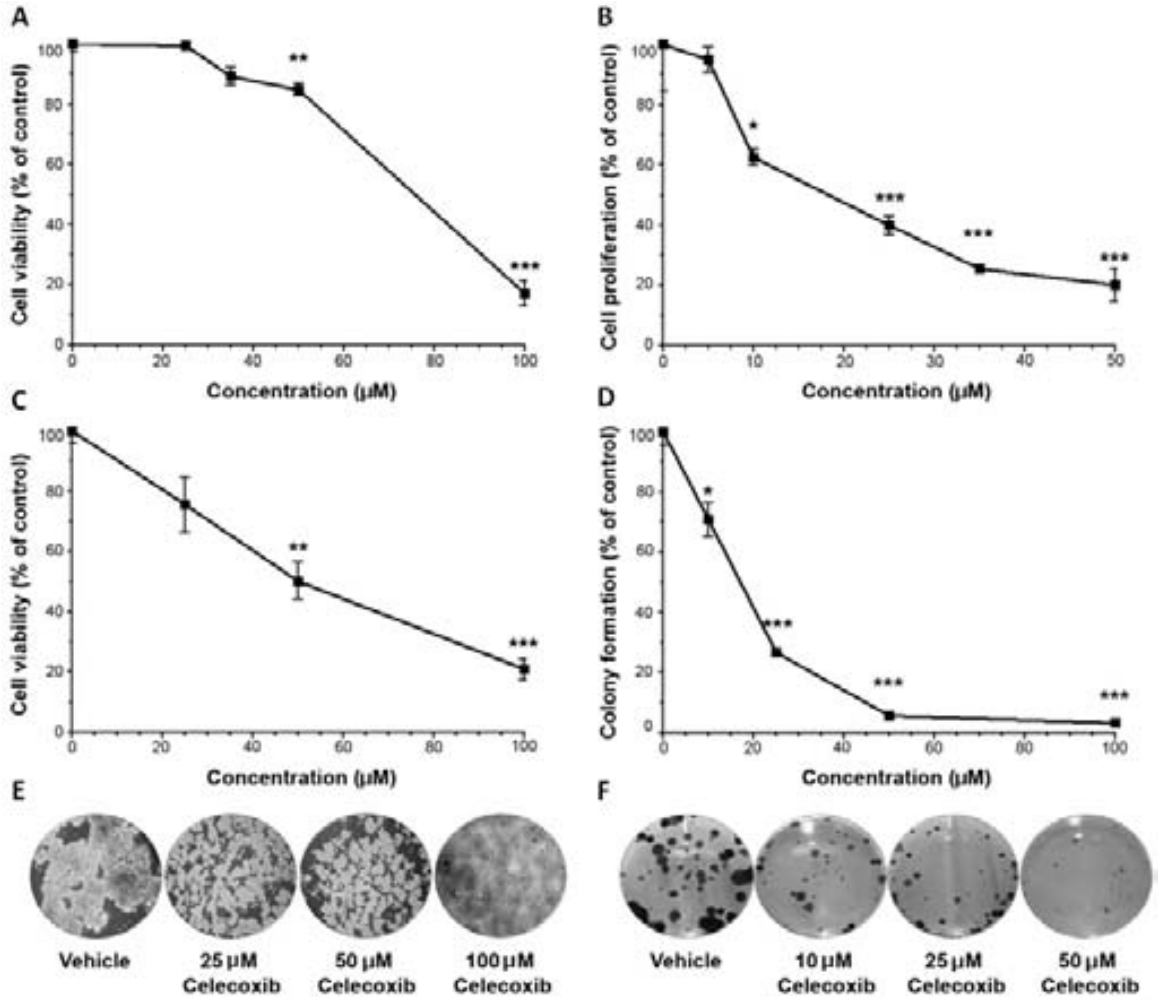

Figure 50. Celecoxib treatment inhibits cell viability and proliferation of human prostate PC-3 cells under anchorage-dependent and independent conditions in a dose-dependent manner. **A.** Celecoxib decreases cell viability under anchorage-dependent conditions. PC-3 cells were treated with medium containing vehicle or 25 to 100 μmol/L celecoxib for 3 days. A trypan blue exclusion assay was performed to evaluate cell viability expressed as a percentage of the differences among the number of viable cells between each treatment group and the solvent-treated control group; **B.** Celecoxib decreases cell proliferation in a dose-dependent manner. PC-3 cells were seeded into a 96-well plate treated with medium containing vehicle or 5 to 50 μmol/L celecoxib for 48 hours. Cell number was assessed by MTT assay; **C.** Celecoxib inhibits cell viability under anchorage-independent conditions. PC-3 cell aggregates were deposited into a 24-well plate and were treated with vehicle or 25 to 100 μmol/L celecoxib for 3 days. Cell death was determined by trypan blue staining; **D.** Celecoxib inhibits colony formation ability in the anchorage-dependent clonogenic assay. PC-3 cells were seeded into a six-well plate at low density. Long-term celecoxib treatment (10 to 100 μM) or vehicle was administered for 14 days before crystal violet staining was performed to evaluate cell colony formation. All results are presented as percentages relative to the value determined with solvent-treated control cultures normalized to 100% for the control. Points, mean of three separate experiments in which each treatment was repeated, bars, and SD (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$); **E.** Representative images of cell viability under anchorage-independent conditions; **F.** Representative images of the anchorage-dependent colony formation assay.

Effect of celecoxib on PC-3 cells under anchorage-independent conditions and on colony formation

To determine whether COX-2 plays a role in tumour cell aggregates viability and in the generation of colonies from tumour initiating cells, the effect of celecoxib on PC-3 cells under anchorage-independent conditions and their ability to form colonies were analyzed.

PC-3 cells were grown as aggregates to mimic the prostate tumour cell dissemination as spheroids and whether COX-2 could affect cell survival in cells grown under anchorage-independent conditions was checked. Treatment with celecoxib (25 to 100 µmol/L) inhibited the growth of PC-3 aggregated cells in a concentration-dependent manner, as measured by cell viability with the trypan blue exclusion (**Figure 50.C**). Celecoxib at a 50 µmol/L concentration caused a 50% ($p < 0.05$) reduction in cell viability compared to solvent-treated cells (**Figure 50.E**).

Next, to determine whether treatment with celecoxib would inhibit the *in vitro* ability of tumour cells to form colonies, in order to test the possible role of COX-2 in the progression of initiating tumours at metastatic sites, a colony-forming assay was performed. PC-3 cells were grown at low cell density (200 cells/ well) in the absence or presence of the indicated concentration of celecoxib (10 to 100 µmol/L) for 14 days followed by crystal violet staining. Similar to results of tumour cell aggregates, long-term celecoxib treatment inhibited anchorage-dependent colony formation in a concentration-dependent manner (**Figure 50.D**). Celecoxib at a 10 µmol/L concentration caused a 29% ($p < 0.05$) decrease in cell colony formation ability compared to solvent-treated cells (**Figure 50.F**).

The results of the *in vitro* colony-forming assay indicate the cancer cells' potential for *in vivo* clonogenic activity [410], and they also could indicate that COX-2 function is important for tumour-aggregated cell growth and for the colony-forming ability of human PC-3 cells.

Effect of celecoxib on the subcellular localization of COX-2 expression in PC-3 cells

PC-3 cells expressed basal levels of cytosolic and nuclear COX-2 protein (**Figure 51**) detected by immunofluorescence, while the celecoxib treatment (25 to 100 µM) for 48 hours showed a higher COX-2 staining in the nuclei fraction compared to control although was not statistically significant. Independent quantification of cytosolic and nuclear fractions was performed in order to study the differential expression and subcellular localization of COX-2 in PC-3 cells after the COX-2 inhibitor treatment. Using the look-up-table with the Spec3 color scale (**Figure 51**, right panels) the fluorescent intensity of COX-2 was more perceptible. Celecoxib at high dose (100 µM) exhibited a marked inhibition of cell viability, as shown in **Figure 50**, therefore, the relative quantification to the COX-2 protein expression was not possible.

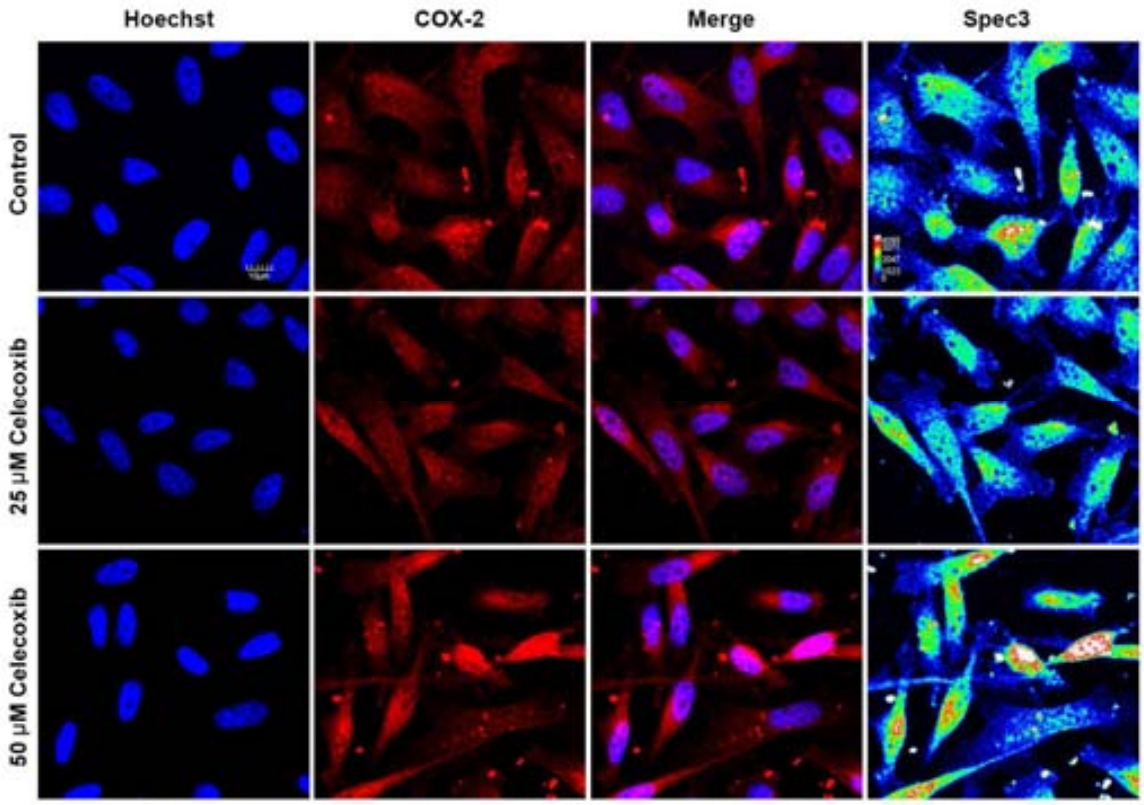

Figure 51. Subcellular localization of COX-2 expressed in PC-3 cells after treatment with celecoxib. Human PC-3 cells were treated with celecoxib (25, 50 and 100 µM) or DMSO for 48 h. Cells were examined by confocal microscopy after immunofluorescence staining with anti-COX-2 (red) and Hoechst (blue) for the nucleus. Overlay of the two images is shown in merge panels. COX-2 fluorescent intensity from cytoplasmatic and nuclear fractions were quantified independently and normalized by the total number of cells. Spec3 panels (right) show the fluorescent intensity using the look-up-table. Scale bar = 10 µm. Note fluorescence staining in 50 µM celecoxib treatment increases the COX-2 expression into the nuclei

Preventive effect of celecoxib on bone metastasis dissemination in athymic nude mice

To validate *in vitro* findings on human PCa cell aggregates simulating tumour cells disseminating from primary tumours to distant organs, the preventive efficacy of the selective COX-2 inhibitor on PCa bone metastasis incidence was assessed.

In this model, PC-3-BM cells were injected into the left ventricles of nude mice. Approximately 98% of the well-injected mice developed bone metastasis. Mice from the celecoxib-treated group (n = 10) were fed the COX inhibitor-supplemented AIN-76A diet (3 mg/kg body weight, oral, daily) seven days before cell inoculation. They maintained the COX inhibitor diet throughout the study, whereas the control mice (n = 10) were only fed the standard diet. All animals were i.c. injected at day 0, and the BLI intensity signal (tumour burden) and number of metastases in the mice were recorded weekly until day 50 post-injection (**Figure 52**), when mice were sacrificed before compromising their welfare.

Consumption of the celecoxib supplemented diet at such a low dose (15 ppm) for 50 days did not cause any differences in the total body weight gain of the celecoxib-treated mice compared to the controls (**Figure 52.B**).

The relative incidence of bone metastases within the two experimental groups was quantified using the IVIS Spectrum System coupled with Xenogen Living Image software, as the mean number of bone metastases per mouse and the mean BLI signal in skeletal sites. After 50 days of celecoxib oral treatment at human standard equivalent dose (15 ppm), the selective COX-2 inhibitor did not show a significant decrease in the establishment of bone metastases for human PCa cells injected i.c. into nude mice (**Figure 52**). However, both the mean number of bone metastases per mouse (**Figure 52.C**) and the mean tumour burden (BLI signal) in bone metastatic sites (**Figure 52.D**) were lower in celecoxib-treated mice compared to controls, though not statistically significant. These results showed that the oral consumption of COX-2 inhibitor at 15 ppm (human equivalent dose) was not enough to significantly inhibit PCa bone metastasis.

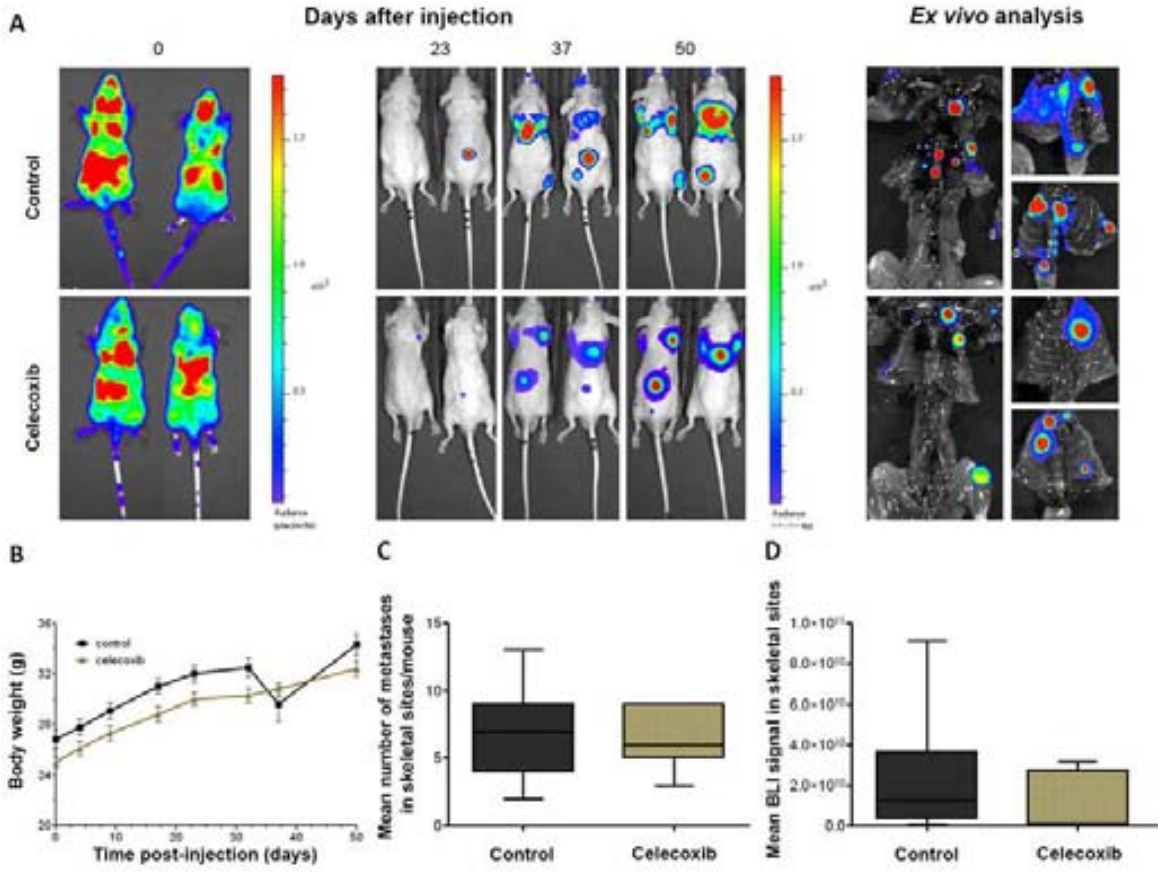

Figure 52. Prevention of PCa bone metastasis with celecoxib. Luciferase-expressing PC-3-BM cells were injected i.c. into nude mice fed on a normal diet (control) or a diet mixed with celecoxib (15 ppm) beginning 7 days before cell inoculation until the end of the experiment on day 50 post-injection. Mice were imaged weekly starting at day 0, and the relative BLI signal of the bodies of the mice was quantified. **a** Representative BLI imaging of nude mice after PCa cell inoculation at day 0 (left panel) until day 50, when mice were sacrificed and *ex vivo* analysis (right panel) performed to detect and quantify bone metastases; **b** From day 0 to day 50, the celecoxib diet had no effect on the weight gain of the prevention group mice compared to the control mice. Points, mean of weight gain, bars, SEM of 10 mice. Before sacrifice, the BLI signal was quantified by measuring the amount of highlighted pixels in the ROIs of each mouse, and total photon flux was quantified using the Living Image Software with the photons/second (ph/s) units; **c** The average number of micrometastases in skeletal sites per mouse at day 50 post-injection was not statistically significant in the celecoxib-treated compared to the control mice; **d** The average BLI signal in skeletal sites per group at day 50 post-injection was slightly lower in the celecoxib-treated mice compared to the controls, though not statistically significant

Celecoxib inhibits tumour progression of PCa cells in bone metastasis

To test the *in vivo* effect of the selective COX-2 inhibitor on the growth established prostate tumours in bone environment, human bioluminescence PCa cells were directly

injected inside the tibia of 5-week old athymic nude mice. Several minutes after cell inoculation, the success of the procedure was verified by imaging with the IVIS® System, where a localized BLI signal was detected and quantified in the hind limbs of the mice (**Figure 53**). A secondary BLI signal could also be detected in the lungs, proving that bioluminescent PC-3 cells properly injected into the bone marrow cavity could reach the lung through hematogenous dissemination as quickly as several minutes post-injection.

One week after cell inoculation, when PCa tumours were well-established in the bone of mice confirmed by the IVIS® System, celecoxib-treated mice began the COX inhibitor-supplemented diet, while the control mice remained on the standard diet throughout the study (**Figure 53.A**). Consumption of the celecoxib supplemented diet (at 15 ppm) for 7 weeks did not show changes in the total body weight gain of the mice from either group (**Figure 53.B**).

BLI signals in the hind limbs were quantified weekly by measuring the amount of highlighted pixels in constant ROIs from day 0 to day 43 post-injection (**Figure 53.C**), the end point of the study. Mice were sacrificed before compromising their welfare, based on the increase of i.t. BLI tumour burden (with two orders of magnitude). Tibias were excised, in order to pathologically confirm that luciferase signals observed by imaging were indeed localized into bones. After 43 days of celecoxib treatment at a human standard dose, there was a significant decrease (167.5 ± 68.9 in difference between ph/s means; $p < 0.05$) in established PC-3 tumours inside the tibias of celecoxib-treated mice compared to the control group (**Figure 54** and **Table 25**).

In a previous pilot experiment, the effect of celecoxib on PCa growth in the bone environment at a higher concentration (1,000 ppm) of celecoxib was investigated. In this study, 5-week old male athymic mice (n = 10) were equally divided into two groups, and luciferase expressing PC-3 cells were i.t. injected as previously described in Materials and Methods. Mice from the control group were fed with the AIN 76A diet, whereas mice from the treated group received the AIN 76A diet supplemented with celecoxib (at 1,000 ppm). Animals in both groups were observed weekly for body weight gain, tumour

progression by BLI, and survival up to 7 weeks. In this study, a strong anti-proliferative effect of celecoxib in the tumour progression of PCa cells inside bones was observed. These results demonstrated that celecoxib had an *in vivo* potential effect on tumour viability and proliferation at high doses. Nonetheless, the aim of the present study was to investigate the efficacy of celecoxib as a suppressive drug on the establishment and progression of PCa bone metastasis at a human equivalent dose.

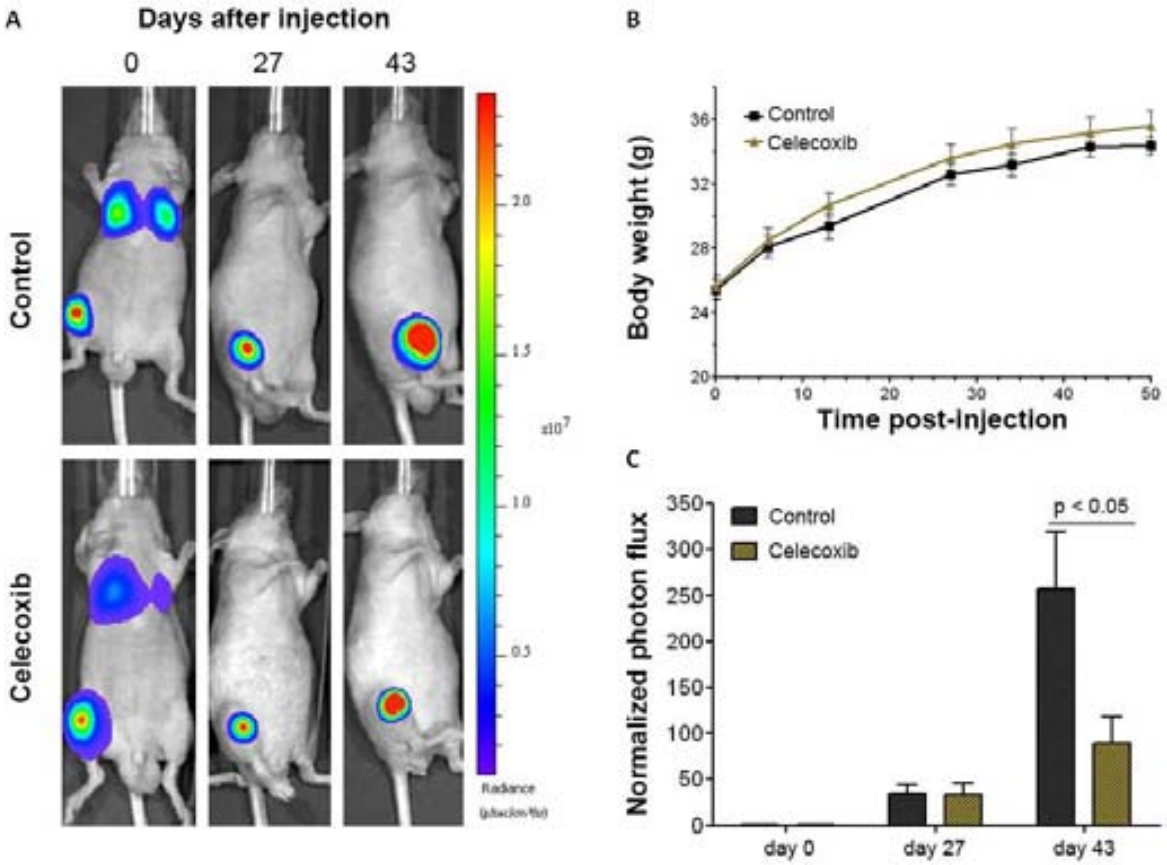

Figure 53. BLI tumour progression of PCa cells i.t. injected into nude mice. To determine the therapeutic effect of celecoxib, luciferase-expressing PC-3 cells were injected i.t. directly into nude mice fed a normal diet (control) or a diet mixed with celecoxib (15 ppm) beginning at day 7 after cell inoculation until the end of the study on day 43. Animals were injected in cohorts of 15 mice. Mice were imaged weekly starting at day 0, and the relative BLI signal (shown as radiance flux in photons per second) in hind limbs was quantified as described in materials and methods. ROIs in all images were kept at a constant area. **A.** Measurement of BLI images from representative control and celecoxib-treated mice over time. Mice receiving celecoxib exhibited a decrease in the tumour growth based on luciferase-expressing PCa cells inside the tibia; **B.** From day 0 to day 43, the celecoxib diet (3 mg/kg body weight, daily) had no effect on the weight gain of the celecoxib-treated mice compared to the control mice. Points, mean of weight gain, bars, SEM of 15 mice. Body weights in all groups were monitored weekly over time; **C.** Histogram of the normalized BLI signal as photon flux from intratibial prostate tumours comparing the treated and control groups over time. The BLI signal at day 0 was set arbitrarily as 1. Columns, BLI signal, bars, SEM

Table 25. Effect of Celecoxib feeding on PC-3 tumour growth inside the tibia of nude mice

Group	Treatment	No. of animals	BLI signal at day 43[a]		
			BLI signal[b]	normalized BLI signal[c]	Difference between means[d]
Control	Fed with standard diet	15	6,7E+08 ± 1,3E+08	256.6 ± 62.6	167.5 ± 68.9[e]
Celecoxib	Fed with 15 ppm of celecoxib in standard diet	15	4,1E+08 ± 7,8E+07	89.2 ± 28.8	

[a] BLI signal detected by IVIS System in intratibially tumors as photon flux (ph/s)
[b] BLI signal detected in intratibially tumors in both treated groups as raw data (ph/s). Mean ± SEM
[c] BLI signal from intratibially tumors normalized to day 0, which was set arbitrarily as 1. Mean ± SEM
[d] Difference between means in celecoxib group compared to control at day 43 post injection. Mean ± SEM
[e] The difference between means is statistically significant (*$p < 0.05$)

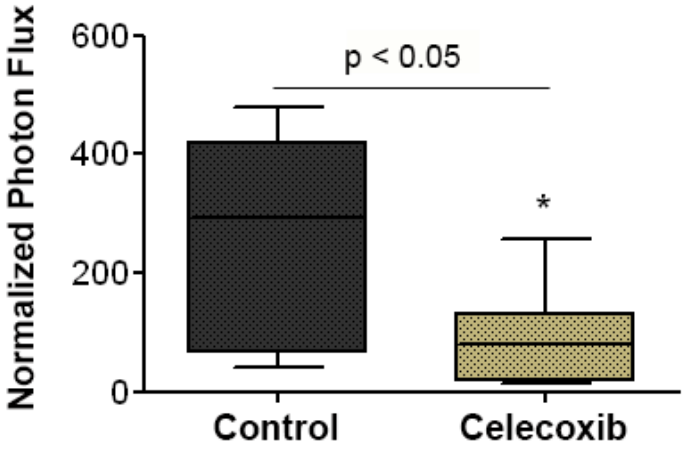

Figure 54. Celecoxib inhibits PC-3 tumour progression in nude mice. Normalized BLI signal of bone metastases in the hind limbs of mice inoculated i.t. with the PC-3.EGFPluc cells. BLI signal intensities from celecoxib-treated and control mice were reported as the average of normalized tumour growth with a significant difference between means of 167.5 ± 68.9 in both groups (*, $p < 0.05$) calculated using Student's t-test with Welch correction.

Celecoxib inhibits the COX-2 expression in PC-3 established tumours

Considering results for the *in vivo* tumour progression inhibition in established tumours in bone niches, whether the growth inhibition detected by BLI correlated to a significant decrease in COX-2 protein expression in these tumours, as a result of the celecoxib treatment by oral consumption, was examined. After sacrifice, intrabone prostate tumours were dissected from both control and celecoxib-treated mice, and the levels of COX-2 were compared by immunoblot detection (**Figure 55.A**). Celecoxib treatment as a therapeutic agent caused a significant COX-2 protein expression decrease in established PC-3 tumours compared to control mice ($p = 0.0021$) (**Figure 55.B**).

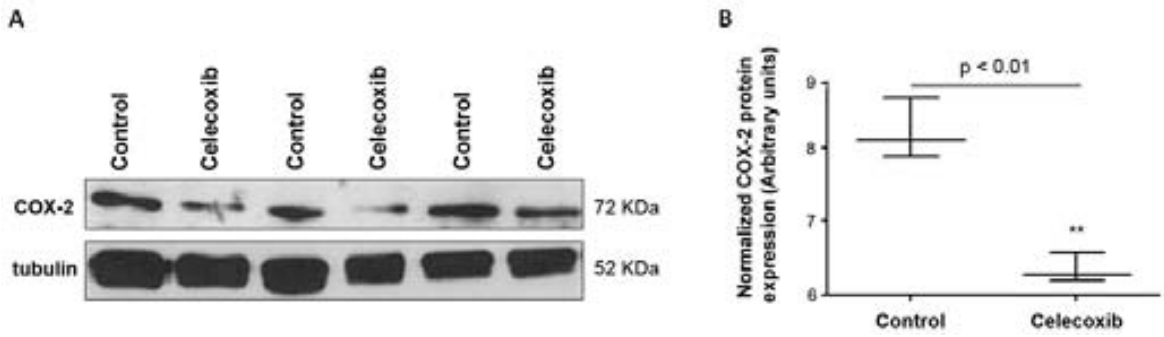

Figure 55. Celecoxib inhibits COX-2 expression in PC-3 i.t. injected tumours. After 43 days of celecoxib treatment, PC-3 tumours growing inside the tibia of nude mice were excised and cell lysates were subjected to western blotting with the COX-2 monoclonal antibody (M19 clone, Santa Cruz Biotechnology Inc.). **A.** The abundance of COX-2 protein band in the region of 72-74 kDa size range decreased significantly in celecoxib-treated mice. The nitrocellulose membrane shown in the upper panel was reprobed with an alpha-tubulin antibody; **B.** Relative quantification of COX-2 protein expression normalized to alpha-tubulin level. Results were expressed as mean ± SEM, $^{**}p < 0.01$

To evaluate the effects of dietary celecoxib on metastatic PCa growth inhibition, histological analysis were performed. Macroscopically tumours (**Figure 56.A**) of nude mice were analyzed and confirmed by *ex vivo* and histological analysis (**Figure 56.A-B**) in order to determine whether tumours were properly injected inside the tibia and consequently, where osteolytic lesions derived from tumour proliferation of PC-3 cells occurred (**Figure 56.C**). Most of the i.t. tumours exhibited an aggressive phenotype with attendant tumour cells invading the surrounding soft tissue (**Figure 56.C**). A global evaluation showed that i.t. prostate tumours from celecoxib-treated mice presented more zones of necrosis compared to those from control mice (around 20-30% and 2-10%, respectively). Moreover, the percentage of mitotic and apoptotic cells were scored (**Figure 56.D-I**) in H&E-stained and IHC sections. A significant increase in the number of mitotic cells ($p < 0.01$, **Figure 56.F**) and concomitant decrease of apoptotic cells ($p < 0.001$, **Figure 56.I**) were found in proliferative zones of control mice compared to those receiving celecoxib. According to the COX-2 immunoblot results (**Figure 55**), the IHC detection of COX-2 showed an inhibition of COX-2 expression in mice receiving the COX inhibitor diet compared to the controls (**Figure 56.J-K**). The IHC quantification presented as percentage of expression between these two groups showed a significant decrease in COX-2 levels in celecoxib-treated mice ($p < 0.001$, **Figure 56.L**) compared to the control group.

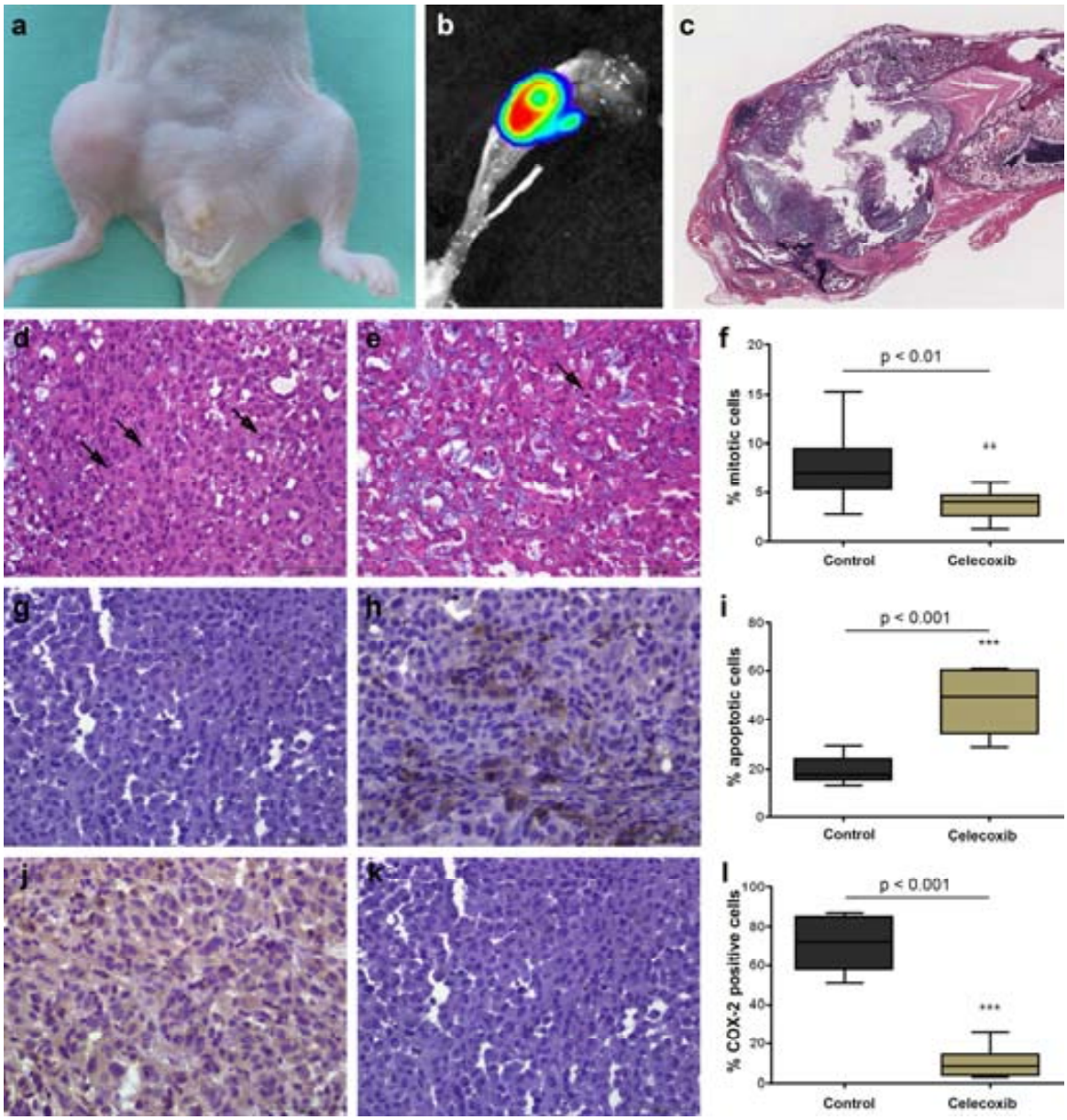

Figure 56. Histologic evaluation of human PC-3 cells i.t. injected into nude mice. **a-b** Representative images of a PC-3 tumour grown i.t. in nude mice at day 43 post-injection (**a**) before sacrifice and (**b**) *ex vivo* BLI image of the tumour visualized with the IVIS® System; **c** Panoramic view showing the PCa tumour growth inside the bone marrow cavity after proper i.t. injection (10x); **d-e** Celecoxib caused an effect on cell viability and proliferation showing differences in scoring mitotic cells in 10 high-power fields (40x) detected by H&E staining in (**d**) control compared to (**e**) celecoxib-treated mice; black arrows indicate examples of mitotic cells; **f** Histrogram represents the percentage of mitotic cells counted in 10 high-power fields (40x) presented as control versus celecoxib (**, $p < 0.01$); **g-h** Lower expression levels of cleaved caspase-3 (positive brown-staining) were found in (**g**) control compared to (**h**) celecoxib-treated mice; **i** Histogram represents percentage of apoptotic cells counted in 10 high-power fields (40x) detected by cleaved caspase-3 (***, $p < 0.001$); **j-k** Higher expression levels of COX-2 (positive brown-staining) were detected in i.t. tumour sections in (**j**) control compared to (**k**) celecoxib-treated mice; **l** Histogram represents percentage of COX-2 positive cells (***, $p < 0.001$).

To correctly evaluate new treatment strategies for bone metastatic disease, appropriate animal models are needed. In this work, two animal models of bone metastasis were analyzed to mimic the crucial steps of the metastatic process, tumour cell establishment and tumour progression. Briefly, for tumour cell-bone seeding, a human PCa cell line was i.c. injected into mice, whereas for tumour progression of pre-established bone metastatic disease, an i.t. injection was chosen. Using these experimental models, both extensively used in the literature, it was possible to investigate the effect of therapeutic agents, such as celecoxib, on the tumour progression of metastatic disease.

Studies have suggested that COX-2 inhibitors may be promising as chemopreventive and therapeutic agents in cancer [388]. The anti-cancer and anti-inflammatory properties of COX-2 inhibitors stem from the blockade of prostaglandins by inhibiting the activity of the rate-limiting enzyme, COX [411]. It is hypothesized that non-steroidal, anti-inflammatory drugs (NSAID), such as celecoxib, sensitize cancer cells to apoptosis by blocking COX (I and II) activity and decreasing prostaglandin levels [412]. In particular, celecoxib was shown to inhibit prostate carcinogenesis in the transgenic adenocarcinoma of a mouse prostate model [388, 413]. *In vitro* and *in vivo* studies with human PCa cells demonstrated the specific role of the selective COX-2 inhibitor in growth inhibition and apoptosis induction enhanced to pro-caspase-6 and -9 expression [414, 415] and its potential effect in combination with atorvastatin [414, 416]. The mechanisms by which celecoxib inhibits the growth of PC-3 prostate tumours and induces apoptosis are not clear, even though Erk1/2 and NF-kβ have been identified as potential targets for the design of chemopreventive agents for the prevention of PCa. It has been shown that the NF-kβ family of transcription factors is constitutively activated in various human malignancies including PCa [417], promoting cell growth and proliferation by regulating the expression of genes, such as *c-myc*, *cyclin D1*, and *IL-6* and inhibiting apoptosis in PCa cells through activation of the expression of anti-apoptotic genes, such as *Bcl-2* [417]. Moreover, RNA interference-mediated COX-2 inhibition resulted in overall cancer cell growth and cell cycle arrest, however, unlike the selective COX-2 inhibitor celecoxib [418], siRNA-mediated COX-2 inhibition was less effective in inducing apoptosis [419].

Results from *in vitro* and *in vivo* assays indicated that celecoxib, at a low dose, had an effect on cell proliferation and the viability of cultured PC-3 cells in monolayer, and even in a more complex system of anchorage-independent conditions. It also decreased PC-3 tumours in bone metastatic niches in nude mice (**Figure 50**, **52** and **53**). However, the objective of this study was not to elucidate the mechanisms of action of celecoxib, which have already been reported.

Treatment of PC-3 cells with celecoxib (50 µmol/L) decreased cell viability and proliferation in monolayer cultured cells to 14% and 80%, respectively (**Figure 50.A-B**), whereas in anchorage-independent conditions, celecoxib (50 µmol/L) caused a 50% reduction in cell viability in PC-3 cells (**Figure 50.C**) and more than 90% reduction of colony forming ability (**Figure 50.D**) in a long-term celecoxib treatment. *In vitro* studies suggest that celecoxib affects growth inhibition and the ability to form colonies in PC-3 cells. Furthermore, all these findings suggest that COX-2 has an important role in cell survival and proliferation but also the selective inhibition of COX-2 mediated by celecoxib resulted in a slight increase in the nuclear subcellular fraction compared to the cytosol.

These results were tested with an initial "physiologic" animal bone metastasis model, based on i.c. injection of PC-3 cells typifying the crucial steps of bone metastasis disease [156]. Celecoxib was selected as a preventive agent based on previous reports that objectified how celecoxib inhibited osteoclast formation in a culture system on human osteoclast precursors at clinical concentrations [420] and how it also inhibited the differentiation of mice bone marrow-derived monocyte/macrophage precursor cells [421]. As there were no reports in the literature using celecoxib to prevent PCa bone metastasis, but there were evidences that COX-2 expression is related to bone metastasis [422], this work studied the potential cancer preventive effect of celecoxib on PCa dissemination to distant organs, such as bones, keeping in mind that the efficacy of any chemopreventive agent against a specific type of cancer primarily depends upon its effect in interrupting the process of carcinogenesis, as well as depending on the

safety, efficacy, availability, acceptability and cost of the proposed chemopreventive agent [423].

In the *in vivo* study, animals were monitored with non-invasive BLI using the IVIS® System that quantified tumour progression over time with a final histological analysis confirming the definitive, local bone metastasis burden. Even though the results showed that there were fewer bone metastases between both prevention and control groups (6.9 ± 1.4, celecoxib; 6.4 ± 0.9, controls; **Figure 52.C-D**), statistical analysis did not show differences in the mean tumour burden after 50 days of treatment with celecoxib. The study suggests that the administration of celecoxib, at a low dose, was not effective enough to prevent the seeding of PCa cells into the host bone of athymic nude mice. However, because no toxicity was observed in the study, it could be possible to increase the dose to improve the efficacy of celecoxib as a preventive agent in PCa bone metastatic disease.

Moreover, the research was focused on evaluating the inhibitory effect of celecoxib, at a low dose, on established bone metastatic disease in athymic nude mice, which had developed experimental bone metastasis through the i.t. injection of PC-3 cells. Although celecoxib showed to inhibit local PCa progression and regression in both subcutaneous PCa tumours and prostatic intraepithelial neoplasia in the transgenic mouse model [388, 424], and it was associated with an induction of apoptosis *in vivo*, the effect of celecoxib on the growth of prostate tumours in bone metastatic disease has not been previously studied. Nevertheless, in other cancer types, such as breast cancer, celecoxib in combination with minocycline hydrochloride has shown an inhibitory effect on osseous metastasis, increasing tumour-cell death [407].

At day 43 after the injection of tumour cells (when the experiment was terminated), all of the celecoxib-treated and control animals had i.t. PCa tumours with osteolytic appearance after histological examination (**Figure 56.C**), as it has been described by others [425, 426]. The oral consumption of celecoxib (3 mg/kg body weight) in the therapeutic group of mice caused a significant decrease (a difference between mean

tumour burden of 167.5 ± 68.9, $p < 0.05$; **Figure 53** and **54**) in PC-3 tumour growth inside the tibias compared to control mice. Subsequently, bone histology revealed that the PC-3 tumours of celecoxib-treated mice had a greater incidence of necrosis, lower number of mitotic cells and higher levels of apoptotic cells (**Figure 56.D-I**) compared to the non-treated mice. At the protein level, COX-2 expression was significantly lower (with 1.87 ± 0.03 of difference between means; $p < 0.01$; **Figure 55**) in PC-3 i.t. tumours after 43 days of celecoxib treatment compared to controls.

These results suggest that celecoxib enhances the dormancy of PC-3 cells that are injected into athymic mice because it could be acting as an anti-proliferative drug decreasing tumour growth, not only through cell viability reducing the number of mitotic or proliferative cells (**Figure 50** and **56**) or maybe inducing senescence [427, 428], but also inducing cell death mediated by different pathways of apoptosis [429-431] or necrosis [432] even though the mechanisms by which celecoxib is acting in tumour cells are not clear.

Clinical trials using celecoxib in PCa patients have shown varied results. One study published that celecoxib caused biological effects on cell proliferation, apoptosis, angiogenesis and hypoxia in PCa tissue [433]. In contrast, 400 mg of celecoxib twice daily for 4 to 6 weeks in men with clinically localized PCa showed that celecoxib had no effect on intermediate biomarkers of prostate carcinogenesis but may alter PCa progression by COX-independent mechanisms [434]. Many clinical studies [435, 436] were carried out to define the cardiovascular risks associated with COXIBs and NSAIDs, but these adverse risks that were dose- and treatment duration-dependent appeared to be compound, more specific to NSAIDs, rather than to COXIBs [437]. Even recently, in the STAMPEDE randomized trial advanced PCa patients who received androgen-deprivation therapy (ADT) plus celecoxib (400 mg twice daily for up to 1 year) were compared to those who received ADT alone. The study concluded that treatment with celecoxib was not sufficiently active [438, 439]. Despite these data, results from this preclinical animal model do not overlap with the clinical results mentioned above, since the STAMPEDE study lacked patient homogeneity, both biologically and clinically, and this could have

hampered the ability to detect a benefit in men, who did not yet have metastasis, or in COX-2-positive patients [440]. Moreover, the population of advanced PCa patients was mixed and varied, and patients were not selected based on their COX-2 over-expression or their risk of metastatic disease. The role of COX-2 inhibition for PCa prevention must still be revealed, and the role of COX-2 inhibition in men with COX-2 over-expressing PCa could be worth investigating in future studies, balancing the potential benefits against the potential cardiovascular risks of these drugs [440].

All data from this work suggest that celecoxib does not prevent or reduce the development of bone metastases, though it does suppress their progression. At human equivalent dose, celecoxib does not interfere with tumour cell seeding in the host bone. It is probable that COX-2 inhibition does not play a major role in the cellular interaction between host stromal cells and prostate tumour cells or that there is the need for other agents that could potentiate the effect. It is also possible that the main effects of celecoxib are the inhibition of cellular proliferation and the stimulation of apoptosis. Neither of these affects tumour-bone seeding. Even though the effects on tumour-bone seeding seem discouraging, celecoxib at a low dose shows an impact in halting the progression of established bone metastatic lesions.

The administration of celecoxib at a standard human dose in preclinical levels seems to arrest the growth potential of human PCa cells when they have metastasized to bone. Thus, celecoxib should be clinically reevaluated as an adjunct to the standard of care for PCa patients with established bone metastatic disease.

Conclusions

1. The direct injection of stable transfected human PC-3 cells inside a human bone graft in immunodeficient mice forms orthotopic tumours in the bone metastatic niche, indicating the usefulness of this model for the pathophysiological studies of human PCa bone metastases.
2. Circulating human PC-3 cells show tissue-tropism for the human bone graft when they are injected intracardiacally in immunodeficient mice. This result indicates that the bone provides a more favourable environment for the growth of PCa cells than other organs or tissues.
3. Circulating human PC-3 cells show a species-tropism for the human bone graft when they are injected intracardiacally in immunodeficient mice. This result suggests that human PCa cells could have a species preference for human tissues as opposed to host mouse tissues.
4. Experimental models using small animals such as mice limit the amount of clinical material (healthy human adult bone) to be implanted. Consequently, the 2D-DIGE proteomic strategy might not be sensitive enough to identify relevant protein candidates of differentially expressed proteins in human bone metastases. These results suggest that the detection of proteins of human bone metastases would be enhanced by increasing the amount of implanted human bone and through the use of other proteomics methods such as iTRAQ.
5. The direct injection of tumour cells into the left ventricle of mice recapitulates the later stages of the metastatic process, from the entry into the bloodstream to the growth in distant sites. For this reason, this model could be the appropriate method for preclinical studies of tumour cell dissemination in advanced stages.
6. The *in vivo* selection process generates a subpopulation of tumour cells with a distinct propensity to metastasize to bone. Three rounds of this *in vivo* selection are sufficient for the enrichment of these cells.

7. Compared to parental PC-3 cells, the selected PC-3 cells with higher *in vivo* preference to metastasize to bone showed an increased migration and invasion capability when they were grown *in vitro* with bone cells.
8. Immediately after the intracardiac injection into the left ventricle, the efficacy of the cell inoculation must be confirmed by a visualization method such as the IVIS® System, to rule out a possible tumour cell injection into the lung or other sites within the thoracic cavity.
9. The differential expression analysis from two populations with distinct metastatic potential derived from the same PCa cell line enables the detection of changes or signatures that may underlie the metastatic capability of those cells.
10. The *in silico* data integration method based on the three "omic" approaches of the discovery phase is a crucial and complex step to describe the bone metastasis signature of PCa.
11. Cell-to-cell interactions and cell contact functions are the main pathways altered in metastases obtained after the GO enrichment analysis, which could explain changes in migration and invasion activities observed in *in vivo* and *in vitro* assays.
12. At microRNA level, miRNA_D, miRNA_E, miRNA_G and miR-21 are differentially expressed in the PC-3-BM cell line compared with parental PC-3 cells. They are also located in the central hub of interactions between miRNAs and proteins.
13. Based on the results from the SILAC experiments, Protein_B, Protein_D, MARCKS and ANXA10 are differentially expressed proteins in the PC-3-BM cell line compared with parental PC-3 cells. These proteins are also highly associated with changes at miRNA and gene level.
14. The above mentioned miRNA and protein molecules, among many others, are promising potential candidates to be further validated in clinical samples for their implication in PCa progression.
15. Direct intratibial cell inoculation is a promising alternative to existing models of bone metastases, as it offers an easier and faster bone metastatic tumour growth. However, it cannot replicate early stages of tumour cell dissemination.
16. The use of the selective COX-2 inhibitor, celecoxib, at the human equivalent dose inhibited tumour progression of PCa cells in the bone metastasis mice model.

However, it did not prevent the development of bone metastases. These results suggest that celecoxib could be reassessed as adjuvant therapy to treat PCa patients with established bone metastatic lesions.

17. These data must be validated in clinical samples to increase the robustness of these results. A high degree of similarity is expected to be found between the cell line model and the clinical specimens.
18. Many of the data generated in this study provide valuable insights into the molecules potentially implicated in the metastatic progression of PCa. Critically, the best candidate molecules could be developed into novel diagnostic and therapeutic tools.

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