

# RANK/RANKL signaling inhibition may improve the effectiveness of checkpoint blockade in cancer treatment

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## ABSTRACT

Binding between the receptor activator of nuclear factor- $\kappa$ B (RANK) and its ligand (RANKL) triggers recruitment of TNF receptor associated factor (TRAF) adaptor proteins and activation of downstream pathways. RANK/RANKL signaling is controlled by a decoy receptor called osteoprotegerin (OPG) which interacts with RANKL. Additional networks regulating RANK/RANKL signaling are active in a context specific manner. RANK/RANKL signaling is essential for the differentiation of bone-resorbing osteoclasts, and is deregulated in pathological processes such as postmenopausal osteoporosis or cancer induced bone destruction. Cells expressing RANK and RANKL are commonly found in the tumor microenvironment. The RANKL/RANK pathway is often overexpressed in tumors of the breast, prostate, endometrium, cervix, stomach, oesophagus and bladder, thyroid and correlated with poor prognosis. RANK signaling plays an important role in the innate and adaptive immune response as it generates regulatory T (Treg) cells and increases production of cytokines. RANK expression induces chemoresistance in vitro through the activation of multiple signal transduction pathways. RANKL blockade improves the efficacy of anti-CTLA-4 monoclonal antibodies against solid tumors and experimental metastases. As RANK inhibition enhances the immune response there is an increasing interest in combining it with immune therapy in an attempt to sensitize immune resistant tumors to immune therapies. Several studies are ongoing to assess this concept. The role of RANK/RANKL inhibition should be further pursued as an immunomodulatory strategy in combination with other treatment modalities.

## 1. Background

Immune therapies have revolutionized oncology over the last years, particularly in patients with melanoma, Hodgkin lymphoma, thoracic end urothelial cancer and mismatch repair deficient tumors (Aspelt et al., 2018). However, with the exception of the first two diseases, only a minority of patients responds to immune therapy. Factors influencing the beneficial effect of immune therapies include mutational burden and neoantigen load, quality and clonality of neoantigens, expression of antigen presenting molecules and immune checkpoints, interferon gamma responsiveness and composition of the microenvironment (hot versus cold tumors (Aspelt et al., 2018). Although combinations of immune therapy (eg CTLA4 and PDL1 blocking) can be synergistic they do not resolve this problem and often induce significant additional toxicity (Santana-Davila and Show, 2018). Tumor cells have multiple

mechanisms for evading immune surveillance such as immunological processes influencing the regulatory T cell function, antigen presentation, modifying the production of immune suppressive mediators (eg VEGF, IKK2), tolerances and immune deviation (Vinay et al., 2015; Tilborghs et al., 2017; van Dam et al., 2016). Therefore there is an increasing interest in combining immune therapy with immune modulating drugs in order to sensitize immune resistant tumors to immune therapies (Casey et al., 2015). Recent data are suggesting that RANK/RANKL inhibition may be an attractive approach to increase the effectiveness of immunotherapy. Signaling between the receptor activator of nuclear factor- $\kappa$ B (RANK) and its ligand (RANKL) is essential for the differentiation of bone-resorbing osteoclasts, and is deregulated in pathological processes such as postmenopausal osteoporosis or cancer induced bone destruction (Santana-Davila and Show, 2018). However, cells expressing RANK and RANKL are also commonly found in the

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tumor microenvironment. RANK signaling plays an important role in the innate and adaptive immune response as it generates regulatory T (Treg) cells and increases production of cytokines. Denosumab is a fully human monoclonal antibody that binds the cytokine RANKL and blocks the interaction of RANKL with RANK (Hanley et al., 2012; Lacey et al., 2012). Denosumab is approved for the “prevention of skeletal related events (pathological fracture, radiation to bone, spinal cord compression or surgery to bone in adults with bone metastases from solid tumours” and for the treatment of osteoporosis (Castellano et al., 2011; Wada et al., 2006). The drug has been widely used alone and in combination with chemotherapy, radiotherapy and targeted drugs. It has an acceptable toxicity profile, making it attractive to be combined with other drugs. Side effects are absent or mild and in most cases easy to manage. Hypocalcemia and hypophosphatemia, muscle cramps, cellulitis, numbness and rarely osteonecrosis of the jaw have been described (Castellano et al., 2011). In the present paper the effects of RANK/RANKL signaling inhibition on the microenvironment of malignant tumors are reviewed. It is hypothesized that this approach may be used to improve the response to immunotherapy.

## 2. RANK/RANKL signaling

RANKL, a tumor necrosis factor alpha superfamily cytokine, was originally identified in T-cells and dendritic cells (DC). It is a type II homotrimeric membrane protein that is encoded by the TNFSF11 gene in humans and is also known as TNF-related activation induced cytokine (TRANCE), osteoprotegerin ligand (OPGL) or osteoclast differentiation factor (ODF). RANKL has three isoforms due to alternative splicing of the same gene (Ikeda et al., 2001; Lam et al., 2001; Luan et al., 2012). The full length RANKL is called RANKL1, while RANKL2 misses a part of the intracellular domain, and in RANKL3 the N-terminal part is deleted. RANKL can be present in a membrane or soluble form (Sigl et al., 2016). It is the only known ligand binding to a membrane receptor named receptor activator of nuclear factor- $\kappa$ B (RANK), a member of the TNF receptor superfamily (TNFRSF11A). RANK is a type I transmembrane protein with a large cytoplasmic domain containing four cysteine-rich repeat motifs and two N-glycosylation sites. Binding between RANKL and RANK induces receptor trimerization which triggers recruitment of TNF receptor associated factor (TRAF) adaptor proteins and activation of downstream signaling pathways (such as TRAF6, NF- $\kappa$ B, AKT/PKB, JNK, ERK, Src and the MAP kinase cascade) (Gonzalez-Suarez and Sanz-Moreno, 2016). RANK/RANKL signaling is controlled by a decoy receptor called osteoprotegerin (OPG) (TNFRSF11B) which interacts with RANKL (Fig 1). OPG lacks transmembrane or cytoplasmic domains, but is a soluble glycoprotein which can exist either as a 60-kDa monomer or as a 120-kD dimer. The dimerization of OPG is necessary for RANK/RANKL inhibition as it increases the affinity of OPG to RANKL dramatically (Schneeweis et al., 2005). OPG expression can be upregulated by several factors such as TRAIL, IL-1 $\beta$ , Wnt/ $\beta$  catenin signaling, TNF $\alpha$  and estrogen, and down regulated by TGF- $\beta$  and PTH (Millian, 2015). The final inhibitory effect of OPG on RANKL is dependent on its binding to these ligands (Renema et al., 2016; Theolaire et al., 2004). Recently, a new receptor for RANKL, LGR4, has been identified (Luo et al., 2016). LGR4 competes with RANK to bind RANKL and suppresses canonical RANK signaling. RANKL binding to LGR4 activates the G $\alpha$ q and GSK3- $\beta$  signaling pathway, an action that suppresses the expression and activity of nuclear factor of activated T cells and cytoplasmic, calcineurin-dependent 1 (NFATC1) during osteoclastogenesis. In addition functional RANK splicing variants have been identified in normal tissues and breast cancer cell lines suggesting that a complex network of additional levels of regulation beyond RANK, LGR4 and OPG exist (Papanastasiou et al., 2012).

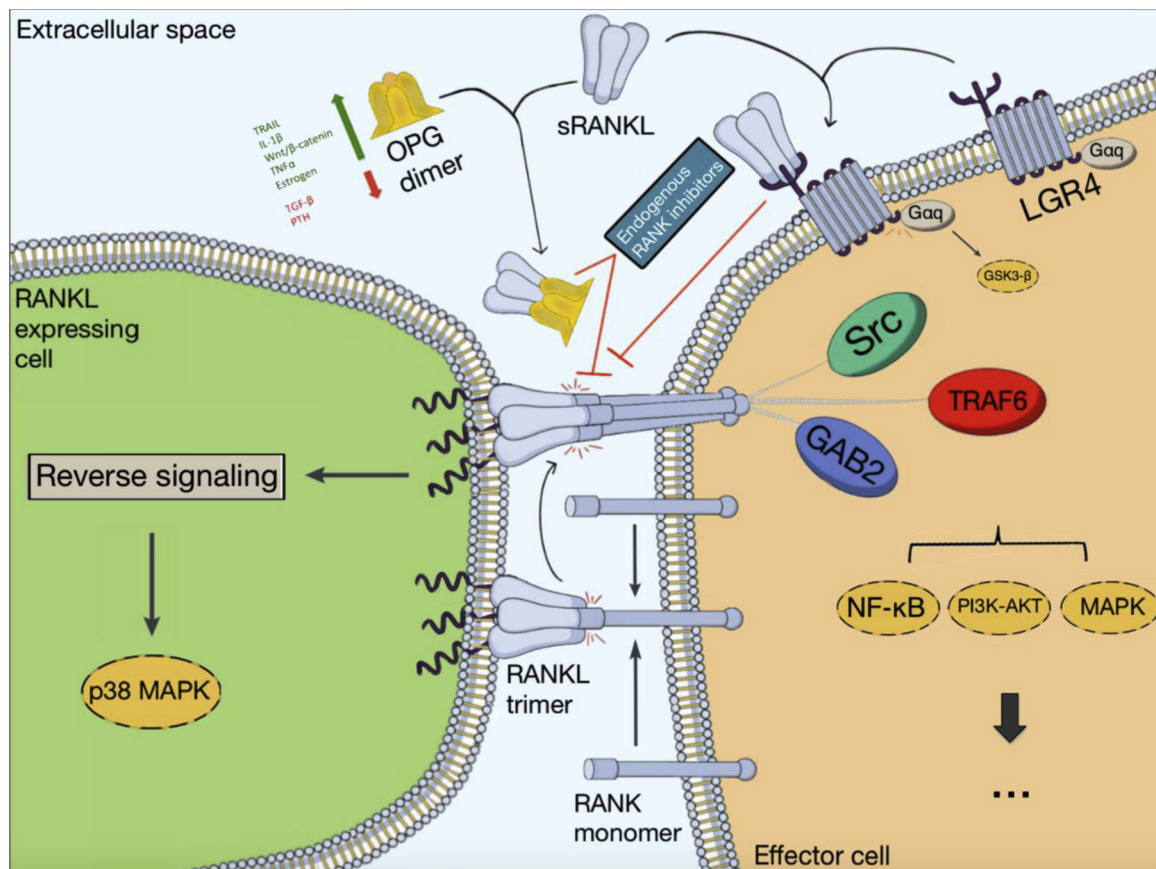
## 3. RANK/RANKL signaling and the microenvironment

RANK and RANKL expressing tumor cells are commonly found in

the tumor microenvironment (Chu and Chung, 2014). RANKL modulates the immune response by inducing T-cell proliferation and dendritic cell survival (Wong et al., 1997). In human breast carcinomas RANKL is found in tumor infiltrating lymphocytes (TILs) and RANK is strongly expressed in tumor associated macrophages (TAMs) (Gonzalez-Suarez et al., 2010). TAMs accumulate in the microenvironment and depending on their M2 or M1 phenotype are involved in tumor growth, angiogenesis and metastasis. RANKL acts as a chemoattractant for these cells. RANK/RANKL signaling in M2 macrophages modulates production of chemokines, promoting the proliferation of T regulating (Treg) lymphocytes thereby creating an immunosuppressive environment. As RANKL is mainly produced by Treg lymphocytes, a vicious circle is established in conjunction with the TAMs mainly expressing RANK (Tan et al., 2011). Tumor-infiltrating Treg cells have been shown to stimulate mammary cancer metastasis through RANKL-RANK signaling (Li et al., 2009). RANKL treatment enhances survival of mature dendritic cells (DCs) and triggers generation of proinflammatory cytokines (IL1, IL6, IL12) which can promote differentiation of CD4 + T cells into TH1 cells providing a major costimulatory factor for CD4 + T cell responses (Li et al., 2009; Chen et al., 2001). RANK is also expressed on NK cells, playing an important role in immunosurveillance. RANKL/RANK is involved in cross talk between the bone and immune systems making osteoclasts to function as antigen-presenting cells thereby activating CD4+ and CD8 + T cells (Zhang et al., 2017). A similar phenomenon is likely to be present in the microenvironment of solid tumors. The crosstalk of tumor cells with the immune system is not completely understood, but the impact of RANK-RANKL signaling on the tumor immune response is likely to be context specific (Tilborghs et al., 2017). Due to sequestering OPG by tumor cells or entrapment of OPG by the proteoglycans and glycosaminoglycans of the extracellular matrix, a microenvironment is created that facilitates the expansion of the tumor cells (Goswami and Sharma-Walia, 2016). In addition OPG binds TRAIL, a key natural pro-apoptotic and anticancer factor. By blocking TRAIL activity OPG can act as an antiapoptotic and pro-proliferative stimulus for cancer cells (Renema et al., 2016; Reid et al., 2009). It has been shown that RANK/RANKL signaling can promote the initial stages of cancer development by inducing stemness and epithelial mesenchymal transition (Palifox et al., 2012). RANKL (eg produced by osteoblasts or bone marrow stromal cells) attracts RANK-expressing cells and induces their migration by activation of specific signaling pathways such as the MAP kinase pathway (Liu et al., 2016). RANKL was also detected in endothelial cells has also been implicated in angiogenesis through a Src and phospholipase C-dependent mechanisms (Min et al., 2003).

## 4. The RANKL/RANK pathway in human cancer beyond bone metastasis

Several studies documented RANK signaling to be important in a variety of cancers. This was nicely reviewed by Renema et al and de Groot et al (Renema et al., 2016; de Groot et al., 2018). Mouse models and randomized studies in humans have shown that combination of antibodies blocking OPG or RANK with chemotherapy, hormone therapy or targeted drugs resulted in stronger decrease of tumor burden in the bone (de Groot et al., 2018). However, it recently became clear that inhibition of RANK signaling has a direct effect on tumor cells beyond the bone. Yamada et al could show that RANKL-overexpressing head and neck squamous cell carcinomas were grossly vascularized, and in a mouse model RANKL promoted tumor angiogenesis in a VEGF independent manner suggesting that VEGF-independent strategies blocking RANKL signaling should be taken into consideration to inhibit angiogenesis (Yamada et al., 2011). In animal non small cell lung cancer (NSCLC) models, denosumab delayed bone metastases and reduced skeletal tumor growth (De Castro et al., 2015). In a posthoc analysis of patients with lung cancer treated with denosumab because of bone metastases, denosumab prolonged overall survival by 1.2



**Fig. 1. RANKL/RANK signaling and its endogenous inhibition.** Binding between RANKL and RANK induces receptor trimerization, which triggers recruitment of TNF receptor associated (TRAF) adaptor proteins and activation of downstream signaling pathways (such as NF-κB, PI3K-AKT and the MAP kinase cascade) (Gonzalez-Suarez and Sanz-Moreno, 2016). This activity can be induced by either membrane-bound RANKL or soluble RANKL (sRANKL). sRANKL is derived from the membrane-bound form through alternative splicing or proteolytic cleavage (for example, by matrix metalloproteinase (MMP) or a disintegrin and metalloproteinase (ADAM) family members) RANK itself lacks kinase activity, and its signaling is initially mediated by adaptor molecules, such as TRAF proteins (including TRAF6), GRB-associated-binding protein 2 (GAB2), and SRC. The signaling cascade is controlled by a decoy receptor called osteoprotegerin (OPG) (TNFRSF11B), which interacts with RANKL. OPG lacks transmembrane or cytoplasmic domains, but is a soluble glycoprotein, which can exist either as a 60-kDa monomer or as a 120-kDa dimer. The dimerization of OPG is necessary for RANK/RANKL inhibition as it increases the affinity of OPG to RANKL dramatically (Schneeweis et al., 2005). OPG expression can be upregulated by several factors such as TRAIL, IL-1β, Wnt/β-catenin signaling, TNFα and estrogen, and down regulated by TGF-β and PTH (Millian, 2015). The final inhibitory effect of OPG on RANKL is dependent on its binding to these ligands (Renema et al., 2016; Theolaire et al., 2004). Recently, a new receptor for RANKL, leucine-rich repeat-containing G protein-coupled receptor 4 (LGR4), has been identified (Luo et al., 2016). LGR4 competes with RANK to bind RANKL and suppresses canonical RANK signaling. RANKL binding to LGR4 activates the Gαq and GSK3-β signaling pathway, an action that suppresses the expression and activity of nuclear factor of activated T cells and cytoplasmic, calcineurin-dependent 1 (NFATC1) during osteoclastogenesis. The endogenous inhibitors target both soluble and membrane-bound forms of RANKL. Additionally, reverse signaling following RANKL ligation has also been reported in studies using in vitro models of osteoblastogenesis and can modulate IFNγ secretion from CD4 + T cells through the p38 MAPK-dependent signalling pathway.

months versus zoledronic acid (De Castro et al., 2015). This issue warrants further investigation in order to elucidate whether the survival benefit is due to better control of bone metastases or related to a direct antitumor effect of denosumab. Naumik et al measured the concentration of OPG and soluble RANKL in bronchoalveolar lavage fluids of 44 NSCLC patients and 15 healthy volunteers taken as control subjects (Naumnik et al., 2018). The OPG content but not sRANKL was higher in the NSCLC group than that in controls. However, the greater the level of sRANKL in NSCLC patients, the shorter the overall survival. The RANKL/RANK pathway was variably expressed in tumors of the thyroid and increased serum OPG was also correlated with poor prognosis in gastric, cervical, oesophageal and bladder carcinoma (Heymann et al., 2008; Mizutani et al., 2004; Zhang et al., 2018; Shang et al., 2015; Ma et al., 2017). Song et al (2014) found that RANK expression was significantly higher in hepatocellular carcinoma (HCC) than in peritumoral hepatic tissue (Song et al., 2014). HCC cell lines express RANK constitutively, and activation of the RANKL-RANK axis significantly promoted migration and invasion ability of HCC cells in vitro. Recently it has been demonstrated that RANK/RANKL-expression is

significantly elevated in endometrial cancer tissue, particularly in tumors of higher stage. RANK levels were positively correlated to expression of N-Cadherin and negatively with E-cadherin suggesting that RANK/RANKL expression is related to epithelial-mesenchymal transition (EMT). Cell line evidence shows that the induced EMT is mediated by the cytokine CCL20 and activation of the MAPK pathway in endometrial cancer cells (Liu et al., 2016; Wang et al., 2015). The RANK-mediated signal network was also found to drive EMT in prostate cancer cells (Chu and Chung, 2014; Christoph et al., 2018). Christoph et al could show, using a PCR after microdissection of 71 prostate cancers, that RANK, RANKL and OPG are directly expressed by prostate cancer cells in the primary tumor and showed a clear correlation with Gleason Score, serum PSA level and advanced disease (Christoph et al., 2018). Schultz et al found that osteolytic prostate cancer cells induce the expression of RANKL in a STAT3-5 dependent manner (Schultz et al., 2014). Therefore there may be a role for RANKL inhibitors to suppress this inflammatory network.

Activation of RANK signaling promotes mammary tumorigenesis as MMTV-RANK transgenic mice are prone to develop mammary tumors

(Pellegrini et al., 2013). It has been postulated that paracrine signaling through RANK/RANKL is responsible for the expansion of mammary stem cells observed during pregnancy and luteal cycles (Gonzalez-Suarez and Sanz-Moreno, 2016; Cross et al., 2006). Pharmacologic inhibition of RANKL or genetic ablation of RANK reduces (particularly estrogen and progesterone receptor negative) mammary tumor and metastasis development in animal models (Ma et al., 2017). Breast cancer cells are able to produce RANKL and stimulate osteoclast differentiation when co-cultured with bone marrow stromal cells (Papanastasiou et al., 2012; Cross et al., 2006; Park et al., 2003). In human breast tumors, high RANK expression levels are associated with altered mammary differentiation, suggesting that increased RANK signaling may contribute to breast carcinogenesis (Gonzalez-Suarez and Sanz-Moreno, 2016). In a large prospective study including 1976 invasive breast cancer cases serum soluble RANKL (sRANKL) was quantified using an ELISA and serum OPG using an electrochemiluminescent assay. No solid evidence was found for an association between sRANKL and breast cancer risk (Sarink et al., 2017). However, tissue levels and not serum levels of components of the RANK/RANKL signaling network may be crucial factors involved. High levels of RANK were found in human primary breast adenocarcinomas that lack expression of the hormone receptors, in tumors with high pathologic grade and proliferation index, and are significantly associated with metastatic tumors. These results suggest that RANK expression in primary breast cancer is associated with poor prognosis (Pellegrini et al., 2013). The RANKL/RANK pathway also plays an important role in breast cancer progression (Palafox et al., 2012). It has been shown in vitro that HIF-1 $\alpha$  induced expression of RANKL initiates increased migration of breast cancer cells via PI3K/AKT signaling (Tang et al., 2011). Zhang et al studied the effect of Casitas B-lineage lymphoma (Cbl-b, an essential regulator of the RANKL/RANK pathway) on the prognosis of RANK-expressing breast cancer patients, as well as on RANKL/RANK pathway (Zhang et al., 2015). The results showed that RANK and Cbl-b expression was separately detected in 154 (154/300, 51.3%) and 165 (165/300, 55.0%) breast cancer tissue samples. In RANK-expressing breast cancer patients, Cbl-b expression was correlated with significantly lower metastasis rate, better disease-free survival (DFS) and breast cancer-specific survival (BCSS). To explore underlying mechanisms these authors performed functional in vitro studies revealing that Cbl-b down-regulated RANK protein expression and inhibited RANKL-induced breast cancer cell migration by negatively regulating the Src-Akt/ERK pathway. This suggests that Cbl-b improves the prognosis of RANK-expressing breast cancer patients by inhibiting RANKL-induced breast cancer cell migration and metastasis. Survival data of a group of 248 breast cancer patients from TCGA, revealed an EGFR<sup>hi</sup>/RANK<sup>hi</sup> subpopulation that showed a statistically significant ( $p = 0.001$ ) reduced overall survival when compared to EGFR<sup>low</sup>/RANK<sup>low</sup> group of patients (Papanastasiou et al., 2018). EGFR and RANK combinatorial in vitro analyses revealed a significant upregulation of AKT and ERK signaling after EGF stimulation in cell lines and also an increase of breast cancer cell invasiveness.

The adjuvant effect of RANK/RANKL inhibition in patients with breast cancer remains unclear. In a recent analysis of the ASBSG-18 study (a prospective study of 3425 patients with hormone sensitive early breast cancer treated with an adjuvant aromatase inhibitor which were randomized to receive placebo or denosumab 60 mg subcutaneously every six months for 5 years) it was shown that disease free survival was significantly better ( $p = 0.026$ ) in the denosumab group at 5 (89.2% vs 87.3%) and 8 years follow-up (80.6% vs 77.5%) (Gnant et al., 2018). A cohort study including 37,604 female MEDICARE beneficiaries confirmed that patients receiving at least 6 months of an oral bisphosphonate, two doses of ibandronate or one dose of zoledronate or denosumab at doses equivalent or higher to those approved for osteoporosis, during the first two years of BC diagnosis, had a significantly better overall survival (81% vs 77%) (Herrera Pena et al., 2018). In the DCARE study 4509 patients with high risk early breast cancer had

either standard (neo)adjuvant therapy with or without denosumab (120 mg SC every month for 6 months, then 3 monthly up to 5 years). Twenty percent of these patients were HER-2 positive, and 95.9% of the recruited population had anthracycline and/or taxane chemotherapy. Denosumab did not improve bone metastasis free survival, disease free or overall survival in the postmenopausal subset (Coleman et al., 2018). In summary, current data suggest that in a postmenopausal population which is only treated with an aromatase inhibitor there is a clear benefit of treatment with denosumab, but this is not the case when adjuvant chemotherapy is given. Mechanistically one can hypothesize that chemotherapy reduces some of tumor suppressive effects of RANK/RANK inhibition in the cancer microenvironment.

## 5. RANK/RANKL signaling and chemo- or radiotherapy

The role of the RANK/RANK signalling in drug resistance remains unclear. Tsubaki et al found that treatment with RANKL induced drug resistance in RANK-expressing but not RANK-negative cell lines (Tsubaki et al., 2016). RANKL stimulation of RANK-expressing cells increased multidrug resistance protein 1 (MDR1), breast cancer resistance protein (BCRP), and lung resistance protein 1 (LRP1) expression and decreased BIM expression through various signaling molecules. RNA silencing of BIM expression induced drug resistance, but the RANKL-mediated drug resistance could not be overcome through the RNA silencing of MDR1, BCRP, and LRP1 expression. These results indicate that the RANK/RANKL system induces chemoresistance through the activation of multiple signal transduction pathways (Sisay et al., 2017). In a mouse model RANKL blockade increases the efficacy of cisplatin chemotherapy (Faget et al., 2018). Thimodeaou et al evaluated early breast cancer patients, who had been treated with anthracycline-based chemotherapy within two randomized trials, by quantitative reverse transcription–polymerase chain reaction on 819 formalin-fixed paraffin-embedded tumor tissue samples for mRNA expression of RANK, OPG, and RANKL (Timothéadou et al., 2017). In the univariate analysis, low RANKL mRNA expression was found to be an unfavorable factor for DFS [hazard ratio (HR) = 1.33, 95% confidence interval (CI) 1.05–1.68, Wald's  $P = .018$ ] and bone metastasis-free survival (HR = 1.67, 95% CI 1.09–2.56,  $P = .018$ ), although it did not retain its significance in the multivariate analysis. RANK/OPG, RANKL/RANK, and RANKL/OPG ratios (using the median value as a cutoff) were not associated with DFS, OS, or bone metastasis-free survival. One can conclude that at the moment there is no objective evidence that RANK/RANKL signaling inhibition has an influence on the effectivity of chemotherapy in humans.

Treatment with RANKL resulted in marked protection from cell death in response to  $\gamma$ -irradiation and doxorubicin (Schramek et al., 2010). Schramek et al could show that  $\gamma$ -irradiation-induced upregulation of the pro-apoptotic molecules BIM, PUMA and NOXA did not occur in the presence of RANKL. Loss of RANK expression on mammary epithelial cells abrogated the protective effects of MPA on  $\gamma$ -irradiation-induced cell death (Schramek et al., 2010). There are no clinical studies addressing the adjunct effect of RANKL inhibitors to radiotherapy. Although associated with several methodological, practical and ethical challenges, randomized controlled trials are needed.

## 6. RANK/RANKL signaling and checkpoint inhibition

In 2014 Smyth et al described a case of a rapidly advancing metastatic melanoma with aggressive and symptomatic bone metastases requiring treatment with the anti-RANKL antibody denosumab for palliation in a patient who was concomitantly treated with the anti-CTLA-4 antibody ipilimumab (Smyth et al., 2016). She had a dramatic partial response and was alive at 62 weeks. Using the B16F10 melanoma preclinical model of experimental metastases, these authors could demonstrate that anti-CTLA-4 and anti-RANKL monoclonal antibodies (mAbs) have modest antimetastatic activities when used as

monotherapy, but metastases suppression was considerably enhanced when these drugs were combined at the time of intravenous melanoma inoculation. The combined effect of anti-CTLA-4 and anti-RANKL is dependent on lymphocytes, as treatment was completely ineffective in RAG2<sup>-/-</sup>γc<sup>-/-</sup> mice lacking all lymphocytes and in mice specifically depleted of natural killer cells. Based on the above observations, Afzal and Shirai evaluated in a retrospective analysis the synergistic effect of immune checkpoint inhibitors and denosumab in metastatic melanoma patients (Afzal and Shirai, 2018). Eleven (29.72%) out of 37 patients assessed received immune checkpoint inhibitors and denosumab, and the others only immune checkpoint inhibitors. Median progression-free and overall survival in the cohort having the combination treatment respectively was 11.6 and 57 months compared with 4.15 and 22.8 months in the controls. Although there are potential biases, this suggests that adding denosumab to immune checkpoint inhibitors may have a beneficial effect on outcome. In an attempt to unravel the underlying mechanisms of this observation, Ahern et al studied the efficacy of a combination of RANKL and CTLA-4 blockade by analysis of tumor-infiltrating lymphocytes, tumor growth, and metastasis in a model using a variety of neutralizing antibodies and genotyped mice (Ahern et al., 2017). RANKL blockade improved the efficacy of anti-CTLA-4 mAbs against solid tumors and experimental metastases. Treg-depleting anti-CTLA-4 mAbs of the mouse IgG2a isotype showed the highest combinatorial activity. The optimal combination depended on the presence of activating Fc receptors and lymphocytes (particularly natural killer and CD8<sup>+</sup> T cells), whereas anti-RANKL alone did not require Fc receptors. The significantly higher T-cell infiltration into solid tumors post anti-RANKL and anti-CTLA-4 was accompanied by increased T-cell effector function. In the recently opened CHARLI trial (NCT03161756) and KEYPAD (NCT03280667) study denosumab in combination with immune checkpoint inhibitors will be evaluated in patients with unresectable or metastatic melanoma and renal cell carcinoma respectively. Two translational studies are under way to study the impact of denosumab on the systemic immunity and local immunologic microenvironment in detail: the PERIDENO (NCT03532087) study in postmenopausal patients with HER2 negative breast cancer in combination with AC-T chemotherapy and our neoadjuvant study with denosumab monotherapy in cervical cancer (ISS 20,177,041).

It is clear that RANK/RANKL inhibition has multiple important effects on the immune environment which may be context specific and difficult to entangle. Neoadjuvant studies could be crucial to unravel its role in a clinical context (van Dam et al., 2017). Immunotherapy can induce spectacular responses, often lasting for a considerable time, but is effective in only in a minority of patients. One of the major challenges for future research is to increase the applicability and effectiveness of this attractive treatment modality by making the tumor more immunogenic. Induction of the abscopal effect by radiotherapy, the use of medication changing the microenvironment and the development of new biomarkers are amongst the potential options which should be explored (van Dam et al., 2018; Tuyraerts et al., 2018).

## 7. Conclusion

The role of RANK/RANKL inhibition should be further pursued as an immunomodulatory strategy in combination with other treatment modalities. The effects of RANK/RANKL signaling inhibition on the microenvironments needs further attention beyond the bone. As denosumab has clear immune stimulating effects and an interesting toxicity profile the drug has an interesting potential to be explored for this indication.

## Authors' contributions

PAvD designed the paper and performed the data acquisition and analysis. Interpretation of the data and drafting and revision of the

manuscript was performed by all authors.

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## Ethics approval and consent to participate

Not relevant for this paper.

## Availability of data and material

References available.

## Consent for publication

All authors consent for publication.

## Competing interests

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