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REVIEW ARTICLE

Combining immune checkpoint inhibitors with denosumab: a new era in repurposing denosumab in oncology?

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Summary

The designation of immune checkpoint inhibitors (ICPi) as scientific breakthrough of the year 2013 marked a turning point in cancer therapeutics, unleashing the host immune system against tumors. ICPi block the cytotoxic T lymphocyte antigen 4 (CTLA-4), the programmed cell death protein (PD) 1 (PD-1), and the ligand of the latter (PD-L1) -the landmark immune checkpoints-abrogating the escape of cancer cells from immunosurveillance. Despite the durable antitumor response elicited by ICPi in an expanding list of cancer types and a substantial fraction of patients, the resistance to this modality - primary and acquired - has inspired research on combinational regimens to reinvigorate immunosurveillance in immune-refractory tumors. Besides various combinations of ICPi with other ICPi, targeted therapies, chemotherapy, and radiation, emphasis is placed on the identification of novel partners of ICPi. Scientists capitalize on repurposing already-approved drugs to overcome the diminishing efficiency of commercial drug research and development. Denosumab, a human monoclonal immunoglobulin antibody inhibiting the receptor activator of nuclear factor kappa-B ligand (RANKL), is excellent candidate for repurposing in oncology, given its anticancer potential and accepted safety profile. Originally approved as anti-osteoporotic agent inhibiting the osteoclast-driven bone resorption, denosumab has demonstrated multifaceted anticancer efficacy, beyond abolishing the osteoclast-dependent RANKL signaling. The present review provides a comprehensive overview of the preclinical and clinical evidence indicating denosumab as effective partner of ICPi, emphasizing the mechanisms underlying the enhanced anticancer efficacy of this combination as compared to monotherapies. Current challenges and future perspectives in incorporating the combination of ICPi with denosumab in clinical practice are discussed..

Key words: *cancer immunotherapy, immune checkpoint inhibitors, drug repurposing, Denosumab, combinational treatment*

Introduction

Over the current decade, immunotherapy has signified a "turning point" in cancer therapeutics [1]. Improved understanding of the ligand-receptor interface between cancer cells and patient's immune cells within tumor microenvironment (TME) has led to harnessing the host immune system, resulting in high response rates, durable responses, and long-term survival in an expanding repertoire of cancer types [2].

Cancer cells co-opte the immune checkpoints - inhibitory immune regulators assigned to ensure immune tolerance - in order to evade immune surveillance. In the context of active immunotherapy, administration of immune checkpoint inhibitors (ICPi) -monoclonal antibodies (mAbs) blocking the immune checkpoints - modulate the balance between stimulation and inhibition of immunity, restoring the antitumor immune response [2]. The

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cytotoxic T lymphocyte antigen 4 (CTLA-4), the programmed cell death protein (PD) 1 (PD-1), and the ligand of the latter (PD-L1), constitute landmark immune checkpoints, the blockade of which has already established a leading role in oncology [3].

With the advent of era of immunogenomics, integration of the genomic landscape of human malignancy with novel insights into tumor-immune interactions has enabled unraveling the immune landscape of cancer, providing fuel for future targeted studies anticipated to revolutionize the field. Innovative immunogenomics analyses of over 10,000 tumors revealed six immune subtypes that encompass multiple cancer types and are hypothesized to determine immune response patterns defining prognosis. The illumination of intracellular and extracellular networks that govern the tumor-immune cell interactions has prompted a shift of immune checkpoint therapy from cancer types to molecular signatures [4].

Despite the groundbreaking outcomes in some clinical trials, only a subset of patients initially respond to ICPi, while a substantial proportion of initial responders finally relapse with lethal drug-resistant disease. Several mechanisms, such as the attenuation of interferon (IFN) receptor signalling and of antigen presentation, allow cancer cells to evade the T-cell-mediated immune surveillance [5]. For instance, a marked reduction of somatic mutations in tumors observed after initial response to checkpoint blockade leads to decreased production of nonself immune antigens (neoantigens) [6]. Additional mechanisms of checkpoint resistance resulting in non-responsiveness to ICPi have been described, involving: (i) mutation, deletion or loss of heterozygosity (LOH) of beta-2-microglobulin (B2M), resulting in failure of assembling major histocompatibility complex (MHC) or Human Leukocyte Antigen (HLA) class complexes and thus reduced neoantigen presentation to T-cell receptor (TCR) complex [7]; (ii) mutations in Janus kinase (JAK) 1 gene or JAK2 gene leading to inhibition of signal transducer and activator of transcription 1 (STAT1), thereby inhibiting the anti-tumor immune signaling of IFNγ [5]; (iii) Fas-ligand driven apoptosis of tumor-infiltrated lymphocytes (TILs) triggered by polymorphonuclear-myeloid-derived suppressor cells [8].

Elucidation of the mechanisms endowing cancer cells with resistance to ICPi has spurred the development of strategies to reinvigorate immune surveillance in immune-refractory tumors. More than 1,100 clinical trials address combinations of ICPi with other ICPi, radiation, chemotherapy, molecularly targeted therapy, metabolic therapy, co-stimulatory signals on antigen presenting cells (APC) (e.g. anti-CD40), adoptive T cell transfer (CAR T or TCR T), oncolytic viruses, or vaccines that target neoantigens [9].

Recent advances in oncoimmunology have revealed many druggable pathways of immunosuppression in the TME that could be targeted in combination with ICPi.

However, scientists decry the decline in commercial drug research and development in current decade [10], capitalizing on drug repurposing -i.e. identification of new indications in already-approved drugs - to overcome the low productivity of drug industry [11,12].

Approved in 2010 by FDA as anti-osteoporotic agent [13], denosumab is perceived as excellent candidate for drug repurposing in oncology on account of its presumed anticancer efficacy and favorable safety profile [14]. Denosumab is a fully human monoclonal immunoglobulin G2 (IgG2) antibody with high affinity and specificity for receptor activator of nuclear factor kappa-B ligand (RANKL). Binding to RANKL, denosumab impedes the RANKL signaling, acting in a way reminiscent of OPG, the decoy receptor for RANKL. The rationale of the anticancer effect of denosumab is the abrogation of the tumor-promoting role of RANKL [14].

The present review provides a comprehensive overview of denosumab as an effective partner of ICPi in cancer therapeutics, highlighting current evidence and emerging challenges.

The role of RANKL in cancer as the rationale for repurposing denosumab in oncology

RANKL is a 316 amino acids (aa) member of TNF family, encoded by TNFSF11 gene, produced primarily by osteoblasts. It was identified in 1997 as a ligand of osteoprotegerin (OPG), a member of tumor necrosis factor (TNF) receptor superfamily produced mainly by cells of osteoblast lineage [15]. RANKL owes its discovery to its ability to interact with its main cognate receptor RANK in order to stimulate the differentiation of osteoclasts in the presence of macrophage colony stimulating factor (M-CSF) and orchestrate the osteoclasts-mediated bone resorption.

Interestingly, RANKL had been discovered in the setting of immune biology, prior to its identification as OPG ligand. Expressed on T cells, RANKL is responsible for the survival of RANK-expressing dendritic cells (DCs), enhancing also the ability thereof to trigger naive T-cell proliferation [16]. Moreover, RANKL is expressed in a plethora of organs, principally in lymph nodes, thymus, lung, and mammary glands, Peyer's patches, intestine,

brain, heart, skin, skeletal muscle, kidney, liver, exerting a pleiotropic effect. Beyond osteoblasts, diverse cells express RANKL, such as bone marrow stromal cells, activated T cells, B cells, fibroblasts, endothelial cells, chondrocytes, and mammary epithelial cells [17]. In fact, RANKL is considered as a key orchestrator of the interplay between bone biology and oncoimmunology, which rationalizes pursuing denosumab in cancer therapeutics.

Denosumab targets the bone-dependent role of RANKL

The RANKL induced osteoclasts driven bone resorption justifies the establishment of denosumab in the treatment of osteoporosis in postmenopausal women and patients with glucocorticoid-induced osteoporosis at a dose of 60 mg every 6 months, subcutaneously [13]. It also paved the way for the incorporation of denosumab in the treatment of bone loss in breast cancer patients receiving aromatase inhibitors and prostate cancer patients receiving androgen deprivation therapy [18,19].

Moreover, RANKL has been shown to orchestrate the "vicious cycle" of tumor-bone interactions, a process central in bone metastases of solid tumors, primary bone tumors, and multiple myeloma (MM), expanding the indications for administration of denosumab in bone oncology.

Hallmark clinical trials establishing the bone-modifying effect of denosumab in cancer are depicted in Table 1 [18-29]. This effect culminates in the FDA approval of denosumab for: (i) prevention of skeletal-related events (SREs) -bone pain, hypercalcemia, pathologic fractures, and neurologic complications– in patients with bone metastases from solid tumors at a dose of 120 mg every 4 weeks (November 2010); (ii) increase of bone mass and counteraction of the high risk of fracture in patients with non metastatic prostate cancer under androgen deprivation therapy and in patients with breast cancer receiving adjuvant aromatase inhibitors at a dose of 60 mg every 6 months (September 2011); (iii) treatment of giant cell tumors that are not amenable to surgery or in the case that the surgery may lead to severe morbidity at a dose of 120 mg every 4 weeks (June 2013); (iv) treatment of hypercalcemia of malignancy resistant to bisphosphonate at a dose of 120 mg every four weeks adding 120 mg on days 8 and 15 of the first month of treatment (December 2014); (v) prevention of SREs in MM at a dose of 120 mg every 4 weeks (January 2018) [30].

Denosumab targets the bone-independent role of RANKL in cancer

Numerous studies sustain the multifaceted role of RANKL in cancer, rationalizing the versatile anticancer effect of denosumab extending beyond a bone-modifying role [31].

In mouse models of mammary tumorigenesis, RANKL/RANK signaling can give rise to pre-neoplasia and tumors [32] and mediate the progestin medroxyprogesterone acetate (MPA) driven breast cancer, being induced by MPA [33]. Building on these findings, studies in mice and humans showed that RANKL expression is induced by progesterone and acts as a downstream effector of progesterone signaling in breast [34-36].

Progesterone has been shown to increase the RNA stability of RANKL, which in turn was indispensable for progesterone-induced proliferation. Indeed, RANKL induced by progesterone is considered a signal emitted by luminal cells to basal cells which respond by upregulating RANK, transcriptional targets and cell cycle molecules [35]. In human BRCA 1 mutation carriers, RANKL expressed on mature luminal cells induced by high circulating levels of progesterone has been shown to interact with RANK expressed on progesterone-responsive RANK expressing luminal progenitor cells, driving the malignant transformation of the latter [36].

RANKL exerts a tumor-promoting - protumorigenic and prometastatic- role in a wide array of malignancies beyond breast cancer, including prostate, colorectal, lung, bladder and gastric cancer [31]. Downstream of RANKL/RANK interaction are activating numerous signaling pathways implicated in epithelial mesenchymal transition (EMT), neo-angiogenesis, cancer cell migration, and invasion [37-40]. Furthermore, RANKL expressed in the metastatic foci acts either as a chemoattractant or as a "soil" factor facilitating the migration of cancer cells [41]. Recent data implicate the leucine rich repeat containing G protein-coupled receptor 4 (LGR4) - a RANKL receptor originally credited with anti-osteoclastic activity - in cancer cells proliferation [42]. Consequently, denosumab can directly attenuate the RANKL-induced tumor growth and metastases.

The dual role of RANKL in immunity complicates the immunomodulatory efffect of denosumab

Accumulating evidence indicates the pivotal role of RANKL/RANK interplay in immunity. Expressed on activated $CD4^+$ and $CD8^+$ T cells and natural killers (NK) cells, RANKL stimulates various processes-landmark of immunity, mediated through monocytes, macrophages and DCs, including DCs survival and maturation, T-cell activation, and NK cell inhibition [43].

The RANKL/RANK interplay may inhibit immunity via: (i) promotion of development of med-

Table 1. Clinical trials indicating the bone-modifying efficacy of denosumab in bone oncology

Title of study (ClinicalTrials.gov Identifier)	*Results (Ref)*
A Randomized, Double-Blind, Placebo-Controlled Study to Evaluate AMG 162 in the Treatment of Bone Loss in Subjects Undergoing Androgen-Deprivation Therapy for Non-metastatic Prostate Cancer (NCT00089674)	Increase of BMD at all sites and reduction of the incidence of new vertebral fractures following treatment with DmAb [18].
A Randomized, Double-Blind, Placebo-Controlled Study to Evaluate AMG 162 in the Treatment of Bone Loss in Subjects Undergoing Aromatase Inhibitor Therapy for Non-metastatic Breast Cancer (NCT00089661)	Significant increase of BMD over 24 months at trabecular and cortical bone after twice-yearly administration of DmAb with overall AE rates similar to those of placebo [19].
DCA114273: A Study Comparing Denosumab With Zoledronic Acid in Subjects of Asian Ancestry With Bone Metastases From Solid Tumors (NCT01920568)	Superiority of DmAb over ZA regarding reduction of uNTx/uCr in overall and Chinese patients without new safety concerns [20].
A Randomized, Double-Blind, Multicenter Study of Denosumab Compared With Zoledronic Acid (Zometa®) in the Treatment of Bone Metastases in Subjects With Advanced Breast Cancer (NCT00321464)	Superiority of DmAb over ZA in delaying time to first on-study SRE (HR, 0.82; 95% CI, 0.71 to 0.95; P=0.01 superiority) and time to first and subsequent (multiple) on-study SREs (rate ratio, 0.77; 95% CI, 0.66 to 0.89; P=0.001). Greater decrease in bone turnover markers with DmAb compared to ZA. Similar overall survival, disease progression, and rates of adverse events and serious adverse events between groups [21].
A Randomized, Double-Blind, Placebo-Controlled, Multi-Center Phase 3 Study of Denosumab on Prolonging Bone Metastasis-Free Survival in Men With Hormone Refractory Prostate Cancer (NCT00286091)	Significant increase of BMFS by a median of 4.2 months related with DmAb compared with placebo (median 29.5 [95% CI, 25.4-33.3] vs 25.2 [22.2-29.5] months (HR, 0.85; 95% CI 0.73-0.98, p=0.028). Significant delay of time to first bone metastasis related with DmAb compared to placebo (33.2 [95% CI, 29.5-38.0] vs 29.5 [22.4-33.1] months; HR, 0.84; 95% CI 0.71-0.98; p=0•032). Similar OS between groups (HR, 1.01; 95% CI 0.85-1.20; p=0.91). Similar rates of adverse events and serious adverse events in both groups, except for osteonecrosis of the jaw and hypocalcaemia [22].
A Randomized, Double-Blind, Multicenter Study of Denosumab Compared With Zoledronic Acid (Zometa®) in the Treatment of Bone Metastases in Men With Hormone-Refractory Prostate Cancer (NCT00321620)	Reduced risk of developing first SSE [HR, 0.78; 95% confidence interval (CI) 0.66–0.93; P=0.005] and first and subsequent SSEs (rate ratio, 0.78; 95% CI 0.65–0.92; P=0.004) related with DmAb compared with ZA [23].
A Randomized, Double-Blind, Multicenter Study of Denosumab Compared With Zoledronic Acid (Zometa) in the Treatment of Bone Metastases in Subjects With Advanced Cancer (Excluding Breast and Prostate Cancer) or Multiple Myeloma. (NCT00330759)	Noninferiority of DmAb to ZA in delaying time to first on-study SRE (HR, 0.84; 95% CI, 0.71 to 0.98; P=0.0007). No statistically significant superiority of DmAb over ZA in delaying time to first on-study SRE (P=0.03 unadjusted; P=0.06 adjusted for multiplicity) or time to first-and-subsequent (multiple) SRE (rate ratio, 0.90; 95% CI, 0.77 to 1.04; P=0.14). Similar OS and disease progression between groups [24] Ad hoc analysis in the subgroup of 1,597 patients with solid tumors, excluding patients with multiple myeloma showed: Significant delay of time to first on-study SRE (HR, 0.81; 95 % CI, 0.68-0.96) and time to first-and-subsequent SREs (RR, 0.85; 95 % CI, 0.72-1.00) related to DmAb compared to ZA Significant delay of time to development of moderate or severe pain (HR, 0.81; 95 % CI, 0.66-1.00), pain worsening (HR, 0.83; 95 % CI, 0.71-0.97), and worsening pain interference in patients with no/mild baseline pain (HR, 0.77; 95 % CI, 0.61-0.96) related to DmAb compared to ZA Adverse event rates were 96 % in both groups [25]

Continued on the next page

Title of study (ClinicalTrials.gov Identifier)	*Results (Ref)*
A combined analysis of 3 pivotal, randomised, double-blind, active-controlled, phase 3 trials, comprising patients with breast cancer, prostate cancer, other solid tumors or MM (NCT00321464, NCT00321620, NCT00330759)	Superiority of DmAb over ZA in delaying time to first on-study SRE by a median 8.21 months, reducing the risk of a first SRE by 17% (HR, 0.83 [95% confidence interval (CI): 0.76-0.90]; P<0.001). Disease progression and overall survival were similar between the treatments. Hypocalcaemia was more common for DmAb. Similar rate of osteonecrosis of the jaw (P=0.13) [26]
A Randomized, Open Label, Active Controlled Study of AMG 162 in Subjects With Advanced Cancer Currently Being Treated With Intravenous Bisphosphonates (NCT00104650)	71% of patients on DmAb compared with 29% patients in the IV BP arm (P<0.001) achieved the primary end point of uNTx levels lower than 50 nmol/L BCE/mM creatinine (uNTx<50) at week 13 64% of patients on DmAb and 37% of patients on IV BP arm maintained uNTx lower than 50 at week 25 Incidence of SREs: 8% and 17% in the DmAb group and IV BP group, respectively. Similar rates of adverse events between treatment groups [27].
An Open-label, Multi-center, Phase 2 Study of Denosumab in Subjects With Giant Cell Tumor of Bone (NCT00680992)	96% of analysable patients with surgically unsalvageable GCTB had no disease progression after median follow-up of 13 months 74% of analysable patients with salvageable GCTB whose surgery was associated with severe morbidity had no surgery and 62% of patients who had surgery underwent a less morbid procedure than scheduled [28].
An Open-Label, Multi-Center, Phase 2 Safety and Efficacy Study of Denosumab (AMG 162) in Subjects With Recurrent or Unresectable Giant Cell Tumor (GCT) of Bone (NCT00396279)	Significant reduction or elimination of RANK-positive tumor giant cells, reduction of the relative content of proliferative, densely cellular tumor stromal cells, and replacement thereof by non proliferative, differentiated, densely woven new bone following treatment with DmAb [29].

BCE: bone collagen equivalents; BMD: bone mineral density; BMFS: bone-metastasis-free survival; BP(s): bisphosphonate(s); DmAb: Denosumab; GCTB: Giant cell tumour of bone; HR: hazard ratio; IV: intravenous; OS: overall survival; RANK: Receptor Activator of Nuclear Factor kB: Ref: reference; SSEs: symptomatic skeletal events; SRE(s): skeletal-related event(s); uNTx: urine N-terminal telopeptide; vs: versus; ZA: zolendronic acid

ullary thymic epithelial cells (mTECs), crucial players of T-cell self-tolerance; (ii) enhancement of tolerance in Peyer's Patch; (iii) generation of regulatory T cells (Tregs); and (iv) promotion of T-cell tolerance and deletion [43].

The RANKL/RANK interaction is critically implicated in host immune response to cancer, encountered in both tumor microenvironment (TME) and lymph nodes. Among TILs, RANK is expressed on myeloid cells, whereas RANKL is expressed on T cells in both TME and locoregional lymph nodes. In a mouse model of cancer, RANKL was mainly expressed by activated T cells with proliferating phenotype, expressing programmed cell death protein 1 (PD-1) and Ki67 as well. Both the RANKL and the RANK are also expressed on stromal cells in lymph nodes and on cancer cells.

In TME, interaction of RANKL with RANK expressed on DCs, tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) leads to diverse clinical outcomes [44]. The RANKL signaling may contribute to immunosuppression through decreased expression of costimulatory molecules on RANK-expressing DCs or macrophages, increased production of T helper 2 (TH2) cell-type cytokines, or promotion of regulatory T (Tregs) cell generation. For instance, tumor-infiltrating $CD4^+CD25^+FoxP3^+$ Tregs expressing RANKL have been correlated with breast cancer aggressiveness [43]. Secondly, the interaction of RANKL abundant in tumor/bone microenvironment with RANK expressed on MDSC converts the latter into osteoclasts [45] exerting an immunosuppressive and/or tolerogenic role [46]. Thirdly, the RANK-expressing TAMs show a transition to a tumor-promoting M2 polarization [47].

On the other hand, RANKL may enhance the immune response through control of T- and B-lymphocyte development, promotion of lymph-node organogenesis, increased DCs survival, cytokine expression, and stimulation of T-cell responses [43]. In the TME, RANKL may promote antitumor immunity [44].

The factors that shift the immune response towards attenuation or enhancement of antitumor immunity remain largely unknown, but they appear to be context-dependent, perplexing the immunomodulatory effect of denosumab.

Denosumab as effective partner of ICPi

The postulation that the abrogation of the RANKL-induced suppression of antitumor immunity via denosumab potentiates the antitumor immune response stimulated by ICPi has prompted research on the efficacy of denosumab as partner

of ICPi. Indeed, numerous preclinical and clinical studies sustain that the combination of RANKL blockade with ICPi reinforces the anticancer efficacy of either ICPi or denosumab as monotherapy. Mechanistic insights into the synergistic anticancer effect of this combination are provided by seminal animal models and are currently under evaluation in the clinical setting.

Lessons from preclinical studies

Convincing data from mouse models of cancer indicate the combination of RANKL inhibition with immune checkpoint inhibition as more efficient strategy for abrogating cancer progression compared to either inhibition alone.

Working on the B16F10 melanoma preclinical model of experimental metastases, Smyth et al showed that the modest antimetastatic activities of anti-CTLA-4 (UC10-4F10, 100 μg intraperitoneal on days −1, 0, and 2) and anti-RANKL (IK22-5, 200 μg on days −1, 0, and 2) mAbs as monotherapy were significantly reinforced following combination of these two mAbs at the time of intravenous melanoma inoculation [48].

Considering the ineffectiveness of treatment in RAG2−/−γc−/− mice lacking all lymphocytes, the combination effect of anti-CTLA-4 and anti-RANKL mAbs was suggested to be lymphocytes-dependent. Moreover, a synergistic effect of NK cells and T cells controlled by Tregs suppressor mechanisms was demonstrated to orchestrate the antitumor activity of anti-CTLA-4 and anti-RANKL mAbs. Further exploration merits the hypothesis that the inhibition of intratumor Tregs by anti-CTLA-4 and anti-RANKL mAbs releases the T effector cells activation [48].

In C57BL/6 polyclonal mice vaccinated with B16-GM-CSF (GVAX), the combination of anti-RANKL mAbs and anti-CTLA-4 mAbs was demonstrated to exert a synergistic effect to improve host survival in response to challenge with 2×10^4 B16 melanoma cells compared to iso control/anti-CTLA-4 mAb/GVAX therapy or anti-RANKL mAb/GVAX. Vaccination with B16-GM-CSF (GVAX) potentiates an anti-B16 melanoma immune response via stimulation of innate immune cells and reinforcement of tumor antigen presentation to T cells. Moreover, a significant increase of $Ki67^+CD4^+$ T cells and $KLRG1^+$ and granzyme B^+ $CD4^+$ T cells was observed in the anti-RANKL/anti-CTLA-4/GVAX-treated mice compared with the control groups. This finding points to a synergistic effect of anti-RANKL and anti-CTLA-4 mAbs to increase the tumor-infiltrating $CD4^+$ T cells expressing cytolytic markers [49].

Increased efficacy of the combination of anti-RANKL with anti-PD-1 mAbs compared to either monotherapy or control immunoglobulin has been demonstrated in the mouse 3LL lung adenocarcinoma model. This effect was observed following either simultaneous administration or sequential administration. In fact, administration of anti-PD-1 mAb prior to anti-RANKL mAb resulted in a significantly more pronounced decrease in tumor volume compared with administration of anti-RANKL mAb prior to anti-PD-1 mAb ($p<0.01$) [50].

In mice bearing experimental B16F10 melanoma lung metastases, the combination of hamster anti-CTLA-4 (UC10-4F10) with rat anti-RANKL (IK22-5) mAbs led to increased resistance to metastases compared with treatment with either antibody alone or control immunoglobulin. This result was dependent on the presence of NK cells but not $CD8^+$ T cells, implicating IFNγ as well. Likewise, this combination showed efficacy in controlling prostate carcinoma RM-1 experimental lung metastases, mediated by NK cells [51].

The optimal synergistic effect of the combination of anti-RANKL with anti-CTLA-4 mAbs concerns the IgG2a isotype of anti-CTLA-4, which is known to selectively deplete intratumoral Tregs involving upregulation of FcγRIV expression on $CD11b^+$ TILs.

The antitumor efficacy of anti-RANKL and anti-CTLA-4 (IgG2a) combination therapy in the tumor models of this study was ascribed to selective $CD8^+$ T cell recruitment and increase in $CD45.2^+$ TILs, but not to a more efficient Tregs depletion. Fundamental element of this effect was the role of the cross-presenting $CD8\alpha^+$ conventional DCs. Moreover, the combination resulted in increased T-cell effector function (cytokine polyfunctionality), which was TME-specific [51].

In 2018, Ahem et al demonstrated that RANKL blockade enhanced the anti-metastatic activity of PD1/PD-L1 blockade and ameliorated the subcutaneous growth suppression compared to monotherapy alone in mouse models of melanoma, prostate cancer, and colon cancer. This anti-metastatic activity relied on NK cells and IFN-γ, while the suppression of subcutaneous tumor growth was dependent on T cells and IFN-γ [52].

In the same study, in tumor-bearing mice, triple blockade of PD1, CTLA-4 and RANKL compared to dual anti-PD1 and anti-CTLA-4 combination therapy resulted in: (i) increased proportion of tumor-infiltrating $CD4^+$ and $CD8^+$ T cells; (ii) increased -though not significant- proliferative status of $CD8^+$ TILs as measured by Ki67 staining; and (iii) improved T cell effector function, leading to significant increase of the Th1-type cytokine

Table 2. Recruiting clinical trials addressing denosumab in oncology (Reference [56])

Cancer type (ClinicalTrials.gov Identifier)	*Phase*	*Primary Outcome Measures*	*Time Frame*	*Intervention*
Smoldering Multiple Myeloma (NCT03839459)		Proportion of subjects with a downgraded risk of progression of smoldering multiple myeloma.	1 year	Drug: Denosumab
Multiple myeloma with renal insufficiency (NCT02833610)		Percent Change of sCTX Levels	2 years	Drug: Denosumab
Castration-resistant Prostate Cancer Metastatic Cancer Bone Metastases (NCT03869762)		Radiographic PFS	12 months	Drug: Denosumab in combination With Enzalutamide (androgen receptor signalling inhibitor)
Stage IV Non-Small-Cell Lung Carcinoma (NCT03669523)		ORR according to the PD-L1-expression rate (threshold at 1%)	24 months	Drug: Denosumab-nivolumab combination
Metastatic Breast Cancer Metastatic Prostate Cancer Bone Metastases (NCT02051218)	3	Time to first on-trial symptomatic skeletal event	At the latest 5 years after randomization	Drug: Denosumab (reduced dosing) Drug: Denosumab (standard dosing)
Early breast cancer (NCT03691311)	1	Antiproliferative and/or pro-apoptotic activity of denosumab evaluated by changes in percentage of tumor cells expressing Ki67 and/or cleaved caspase 3 between Biopsy A and Biopsy B.	From first biopsy until surgery intervention, (around 4 wk after enrolment)	Drug:Denosumab
Breast Cancer (NCT02900469)	1	Pharmacodynamic markers of RANKL inhibition	Change from baseline RANKL inhibition determination at 1 month	Biological: Denosumab Procedure: Surgery
Melanoma Stage Iv Melanoma Stage Iii Melanoma (NCT03161756)	1 2	• Median PFS • Grade 3 and 4 Selected irAEs of interest	Approximately 5 years Approximately 2 years	• Drug:Denosumab • Drug: Nivolumab • Drug: Ipilimumab
Melanoma Stage III, IV (NCT03620019)		Evaluation of the antitumor effect of Denosumab alone and combined with PD-1 inhibitor	Variable dependent on primary outcome[a]	Drug: Denosumab alone and in combination with an anti-PD1 agent (pembrolizumab or nivolumab)
Renal Cell Carcinoma, Clear Cell Metastatic Kidney Cancer (NCT03280667)	2	Objective tumour response	6 months	Drug: Denosumab- Prembolizumab combination
Breast Cancer (NCT02613416)	2	Quantification of breast density by MRI	6 months	Drug: Denosumab
Aneurysmal Bone Cysts Giant Cell Granuloma Osteoblastoma Chondroblastoma Chondromyxoid Fibroma (NCT03605199)	2	Evaluation of efficacy of denosumab[b]	3 years	Drug: Denosumab

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Cancer type (ClinicalTrials.gov Identifier)	*Phase*	*Primary Outcome Measures*	*Time Frame*	*Intervention*
Urothelial Carcinoma Kidney Cancer Ureter Cancer Bladder Cancer (NCT03520231)	2	Difference in mean percentage change in sCTX between investigational drug arm and placebo arm	Baseline to Week 10	Drug: Denosumab in combination with first Line platinum-based chemotherapy
Breast Cancer (NCT03324932)	3	Percentage change in BMD for the lumbar vertebrae (L1-L4) on DXA	12 months after start of this study	Drug: Denosumab
Metastatic Renal Cell Carcinoma (NCT03408652)	3	Time to first SRE	54 months after inclusion for each patient	Drug: Denosumab
Metastatic Breast Cancer Metastatic Prostate Cancer Bone Metastases (NCT02051218)	3	Time to first on-trial symptomatic SRE	At the latest 5 years after randomization	Drug:Denosumab Reduced dosing 3x Denosumab 120mg sc. q4w followed by Denosumab 120mg sc. q12w
NCCL carcinoma Bone metastases (NCT03669523)	2	ORR according to PD-L1 expression	24 months	Drug: Denosumab in combination with nivolumab
BRCA1/2 Gene Mutation Carriers (NCT03382574)	Early Phase 1	Efficacy of denosumab compared to no treatment in the pre-surgical setting as regards the Ki67 proliferation index fallopian tube fimbrial epithelial cells in premenopausal BRCA1/2 mutation carriers undergoing risk-reducing salpingo-oophorectomy, with or without hysterectomy	Up to 12 months	Drug: Denosumab
Carcinoma, Non-Small-Cell Lung with bone metastases (NCT03958565)		Percentage normalization of urine NTX	3 months post-treatment	Chemotherapy /immunotherapy along with new onset therapy with IV ZA or denosumab for bone disease

[a] Time Frame:
(i) 21 days and 16 weeks after start of denosumab when evaluating the antitumor effect of denosumab combined with anti-PD-1 inhibitor through assessment of change in density of tumor-infiltrating cluster of differentiation (CD8+) cells (TILs) in tumor tissue;
(ii) 16, 28 and 40 weeks after start of denosumab when evaluating the antitumor effect of denosumab combined with anti-PD-1 inhibitor through assessment of change in recent thymic emigrant cells in peripheral blood;
(iii) 3 weeks after start of denosumab when evaluating the antitumor effect of denosumab alone through assessment of change in density of tumor-infiltrating cluster of differentiation (CD8+) cells (TILs) in tumor tissue;
(iv) 3 weeks after start of denosumab when evaluating the antitumor effect of denosumab alone is through assessment of change in recent thymic emigrant cells in peripheral blood
[b] Proportion of subjects who do not require surgery during the study; Proportion of subjects undergoing the planned versus performed type of surgery during the study; Radiological response; Disease progression based on clinical disease assessment; Combined pain scores
BMD: bone mineral density; Col-I, collagen 1; CTCs: circulating tumor cells; DXA: dual-energy X-ray absorptiometry; irAEs: immune related adverse events; IV, intravenous; MRI, Magnetic Resonance Imaging; NTX: N-terminal telopeptide; OPG: osteoprotegerin; ORR: Overall Response Rate; PD-1: Programmed cell death protein 1; PFS: progression-free survival; q4w: every 4 weeks; q12w: every 12 weeks; RANKL, receptor activator of nuclear factor kB ligand; sc: subcutaneous; sCTX: serum c-telopeptide; SRE: Skeletal Related Event; wk: weeks; ZA: zoledronic acid

polyfunctionality, mirrored in increased co-expression of IFNγ and TNFα. The ability of the triple combination to elicit superior anti-tumor responses compared to dual treatment was independent of the anti-CTLA-4 isotype driven engagement of FcR [52].

Moreover, this study revealed the optimal sequencing of antibodies, which is the administration of ICPi prior to or concurrently with anti-RANKL mAbs due to the ability of the former to enhance the RANKL expression on $CD8^+$ T and $CD4^+$ T cell TILs, priming TME to respond to RANKL blockade [52].

Lessons from clinical studies

In 2016, a seminal case report described the remission of aggressive metastatic melanoma with symptomatic bone metastases 62 weeks after initiation of simultaneous administration of ipilimumab (anti-CTLA-4 mAb) with denosumab [48].

Interestingly, one year earlier, a complete response to therapy had been reported in a patient with metastatic melanoma who received denosumab before and concomitantly with re-initiation of ipilimumab that was initially held due to steroid-refractory colitis [53].

In 2017, an observational study using the Flatiron Health's EHR database from approximately 255 US cancer clinics enrolled advanced melanoma or non-small-cell lung carcinoma (NSCLC) patients who received denosumab within 30 days of administration of anti-CTLA-4 mAbs (ipilimumab) or anti-PD-1 mAbs (pembrolizumab, nivolumab) and had a minimum of 6 months of follow up. Real-world tumor response (rwTR) was evaluated based on scans available up to 30 days after administration of combined treatment. The mean duration of treatment with combination of denosumab with ICPi was 4.0 months for melanoma (n=66) patients and 3.1 months for NSCLC patients (n=241). The rwTR was evaluated for two-thirds of patients, assessing complete response (CR), partial response (PR), stable disease (SD), or disease progression (PD). Significant association of longer mean duration of simultaneous administration with overall response rate (ORR; CR+PR) in melanoma (p=0.0172), NSCLC (p<0.0001), and combined cohorts (p<0.0001) was observed. The disease control rate (ORR plus SD) was 56% and 58% for melanoma and NSCLC patients, respectively.

In a retrospective evaluation of malignant melanoma patients, combination of denosumab with ICPi improved median overall survival (OS) and median progression-free survival (PFS) compared with ICPi monotherapy [54].

Likewise, recently, combination of denosumab with ICPi demonstrated a promising efficacy in metastatic melanoma patients. Within a median follow-up of 19.8 months, the objective response rate was 54% in patients receiving triple combination of nivolumab with ipilimumab and denosumab and 50% in patients receiving combination of anti-PD-1 mAb with denosumab with no unexpected treatment-related adverse events [55].

Challenges and future perspectives

First and foremost, to embrace the combination of ICPi with denosumab, it is imperative to endorse the efficacy of repurposing denosumab in oncology. To this end, the results of relevant ongoing clinical trials -recruiting (Table 2) and not yet recruiting (Table 3) patients- are awaited [56].

The combination of ICPi with denosumab is a great exemplification of precision medicine, highlighting the necessity to identify the cancer types and the cancer patients anticipated to show the optimal response to this strategy.

To date, most data sustaining the clinical efficacy of combination of denosumab and ICPi compared with either agent as monotherapy are derived from studies on melanoma -the archetype of immunogenic tumors. In fact, the combination of ICPi with denosumab is currently evaluated in unresectable stage III and IV melanoma (NCT03161756) [56]. Given that both denosumab and ICPi appear to be effective in malignancies such as NSCLC [57,58] and urothelial cancers [59,60], ongoing clinical trials are addressing the combination of these agents in stage IV NSCLC with bone metastases (NCT03669523) and renal cell carcinoma and clear cell metastatic kidney cancer (NCT03280667) [56]. Most importantly, the emerging applicability of immunotherapy in breast and prostate cancer [61,62], in the treatment of which denosumab possesses a leading role, suggests the evaluation of the combination in these cancers.

The most challenging issue is the establishment of reliable prognostic and predictive biomarkers that will enable appropriate patient selection. So far, the PD-L1 expression is the most widely applied (and the only FDA-approved one) predictive biomarker [63,64]. However, the emerging importance of TME has indicated numerous biomarkers as surrogate end points of response to immunotherapy, including (i) tumor mutation burden (TMB) [65]; (ii) DNA mismatch repair (MMR) deficiency assessed by the MMR deficiency induced microsatellite instability (MSI-H) [66]; (iii) Immunoscore (an immune test measuring Tcell infiltration in formalin-fixed paraffin-embedded (FFPE) [67];

Table 3. Active not yet recruiting clinical trials addressing denosumab in oncology (Reference [56])

Cancer type (ClinicalTrials.gov Identifier)	*Phase*	*Primary Outcome Measures*	*Time Frame*	*Intervention*
Breast Cancer (NCT02682693)	2	• pcR rates of neoadjuvant treatment with or without denosumab in addition to nab-paclitaxel and EC • pcR rates of nab-Paclitaxel weekly for 12 weeks or 2 of 3 weeks for 12 weeks	24 weeks 12 weeks	Drug: Denosumab nab-Paclitaxel Epirubicin Cyclophosphamide Carboplatin Trastuzumab Pertuzumab
Giant Cell Tumor of the Bone (NCT03301857)	4	Rate of adverse events	Length of study: through the earliest date of 5 years after signing the informed consent form, death, withdrawal of consent, or lost to follow-up.	Drug: Denosumab
Prostate Cancer (NCT02721433)	4	Quality of life scores (EORTC QLQ-C30 Functional Domain Physical Subdomain)	1 year	• Drug: Pamidronate • Drug: Denosumab • Drug: Zoledronate
Childhood Osteosarcoma Metastatic Osteosarcoma Recurrent Osteosarcoma (NCT02470091)	2	Disease control rate (Cohort I) Disease control rate (Cohort II) RECIST response	4 months 12 months 4 months	• Biological:Denosumab • Other: Laboratory Biomarker Analysis • Other: Pharmacological Study
Giant cell tumor of bone (NCT03358212)	NA	Recurrence without metastasis	Up to 2 years	Drug: Denosumab
Unresectable Bone giant cell tumor (NCT03620149)	2	PFS ONJ incidence	5.8 years after enrolment of first patient	Drug: Denosumab
Predominant bone metastatic radioiodine rafractory differentiated thyroid carcinoma (NCT03732495)	2	Efficacy of lenvatinib associated with denosumab	24 months	Drug: Denosumab plus Lenvatinib
Nasopharyngeal Carcinoma EBV Related Carcinoma recurrent/metastatic (NCT03923842)	2	Plasmatic EBV DNA change	Change in circulating EBV DNA levels from baseline (prior to first denosumab administration on day -15 of first CT	Denosumab plus Gemcitabine
High Risk SMM and SLiM CRAB Positive, Early Myeloma (NCT03792763)	2	Time to progression (Time from randomization to transformation to symptomatic, active MM)	78 months	Denosumab

AE: adverse events; CRAB: high calcium (C), renal dysfunction (R), anemia (A), and bony (B); CT: chemotherapy; EBV: Epstein–Barr virus; EC: Epirubicin and Cyclophosphamide; EORTC: European Organization for Research and Treatment of Cancer; NA, not available; NCCL: Non-Small-Cell Lung Carcinoma; ONJ: Osteonecrosis of the jaw; ORR, Overall Response Rate; pcR: pathological complete response; PD-L1: programmed cell-death ligand 1; PFS: Progression free survival; sCTX: serum C-terminal telopeptide; SLiM: Myeloma with 60% or more clonal plasma cells in the bone marrow (S), a kappa-to-lambda or lambda-to-kappa ratio of greater than 100 (Li) more than one focal lesion on MRI (M); SMM: Smoldering Multiple Myeloma

(iv) density of $CD8^+$T-cells at the invasive margin of pre-treatment tumor samples correlating with anti-PD1 (pembrolizumab) response [68]; (v) Tregs, which are negative regulatory cells responsible for peripheral immune suppression [69]. Whether these biomarkers could serve as biomarkers of the effectiveness of ICPi combined with denosumab is yet to be explored.

The suppression of bone turnover markers, which indicates the anti-osteoclastic activity of denosumab [70], merits further evaluation as potential biomarker of immunological responses. For instance, assessment of serum C-terminal telopeptide (CTX) levels was incorporated in the setting of neoadjuvant breast cancer trial D-BEYOND (NCT01864798), but no analysis thereof in relation to post-treatment changes in immunological responses, such as the TILs density, was conducted [9].

Regarding the evaluation of serum RANKL levels, many issues remain to be resolved before embracing its applicability as a potential biomarker: (i) methodological pitfalls, such as variation in RANKL assays; (ii) the clarification of whether serum RANKL serves as indicator of the expression of RANKL in TME; (iii) the timing of RANKL assessment given the dynamic nature of RANKL expression by lymphocytes. Furthermore, the equilibrium among OPG/RANKL/TNF related apoptosis inducing ligand (TRAIL) in TME merits consideration [71].

Considering that more than one ICPi may be indicated for the same cancer type (e.g. advanced/metastatic lung cancer) [72], prioritizing ICPi to be combined with denosumab is a challenge that should be overcome.

The increasingly reported antitumor aspect of RANKL signaling [72-77], might be considered when abolishing the RANKL/RANK interaction through denosumab.

Moreover, active surveillance for rare but real toxicities of denosumab, such as osteonecrosis of the jaw, atypical femoral fractures, hypocalcemia, and multiple vertebral fractures following discontinuation of denosumab, is needed [78]. Despite the absence of evidence indicating a compromise of immune response related with denosumab [79-82], the recent warning about the correlation of denosumab with increased secondary malignancies [83] emerging from four clinical trials necessitates further research [84, 85, 21, 22, 24]. In parallel, clinicians should be mindful of the manageable but non negligible toxicity profile of ICPi [72, 86, 87]. Taken together, further efforts to assess any additive toxicity of the combination of ICPi with denosumab and to evaluate the benefit/risk ratio are needed.

Conclusion

The current decade has witnessed innumerous landmark events in cancer therapeutics. Among these, the FDA approval of denosumab in 2010 for prevention of SREs associated with solid tumors laid the groundwork for repurposing denosumab in oncology. Ipilimumab was the first ICPi to be FDA approved in 2011 for treatment of metastatic melanoma, paving the way for other milestones, including designation of cancer immunotherapy as "breakthrough of the year 2013" by the Science magazine, the America's leading scientific journal. In 2016, the first report of the denosumab-induced potentiation of ICPi efficacy [48] launched an exciting field of research.

With six ICPi having gained regulatory approval for cancer treatment and several others being under investigation, the landscape of cancer therapy is evolving rapidly. The combination of ICPi with denosumab has opened up new possibilities in cancer therapeutics ascribed to synergistic effect of the abrogation of the immunosuppressive RANK signalling with the ICPi induced reinvigoration of antitumor immunity. The path forward for the embracement of this combinational strategy in clinical practice entails the resolution of some outstanding issues, including: (i) validation of predictive biomarkers; (ii) optimal patient selection; (iii) assessment of long-term outcomes; (iv) assurance of acceptable and manageable toxicity profile; and (v) optimal design of clinical trials.

Conflict of interests

The authors declare no conflict of interests.

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