UNIVERSIDADE DE LISBOA

Faculdade de Medicina

Tumor infiltrating lymphocytes and PD-1/PD-L1 expression in primary tumors and bone metastases from solid cancers

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Dissertação especialmente elaborada para obtenção do grau de Mestre em Oncobiologia

Especialização em Investigação em Oncobiologia

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A impressão desta dissertação foi aprovada pelo Conselho Científico da

Faculdade de Medicina da Universidade de Lisboa

"No greater opportunity or obligation can fall the lot of a human being than to be a physician. In the care of suffering he needs technical skill, scientific knowledge and human understanding. He who uses these with courage, humility, and wisdom will provide a unique service to his fellow man and will build an enduring edifice of character within himself. The physician should ask of his destiny no more than this and he should be content with no less"

Tinsley R. Harrison

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This work is part of an endless journey. As such, herein is presented another chapter from the same book initiated almost two decades ago, when I first started in medical school. Then as now, this journey would not be possible to accomplish without the necessary drive and strong support. These acknowledgments are entitled to all those people that fuel my ongoing will to keep learning and developing myself.

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Resumo

As metástases ósseas são comuns em doentes com tumores da mama, próstata, pulmão ou rim. Estas metástases representam uma importante causa de morbilidade e de aumento dos custos inerentes aos cuidados de saúde, secundariamente aos eventos esqueléticos que poderão originar, de que são exemplo as fraturas patológicas, lesões neurológicas agudas secundárias a compressão da medula espinhal, hipercalcémia ou a simples necessidade de radioterapia para tratamento sintomático.

O tecido ósseo é um terreno particularmente fértil, sendo um dos locais preferenciais para que metástases possam ocorrer. O processo que regula a ocorrência de metástases ósseas é complexo, requerendo uma ampla rede de interações entre as células tumorais e o microambiente ósseo onde o tumor irá proliferar. No entanto, ainda se desconhece em pormenor o mecanismo responsável pelo tropismo ósseo dos tumores, sendo certo que as características e abundância de vasos sanguíneos existentes na medula óssea desempenham um papel central. Em sentido contrário, a fisiopatologia das metástases ósseas foi amplamente investigada e decifrada. Em última análise, o desenvolvimento de metástases ósseas será responsável pelo desequilíbrio da homeostasia óssea, nomeadamente no que respeita à sua formação e reabsorção, gerando fragilidade e consequente potencial para eventos esqueléticos com importante impacto no prognóstico e qualidade de vida dos doentes.

Simultaneamente, a imunoterapia tem-se revelado como uma poderosa arma terapêutica no tratamento de diferentes neoplasias. Por este motivo, tem-se atribuído uma importância crescente à necessidade de caracterizar imunologicamente os tumores primários e suas metástases. Contudo, persiste um desconhecimento significativo relativamente a este tema, e que é ainda mais proeminente no caso específico das metástases ósseas.

Os princípios básicos que regulam a imunoterapia residem na capacidade que o sistema imunológico detém para reconhecer e destruir células tumorais. No entanto, os tumores apresentam a capacidade única de modificarem as suas moléculas de superfície de modo a não serem detetados pelas células do sistema imunológico. Nesta sequência de eventos, a imunoterapia permitirá recuperar a capacidade do sistema imune reconhecer e atuar contra o tumor. Este processo poderá ser desencadeado utilizando fármacos como os denominados inibidores de *checkpoint* imunitários, nomeadamente inibidores da *programmed cell death protein 1* (PD-1), expressa na superfície das células imunológicas T, e inibidores do *programmed cell death protein ligand 1* (PD-L1), essencialmente expresso pelas células tumorais. Os inibidores de *checkpoint* irão impedir a ativação do PD-1 pelo PD-L1, promovendo uma resposta imunológica anti-tumoral. É neste particular contexto que o estudo do microambiente tumoral, em particular a deteção de células imunológicas intra-tumorais e expressão de PD-1 e PD-L1 se tornam relevantes.

Apesar da intensa investigação que caracteriza esta área do conhecimento, a literatura atual apresenta dados contraditórios, com alguns autores reportando diferenças significativas entre a expressão PD-1 e PD-L1 nos tumores primários e respetivas metástases à distância, enquanto outros relatam valores perfeitamente sobreponíveis. Para além destes factos, o perfil imunológico apresentado pelas metástases à distância parece ser extremamente heterogéneo, com diferentes níveis de expressão de PD-1 e PD-L1 observados entre as diferentes metástases embora, uma vez mais, não seja consensual entre diferentes estudos. É plausível e provável que o microambiente específico de cada foco de metastização promova diferentes estímulos sobre as metástases locais, influenciando o perfil imunológico apresentado. No entanto, todos estes achados apresentam um elevado nível de incerteza que merece ser escrutinado, tendo como objetivo final a melhor compreensão do fenómeno que encerra o perfil imunológico de um determinado tumor primário e sua metástase.

De modo a elucidar as dúvidas existentes no que respeita às variações de perfil imunológico entre o tumor primário e a(s) metástase(s) correspondentes, serão necessários estudos centrados numa determinada neoplasia e locais específicos de metastização à distância. Neste sentido, esta dissertação pretende fazer uma revisão da literatura focada nas evidências existentes para as metástases ósseas; bem como, à luz do conhecimento atual, propor estudos que possam contribuir

para elucidar o seu perfil imunológico e correlação com o respetivo tumor primário.

Palavras-Chave: Metástases ósseas; *tumor infiltrating lymphocytes*; *programmed cell death protein 1; programmed cell death protein ligand 1*; microambiente tumoral; perfil imunológico tumoral

Abstract

Bone is a common site for metastases in patients with cancer, with some tumors such as breast, prostate, lung or renal cell carcinoma frequently originating metastases into this anatomical site. Bone metastases are a major cause for patients' morbidity and increased healthcare costs, since they can originate skeletal-related events as pathologic fractures, acute spinal cord compression, hypercalcemia or the need for radiotherapy.

With immunotherapy as an emerging powerful weapon to optimize the clinical approach to different cancers, greater concern has been raised regarding the characterization of the immune profile of primary tumors and distant metastases. However, there is a striking lack of knowledge enclosing this subject, which is even more prominent for the particular case of bone metastases.

Despite an intense investigation in this field, current literature is full of conflicting findings with some authors reporting significant differential expression of programmed cell death protein 1 (PD-1) and programmed cell death protein ligand 1 (PD-L1) between primary tumors and distant metastases, while others could not find any relevant difference. In addition, among distant metastases, the immune profile seems to be extremely heterogeneous, with different PD-1 and PD-L1 expressions observed for different metastatic sites. Again, these findings are also contradictory depending on the studies.

It is most likely that specific metastatic microenvironments promote different stimuli for local metastatic development, which will influence the immune profile. However, all these findings present a high level of uncertainty that must be scrutinized to better understand the immune profile phenomenon for any given tumor and metastatic site.

Additional studies focusing in particular tumors and specific metastatic sites are lacking. As such, this thesis intends to deeply review this topic; as well to present future study hypothesis, which could help to clarify the immune profile characterization among bone metastases and its correlation with the respective primary tumor.

Key-words: Bone metastasis; tumor infiltrating lymphocytes; programmed cell death protein 1; programmed cell death protein ligand 1; tumor microenvironment; tumor immunoprofiling

List of abbreviations, acronyms and symbols

APC - Antigen presenting cells

BM – Bone metastases

BMP - Bone morphogenic protein

BPs - Bisphosphonates

BTA - Bone-targeted agents

CAR - Chimeric antigen receptor

CCL12 - Chemokine ligand 12

CCL20 – Chemokine ligand 20

CCL22 - Chemokine ligand 22

CNS - Central nervous system

CTGF - Connective tissue growth factor

CTLA-4 - Cytotoxic T-lymphocyte-associated protein 4

CXCL16 - Chemokine ligand type 16

CXCR4 - Chemokine receptor type 4

CXCR6 - Chemokine receptor type 6

CXCR7 - Chemokine receptor type 7

DKK1 - Dickkopf Wnt signaling pathway inhibitor 1

DC – Dendritic cells

DTC - Disseminated tumor cells

ECM - Extracellular matrix

EMT - Epithelial-to-mesenchymal transition

ET-1 - Endothelin 1

FGF - Fibroblast growing factor

GDF-15 - Growth differentiation factor 15

HSC - Hematopoietic stem cell

ICI - Immune checkpoint inhibitor

IGF - Insulin growing factor

IL-6 - Interleukin 6

IL-10 - Interleukin 10

LAG-3 - Lymphocyte-activation gene 3

M-CSF - Macrophage colony-stimulating factor

MCP-1 - Monocyte chemoattractant protein-1

MHC - Major histocompatibility complex

MMP - Matrix metalloproteinase

NSCLC - Non-small cell lung cancer

OPG - Osteoprotegerin

PD-1 - Programmed cell death receptor 1

PD-L1 - Programmed cell death ligand 1

PD-L2 – Programmed cell death ligand 2

PSA - Prostate-specific antigen

PTHrP - Parathyroid hormone-related protein

RANK - Receptor activator of nuclear factor-kappa B

RANKL - Receptor activator of nuclear factor-kappa B ligand

RCC - Renal cell carcinoma

RT - Radiotherapy

SCLC - Small-cell lung cancer

SRE - skeletal-related events

TGFβ - Tumor growth factor β

TIL - Tumor infiltrating lymphocytes

TIM-3 - T-cell immunoglobulin and mucin protein 3

TMA - Tissue microarray

TNBC - Triple-negative breast cancer

TNF - Tumor necrosis factor

TRAP - Tartrate-resistant acid phosphatase

uPA - Urokinase-type plasminogen activator (uPA)

US – United States

VEGF - Vascular endothelial growing factor

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1. Introduction

Bone is a common site for metastases in cancer patients, with prostate, breast, lung, thyroid and renal cell carcinomas (RCC) being the most frequent cancer types originating metastases into the bone [1]. Additionally, the phenomenon of bone metastases (BM) presented a progressive increase over the last decades, since nearly half of 1.4 million people diagnosed with cancer every year in the United States (US) suffer from a type of cancer that frequently metastasizes to bone with. [2]. As such, over 400,000 new patients with BM are estimated to be annually diagnosed in the US alone [3].

Meanwhile, BM are a major cause for patients' morbidity, which includes skeletal-related events (SREs) such as pathologic fractures (Figure 1), spinal cord compression, severe pain and/or impaired mobility, which in turn, will demand additional treatments such as radiation and/or surgery [4]. As a result, BM have a substantial impact on patients' quality of life and contribute to increased healthcare costs [5], representing an important focus for further investigation and improvement, since the treatment for these patients is essentially palliative [6,7]. Moreover, BM decrease tumor-related survival [8-10]. Therefore, there is a need to expand therapeutic opportunities to decrease morbidity and prolong survival of patients with BM.

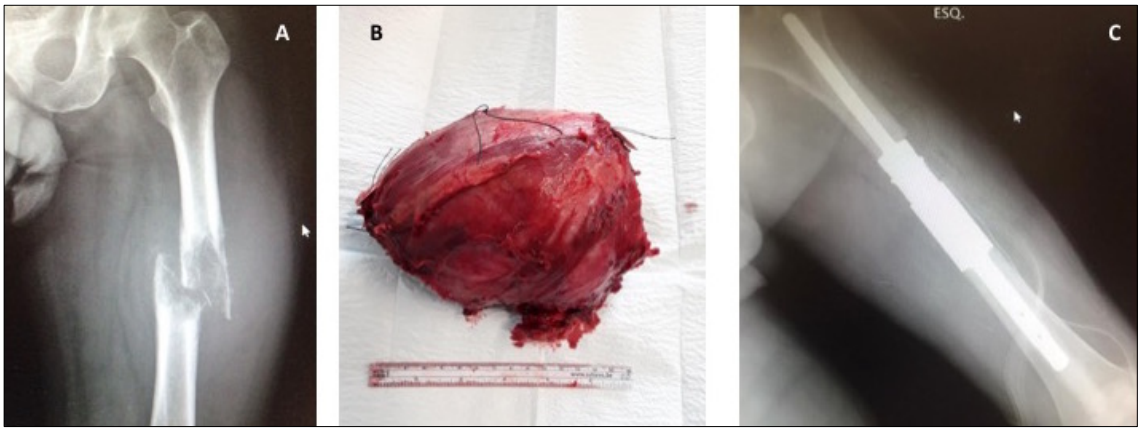

Figure 1 - Pathologic fracture as skeletal-related event. A – Left femur radiograph showing a pathologic fracture subsequent to a bone metastasis from non-small cell lung cancer (NSCLC); B – Photograph of the clinical specimen (resected metastasis); C – Left femur radiograph showing the final reconstruction obtained with an intercalary modular prosthesis.

The advent of immunotherapy opened a new window of opportunities to improve outcome for multiple cancers. Since the seminal work developed by Hodi *et al* [11], we have witnessed an exponential grow in studies evaluating the role for immune checkpoint inhibition when approaching advanced cancers [12,13]. Almost ten years after the first approval of an immune checkpoint inhibitor (ICI) to treat a solid tumor, a wide range of molecules have been developed for different cancers and gained an important role in clinical practice [14,15].

With ICI efficacy linked to tumors infiltration by lymphocytes and expression of programmed cell death receptor 1 (PD-1) and programmed cell death ligand 1 (PD-L1), this raised interest to further investigate these aspects in distant metastases. This excitement is justified by the positive association between PD-L1 expression and the efficacy of ICI among some malignant tumors [16].

However, the immune profile of metastatic disease is still relatively unknown. Additionally, the relation between lymphocyte infiltration, PD-1 and PD-L1 expressions in the primary tumor, when compared with matched metastases is not clear. This phenomenon is particularly true for BM [17-19]. In fact, one of the first studies ever published about this subject was conducted by Callea *et al*, who found no difference between the expression of PD-L1 in primary tumor or metastatic sites in clear cell RCC (ccRCC) [18]. Likewise, Kim *et al* found a concordance in PD-L1 expression between primary and metastatic lung adenocarcinoma [17]. On contrary, Giraldo *et al* reported differential expression of PD-L1 between the primary tumor and metastases in RCC [20]; and Manson *et al* reported an important discordance between PD-1 and PD-L1 expression when comparing primary breast tumors and correspondent distant metastases [18].

All these studies reported a wide range of inter tumoral and inter metastatic site differences, and included metastases from several distant anatomical sites (brain, lung, liver, gastrointestinal or bone) to compare with primary tumors, not focusing in any particular metastatic site. As such, further investigation to better understand the different metastatic microenvironments is still lacking. Stemming from this need, this dissertation aims to compile a state-of-the-art review regarding tumor-infiltrating lymphocytes (TILs), PD1 and PD-L1 expression in solid cancers, and in the particular setting of bone metastases. The main goal is to contribute to understand better the bone metastatic microenvironment - and

its immune profile - which may represent a leap forward to allow better clinical and therapeutic approaches, contributing to diminish SREs and improve patients' quality of life.

2. Bone physiology

The bone is an extremely dynamic living tissue with structural, protective and movement functions, also characterized for being a reservoir for minerals and energy [21]. Bone has two major components: the bone matrix, which is comprised of inorganic salts; and an organic matrix (with cellular and non-cellular components) [21]. Additionally, the bone houses the bone marrow, which is the main site for postnatal hematopoiesis. Within the bone there is a rich population of resident cells (cellular component of the organic bone matrix), being the most well known the bone-remodeling osteoblasts and osteoclasts (which maintain structural integrity and bone health), and the osteocytes that regulate the bone remodeling process [21].

The basic bone multicellular unit is represented by osteoblasts, osteoclasts, bone lining cells, and osteocytes, and is an important anatomical structure that contributes to bone homeostasis [22,23]. However, bone microenvironment also contains adipocytes, fibroblasts, reticulocytes, chondrocytes, endothelial cells, pericytes, nerve cells, and immune cells, as well as hematopoietic and

mesenchymal stem cells [22,23]. All these cells will play a role in bone homeostasis, which is often not clear or fully understood.

Osteocytes modulate bone turnover primarily through the regulation of osteoblasts and osteoclasts. In conditions favorable for bone resorption, osteocytes inhibit osteoblast differentiation and function, through secretion of factors such as the Wnt signaling antagonists sclerostin and Dickkopf Wnt signaling pathway inhibitor 1 (DKK1) [24,25]. In these conditions, osteocytes will also promote bone resorption through macrophage colony-stimulating factor (M-CSF) and monocyte chemoattractant protein 1 (MCP-1), which will recruit osteoclast precursors, and by producing receptor activator of nuclear factor-kappa B ligand (RANKL), the key osteoclast activator [26,27]. In addition to regulating osteoblasts and osteoclasts, osteocytes themselves can also remodel bone surrounding the perilacunar space, expressing many of the proteins used by actively resorbing osteoclasts like tartrate-resistant acid phosphatase (TRAP), cathepsin K, and matrix metalloproteinases (MMPs) [28].

Bone osteoclasts are large multinucleated cells that result from the fusion of bone marrow–derived monocytes/macrophages. Osteoclast differentiation requires RANKL and M-CSF, which are primarily produced by osteoblasts and osteocytes, but also by immune cells [29]. Upon activation, mature osteoclasts form an actin ring that tightly adheres to the bone surface and secrete acid, collagenases, and other proteases that demineralize the bone matrix and degrade proteins [29].

Osteoblasts in the other hand are cells derived from skeletal bone marrow stromal cells, and will play the opposite role of osteoclasts, secreting collagen, which will mineralize into new bone. As above mentioned, osteoblasts secrete numerous factors, including RANKL, which will affect the process of osteoclast activation and differentiation [29]. Also, osteoblasts are responsible for the production and secretion of osteoprotegerin (OPG), that will block the activity of RANKL, interleukin 6 (IL-6), connective tissue growth factor (CTGF) and tumor growth factor β (TGFβ), which will play a key role in cell proliferation within bone microenvironment [30-32].

Normal bone physiology and homeostasis implies a constant communication between resident bone cells. However, the arrival of cancer cells into bone microenvironment disrupts this communication. In the following sections, we will approach the pathophysiology of BM and how the invasion by tumoral cells within bone microenvironment can generate osteolytic, osteoblastic or mixed lesions.

3. Pathophysiology of bone metastases

Tumor metastases represent a complex process, which requires a wide range of interactions between tumor cells and the microenvironment where the tumor will grow [33]. Nonetheless, tissues are not usually friendly for metastases [34,35]. In fact, the metastatic process is often inefficient, since despite the

presence of tumor cells in the bone marrow, in many cases a clinical detectable metastatic disease will not develop [34,35].

The bone is one of the preferential niches for tumor metastases to occur and this phenomenon is probably due to the particularities presented within bone microenvironment, which is an attractive soil for cancer cells [33,36]. The preferential colonization of tumor cells to bone partly relies on the fenestrated capillaries within the bone, the bone matrix, and cells in the bone marrow such as osteoblasts, osteoclasts or osteocytes [37-39]. The reasons behind this particular attraction of cancer cells to bone are still not fully understood, however, we can find a molecular component, as we can see in breast cancer cells with bone tropism genomic signature [40,41]. Also, the hematopoietic stem cell (HSC) niche is an appealing site for cancer cells, since they induce HSC mobilization to occupy that niche, and due to the activation of myeloid cells which will ensure an immunosuppressive action within bone microenvironment, to favor metastases development [42,43].

The process of BM begins with the migration of tumor cells from the circulating blood through the blood vessel wall to the extracellular space of bone [44]. During this process, the epithelial-to-mesenchymal transition (EMT) has a crucial role. EMT includes a series of orchestrated events in which cell-cell and cell-extracellular matrix (ECM) interactions are modified allowing the release of epithelial cancer cells from the surrounding tissue, the cytoskeleton is reorganized to allow movement in three dimensions, and a new transcriptional

program is induced to maintain the mesenchymal phenotype [44]. In such way, epithelial cells, which characterize carcinomas, acquire mesenchymal features with loss of cell-cell adhesion, high mobility, invasiveness, and high resistance to apoptosis, leaving the primary tumor and travelling through the bloodstream until the final metastatic site [45]. Once in the bone marrow, disseminated tumor cells (DTC) will benefit from a high vascularization, which will promote survival and proliferation of tumor cells. On the other hand, the yellow marrow of the bone will also contribute to tumor cells growth [46]. Similarly to immune cells, cancer cells have the particular ability to detect chemokine gradients towards the bone marrow. The overexpression of chemokine receptor type 4 (CXCR4) and chemokine receptor type 7 (CXCR7) in breast and prostate cancer seems to increase their ability to exit the vascular compartment and colonize the bone [40,47]. Moreover, CXCR4 stimulates the chemokine ligand 20 (CCL20) production, which will also promote tumor growth and invasion [48,49]. In patients with RCC, CCL20 levels where higher for those with BM [50]. Osteocytes have the ability to express chemokine ligand 16 (CXCL16), promoting the migration of prostate cancer cells, where CXCR6 (the receptor for CXCL16) is highly expressed [51,52]. In similar fashion, differentiation osteoclasts release chemokine ligand 22 (CCL22), which binds with CXCR4 (expressed in breast cancer cells), and osteoblasts express chemokine ligand 12 (CCL12), that stimulates tropism for cells where CXCR7 is expressed, such as in breast cancer [53,54].

Blood vessels on the bone marrow have the feature of being fenestrated, lacking the usual supporting structure of capillaries, which facilitates extravasation through the vessel wall [44]. Extravasation is highly dependent on superficial adhesion molecules such as E- and N-cadherins, with cancers expressing E-cadherin producing more BM compared with other metastatic foci [55,56]. The interaction between the neoplastic cells and bone extracellular matrix will occur mainly through integrins, which will bind to collagen type I, sialoprotein, vitronectin and osteopontin [57,58]. After being established in the bone metastatic niche, tumor cells can evolve and produce a symptomatic and clinical detectable disease, or instead, remain dormant for years, depending on the surrounding microenvironment [59,60].

Also, decades of studies allowed to identify an extremely rich pre-metastatic niche in bone, which refers to a strong supportive environment to facilitate the invasion, location, survival, and proliferation of metastatic tumor cells [61,62]. The scientific evidence shows that the pre-metastatic niche can be developed even before tumor dissemination to bone occur, and will be supported by tumor-derived factors released from the primary tumors [63]. Additionally, the formation of a pre-metastatic niche relies on a suppressive immune system, since primary tumors recruit myeloid cells, which in turn will allow tumor cells to evade immune surveillance, leading to metastasis [64,65].

Despite little is known about the interaction between immune system and quiescent disseminated tumor cells (DTCs), there are some clues about how

DTCs overcome immunesurveillance and eventually develop into clinically relevant metastases [66]. It has been reported a downregulation of major histocompatibility complex (MHC) class I on DTCs, inhibiting CD8+ T cell recognition; the recruitment of immunosuppressive regulatory T cells (Treg); quiescence mediated by protection from oxidative stress and immune killing [67,68]; suppressed immune responses through expression of checkpoint ligands and secretion of immunosuppressive cytokines; and evidence of a low number and dysfunctional tumor specific T cells [69].

The development of BM will unbalance bone formation and resorption, originating mainly two major categories of BM: osteolytic or osteoblastic (Figure 2) [70]. Whereas prostate cancer shows preferential osteoblastic BM, lung cancer or RCC usually produce osteolytic lesions [70].

Nonetheless, we should also note the growing evidence regarding coexistence of osteolytic and osteoblastic metastases, leading to mixed-type metastases [70]. The characteristics of a given BM is of clinical importance, since they will imply a different risk for SREs, different therapeutic strategies, and distinct impact in patients' quality of life.

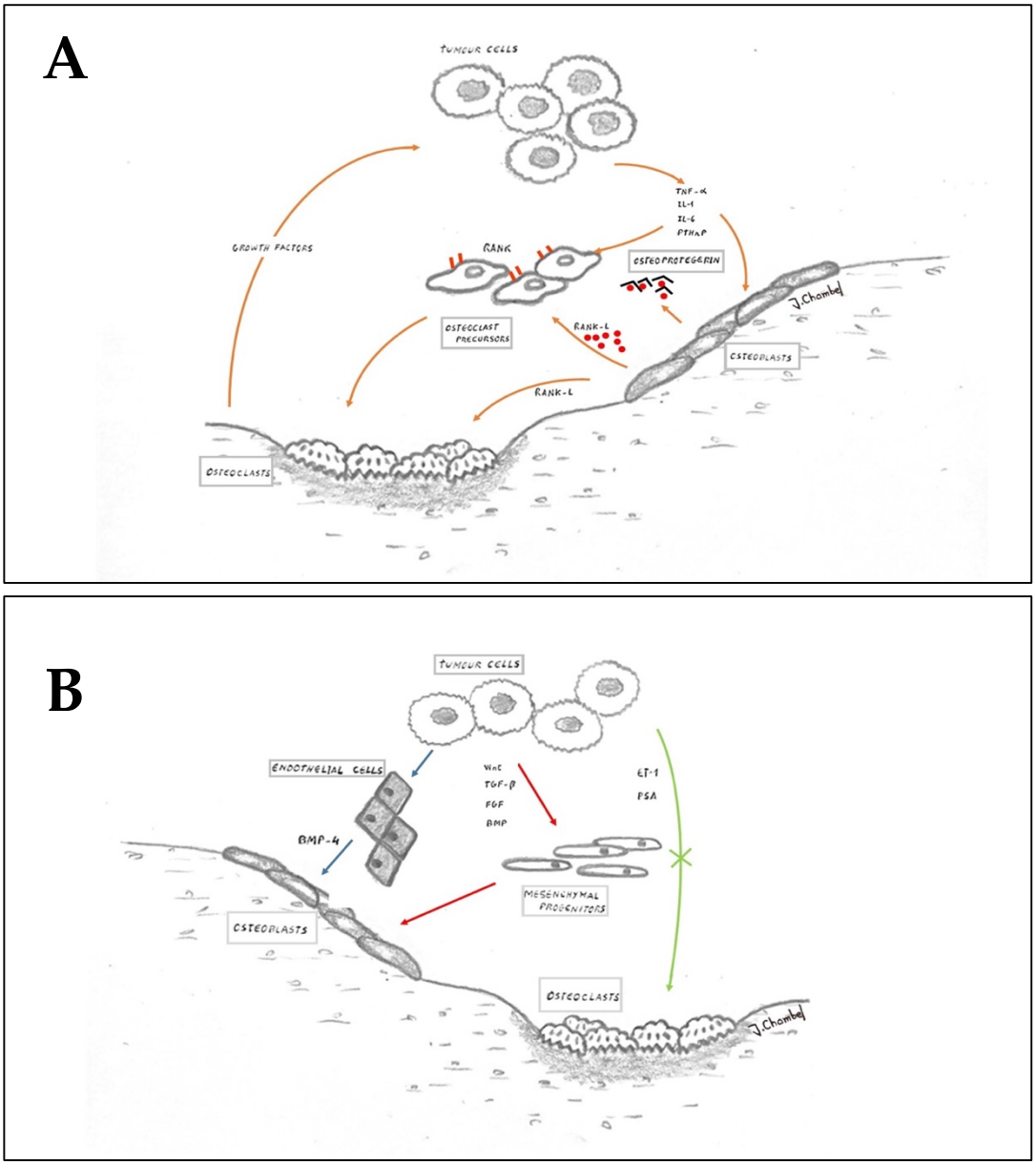

Figure 2 - Basic underlying pathogenesis for osteolytic (A) and osteoblastic bone metastases (B).

3.1 Osteolytic bone metastases

Several regulatory molecules drive osteoclast activation; however, M-CSF and RANKL are particularly important in this process. RANKL, produced by osteoblasts, binds to receptor activator of nuclear factor-kappa B (RANK) on the osteoclast precursor surface, which stimulates downstream signaling molecules

and promotes the maturation of osteoclast precursors into functional osteoclasts [71,72].

RANK is a surface receptor of the tumor necrosis factor (TNF) family, which plays a crucial role in osteoclast differentiation, activation, and function [73]. Also, and despite RANK being primarily expressed in osteoclasts (and their progenitors), it also seems to participate in tumor metastatic process [74,75]. At least for breast cancer, RANKL seems to exert a pro migratory effect on tumor cells promoting their metastases into bone. Additionally, breast cancer cells produce parathyroid hormone-related protein (PTHrP), which in turn stimulates RANKL production in osteoblasts, generating an osteoclast activation promoting osteolytic cancer metastases [76-79].

The RANKL/RANK axis is also influenced by osteoprostegerin (OPG), a member of the tumor necrosis factor receptor superfamily, which is also secreted by osteoblasts and bone marrow stromal cells. OPG has the ability to bind to RANKL, blocking the interaction with RANK [80]. Additionally, many other players such as TNF-α, ILs or calcium-sensing receptors and their correspondent signaling pathways, will also be involved in the complex cross-talk which will originate osteolytic BM [33,70].

3.2 Osteoblastic bone metastasis

Although an important effort has been made to better understand the nature of osteoblastic BM, still little is known about this condition. Nonetheless, it seems that this type of BM is associated with tumor production of osteoblastic factors, which stimulates osteoblast proliferation and differentiation. Endothelin 1 (ET-1), growth differentiation factor 15 (GDF15) and bone morphogenic proteins (BMPs) are the main players in action [81,82]. However, other molecules, such as fibroblast growing factors (FGFs), urokinase-type plasminogen activator (uPA), prostate-specific antigen (PSA), insulin growing factors (IGFs) and the vascular endothelial growing factor (VEGF) are involved in this complex and largely unknown process [83-86].

Regarding osteoblastic BM, prostate cancer cells presents a unique ability to stimulate abnormal new bone formation. The Wnt signaling pathway and fibroblast growing factor receptor activated by ET-1 have been shown to have a crucial role in this process (Figure 2) [87]. Further investigation in this field will be necessary to fully understand the complexity of the osteoblastic bone metastasis formation.

4. Principles of Immunotherapy

The basic principle behind the use of immunotherapy to fight cancer relies in the fact that immune system has the ability to recognize and kill tumoral cells. In fact,

lymphocytes have the ability to infiltrate tumors, with activated T-cells entering the bloodstream and afterwards migrate across the endothelial barrier into the tumor bed [88]. This phenomenon takes place due to the presence of tumor-specific neoantigens such as mutated proteins that are released by tumor cells. These tumor-specific antigens are captured by antigen presenting cells (APCs), such as dendritic cells (DC), for processing and presentation generating lymphocyte activation and action [89]. These are the principles for the immunosurveillance of cancer, and also the reason why there is a proliferation of cancer cells in the absence of an immune system [90]. However, immunosurveillance will generate pressure into tumoral cells, which in turn will promote immunoediting and potential tumoral escape [91]. The phenomenon of tumoral evasion from the immune system is already known to be based on different events. First of all, a down-regulation of the expression of MHC class I in APCs is promoted, which is a fundamental key-step to expose tumoral antigens to the immune cells; secondly, an overexpression of inhibitory molecules (cytokines, oxygen reactive species and surface ligands) will occur, being PD-L1, programmed-cell death ligand 2 (PD-L2), TGFβ, interleukin 10 (IL-10), hydrogen peroxide or nitric oxide the most prominent; finally, and with time, tumoral immune infiltrate will also change, with accumulation of suppressor cells which will impair killer lymphocytes [92]. These are the main obstacles to cancer immune response, which have been exploited by immunotherapy.

The general idea behind any immunotherapy is to target the anti-tumor

responses, either through passive or active strategies [93,94]. Different options have been attempted in order to stimulate an immune response in cancer patients: vaccines based on tumor-specific antigens; cytokines to enhance lymphocyte activation and induce MHC expression; CAR (chimeric antigen receptor) T-cells, which are based on T-cells removed from a patient and transfected with a construct encoding a chimeric antigen receptor that are reinfused to mediate tumor killing T-cells; and activation or blockade of immune checkpoints, using highly-specific monoclonal antibodies that can enhance the lymphocyte activation [93,94]. Among all the attempts, the most relevant and promising was obtained with the ICI.

T-cell activity is regulated by immune checkpoints that limit autoimmunity, inhibitory and stimulatory interactions that have the ability to mediate T-cell immune responses [95]. The most actively studied immune checkpoint pathways are the ones involving CTLA-4, PD-1, lymphocyte-activation gene 3 (LAG-3), and T-cell immunoglobulin and mucin protein 3 (TIM-3), which regulate T-cell activity at different stages of the immune response [95].

Immune checkpoints function through receptor-ligand interactions, where the receptor is expressed on the T-cell and the ligand on antigen-presenting cells or peripheral tissues. These checkpoints are potential targets that tumors may exploit to evade the immune response [95]. In fact, PD-1 on T-cells seems to be inactivated by PD-L1 and PD-L2 produced by tumor cells (but also immune cells)

and, as such, blocking the PD-1/PD-L1 axis could prevent the inhibition of the immune response, achieving clinically relevant anti-tumoral responses (Figure 3) [2,3]. Nonetheless, the first major breakthrough with ICI was observed with the blockage of CTLA-4 [1].Since the clinical confirmation for the efficacy provided by ICI, measuring the expression of molecules like PD-1/PD-L1 or evaluating TILs is a much more common practice, in particular for those tumors with a high mutational burden and where ICI represent another clinical valid therapeutic option [96].

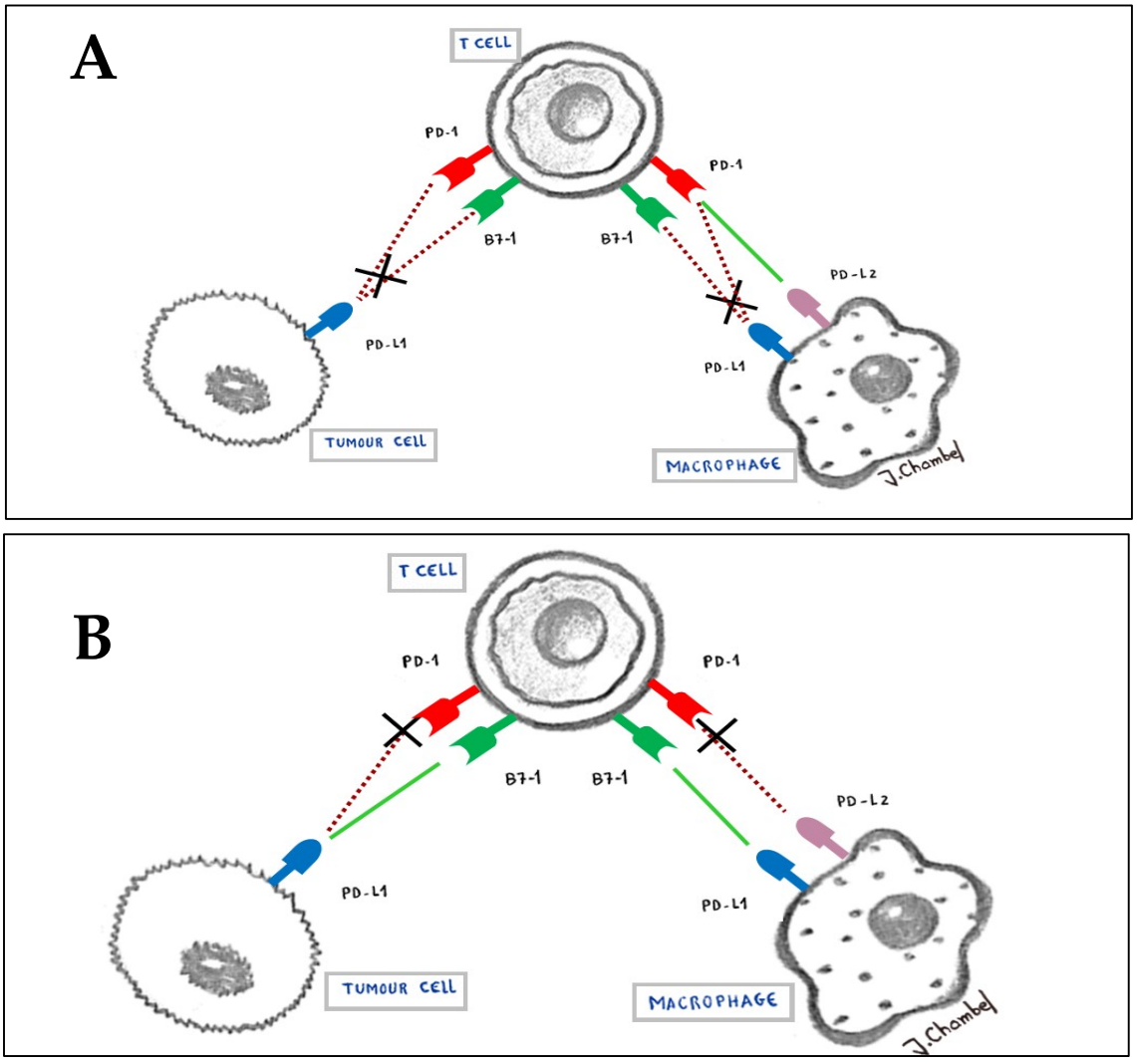

Figure 3 - Checkpoint inhibitors targets used in modern immunotherapy: PD-L1 blockade (A) and PD-1 blockade (B).

5. Checkpoint Inhibitors for solid tumors

Science has paved a long way since immunotherapy was first attempted. In fact, William Coley, back in 1893, was the first one to use principles of immunotherapy to treat cancer patients, injecting bacterial toxins into cancer cells, in order to stimulate anti-tumor immune responses [97]. The transition to targeted immunotherapy as we know it today was only possible in the late 70's, after the development of the hybridoma technology, which supported the production of monoclonal antibodies [98]. More recently, a number of antibodies targeting cellular immune checkpoints as the PD-1, PD-L1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) have been developed to promote the activation of T-cells and promote tumor control. This treatment strategy has been shown to be particularly effective in those tumors with high mutation burden (Figure 4) [99].

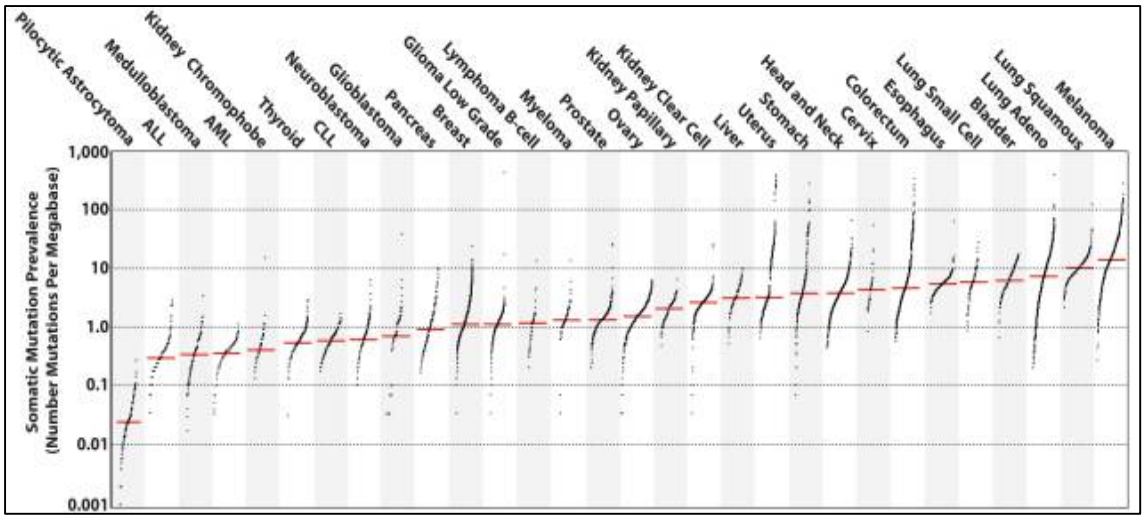

Figure 4 – Graphic representation of the mutational burden per tumor [100].

T-cells are key players in anti-tumor immunity and, therefore, the bulk of cancer immunotherapy research has focused on inducing T-cell-mediated anti-tumor responses [99]. CTLA-4 and PD-1 are co-inhibitory receptors found on the cell surface of T-cells. Upon binding to their corresponding ligands (CD80/86 and

PD-L1/L2, respectively), T-cells become anergic [101]. In the context of the tumor microenvironment, the aberrant expression of immune checkpoint ligands (on tumor and immune cells), together with chronic exposure to tumor antigens, can lead to the undesirable suppression of T-cell activity [102]. Therefore, the blocking of such mechanisms can unleash a renovated anti-tumor immune response [103,104].

Treatment with checkpoint blocking antibodies has been approved for a number of cancers including melanoma, urothelial bladder cancer, head and neck squamous cell carcinoma, non-small cell lung cancer (NSCLC) and classical Hodgkin lymphoma, while positive indications have been found for many other malignancies [1,3,105-108].

Immunotherapies have a high synergistic potential with standard chemotherapy and radiotherapy, as these are known to induce immunogenic cell death [109,110]. This synergy might be especially valuable for tumors with low mutation burden. The rationale encompasses the targeting of fast-dividing tissues by impairing mitosis and inducing DNA damage. This leads to the release of tumor antigens and acquisition of damage-associated molecular patterns, which activate antigen presenting cells (APCs) [111]. Macrophages are also attracted to consume the damaged tumor cells, which further enhances the anti-tumor response of T-cells upon presentation of the tumor antigens [112]. In melanoma patients, an improved clinical response rate was observed upon treatment with a combination of anti-CTLA-4/PD-1 with radiotherapy, compared

to treatment without radiation [110]. Similar effect was observed in a treatment-refractory metastatic lung adenocarcinoma patient after therapy with radiotherapy and ipilimumab [113].

In summary, cancer immunotherapy has experienced remarkable advances in recent years, with striking clinical responses in several types of solid cancers, particularly those with a high mutation burden [99,100]. Particular success has been seen in melanoma and NSCLC with pembrolizumab and nivolumab, with or without ipilimumab [114]. However, the list of cancer subtypes treated with ICI is rapidly expanding and currently includes melanoma, NSCLC, small-cell lung cancer (SCLC), head and neck carcinoma, RCC, ovarian cancer, cervical cancer, urothelial cancer, colorectal cancer, upper gastrointestinal cancers and hepatocellular carcinoma. In addition, the increasing studies on ICI in breast cancer identified HER2-positive and triple-negative breast cancers (TNBC) as the most immunogenic subtypes, and therefore, with most potential for immunotherapy based treatments [115-117].

Overall, the success achieved with immunotherapy paved the way towards new clinical practices and new hope for cancer patients. We should expect a great deal of exciting news in a near future regarding these subjects.

6. Immune profile of metastases from solid tumors

Despite the study on tumor immune profile, intra-tumor heterogeneity, which fosters tumor evolution and metastases, theoretically can allow the development of metastases with different immune profiles [118]. Cancer sequencing has already unraveled the genetic heterogeneity between primary and metastatic tumors caused by clonal evolution [119]. Additionally, Garcia-Mulero *et al.* found that the primary origin of BM affects the immune phenotype, which will present different levels of immunogenicity [120]. Also, and compared with the primary lesions, bone metastases showed more abundance of stromal cells, enrichment in fibroblast, and significant differences in B lineage infiltration score [120].

The following sections will address these findings in the current literature for breast, lung, renal cancers and malignant melanoma, focusing whenever possible on the immune profile expressed by BM. Similar findings reporting differential expressions for PD-L1 and PD-1 were documented for several other solid tumors, like endometrial cancer, head and neck squamous cell carcinoma or ovarian cancer [121-123].

6.1 Breast cancer

In 2018, Dieci *et al.* promoted a study to evaluate the immune characterization of breast cancer metastases [124]. They included 94 patients with metastatic breast cancer where distant metastases samples were available for evaluation. Among

the immune parameters selected, they included TIL quantification and PD-L1 expression. TILs were not significantly different across biopsy sites, however, lung metastases samples showed the highest levels among all sites. Matched primary tumors were available for only 55 patients and, again, in this subgroup, TILs were not significantly different in primary versus matched metastases [124]. In the same study, PD-L1 was evaluated on tumor cells and on stromal/immune cells. PD-L1 expression was found to be predominant in stromal/immune cells rather than tumor cells, with PD-L1 expression presenting no association with overall survival (OS) [124]. In this series, the authors used metastases from several metastatic sites, mainly from the lung, liver and central nervous system (CNS), but bone was not one of the major metastatic sites considered.

Ogiya *et al.* compared the immune microenvironment between primary tumors and brain metastases in patients with breast cancer. These authors concluded that brain metastases have a decreased TIL count compared to primary breast tumors, and that there was no significant difference in PD-L1 expression between primary tumors and correspondent brain metastases [125].

Tawfik *et al.* studied the expression of PD-1 and PD-L1 in breast cancer and paired metastases in regional lymph nodes and non-paired distant metastases [126]. They concluded that PD-L1 was differentially expressed between primary breast cancer and regional lymph nodes, mainly driven by triple negative status; however, a near-total absence of PD-L1 expression was seen for distant metastases compared with the primary tumor and lymphatic metastases [126].

Once again, and among the distant metastases evaluated, bone was not a major metastatic site considered for immune profile study.

Manson *et al.* also reported a discordant expression for PD-1 and PD-L1 between primary tumors and their matched distant metastases, at least for one-third to a half of the breast cancer patients [13]. Nonetheless, among 106 primary female breast cancers and their matched distant metastases from various distant anatomical sites, only four cases were obtained from bone. These authors observed some expression differences between different anatomic sites, in particular for PD-1 and immune PD-L1. That was the case for bone metastases, where all four cases were negative for PD-1 [13].

Rosenblit *et al.* compared PD-L1 expression between primary tumors and metastatic lesions in triple negative breast cancers (TNBC) [127]. These authors found a variable PD-L1 positivity among the different metastatic locations, with substantially lower positivity rates in liver, skin and bone metastases compared with primary breast lesions or even lung, soft tissue or lymph node metastases. These findings favors a difference in the immune microenvironment across metastatic sites [127].

More recently, Boman *et al.* promoted a systematic review where they analyzed the discordance of PD-L1 status between primary and metastatic breast cancer. They found a significant discordance between PD-L1 status in primary and metastatic breast cancer, which reinforces the idea of the importance for appropriate tissue sampling when selecting patients for immunotherapy. In

other words, distant metastases biopsies should also be performed in order to better characterize the immune profile of the tumor [128]. This systematic review reflects the data collected from 972 patients; however, only 64 cases were from BM.

As above stated, TNBC is the most immunogenic breast cancer subtype, and efficacy of anti-PD-1 drugs has already been demonstrated in clinical trials, however, the impact of ICI specifically on breast cancer BM is still to be determined [127,129].

6.2 Lung cancer

Kim *et al.* studied the differential expression of PD-L1 between primary tumor and metastases in lung adenocarcinomas [11]. A cohort of 161 paired primary lung adenocarcinomas and metastatic tumor tissues was used, with lymphatic nodes as the major site of metastases in this study. The overall concordance rate for PD-L1 expression between primary and metastatic tumors was globally high, concluding that the evaluation of PD-L1 in either primary or metastatic tumors would be helpful for guiding anti–PD-1/PD-L1 immunotherapy [11].

In other study, Uruga *et al.* also compared PD-L1 expression between the primary tumor and lymph node metastases, reporting concordance in most of the cases [130]. Once again, the BM immune profile was not investigated. Liu *et al.* also supported similar PD-L1 expressions between the primary tumor and lymph

node metastases [131].

Meanwhile, ICI in monotherapy or combined with chemotherapy were already been approved as the standard treatment for advanced NSCLC, with several clinical studies reporting prolonged survival and improved quality of life [132,133]. However, inconsistent efficacy of ICIs on bone lesions outcomes were reported, with some authors presenting an optimal bone disease control, while others showed progression even during the treatment [134]. In this scenario, Nakata *et al*. reported that an increased number of BM at the beginning of treatment could be associated with a higher risk for BM progression after treatment with ICI, namely nivolumab [135].

For patients with lung cancer and BM, the prognosis seems consistently dismal, regardless the use of ICI in monotherapy or associated with other drugs. Tamiya *et al.* observed no differences in progression-free survival (PFS) for patients with advanced NSCLC with or without BM treated with nivolumab [136]. The same findings were supported by the study promoted by Kawachi *et al.* [137].

6.3 Renal cancer

To evaluate the expressions of PD-L1 in primary RCC and distant metastases, Jilaveanu *et al.* studied a tissue microarray (TMA) from 34 matched pairs of nephrectomy and correspondent metastatic sites[10]. In this series, metastatic tissues had greater PD-L1 expression than the primary tumors, and as such, the

primary tumor does not seem an adequate surrogate for determining PD-L1 expression in metastatic sites [10]. In this study, lung metastases represented the vast majority of the cases, however, other visceral sites, skin, soft tissue and even soft tissue components of BM were included [10].

Callea *et al.* also compared PD-L1 expression in a series of primary RCC and their metastases[12]. They found a discordant tumor cell PD-L1 staining between primary tumors and metastases in 20% of cases. In this setting, these authors concluded that the heterogeneity of PD-L1 expression in RCC may require independent analysis of metastatic lesions [12]. Among the 76 metastases samples used, only 12 cases were BM.

Again, in the Basu *et al.* report, where eight cases of BM were evaluated among 50 samples, some discordance in PD-L1 (but also PD-1 and PD-L2) was found between RCC primary tumors and metastatic tissues [138].

Immunotherapy had a tremendous impact on RCC, with the latest European RCC standards upgrading ICI to the first-line standard treatment options [139]. Despite the obvious clinical advantages presented by ICI in RCC treatment, the effect on BM is not clear, despite ICI combined with other treatments seems to achieve better results than ICI monotherapy [140]. Regarding the prognostic significance of ICI therapy for RCC patients, several studies reported no differences in PFS or therapeutic response rate between patients with and without BM [141,142].

6.4 Malignant melanoma

Anti-PD-1/PD-L1 inhibitors have significantly improved clinical outcome in metastatic melanoma patients [11]. For this particular cancer, Kakavand *et al.* reported a high level of PD-L1 expression in sentinel lymph nodes, providing a strong rationale for anti-PD-1 therapy [143]. In addition, Berghoff *et al.* demonstrated the presence of considerable lymphocytic infiltrates and PD-L1 expression in brain melanoma metastases [144]. However, none of these studies compared metastases and primary tumors. Additionally, and to the best of our knowledge, there are no reports regarding BM from melanoma, or even other metastases location than lymph nodes or brain, regarding the immune characterization. The lack of literature is probably because bone is rarely the first metastatic site of melanoma, opposite to the central nervous system and lung metastasis. Moreover, there is an absence of studies addressing the impact of BM in advanced melanoma treated with ICI, with only one case describing a decrease in osteoblastic bone lesions after pembrolizumab [145].

7. Bone metastases immune profile

A Pubmed search with the terms "*bone metastases and PD-L1 expression*" without time restrictions, will display a low number of publications (73), from which only 11 provide information regarding carcinoma BM microenvironment and immune profile (Table 1). In addition, another interesting publication by Wang

et al. was found using this research criteria, however, this paper was disregarded because it was fully written in Chinese [154]. As such, it is clear that bone is not among the most frequent evaluated metastatic sites concerning TIL, PD-1 or PD-L1. Despite this striking lack of knowledge involving BM microenvironment, some relevant information is available.

Among the studies focusing in the immune profile of BM, apparent low expression of PD-1 and PD-L1 seems to occur, probably reflecting a particular tumoral microenvironment. In fact, Pontarollo *et al.* reported a lower expression in NSCLC BM in comparison with the levels of PD-L1 expressed for lung tumors [150]. Manson *et al.* also reported the absence of PD-1 expression in BM from breast cancer, however, only four cases were evaluated in this series [13]. On contrary, Wang *et al.* reported identical PD-L1 expression in BM from advanced NSCLC when compared with other metastatic sites [146]. Ihle *et al.* studied BM microenvironment in prostate carcinoma, finding a distinct level of T cell populations for lytic and blastic metastases within the bone, being higher for the latter [148]. Also, blastic metastases seem to present higher levels of PD-L1 expression. Nonetheless, primary prostate tumors sistematically present low levels of immune cell infiltration and neoantigen expression, being considered a *cold* tumor which makes any immunotherapy approach challenging [148].

Furthermore, there is growing evidence that anti-resorptive drugs usually used to manage BM, such as bisphosphonates (BPs) and denosumab, can influence the microenvironment and immune profile of BM [155]. In the advanced setting, both

BPs and denosumab reduce the skeletal complications associated with BM, with BPs such as Pamidronate and Zoledronic Acid (ZA) being used in clinical practice for several decades. Recently, some reports suggest that ZA acts as an immune modulator by significantly inhibiting expansion of regulatory T cells [156]. Denosumab on the other hand, stills do not have reports on anti-tumor immune cells effects, however, numerous preclinical and clinical studies sustain that the combination with ICI reinforces the anticancer efficacy compared with monotherapy [157]. Additional studies are ongoing in the clinical setting to elucidate the mechanisms behind these preliminary findings. In fact, RANK/RANKL axis seems to be somehow involved in still to clarify immune processes [158], with Gomez-Aleza *et al.* reporting how the loss of RANK signaling in mouse tumor cells increases leukocytes, lymphocytes, and $CD8^+$ T cells, and reduces macrophage and neutrophil infiltration, promoting the anti-tumor effect of immunotherapies in breast cancer through a tumor cell mediated effect [159]. Moreover, RANK pathway inhibition has been shown to modulate the immune environment and enhance the efficacy of ICI against solid tumors [160].

All together, these features highlight the need for further investigation, focusing on the metastatic bone microenvironment, to better understand the complexity of the metastatic process into the bone and the immune profile which can be found in each particular solid tumor.

Table 1 – Publications on bone metastases microenvironment and immune profile obtained with search criteria "*bone metastases and PD-L1 expression*" on Pubmed database

Authors	Year	Article Type	Primary tumor	References
Wang *et al.*	2019	Retrospective Case series	NSCLC	Ref. 146
Zhang *et al.*	2019	Prospective Case series	RCC	Ref.147
Ihle *et al.*	2019	Retrospective Case series	Prostate	Ref. 148
Hong *et al.*	2020	Retrospective Case series	NSCLC	Ref. 149
Garcia-Mulero *et al.*	2020	Retrospective Case series	Breast, colon, NSCLC, kidney, prostate, skin melanoma	Ref. 120
Pontarollo *et al.*	2020	Retrospective Case series	NSCLC	Ref. 150
Rozenblit *et al.*	2020	Retrospective Case series	Breast Cancer	Ref. 127
Boman *et al.*	2021	Systematic Review	Breast Cancer	Ref. 128
Qiao *et al.*	2021	Retrospective Case series	NSCLC	Ref.151
Palicelli *et al.*	2021	Systematic Review	Prostate	Ref.152
Nakasato *et al.*	2021	Case Report	Bladder Cancer	Ref. 153

NSCLC – Non-small-cell lung cancer; RCC – Renal cell carcinoma

7.1 Future research

Despite all the interest to study TILs, PD1 and PD-L1 expression in metastases from different solid cancers, an investigation clearly focusing in BM is still lacking. In this scenario, we herein propose the need to explore the TILs, PD-1

and PD-L1 expression using a significant number of paired samples from a given primary solid cancer and the correspondent BM. We believe that this approach will proportionate a better understanding for BM microenvironment. This knowledge can represent a leap forward to allow a better clinical approach and contribute to diminish SREs and improve patients' quality of life. It would be important to compare TILs, PD-1 and PD-L1 expression in paired samples from a given primary solid cancer and respective BM; as well as to correlate TILs, PD-1 and PD-L1 expression in BM with SREs and clinical intervention (medical treatment, radiotherapy and/or surgery) outcomes. The major limitation of such a study will be to identify a suitable and powerful cohort of paired samples. A multi-institutional effort, using Pathology databases from all the main hospitals treating BM in Lisbon metropolitan area, retrieving the data from last decade (2012-2021), could render a significant number of samples**.** Matched pairs of primary tumors and BM could be assessed for TILs in H&E stained tissue section, while PD-1 and PD-L1 expression would be evaluated using immunohistochemistry (IHC). Of course, such an effort requires comprehensive collection of clinical data. Information regarding BM free-survival; hormonal status in the particular case of breast cancer; and detailed insight regarding SREs (and applied treatment) is crucial, so it can be correlated with TILs, PD-1 and PD-L1 expression, for both primary tumor and respective BM. Additional information concerning presence of Treg cells and mesenquimal derived stem

cells in the BM microenvironment should be added to allow eventual further correlations.

Despite the limitations within this study proposition, it has the virtue of being adapted to our own clinical practice and reality, which is fundamental to ensure the feasibility for such project. Furthermore, such research could not only represent the first step for an optimized future clinical approach, but also the launching pad for better-designed protocols in future endeavors.

8. Conclusions

The relation between tumor infiltration by lymphocytes, PD-1 and PD-L1 expression in the primary tumor and matched BM, is still an unsolved matter. In addition, clinical meaning and possible repercussions for these features are still to unveil. The current literature provides some conflicting findings for almost every solid tumor regarding its immune profile. While some authors report significant differential expressions in PD-1 and PD-L1 between primary tumors and distant metastases, others could not find any relevant differences [10-14, 126-131, 146, 150, 161-163].

Cancer itself is a heterogeneous disease that varies in presentation, morphological features, behavior, and response to therapy. Current evidence indicates that spatial and temporal intratumor heterogeneity is present in breast cancer, lung cancer and many others neoplasms [164-168]. This heterogeneity

will pose not only critical challenges for the diagnosis, prediction of behavior and management of cancer, but will also affect pathogenesis, evolution, and progression [164-168]. In this scenario is not surprising that distant metastases could present a distinct immune profile in comparison with the primary tumor. Furthermore, we can even expect to see different immune profiles within the same tumor [164-168].

Another important issue that should not be forgotten, is the imminent role and effect from the host itself on tumor cells, and consequently, in the immune characterization that they could present. In fact, even in the presence of high level of TILs and PD-1/PD-L1 expressions, the therapeutic effectiveness of ICI can remain poor, highlighting the need for a deeper understanding of the complex and varied molecular mechanisms driving the expression and activation of the PD-L1/PD-1 signaling pathway [169]. This condition may be associated with resistance against immune checkpoint blockade therapy. In this regard, to understand how PD-L1 levels are regulated would be of great importance, however, the mechanisms by which PD-L1 expression is regulated are complex and they can occur at different levels from signaling pathways to post-transcriptional levels [170]. Desirably, the intense investigation ongoing in this field can bring some light into this matter soon.

Until date, the vast majority of the studies regarding TILs and PD-1/PD-L1 expressions, used primary tumors and metastases biopsy samples, which present the risk of not being representative of the tumor, since there is a great deal of

heterogeneity among the tumor itself and between metastases located in different sites [13,127,150]. Other relevant aspect entails the low number of studies with matched samples between the primary tumor and correspondent metastases. Furthermore, we found in this literature review a low number of information regarding BM, despite the fact that bone is one of the most common sites to find metastases [12,13,128,138]. In addition, it is most likely that microenvironments within metastatic sites promote specific stimuli for different local metastatic development, which will influence the immune profile presented. Indeed, the immune profile presented by distant metastases seems to differ among different studies for the same tumor. In summary, all these findings reflect a high level of uncertainty that must be scrutinized to better understand the phenomenon.

In order to clarify these aspects, additional studies focusing on particular tumors and particular metastatic sites will be necessary. If possible, these studies should include a significant number of paired matches between primary tumor and correspondent metastases, with TIL, PD-1 and PD-L1 analyses available from a representative sample, in order to minimize the heterogeneity effect within the tumor.

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