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Oestrogen and zoledronic acid driven changes to the bone and immune environments: Potential mechanisms underlying the differential anti-tumour effects of zoledronic acid in pre- and post-menopausal conditions

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ABSTRACT

Late stage breast cancer commonly metastasises to bone and patient survival averages 2–3 years following diagnosis of bone involvement. One of the most successful treatments for bone metastases is the bisphosphonate, zoledronic acid (ZOL). ZOL has been used in the advanced setting for many years where it has been shown to reduce skeletal complications associated with bone metastasis. More recently, several large adjuvant clinical trials have demonstrated that administration of ZOL can prevent recurrence and improve survival when given in early breast cancer. However, these promising effects were only observed in post-menopausal women with confirmed low concentrations of circulating ovarian hormones. In this review we focus on potential interactions between the ovarian hormone, oestrogen, and ZOL to establish credible hypotheses that could explain why anti-tumour effects are specific to post-menopausal women. Specifically, we discuss the molecular and immune cell driven mechanisms by which ZOL and oestrogen affect the tumour microenvironment to inhibit/induce tumour growth and how oestrogen can interact with zoledronic acid to inhibit its anti-tumour actions.

1. Introduction

Late stage breast cancer commonly metastasises to bone, lung, liver and brain with bone being the predominant site. There are currently no curative treatments for metastatic breast cancer and patient survival averages 2–3 years following initial diagnosis of bone involvement [1]. One of the most successful bisphosphonates for the treatment of bone metastases is zoledronic acid (ZOL). This drug has several roles in breast cancer management not just in the advanced metastatic setting but also in the early curative setting. In the advanced setting, ZOL has been shown to reduce the skeletal complications associated with bone metastases. In the early setting, ZOL prevents the bone loss that is associated with curative systemic cancer therapies, reducing bone loss and fracture rates in post-menopausal women, and the high level of treatment-induced bone loss in premenopausal women.

More recently, the efficacy of bisphosphonates to prevent breast cancer recurrence and improve survival, when given in early breast cancer, has been evaluated in large adjuvant breast cancer trials. These trials have demonstrated promising results for post-menopausal women (natural or biochemical) showing that these drugs are effective at preventing recurrence, however, the same treatment results in worse outcome and increased recurrence in pre-menopausal women [2–6]. During the menopause there are dramatic shifts in circulating concentrations of steroid hormones with reduced oestrogen, inhibin and concomitant increases in follicle stimulating hormone (FSH) [7]. Evidence from clinical and pre-clinical studies indicate that, in an adjuvant setting, the anti-tumour efficacy of ZOL correlates specifically with circulating concentrations of oestrogen, we therefore hypothesise that oestrogen can inhibit anti-tumour effects of this bisphosphonate. In this review we focus on clinical evidence that oestrogen is the key hormone that influences the anti-tumour effects of ZOL and the pre-clinical evidence describing the physiological mechanisms driving this phenomenon. Specifically, we discuss the molecular and immune cell driven mechanisms by which ZOL and oestrogen affect the tumour microenvironment to inhibit/induce tumour growth and how oestrogen can interact with zoledronic acid to inhibit its anti-tumour actions.

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2. Evidence from clinical studies

2.1. Incidence/prevalence of bone metastasis after early breast cancer according to menopausal status

Bone is the most common site of metastasis in advanced breast cancer with almost 70% of metastatic patients experiencing bone involvement [8]. Although, these patients have considerably increased morbidity and decreased survival and quality of life, a recent study showed that women with only bone metastases have better survival compared to women with visceral metastases [9]. This study included 7064 women with breast cancer who were diagnosed between 1975 and 2006 at Guy's Hospital in London, UK and were followed up for disease recurrence patterns until the end of 2010. Results showed that the incidence of bone metastases was higher in women younger than 40-year-old compared to the rest of the age groups (29.6% < 40-year-old, 23% 40–49-year-old, 25.1% 50–59-year-old, 21.7% 60–69-year-old and 15.3% > 70-year-old) and significantly higher in women who were diagnosed with breast cancer before the age of 40 compared with those who were diagnosed when over 70. Earlier data from Braun et al., in which they evaluated data from 9 different studies (n = 4703 patients in total) assessed the importance of micro-metastasis in bone marrow aspirates at the time of early breast cancer diagnosis of women with stage I, II and III disease [10]. The study indicated that the incidence of bone marrow disseminated tumour cells (DTC) was higher in pre-menopausal women and that the bone marrow of these women may be more attractive to tumour cells than the bone marrow of post-menopausal women (32.7% and 29.5% respectively; P = 0.02). Furthermore, a greater significance was seen when comparing age with disseminated tumour cells in bone. Tumour cells were detected in the bones of 34.5% of 20–35 year olds compared with 27.8% of women over 65 (P = 0.001). Taken together, these data imply that the longer a woman has been post-menopausal the less attractive the bone microenvironment may be for tumour cells [10].

2.2. Use of zoledronic acid to treat disseminated tumour cells (DTCs) in bone according to menopausal status

Breast cancer patients can experience recurrence of their disease many years after the removal of the primary breast cancer. This is due to the long-term survival of DTCs which stay dormant for years before they are stimulated to proliferate into overt metastases. Studies showed that 30–40% of breast cancer patients have DTCs in their bone marrow and the presence of these are an adverse prognostic risk factor for distant metastasis and mortality [10]. DTCs have been detected following cytotoxic chemotherapy treatments, demonstrating their ability to resist conventional treatments and their persistence post chemotherapy is strongly associated with poor outcomes [11 12]. Therefore, multiple clinical studies have evaluated the effects of alternative treatments and more specifically the effects of bone targeted agents, bisphosphonates, on DTC survival in bone, with promising results.

In a non-randomised phase 2 trial, 31 women (36.9% pre-menopausal/63.1% post-menopausal) with early breast cancer and persistent DTCs in their bone marrow, received 4 mg of ZOL 4 weekly for 6 months, after the completion of their adjuvant treatment compared with 141 controls (41.9% pre-menopausal/58.1% post-menopausal) [13]. Follow up bone marrow aspirations showed elimination of DTCs in 87% of ZOL treated patients compared to 73% of controls, but the reduction in the total number of DTCs between the first (pre ZOL) and the second (6 months post ZOL) bone marrow aspirate was statistically significant only in ZOL group (p = 0.02 vs p = 0.14). The elimination of DTCs in control group was thought to be due to immune related clearance as well as the anticancer systemic and endocrine treatment that patients received. Also, negative aspirations were associated with better survival. In 2013, in a randomised controlled open-label multicentre study, 86 women with DTC-positive bone marrow were randomised to receive standard adjuvant systemic treatment with or without ZOL every 4 weeks for 24 months (35% pre-menopausal/65% post-menopausal and 37% pre-menopausal/63% post-menopausal respectively) [14]. After the completion of the ZOL treatment, all treated patients (n = 40) had negative bone marrow results compared to 16% of controls who had persistent DTCs in their bone marrow (n = 46). Effects of ZOL were also assessed in combination with neoadjuvant treatment in a phase-2 randomised single centre study [15] Women with early breast cancer were allocated to receive ZOL 4 mg every 3 weeks (n = 60) or no ZOL (n = 60), for 12 months, along with 4 cycles of neoadjuvant epirubicin plus docetaxel and 2 cycles of adjuvant epirubicin and docetaxel. After 3 months of treatment, the number of patients with detectable DTCs reduced more in the ZOL group compared to the chemotherapy only group. Also, it was more likely for the ZOL treated patients to remain DTC negative after this period of time. However, at 12 months, there was no difference in the number of DTC-positive patients between the two groups most likely due to the small number of patients or the different adjuvant treatments that patients received after the first 3 months. The largest study which investigated the impact of ZOL on DTCs, was published in 2014 [16]. 3141 patients with early breast cancer underwent bone marrow aspirates and 803 of them had detectable DTCs. Both DTC-positive and negative patients received systemic treatment and adjuvant ZOL based on national guidelines (bisphosphonates were also offered in the context of 3 clinical studies, GAIN, NATAN and SUCCESS A). Retrospective analysis showed that bisphosphonates were associated with better DFS (disease free survival) and OS (overall survival) in DTC-positive participants. Although, all of the studies that have been discussed here have included both pre- and post-menopausal women, Hartkopf et al. was the only one that performed subgroup analysis in order to identify the effects of bisphosphonates on DTCs based on the menopausal status. Post adjuvant bisphosphonate treatment, DFS was found to be better in both pre- and post-menopausal women with detectable DTCs (p = 0.018 and p = 0.014 retrospectively), in contrast with OS which was better only in post-menopausal women with detectable DTCs (p = 0.009). However, these results should be interpreted with caution due to the retrospective nature of the study also due to the fact that patients who received bisphosphonates were mostly post-menopausal with detectable DTCs [16].

Despite the fact that clinical studies have illustrated the benefits of ZOL on elimination of DTCs from bone marrow of women with early breast cancer, more trials are needed in order to confirm its clinical benefits in pre-menopausal patients. Additionally, available studies have either had a small number of participants or not been randomised which makes interpretation of their outcomes more difficult and the need for further trials more crucial.

2.3. Use of bisphosphonates to prevent disease recurrence and improve survival according to menopausal status

Adjuvant systemic treatment is offered to patients with early breast cancer with the aim to eradicate any micrometastases present at the time of diagnosis. Adjuvant bisphosphonates are now part of most standard adjuvant anticancer treatment assisting the elimination of micrometastases and increasing the chances of cure in post-menopausal women following the results of large phase 3 trials and a patient data meta-analysis.

The clinical benefit from the inclusion of bisphosphonates in adjuvant treatment was first described by GAIN, the Danish collaborative study, ABCSG-12 and the AZURE trial [2,4,17,18]. These trials reported improvement in disease free survival (DFS) with adjuvant bisphosphonates in women with low levels of systemic female hormones (oestrogen and inhibin A) at the start of the adjuvant breast cancer treatment.

In ABCSG-12, 1803 pre-menopausal women received adjuvant goserelin with endocrine treatment (tamoxifen or anastrozole), with or

without ZOL (4 mg every 6 months for 3 years) and after 94.4 months of medial follow up, relative risks of disease recurrence was reduced in the ZOL arm [6]. When the data form the AZURE, trial was published (3360 women randomised to receive standard adjuvant systemic treatment with or without ZOL (4 mg every 3–4 weeks for six doses, then every 3 months for eight doses, followed by every 6 months for five doses, for a total period of 5 years), the overall results showed that DFS and OS were similar in both ZOL and control group. However, women who were 5 years into menopause appeared to benefit, with IDFS (invasive disease free survival) of 78.2% in the ZOL arm compared to 71% in the control arm, and OS at 5-year of 84.6% and 78.7% in ZOL and the control group, respectively [3]. ZO-FAST, another large study, which was designed to assess ZOL as a bone protector in post-menopausal patients on adjuvant aromatase inhibitors (AIs) for early breast cancer, also reported less disease recurrences in the ZOL group (4 mg 6 monthly for 5 years) [19].

In 2015, the early Breast Cancer Trial Collaborative Group (EBCTCG) published a meta-analysis of 26 randomised trials of adjuvant bisphosphonates in early breast cancer which included data from 18,766 women (EBCTCG, 2015) [20]. In the combined, meta-analysis, the most apparent effect of bisphosphonates was in bone recurrence, irrespective of menopausal status. However, subgroup analysis revealed a clear benefit in post-menopausal women with decreased overall recurrence (RR 0·86, 95% CI 0·78–0·94; 2p = 0·002), distant recurrence (RR 0·82, 0·74–0·92; 2p = 0·0003) and mortality (RR 0·82, 0·73–0·93; 2p = 0·002).

In pre-menopausal patients, data from AZURE and ABCSG-12 and the subsequent meta-analysis suggested benefit from adjuvant bisphosphonates in those patients who were on ovarian suppression therapy at the start of their bisphosphonate treatment. In contrast, pre-menopausal women rendered post-menopausal due to chemotherapy did not have the same benefit from bisphosphonates. This suggests that menopausal status at the initiation of adjuvant bisphosphonates is important. It has also been hypothesised that early interaction between bisphosphonates and endocrine/paracrine factors in the bone microenvironment may impact the survival of DTCs in the bone marrow microenvironment [21].

2.4. Can ZOL influence tumour cells outside bone according to menopausal status?

Neo-adjuvant systemic chemotherapy, given to women with early breast cancer prior to their breast cancer surgery aims to downsize the primary breast tumour to make women who are inoperable operable and/or avoid of mastectomy. This treatment strategy also enables sensitivity to systemic treatment to be assessed and pathological complete response (pCR) following neo-adjuvant chemotherapy is associated with better long-term outcomes [22].

The use of ZOL as part of the neo-adjuvant chemotherapy and its effects on response were summarised in a meta-analysis of four randomised neo-adjuvant trials [23]. Pathological complete response in the breast tumour (pCRb) and proximal lymph nodes (pCR) of women with stage II/III early breast cancer, were assessed in 735 and 552 women, respectively treated with ZOL compared to control. Outcome from the overall population analysis did not show any benefit in pCRb or pCR from the inclusion of ZOL in the neo-adjuvant systemic treatment, however, in the subgroup analysis according to menopausal status, post-menopausal women tended to have better pCRb (10.8% versus 17.7%, OR 2.14, 95% CI 1.01–4.55, p = 0.048) and pCR (7.8% versus 14.6%, OR –2.62, 95% CI 0.90–7.62, p = 0.076) with ZOL. However, the data was not robust or sufficient enough in order to change the standard of care in the neo-adjuvant setting. Further research with longer follow ups and larger samples is necessary to provide a clearer picture of the role of ZOL in influencing pCR rates dependent upon menopausal status.

2.5. Influence of bisphosphonates on established metastatic disease in bone according to menopausal status

In advanced breast cancer with bone metastasis, bisphosphonates have been shown to reduce the risk for skeletal related events (SREs) and also to prolong the onset of SREs, when they were combined with standard anticancer treatment [24]. In two large meta-analyses (n = 2189, n = 2806), bisphosphonate treated breast cancer women with bone metastases had lower risk of developing SREs (17% and 15% respectively), in comparison to placebo treated group [25,26]. The majority of clinical studies have focused on the use of ZOL in the metastatic setting with a Japanese study (n = 228) demonstrating 39% reduction in SREs with ZOL in contrast to placebo [27]. In all the published studies, ZOL and bisphosphonates in general, were used in pre- and post-menopausal, and their ability to prevent SREs in metastatic breast cancer does not seem to be affected by the menopausal status.

3. Evidence from pre-clinical studies

3.1. Effects of zoledronic acid on tumour growth in the bone microenvironment under pre- and post-menopausal conditions.

Tumour cells are thought to home to specific metastatic niche(s) within the bone; the endosteal niche (made up of osteoblasts and haematopoietic stem cells) and the peri-vascular niche [28–31]. Once disseminated in these niches' tumour cells are held in a dormant state until either physiological/biological stimulus cause expansion of the niche or dysregulation in immune response which enables outgrowth of these disseminated tumour cells into overt metastasis. Once tumours start to proliferate in bone they release growth factors that stimulate maturation of osteoclasts leading to osteoclastic bone resorption, in turn the resorbed bone releases tumour stimulating growth factors that further stimulate tumour growth, a process commonly known as the vicious cycle (reviewed in [32]). ZOL is a potent third generation bisphosphonate with high affinity to bone. Its primary mechanism of action is to prevent osteoclast maturation and cause osteoclast apoptosis via inhibition of the mevalonate pathway, thereby inhibiting progression of the vicious cycle [33]. Because of the strong feedback mechanism that exists between osteoclasts (required for resorption) and osteoblasts (required for bone generation) reducing osteoclast activity with ZOL also results in reduced osteoblast activity thereby inhibiting expansion of the endosteal niche and tumour growth in this site (Fig. 1). Furthermore, through inhibition of the mevalonate pathway in endothelial cells, ZOL inhibits sprouting of new blood vessels preventing expansion of the peri-vascular niche [34]. Despite these anti-cancer properties in bone, evidence from preclinical models have demonstrated that ZOL is only able to exert optimal anti-tumour effects under low circulating concentrations of ovarian hormones including oestrogen. In mouse model systems, ovariectomy (OVX) induced menopause increased bone resorption and triggerd the growth of disseminated, MDA-MB-231, breast cancer cells in bone. Administration of ZOL prevented OVX induced increases in bone resorption and growth of disseminated cancer cells into the bone but had no effect on tumour growth in the bones of pre-menopausal (sham operated) mice [35]. These results support the findings observed in the AZURE, ABCSG-12, and ZO-FAST clinical trials where adding ZOL to standard of care therapy only benefitted women with established menopause and thus low circulating ovarian hormones [36].

Initially, the mechanism for the differential anti-tumour effects of ZOL under pre- and post- menopausal conditions was proposed to be mediated by altered osteoclast activity driven by menopause related hormones [35]. Deprivation of key ovarian hormones (including oestrogens) following OVX results in a concomitant increase in Receptor activator of nuclear factor kappa-B (RANK) ligand (RANKL) in the bone microenvironment and subsequent stimulation of osteoclast activity

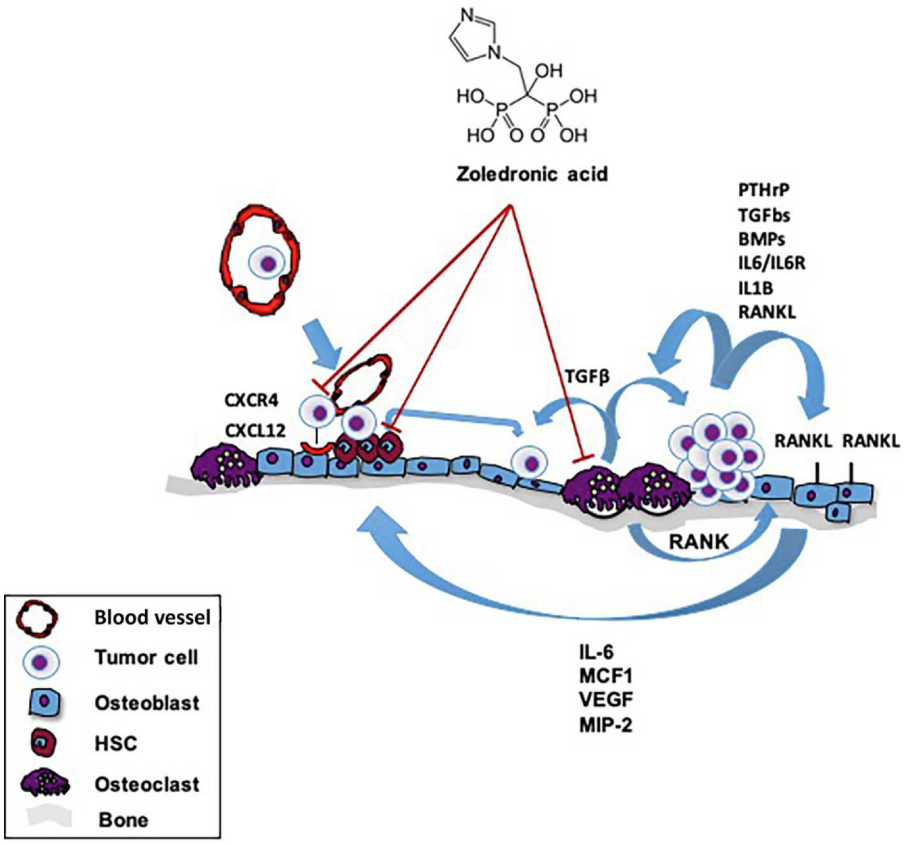

Fig. 1. Effects of Zoledronic acid on the bone metastatic niche and the "vicious cycle of bone metastases". Breast cancer cells arrive in the haematopoietic stem cell and peri-vascular niches in bone. Stimulation of these niches promotes metastatic outgrowth of tumour cells which emit growth factors stimulating activity of osteoclasts and osteoblasts. In turn, bone resorption leads to the release of bone bound growth factors into the local microenvironment that further stimulate tumour growth and expansion of the metastatic niches. ZOL interrupts this process, directly inhibiting expansion of the metastatic niches and impeding activity of osteoclasts through inhibition of the mevalonate pathway. Blue arrows indicate pro metastatic processes, red bars indicate metastatic inhibition by ZOL. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[37,38]. The RANK-RANKL pathway is essential for osteoclast maturation, activation and lifespan maintenance, and disruption of this pathway was shown to disrupt osteoclastic bone resorption and prevent outgrowth of disseminated tumour cells in the bones of OVX mice [39], highlighting the importance of osteoclasts in bone metastasis. However, data obtained from in the clinic has led to the hypothesis that other cell types may be equally or more important than osteoclasts in driving tumour growth in bone; In the ABCGS-18 trial in which all patients were post-menopausal administration of 60 mg of the RANKL specific antibody, Denosumab, every 6-months reduced disease recurrence [40]. But in the larger, D-Carestudy where the dose of denosumab was increased to 120 mg and administration was more frequent no reductions in disease recurrence were observed irrespective of the patients menopausal status [41]. These data suggest that reduction but not removal of osteoclast activity is beneficial to prevent bone metastases [42]. Indeed, there are a large number of bone marrow cell types, including those of osteoclastic linages that express oestrogen receptors and have been shown to also promote a resorptive phenotype under low oestrogen conditions [43]. In addition, low oestrogen is associated with an increase of inflammatory molecules in the bone microenvironment such as interleukin (IL) -1, IL-6, and Tumour Necrosis Factor-alpha (TNF-α), which have a direct effect on osteoclast activity [44]. Therefore, the effect of ZOL in the bone microenvironment might depend on a number of different factors associated with oestrogen and the menopausal status, not just the actions on the osteoclast activity.

3.2. Effects of ZOL in bone microenvironment

Due to its high resemblance to a pyrophosphate molecule, ZOL binds strongly to areas of new remodelling where the hydroxyapatite is exposed. Although serum concentrations of ZOL decrease rapidly over a period of few hours, its presence in bone can last several years [45]. Once ZOL is bound to the hydroxyapatite, it gets released during the remodelling process by active osteoclasts. Uptake of ZOL by the osteoclast leads to inhibition of farnesyl diphosphate synthase, a critical enzyme in the mevalonate pathway, preventing post-translational prenylation of GTPases such as RAP and RAS resulting in apoptosis of the osteoclast via caspase 3 activation [45].

In the bone environment, ZOL has modulatory effects beyond those observed in osteoclasts, including reducing the vascular niche, inhibiting maturation of bone cell progenitors and killing mature cells. The bone is a highly vascularised tissue in which the vascular niche is closely linked with bone maintenance and tumour cell dormancy/outgrowth. ZOL has been shown to decrease the number of human vascular endothelial cells, *in vitro*, in a dose dependent manner through increased cellular apoptosis [34]. In addition, ZOL reduces vascular endothelial cell activity and migration and changes in the morphology of this cell type [34]. Although the mechanisms of how ZOL induced these changes were not investigated, it is likely that the mevalonate pathway was the main target of this drug on the vascular endothelial cells. These findings, however, were not recapitulated in *in vivo* models where blood vessels are only exposed to ZOL for short periods of time as would be the case in breast cancer patients [46]. When looking at the vasculature and surrounding perivascular cell populations in BALB/c nude mice mature mice (mimicking a pre-menopausal situation) no significant differences in the number of vessels of ZOL treated and untreated animals were observed. Although *in vitro* and *in vivo* results contrast each other, the *in vivo* study focused solely on osteoprogenitor-related vessels (type-H) and activity and viability of these vessels were not assessed [47]. A similar study performed in younger (4-week old) immunocompetent mice (mimicking a low oestrogen environment) showed increased numbers of vessels following treatment with ZOL [48] no alteration in activity were observed. However, in adult mice (4-month old; mimicking pre-menopausal conditions) no significant differences in blood vessels were observed between the ZOL treated and control groups. Taken together, these results suggest that ZOL does not

alter the functionality or number of vessels in mature, pre-menopausal mice (after 12-week old), however, ZOL has an increased effect in young mice (4 and 6-week old) which may be in correlation with the high level of development of the mouse skeletal tissue during this period and or associated with low circulating concentrations of oestrogen in pre-pubescent animals. In patients populations, ZOL has been reported to reduce the serum concentrations of VEGF, therefore, a trend towards a decrease number of blood vessels would be expected [49], however, no studies have been carried on human tissues to further confirm whether ZOL increases or has no effect on the bone vasculature or if this bisphosphonate has different effects in pre- and post-menopausal women.

ZOL also directly affects haematopoietic and mesenchymal niches in the bone microenvironment. Using an immunocompetent mouse model, Soki et al., demonstrated that Haematopoietic stem cells are significantly increased in number after a treatment with ZOL but their mobility and re-localisation patterns remain unaltered [48]. It was previously assumed that the haematopoietic niches where highly dependent on osteoclasts cells for the niche mobilization to the periphery from the bone marrow cavities. This mechanism was based on the formation of the bone marrow cavity by an active osteoclast, therefore, after exposure to ZOL and subsequent impaired osteoclastic activity, it would be hypothesised that mobility of the haematopoietic niche would be reduced [50,51]. However, these results indicate that mobilization may not be dependent on osteoclast activity but rather an alternative mechanism to be determined. In contrast, it is the mesenchymal niche where a decrease in the number of osteoprogenitors (Osteoblast-specific transcription factor Osterix (Osx) positive) and an increment in earlier, less committed, osteoprogenitors is observed following administration of ZOL [46]. There is substantial data showing that ZOL reduces osteoblast activity, overall numbers of osteoblasts in bone and genes associated with osteoblast formation [35,52]. However, this is likely to be an oversimplification and it appears that under the selective pressure of ZOL the osteoblasts that remain in bone re-locate to areas in bone where they are most needed. In mouse models, the numbers of osteoblasts significantly increased after a single dose of ZOL but only in regions of intense bone remodelling, commonly the metaphysis in long bones (tibia and femur). Whereas in regions of low rates of bone remodelling, such as cortical bone or low trabecular (for tibia), the number of osteoblasts is significantly decreased [46,53]. All of this evidence point to ZOL having dramatic effects on the bone microenvironment, inhibiting bone resorption and limiting expansion of the bone metastatic niches, these are processes thought to be responsible for reducing the ability of tumour cells to colonise and grow in bone. It has been postulated that oestrogen may inhibit ZOL from exerting these anti-tumour effects through its own bone anabolic actions.

3.3. Effects of low oestrogen in the bone microenvironment

Oestrogens are a group of steroidal hormones of which the most abundant in women and rodents is oestrogen. Oestrogens play key roles in the development and maintenance of the skeletal tissue [43]. Oestrogen acts through the dimerization of two different types of receptors, oestrogen receptor-alpha (ERα) or -beta (ERβ). These receptors are widely expressed in a great number of cell types within the bone microenvironment and their function depends on the type of receptor activation, the level of ER expressed by the cell, the cell type, and/or the nature of ER-dependent promoter regulatory sequences [43]. For instance, physiological concentrations of oestrogens extend the lifespan of osteoblasts mainly by activation of ERβ, whereas they will decrease osteoclast lifespan via activation of ERα [54–56]. However, the mechanisms by which low concentrations of oestrogen affect cells within the bone microenvironment either directly or indirectly remain to be elucidated.

Bone cells are derived from two different cell linages; osteoblasts are derived from a mesenchymal linage whereas osteoclasts proceed from myeloid haematopoietic cells. Both lineages have been reported to express ER and are affected directly or indirectly by circulating oestrogen hormones. Early osteoblastic progenitors have been shown to alter their self-renewal mechanisms in presence of oestrogen. In physiological levels of oestrogen, osteoblast progenitors are prevented from proliferating and undergoing self-renewal processes, potentially by direct activation of ERα, allowing them to commit into differentiation [57,58]. Moreover, oestrogen increases the transcriptional levels of adhesion molecules such as N-cadherin. These findings demonstrate that under pre-menopausal concentrations of oestrogen, once osteoprogenitors differentiate into osteoblast, they increase mobility molecules to favour their migration to areas where resorption is taking place, however, the loss of oestrogen impairs this ability. Osteoblast progenitors respond differently to oestrogen stimulus compared with mature osteoblasts. Osteoblastic progenitors expressing Osteoblast-specific transcription factor Osterix 1 (Osx1) (expressed in early osteoblast differentiation) require activation of Wnt/β-catenin via ERα in order to differentiate into functional periosteal cells and achieve optimal cortical bone accrual. In contrast, mature osteoblast and osteocytes do not respond to this stimulus [59], instead they are prevented from undergoing apoptosis by inhibiting activation of caspase-3/7 [54].

The effect of oestrogen on osteoclasts lineages is still a matter of debate, however, evidence suggests that oestrogen affects maturation, activation and life span of osteoclasts through direct and indirect mechanisms. For example, numbers of osteoclasts and osteoclast progenitors are significantly increased in a mouse model knockout for ERα in monocytes/macrophages, compared to wildtype mice, but only in the cancellous site of the bone [56]. This study demonstrated that removal of oestrogen signalling in the osteoclast lineage only affects the population of osteoclast and osteoclast progenitors located in the cancellous area of the bone decreased their lifespan. [56]. In addition, osteoclastogenic factors such as IL-1, IL-6, TNFα, and macrophage colony-stimulating factor (M-CSF) are secreted by diverse haematopoietic and osteoblastic cells. The production of these factors is well documented to be dependant of oestrogen concentrations, with high concentrations of this hormone repressing osteoclastigenesis and bone resorption [44]. In contrast, under post-menopausal concentrations of oestrogen, increased production of IL-1 is observed which leads to an increased differentiation of osteoclastic progenitors as well as the production of RANKL by immune cells, primarily T-cells, and osteoblastic cells resulting in augmented numbers of active osteoclasts [44,60]. Oestrogen also plays an additional antiresorptive role in by directly inducing apoptosis in active osteoclasts via transforming growth factor-β1 (TGF-β) in a dose-dependent manner [55]. Although this mechanism was observed in osteoclast colonies *in vitro*, mouse model studies suggest that this mechanism is specificity important in the regulation of osteoclasts in the trabecular area of the bone [56]. Importantly, data suggest that oestrogen has dramatic effects on a wide variety of cell types with known links to driving tumour growth in bone including osteoblasts, haematopoietic stem cells and osteoclasts. Interestingly, the most striking oestrogen driven effects on bone resorption were observed in the trabecular region of bone where ZOL has its most potent anti-resorptive effects [54].

Taken together ZOL and oestrogen both have effects in bone that preserve bone, but these are trough different mechanisms. ZOL inhibits bone resorption through active killing of osteoclasts which has the knock-on effect of reducing osteoblast activity, whereas, oestrogen has bone anabolic effects through increasing the lifespan of osteoblasts and decreasing the lifespan of osteoclasts (but increasing osteoclast precursors, therefore increasing the turnover of osteoclasts in bone). It is therefore possible that ZOL may not be as effective at reducing bone resorption and the vicious cycle of bone metastases because of the increased numbers of osteoclasts generated. When ZOL kills the existing osteoclasts under high concentrations of oestrogen, these may be replaced at a sufficiently high rate to allow continued bone resorption. Although this simple, suggested, mechanism may partly explain the

reduced anti-tumour effects observed following adjuvant administration of ZOL in pre-menopausal women, the most striking effects were observed outside of bone with pre-and post-menopausal women experiencing opposite outcomes; increased and decreased relapse respectively [2,61]. It is therefore likely that systemic effects of oestrogen and ZOL are responsible for these differences and both of these substances have profound effects on the immune system.

3.4. Immune landscape of breast cancer

The innate and adaptive immune systems have profound effects on breast cancer progression and metastasis. Genetic and epigenetic modifications in cancer results in changes in cell surface proteins leading to expression of cancer associated antigens that can be recognised by complement proteins of the immune system [62,63]. Therefore, tumour cells must evade immune recognition in order to survive. Breast cancer cells employ immune modulatory techniques, such as up-regulation of PD-L1, subsequent stimulation of the immunosuppressive regulatory T (Treg) cell populations and the polarisation of macrophage populations into pro-tumour subtypes. Indeed, a study into natural killer (NK) cell activity in NK cells isolated from breast cancer patients found that activity dropped from 62.5% to 24.4% in patients with breast cancer when compared to healthy individuals [64]. The presence of specific immune cell subsets within the tumour microenvironment indicate a positive or negative survival benefit. Eosinophils, neutrophils, B lymphocytes and $CD4^+$ or $CD8^+$ T cell infiltration are associated with a good prognosis. Whereas, tumour associated macrophages (TAMs), T regulatory cells (Tregs) and activated mast cells are correlated with a poor prognosis. Both oestrogen and Zol have profound immune modulatory effects with the potential to alter survival of breast cancer and we suggest that immune modulation instigated by high circulating concentrations of oestrogen (observed in pre-menopausal women) may antagonise the anti-tumour immune response stimulated by ZOL.

3.5. Effects of ZOL on anti-cancer immunity

In addition to its effects on bone cells, ZOL has profound effects on the immune system, including a plethora of immune cell subsets expressing the mevalonate pathway enzymes, leading to a general increase in anti-tumour immunity [65]. Specifically, ZOL can influence macrophage polarisation in the tumour microenvironment, γδ T cell activation by accumulation of its activating antigen on target cells, NK cell activity, Treg cell activation and infiltration, PD-L1 expression and effector T cell function. Therefore, understanding the interactions of ZOL with these immune cell subsets in breast cancer metastasis could help shed light on the mechanisms whereby adjuvant ZOL prevents metastasis in post-menopausal women.

3.5.1. Gamma delta T cells

γδ T cells are a subset of immune-surveillance cells that detect self-prenyl pyrophosphate metabolites to enable immune response to microbes and tumours. The majority of these cells have the Vγ9Vδ2 T cell receptor and they are not restricted by dependence on MHC class proteins, but instead require butyrophilin 3A1 from the immunoglobulin family which is expressed on all human cells [66]. This effectively allows any healthy human or tumour cell to present antigens, without the need for MHC proteins which are often downregulated on breast cancer cells. γδ T cells respond to the eukaryote isopentenyl pyrophosphate (IPP), which is produced through the mevalonate pathway, and can often accumulate in unregulated tumour cells.

ZOL inhibits the activity of farnesyl pyrophosphatase, a downstream enzyme of IPP in the mevalonate pathway, leading to accumulation of IPP and activation of γδ T cells and subsequent tumour cell apoptosis [67]. In *In vitro* studies, administration of ZOL to peripheral blood mononuclear cells resulted in a significant expansion of the γδ T cell subset [68]. Interestingly, ZOL has been reported to accumulate IPP in circulating monocytes, leading to activation of circulating γδ T cells resulting in a greater immunostimulatory effect by increasing tumour targeting via γδ T cell degranulation and the release of cytotoxic granules [69]. However, these peripherally activated γδ T cells lacked the ability to migrate to tumours [70], suggesting the direct activation of γδ T cells in the tumours by tumour cell derived IPP is necessary to exert anti-tumour activity.

γδ T cells express PD-1 as a mechanism to differentiate between self and non-self, *in vitro* experiments in the laboratory have demonstrated that when in contact with tumour cells γδ T upregulate PD-1 making their interactions with PD-L1 more frequent, increasing T cell exhaustion and reducing their potential cytotoxicity [71]. γδ T cells isolated from tumour bearing mice and stimulated with mitogen respond poorly compared with those isolated from healthy mice which proliferate readily. This inhibited proliferation was rescued by treatment with a monoclonal antibody against PD-L1. Furthermore, ZOL has been shown to rescue PD-L1 inhibition as incubation of PD-1 expressing γδ T cells with PD-L1 expressing tumour cells pre-treated with Zol led to an increase in γδ T cell activation when compared to untreated tumour cells, suggesting that IPP signalling could overcome PD-L1 inhibition [72].

3.5.2. Treg

T regulatory cells (Tregs) are essential in maintaining peripheral tolerance by suppressing T effector cell activity. $CD4^+$ $Foxp3^+$ Tregs are the most widely studied and can be categorised into natural (nTreg), which are committed cells from the thymus, or induced (iTreg) which are converted from naïve $CD4^+$ T cells in the periphery, dependent on TGFβ and IL-2. Tregs express both PD-1 and PD-L1 directly effecting Treg generation [73,74]; *in vivo* mouse models demonstrated that the conversion and maintenance of iTreg was dependent on PD-L1, via inhibition of the mTOR/AKT pathway [73]. Tregs play an important role in immunosuppression in the tumour microenvironment, allowing tumours to evade immune surveillance by suppressing $CD4^+$ $CD25^-$ $CD8^+$ effector T cells. Therefore it is unsurprising that Tregs have been found to be upregulated in both mouse and human tumours [75] where Treg infiltration is characteristic of more invasive tumours and predicts diminished survival.

In mice treated with ZOL, the levels of circulating $CD4^+$, $CD8^+$, $Foxp3^+$ Tregs were significantly decreased, providing evidence of a further mechanism of ZOLs immunomodulatory nature [76]. Treg derived RANKL in the tumour microenvironment is central to stimulating tumour growth, since tumour growth can be stimulated by exogenous RANKL in the absence of Tregs. The importance of immunomodulation by Tregs in breast cancer progression has been determined in a series of *in vitro* experiments [77]. Treatment with 10 μM ZOL significantly inhibited proliferation of Tregs, whilst having no significant effect on lymphocyte proliferation. The migratory capacity of Tregs in response to conditioned media from MDA-MB-231 cells was also significantly inhibited by ZOL suggesting that there would be less tumour infiltration by Tregs. In addition to the increased migratory capacity of MDA-MB-231 cells by Tregs, the study also found this migration could be inhibited by ZOL through downregulation of RANKL from infiltrating Treg cells [77]. Further studies confirmed these findings, showing that treatment of Tregs with ZOL reduced expression of STAT5, TGFβ and CD25, functionally leading to a reduced capacity of Tregs to supress T and NK cell proliferation. Analysis of molecular changes in the human breast cancer cell line MDA-MB-231 following treatment with ZOL revealed a downregulation of CCR4 and CTLA4 [77], both of which have previously been shown to be vital in the recruitment of Tregs into the tumour microenvironment [78]. This finding was functionally confirmed by a migration assay showing that anti-CCL2 or CCL5 antibodies significantly inhibited Treg migration in response to MDA-MB-231 conditioned medium [79]. Interestingly, PD-1 expression on Tregs was significantly downregulated by ZOL, suggesting a reduced capacity for naïve T cell conversion into mature Tregs, and a loss of $CD8^+$ effector T

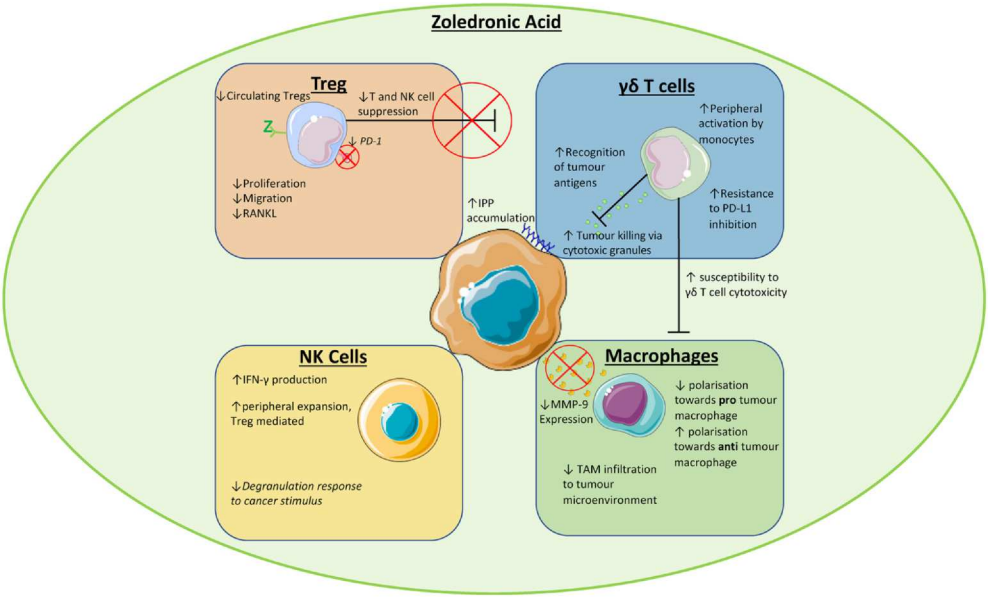

Fig. 2. Effects of ZOL on the immune response to breast cancer. ZOL causes a decrease in immune suppression within the tumour microenvironment, mediated through a decrease in Treg infiltration and activity, an increase in macrophage polarisation towards an anti-tumour phonotype and an increase in γδ T cell antigen recognition.

cell expression.

3.5.3. Macrophages

Macrophages, part of the innate immune system, are mononuclear cells of the myeloid lineage that originate from monocytes in the bone marrow. Once they leave the bone marrow and enter a tissue, they can be stimulated to differentiate into macrophages, which can have opposing effects on bone metastasis. Macrophages are essential in promoting a specific adaptive immune response by collaborating with T and B cells to induce or inhibit T cell recruitment and activation [80]. Depending on the cytokines acting on the precursors, macrophages can differentiate into two broad classes. Firstly, pro-inflammatory anti-tumour macrophages, secreting IL-1, IL-6, IL-12 and IFN-γ to recruit T cells and eliminate tumour cells [81]. Secondly, tumour associated macrophages (TAMs) which are considered one of the most important regulators of tumour progression and bone metastasis [82] since they secrete cytokines such as IL-10 and TGF-β which decrease $CD4^+$ and $CD8^+$ T cell activity on the tumour microenvironment [83].

ZOL can alter the polarisation and function of macrophages within the tumour microenvironment. ZOL has been shown to be taken up by macrophages both *in vivo* and *in vitro*, with 5 μM over 24 h causing significant increases in unprenylated Rap1A *in vitro*, a marker for inhibition of the mevalonate pathway [84]. Furthermore, macrophages isolated from breast cancer xenografts also had significantly higher unprenylated Rap1A following a single dose of Zol [84]. When mice were treated with ZOL for 3 weeks and their tumours were analysed by histological methods, it was revealed that ZOL had inhibited TAM infiltration and reduced vascularisation, caused by a reverted polarisation of macrophages away from the TAM polarisation and towards the anti-tumour polarisation [85].

Interaction between tumour cells and macrophages enhances MMP-9 expression in TAMs, an enzyme which plays a major role in the invasion and metastasis of breast cancer cells due to its role in ECM remodelling [86]. Furthermore, MMP-9 is required by myeloid derived suppressor cells (MDSC) for their immunosuppressive functions within the tumour microenvironment. When mice were treated with ZOL, the levels of MMP-9 and the number of TAMs were significantly decreased, followed by a reduced expansion of MDSC. This was found to be partly due to a decrease in bone marrow progenitor derived MMP-9 [87]. A series of *in vitro* experiments modelling the cancer cell macrophage interaction on the presence of ZOL found that ZOL could selectively supress the expression of MMP-9 in the tumour associated macrophage population, as well as shifting macrophage polarisation to effectively drive proliferation of activated γδ T cells [88].

Interestingly, ZOL also affected the interactions of macrophages and γδ T cells in the tumour microenvironment. Human monocytes were differentiated into either anti-tumour macrophages or TAMs before being treated with ZOL *in vitro*. ZOL treatment rendered the macrophages susceptible to γδ T cell cytotoxicity in a perforin dependent manner, independently of macrophage polarisation [89]. This could go some way to explaining a mechanism whereby TAMs are cleared from the tumour microenvironment.

3.5.4. NK cells

Natural Killer (NK) cells are part of the innate immune system and are also a vital immune cell type having cytotoxic effects on breast cancer cells by targeting stress induced markers [90]. Research into the effects of ZOL on NK cell function in breast cancer metastasis is limited, with conflicting results highlighting the need for further research into this field. When NK cells and dendritic antigen presenting cells were co-cultured together in the presence and absence of ZOL, ZOL was shown to stimulate IFN-γ production in NK cells. This was the result of interactions with dendritic cells where ZOL induced depletion of endogenous prenyl pyrophosphates which lead to the secretion of IL-18 and IL-1β to activate NK cells [91]. Furthermore, this was shown to be dependent on caspase 1 expression in NK cells. When studying Treg function in patients with metastatic cancer, ZOL selectively decreased Treg infiltration. Interestingly, this led to a significant increase in NK cell proliferation in peripheral blood of patients with metastatic cancer, possibly mediated via Treg signalling, since ZOL treated Tregs had a reduced capacity for inhibiting NK cell proliferation [92]. In contrast, one study investigating the *in vitro* effects of ZOL on NK cells from healthy donors and Ewing sarcoma patients found that ZOL significantly impaired expansion of NK cells. In this study, ZOL impaired the degranulation response of NK cells to Ewing sarcoma stimulus suggesting a decrease in functional activity in which ZOL may help increase the survival of tumour cells in this context [93].

Overall data show that ZOL has substantial anti-tumour immune modulatory effects, acting on a plethora of immune cells within the tumour microenvironment and leading to a general increase in immune cell infiltrate (Fig. 2). Treg cells, the regulators of T cell immune response, are significantly inhibited by ZOL leading to a block in immune inhibition within the tumour microenvironment and having secondary consequences by allowing the action of CD8 T cells and NK cells. These

immunomodulatory effects of ZOL could explain why adding this drug to standard of care following primary tumour resection leads to improved disease-free survival and reduced bone metastasis in post-menopausal women. Why these improved survival effects are not seen in pre-menopausal women may be due to oestrogen driven alterations in immune regulation interfering with ZOL driven anti-tumour immune responses.

3.6. Immune modulatory effects of oestrogen and interactions with ZOL

Oestrogen is immunogenic, influencing the activity and survival of a plethora of different immune cells expressing the ER, perhaps explaining the sex-differential response of the immune system to pathogens. ER is expressed on dendritic cells, macrophages, mast cells, neutrophils, NK cells, B cells, $CD4^+$ T cells, $CD8^+$ T cells and Tregs [92]. Not all these cells have anti-tumour properties, with Tregs, mast cells and some tumour associated macrophages being linked to a poor prognosis. Therefore, oestrogen driven immune modulatory effects on breast cancer metastasis and patient outcome is a complex one. The contributions of the individual immune cell types to this process is summarised in the subsections below.

3.6.1. PD-L1

Oestrogen is a strong oncogenic driver in breast cancer, and it has been shown to modulate immune response. Oestrogen increases PD-L1 protein expression in ER + ve MCF-7 cells via Akt/PI3K activation [94]. This expression was mediated via an increase in mRNA stability since blocking gene transcription had little effect. When co-cultures with T cells, oestrogen upregulated PD-L1 in ER + ve breast cancer cells resulting in reduced T cell function, indicated by diminished expression of IFN-γ and IL-2 suggesting T-cell exhaustion. These effects were independent of the direct action of oestrogen on T cells [94]. Increased oestrogen driven expression of PD-L1 may, in part, explain the reduced sensitivity to the anti-tumour effects of ZOL in pre-menopausal women as ZOL induces tumour cell killing through increased activation of T cells and high concentrations of oestrogen may prevent these ZOL activated T cells from exerting their anti-tumour activity.

3.6.2. Tregs

Immunosuppressive Treg cells, express ERs which enhance their function and ability to expand the Treg cell compartment [95] (Fig. 3). Indeed, pre-menopausal physiological concentrations of oestrogen given to ovariectomised mice stimulated the conversion of $CD4^+$ $CD25^-$ T cells into $CD4^+$ $CD25^+$ cells while also increasing expression of Foxp3 and IL-10, which are important Treg cell markers. Furthermore both *in vitro* and *in vivo* studies show that upregulation of Treg cells leads to enhanced suppression of T cell proliferation and in mixed lymphocytes. Interestingly, Treg cells have been shown to interact with the bone resident osteoclasts and modify their bone modulatory functions *in vitro*, with oestrogen enhancing this effect. Co-cultures of bone marrow cells and isolated human Tregs in the presence and absence of oestrogen found that osteoclast differentiation was supressed more efficiently in the presence of oestrogen, an effect which was mediated via Treg derived IL-10 and TGF-β1 [96].

Oestrogen induced PD-L1 expression in the metastatic breast cancer microenvironment augments the immunosuppressive niche by inducing iTreg conversion. This is particularly important for bone metastasis as evidenced in a number of studies showing that Tregs are increased in patients with bone metastasis, with signalling via CXCR4/CXCL12 mediating Treg trafficking to bone suggesting that the bone microenvironment is a preferential site for functional Tregs [97]. Treg trafficking to bone results in adverse effects for breast cancer patients, actively promoting metastasis at this site.

Treg cells are a major source of RANKL in the tumour microenvironment and in the bone, expressing up to four fold more RANKL than $CD4^+CD25^-$ T cells [98]. This suggests that Tregs are important for maintaining RANKL expression in the tumour microenvironment of metastatic breast cancer. Tregs increased the metastatic potential of breast cancer cells and exhibited an adverse effect on prognosis. Furthermore, the adverse effects of Tregs was replaceable with addition of exogenous RANKL [98].

Oestrogen has been shown to regulate Treg cell differentiation and suppressive activity by inducing PD-1 expression. Intracellular PD-1 expression in Tregs was shown to be oestrogen sensitive and moderated via the Treg ER. Oestrogen signalling upregulates PD-1 expression, since ER KO deceased oestrogen mediated PD-1 expression [99] (Fig. 3). When PD-1 was knocked out of Treg cells, they lost their functional suppressive activity highlighting the importance of both oestrogen and the PD-L1/PD-1 axis in immune suppression via Treg cells [99]. This is in stark contrast to the effects of ZOL on PD-1 expression. ZOL downregulates PD-1 expression on Tregs, which both reduces iTreg conversion and reduces the ability of Tregs to functionally supress T effector cells in the tumour microenvironment [100]. Furthermore, ZOL was shown to inhibit the proliferation and migratory capacity of Treg cells which would reduce their capacity to infiltrate the tumour microenvironment. Oestrogen again does the opposite by

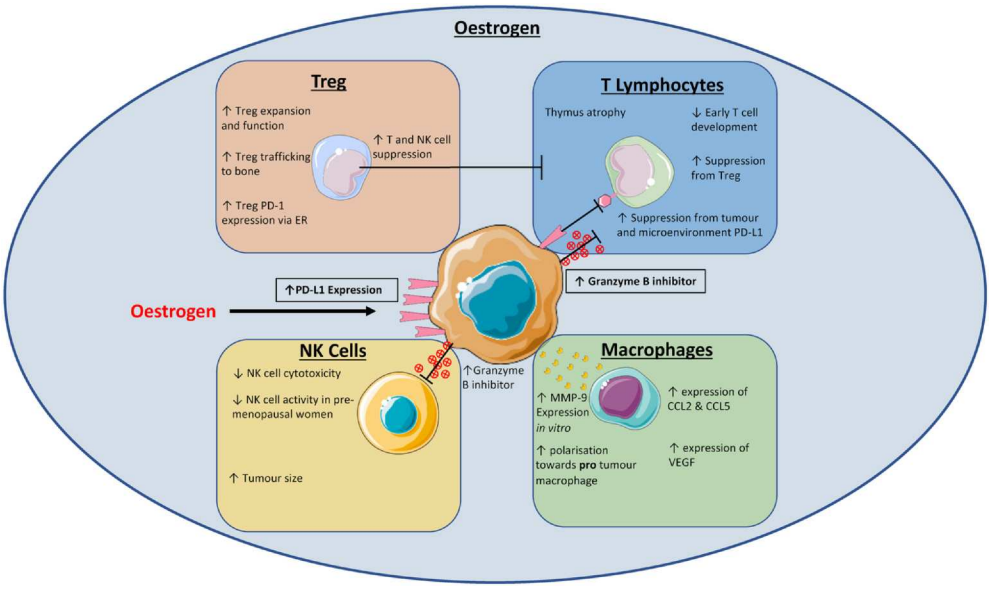

Fig. 3. The effect of oestrogen on the immune response to breast cancer. Oestrogen increases immune suppression within the tumour environment, mediated through increased Treg infiltration and action and an increased expression of PD-L1. Furthermore, oestrogen leads to a change in macrophage polarisation towards a pro-tumourigenic phenotype.

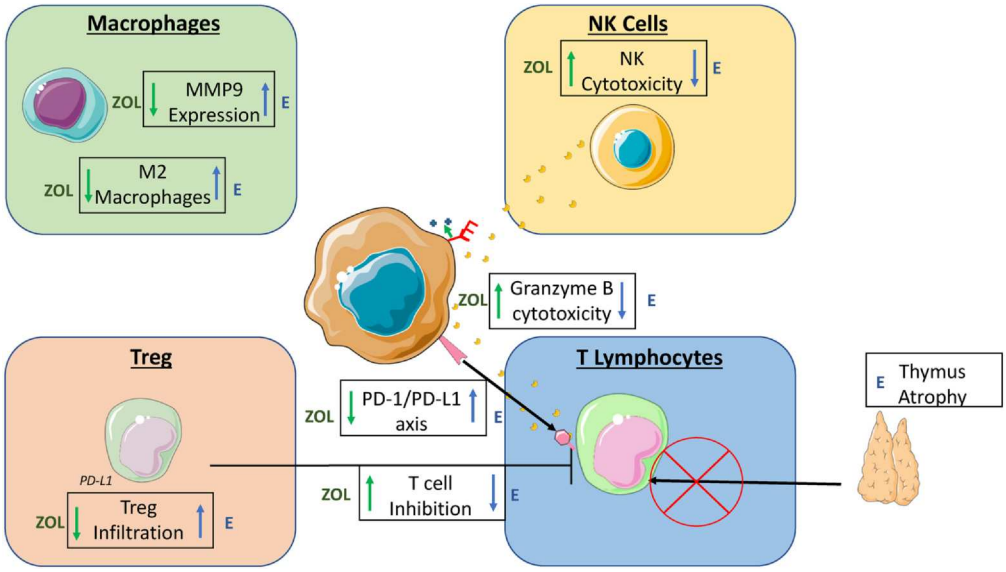

Fig. 4. The overlapping effect of oestrogen and ZOL on the immune response to breast cancer. Oestrogen and ZOL have overlapping and contrasting effects on the immune cell subsets and their cytotoxic effects on breast cancers. These overlapping effects could contribute to the progression of breast cancer metastasis in pre-menopausal women observed in clinical trials and inform future therapies and research into these effects.

increasing Treg cell infiltration into tumours via CXCR4/CXCL12 signalling. It is therefore possible that reduced bone metastasis observed in both pre- and post-menopausal women treated with adjuvant/neo-adjuvant Zol is due to inhibition osteoclastic bone resorption and bone turnover (as discussed previously). However, under high oestrogen conditions, increased PD-1/PDL-1 expression may prevent tumour cells disseminated in other organs from being detected and killed by T cells. As discussed, Zol has been shown to be a potent activator of T cells [76–79]. Thus under conditions of low oestrogen, this bisphosphonate may be able to stimulate an immune response to disseminated tumour cells reducing metastatic outgrowth in distal organs outside of bone accounting for the differential effects of adjuvant Zol on soft tissue metastasis under pre- and post-menopausal conditions (Fig. 4).

3.6.3. T lymphocytes

CD4 and CD8 T lymphocytes are vital in immune protection from breast cancer metastasis with CD4 assisting B cells in antibody mediated protection and CD8 effector cells important in direct cellular responses. Studies have shown that increased $CD8^+$ T lymphocytes in the breast cancer microenvironment correlate with improved overall survival [101]. $CD8^+$ T cells have also been shown to be a vital defence against bone metastasis, since escape from T cell detection by MHC downregulation promotes bone metastasis [102]. However, anti-metastatic effects associated with bone metastasis are likely to be associated with early metastatic events as very few $CD8^+$ T cells are detected in the bone microenvironment [103]. It is therefore likely that alterations in T cell activity affect tumours in soft tissue more than in bone. Although CD8+ T cells can be silenced in the breast cancer microenvironment by oestrogen induced Tregs, they also express ERα and are profoundly influenced by oestrogen signalling (Fig. 3). Furthermore, it has been known for some time that oestrogen can block early T cell development in the thymus [104] leading to decreased T cells in mice following oestrogen administration [105]. This has been shown to be due to thymic atrophy in the presence of oestrogen, due to decreased production of $Flt3^+Sca\text{-}1^+c\text{-}Kit^+$ thymic homing progenitors and reduced proliferation of thymocytes [106].

Oestrogen can also affect $CD8^+$ T cell tumour killing function within the tumour microenvironment. The main way for cytotoxic $CD8^+$ T cells to exert their effects is via granule mediated exocytosis of serine proteases such as granzyme B to initiate caspase mediated apoptosis in the target tumour cell [107]. Interestingly, when the ER^+ breast cancer cell line, MCF-7, was treated with oestrogen it induced expression of the granzyme B inhibitor PI-9, protecting these cells from $CD8^+$ T cell targeting, as well as from NK cell mediated apoptosis [108].

The effect of oestrogen on $CD8^+$ lymphocytes highlights another overlapping role of oestrogen and ZOL (Fig. 4). In post-menopausal women, an anti-tumour effect of ZOL can be mediated in part by a decrease in Treg cell infiltration allowing expansion of $CD8^+$ T cells in the tumour microenvironment. However, in pre-menopausal women, $CD8^+$ T cell function in the tumour microenvironment may be further inhibited by expression of granzyme B inhibitor inhibiting tumour death. Therefore, even if Treg infiltration is reduced by ZOL, the resulting $CD8^+$ T cell infiltration may still have a diminished function leading to less tumour killing in soft and increased metastases in these sites.

3.6.4. Macrophages

Tumour associated macrophages play a vital role in breast cancer progression and bone metastasis. Indeed, they have been shown to be involved in a plethora of processes facilitating metastasis: invasion, vascularisation, intravasation, circulating tumour cell survival, extravasation and pre-metastatic niche formation [109]. Macrophages and osteoclasts of the bone microenvironment are derived from the same precursors, suggesting that macrophages may be sensitive to oestrogen signalling as osteoclasts are. Furthermore, many inflammatory diseases display a sex difference in their phenotype, which has been suggested to be via macrophage interactions with oestrogen. In a mouse asthma model, oestrogen signalling was shown to increase IL4 and M2 macrophage gene expression following OVX and oestrogen administration, contributing to the gender differences in disease progression [110]. Macrophages express ERα and β, with TAMs expressing ERs across many cancer types [111]. Oestrogen signalling on macrophage ERs in the tumour microenvironment can affect macrophage polarisation (Fig. 3). The mouse polyomavirus middle T ER-positive breast cancer model was treated with oestrogen, which led to significantly higher M2 macrophage infiltration when compared to untreated controls which had higher M1 macrophage tumour infiltration [112]. This effect was found to be mediated via increased expression of CCL2 and CCL5 in macrophages from the oestrogen treated group [112]. Oestrogen treated macrophages also expressed and secreted more VEGF, an important molecule in metastasis progression since it both directly stimulates tumour growth and assists the angiogenic switch in growing tumours [112]. Furthermore, oestrogen has been reported to upregulate expression of MMP-9 in macrophages *in vitro*, an important enzyme playing a role in extracellular matrix remodelling and tumour invasion

[113].

There are several overlapping effects of oestrogen and ZOL on macrophages (Fig. 4), the first of which being the effect on MMP expression. Oestrogen has been shown to upregulate MMP-9 expression *in vitro*, whereas ZOL decreases expression of MMP-9, thereby reducing TAM infiltration and tumour invasion and metastasis. These opposing effects on MMP-9 expression underlines another way that oestrogen could affect the anti-tumour properties of ZOL [87,113]. Oestrogen induced increase in MMP-9 could increase the metastatic potential of breast cancer cells and could explain the discrepancies in pre- and post-menopausal women as observed in clinical trials. Furthermore, ZOL and oestrogen have been shown to have opposing effects on macrophage polarisation with oestrogen upregulating pro-tumour macrophage infiltration via increased CCL2 and CCL5 expression and ZOL inhibiting macrophage tumour infiltration by stimulating polarisation towards an anti-tumour phenotype.

3.6.5. NK cells

Natural Killer (NK) cells represent another important immune mediated tumour killing cell present in the bone marrow as part of the innate immune system. Indeed, when the NK cells are depleted in cancer models, bone metastasis and uncontrollable proliferation were observed [102]. NK cells target the tumour by recognising stress induced markers and the downregulation of "self" ligands by binding to recognisable cancer cell surface ligands, such as the cell stress marker NKG2D [90]. NK cells target the downregulation of MHC molecules to identify themselves as self, killing the cells by granule mediated exocytosis and Fas-Fas ligand interactions [114]. However, it has been shown that a partial loss of HLD class 1 proteins renders the tumours resistant to both T cell and NK cell attack, suggesting expression level is vital for NK cell function [115]. Interestingly, ERα and ERβ have both been described in NK cells, and have significant functional effects on these cells, with ERβ first being suggested as the main mechanism for oestrogen response [116]. In mouse models, oestrogen has been shown to reduce NK cell cytotoxicity in a dose dependent manner in a range of castrated mouse strains [80,117] (Fig. 3). A clinical study into the effects of oestrogen replacement on NK cell activity in post-menopausal women found that oestrogen significantly impaired NK cell activity when compared to post-menopausal women without oestrogen replacement therapy [118]. This suppressive effect of oestrogen on NK cell activity has been linked to an enhancement of pulmonary metastasis in melanoma and fibrosarcoma. Furthermore, in mouse models of breast cancer, oestrogen treatment significantly supressed NK cell activity and increased tumour size [119]. However, it is unclear whether this was a direct result of NK cell depletion or the broad effects of oestrogen. This highlights a contrasting effect of ZOL and oestrogen on NK cell anti-tumour activity (Fig. 3), however it also highlights the need for further research into this field. Immunotherapy treatments targeting NK cells are getting closer to clinical use and ZOL is an important molecule used in the clinic. Therefore, understanding the interactions of ZOL and oestrogen on the activity of NK cells is a vital area of research.

3.7. Additional factors to consider

The anti-metastatic effects of adjuvant Zol in post-menopausal women appear to be limited to breast cancer. No benefits have been observed in post-menopausal patients with lung cancer or in men following chemical or physical castration with prostate cancer [120]. Furthermore, administration of the anti-RANKL antibody, denosumab, has similar effects to Zol in that it reduces osteoclastic bone resorption, increases $CD8^+$ T cells and PDL-1 expression [121]. It has therefore possible that other, breast cancer specific, factors aside from reduced circulating concentrations of ovarian hormones may drive or contribute to response to bisphosphonates in early breast cancer. Analysis of tumour tissue arrays from 1739 patients enrolled on the AZURE trial (detailed above) has demonstrated direct correlations with expression of the transcription factor MAF in primary tumours and invasive disease free survival following administration of Zol [122]. Patents who's tumours were negative for the expression of MAF demonstrated better outcome following administration of Zol compared with patients with MAF expressing primary tumours (HR: 0.52, 95% CI 0.36–0.75) [122] Interestingly, MAF did not predict patient outcome in the control group and no correlations between MAF and menopausal status were observed indicating that this molecule may be an independent prognostic marker for predicating likely benefit from adjuvant Zol [122]. In addition, using samples from the same AZURE data sets researchers have identified other biomarkers that can predict anti-tumour effects of Zol independently of menopausal status. Patients whose primary tumours express both macrophage-capping protein (CAPG) and PDZ domain containing protein (GIPC1) were more likely to develop first distant recurrence in bone (HR: 4.5, 95% CI, 2.1–9.8; $P = 0.01$) and had reduced overall survival (HR: 1.8, 95% CI, 1.01–3.24; $P = 0.45$). Zol had a substantial increased effect in patients with high expression of both proteins leading to a 10 fold reduction in the hazard ratio for first instance recurrence in bone, compared with control ($P = 0.008$) [123]. Expression of IL-1B in primary tumours was also shown to correlate with subsequent relapse in bone (HR: 1.85; 95 CI: 1.05–3.26; $P = 0.02$) or other sites (HR: 2.09, CI: 1.26–3.48; $P = 0.0016$) and expression of this proinflammatory cytokine was associated with reduced overall survival in patients treated with zoledronic acid compared with control ($P = 0.002$) [124]. Whether alone, or in combination, these potential biomarkers make better predictors of the potential anti-tumour effects if Zol compared with menopausal status remains to be established. Further research is required to establish potential mechanisms by which these factors may interact with Zol to influence the metastatic process. It will also be useful to establish if breast cancers express specific factors that render them more sensitive to bisphosphonates under post-menopausal conditions or if non-responsiveness to anti-tumour effects of bisphosphonates observed in lung and prostate cancer are due to other factors such as differential response to immune regulation.

4. Conclusions

The ability of breast cancer to evade the immune system is vital for its progression in both primary tumour growth and its ability to metastasise. Oestrogen and ZOL both have profound and often opposing effects on the immune system and the ability of breast cancer cells to evade immune detection. Clinical trials have demonstrated that menopausal status affects patient's response to adjuvant ZOL: Both pre- and post-menopausal women develop fewer bone metastases following administration of ZOL suggesting that ZOL is able to exert effects on the bone microenvironment that make it non-permissive for tumour growth irrespective of the effects of oestrogen. It has been suggested that under pre-menopausal concentrations of oestrogen, inhibition of the bone microenvironment by ZOL, may cause tumour cells to escape to move to other sites, whereas under post-menopausal concentration of oestrogen, ZOL either kills tumour cells or holds these in a dormant state within the bone. These suggestions could in some part explain why pre-menopausal women experience increased soft tissue metastasis following adjuvant ZOL. This hypothesis remains to be answered and tumour progression both inside and outside of the bone is tightly regulated by immune cells suggesting that both bone cell mediated, and immune cell mediated response to ZOL may play intricate roles in regulating tumour growth at different sites.

CRediT authorship contribution statement

CG, VCL, ET, PO - Investigation and writing original draft. PO, MM and CW - supervision, review and editing. PO and CW - acqusition of funding.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbo.2020.100317.

References

[1] R. Coleman, et al., Effects of bone-targeted agents on cancer progression and mortality, J. Natl. Cancer Inst. 104 (14) (2012) 1059–1067.
[2] R. Coleman, et al., Adjuvant zoledronic acid in patients with early breast cancer: final efficacy analysis of the AZURE (BIG 01/04) randomised open-label phase 3 trial, Lancet Oncol. 15 (9) (2014) 997–1006.
[3] R.E. Coleman, et al., Breast-cancer adjuvant therapy with zoledronic acid, N. Engl. J. Med. 365 (15) (2011) 1396–1405.
[4] M. Gnant, et al., Adjuvant endocrine therapy plus zoledronic acid in premenopausal women with early-stage breast cancer: 5-year follow-up of the ABCSG-12 bone-mineral density substudy, Lancet Oncol. 9 (9) (2008) 840–849.
[5] M. Gnant, et al., Adjuvant endocrine therapy plus zoledronic acid in premenopausal women with early-stage breast cancer: 62-month follow-up from the ABCSG-12 randomised trial, Lancet Oncol. 12 (7) (2011) 631–641.
[6] M. Gnant, et al., Zoledronic acid combined with adjuvant endocrine therapy of tamoxifen versus anastrozol plus ovarian function suppression in premenopausal early breast cancer: final analysis of the Austrian Breast and Colorectal Cancer Study Group Trial 12, Ann. Oncol. 26 (2) (2015) 313–320.
[7] A. Allshouse, J. Pavlovic, N. Santoro, Menstrual cycle hormone changes associated with reproductive aging and how they may relate to symptoms, Obstet. Gynecol. Clin. North Am. 45 (4) (2018) 613–628.
[8] R.E. Coleman, Skeletal complications of malignancy, Cancer 80 (8 Suppl.) (1997) 1588–1594.
[9] M. Harries, et al., Incidence of bone metastases and survival after a diagnosis of bone metastases in breast cancer patients, Cancer Epidemiol. 38 (4) (2014) 427–434.
[10] S. Braun, et al., A pooled analysis of bone marrow micrometastasis in breast cancer, N. Engl. J. Med. 353 (8) (2005) 793–802.
[11] S. Becker, et al., Primary systemic therapy does not eradicate disseminated tumor cells in breast cancer patients, Breast Cancer Res. Treat. 106 (2) (2007) 239–243.
[12] W. Janni, et al., The persistence of isolated tumor cells in bone marrow from patients with breast carcinoma predicts an increased risk for recurrence, Cancer 103 (5) (2005) 884–891.
[13] B. Rack, et al., Effect of zoledronate on persisting isolated tumour cells in patients with early breast cancer, Anticancer Res. 30 (5) (2010) 1807–1813.
[14] M. Banys, et al., Influence of zoledronic acid on disseminated tumor cells in bone marrow and survival: results of a prospective clinical trial, BMC Cancer 13 (2013) 480.
[15] R. Aft, et al., Effect of zoledronic acid on disseminated tumour cells in women with locally advanced breast cancer: an open label, randomised, phase 2 trial, Lancet Oncol. 11 (5) (2010) 421–428.
[16] A.D. Hartkopf, et al., Prognostic relevance of disseminated tumour cells from the bone marrow of early stage breast cancer patients – results from a large single-centre analysis, Eur. J. Cancer 50 (15) (2014) 2550–2559.
[17] G. von Minckwitz, et al., German adjuvant intergroup node-positive study: a phase III trial to compare oral ibandronate versus observation in patients with high-risk early breast cancer, J. Clin. Oncol. 31 (28) (2013) 3531–3539.
[18] B. Kristensen, et al., Bisphosphonate treatment in primary breast cancer: results from a randomised comparison of oral pamidronate versus no pamidronate in patients with primary breast cancer, Acta Oncol. 47 (4) (2008) 740–746.
[19] N.J. Bundred, et al., Effective inhibition of aromatase inhibitor-associated bone loss by zoledronic acid in postmenopausal women with early breast cancer receiving adjuvant letrozole: ZO-FAST Study results, Cancer 112 (5) (2008) 1001–1010.
[20] A. Mathew, N.E. Davidson, Adjuvant endocrine therapy for premenopausal women with hormone-responsive breast cancer, Breast 24 (Suppl 2) (2015) S120–S125.
[21] C. Wilson, I. Holen, R.E. Coleman, Seed, soil and secreted hormones: potential interactions of breast cancer cells with their endocrine/paracrine microenvironment and implications for treatment with bisphosphonates, Cancer Treat. Rev. 38 (7) (2012) 877–889.
[22] D. Zardavas, M. Piccart, Neoadjuvant therapy for breast cancer, Annu. Rev. Med. 66 (2015) 31–48.
[23] J.R. Kroep, et al., Effects of neoadjuvant chemotherapy with or without zoledronic acid on pathological response: a meta-analysis of randomised trials, Eur. J. Cancer 54 (2016) 57–63.
[24] B. O'Carrigan, et al., Bisphosphonates and other bone agents for breast cancer, Cochrane Database Syst. Rev. 10 (2017) CD003474.
[25] N. Pavlakis, R. Schmidt, M. Stockler, Bisphosphonates for breast cancer, Cochrane Database Syst. Rev. 3 (2005) CD003474.
[26] M.H. Wong, M.R. Stockler, N. Pavlakis, Bisphosphonates and other bone agents for breast cancer, Cochrane Database Syst. Rev. 2 (2012) CD003474.
[27] N. Kohno, et al., Zoledronic acid significantly reduces skeletal complications compared with placebo in Japanese women with bone metastases from breast cancer: a randomized, placebo-controlled trial, J. Clin. Oncol. 23 (15) (2005) 3314–3321.
[28] Y. Shiozawa, et al., Human prostate cancer metastases target the hematopoietic stem cell niche to establish footholds in mouse bone marrow, J. Clin. Invest. 121 (4) (2011) 1298–1312.
[29] E.A. Pedersen, et al., The prostate cancer bone marrow niche: more than just 'fertile soil', Asian J. Androl. 14 (3) (2012) 423–427.
[30] Y. Kienast, et al., Real-time imaging reveals the single steps of brain metastasis formation, Nat. Med. 16 (1) (2010) 116–122.
[31] W.S. Carbonell, et al., The vascular basement membrane as “soil” in brain metastasis, PLoS ONE 4 (6) (2009) e5857.
[32] M. Futakuchi, K. Fukamachi, M. Suzui, Heterogeneity of tumor cells in the bone microenvironment: Mechanisms and therapeutic targets for bone metastasis of prostate or breast cancer, Adv. Drug Deliv. Rev. 99 (Pt B) (2016) 206–211.
[33] I. Holen, R.E. Coleman, Anti-tumour activity of bisphosphonates in preclinical models of breast cancer, Breast Cancer Res. 12 (6) (2010) 214.
[34] M. Lang, et al., Influence of zoledronic acid on proliferation, migration, and apoptosis of vascular endothelial cells, Br. J. Oral Maxillofac. Surg. 54 (8) (2016) 889–893.
[35] P.D. Ottewell, et al., Zoledronic acid has differential antitumor activity in the pre- and postmenopausal bone microenvironment in vivo, Clin. Cancer Res. 20 (11) (2014) 2922–2932.
[36] P. Hadji, et al., The impact of menopause on bone, zoledronic acid, and implications for breast cancer growth and metastasis, Ann. Oncol. 23 (11) (2012) 2782–2790.
[37] W. Ma, et al., Ortho-silicic acid inhibits RANKL-induced osteoclastogenesis and reverses ovariectomy-induced bone loss in vivo, Biol. Trace Elem. Res. (2020).
[38] Z. Qian, et al., Cytisine attenuates bone loss of ovariectomy mouse by preventing RANKL-induced osteoclastogenesis, J. Cell Mol. Med. (2020).
[39] P.D. Ottewell, et al., OPG-Fc inhibits ovariectomy-induced growth of disseminated breast cancer cells in bone, Int. J. Cancer 137 (4) (2015) 968–977.
[40] M. Gnant, et al., Adjuvant denosumab in postmenopausal patients with hormone receptor-positive breast cancer (ABCSG-18): disease-free survival results from a randomised, double-blind, placebo-controlled, phase 3 trial, Lancet Oncol. 20 (3) (2019) 339–351.
[41] R. Coleman, et al., Adjuvant denosumab in early breast cancer (D-CARE): an international, multicentre, randomised, controlled, phase 3 trial, Lancet Oncol. 21 (1) (2020) 60–72.
[42] A.T. Stopeck, et al., Denosumab compared with zoledronic acid for the treatment of bone metastases in patients with advanced breast cancer: a randomized, double-blind study, J. Clin. Oncol. 28 (35) (2010) 5132–5139.
[43] S.C. Manolagas, C.A. O'Brien, M. Almeida, The role of estrogen and androgen receptors in bone health and disease, Nat. Rev. Endocrinol. 9 (12) (2013) 699–712.
[44] M.N. Weitzmann, R. Pacifici, Estrogen deficiency and bone loss: an inflammatory tale, J. Clin. Invest. 116 (5) (2006) 1186–1194.
[45] H. Neville-Webbe, R.E. Coleman, The use of zoledronic acid in the management of metastatic bone disease and hypercalcaemia, Palliat. Med. 17 (6) (2003) 539–553.
[46] R. Hughes, et al., Bone marrow osteoprogenitors are depleted whereas osteoblasts are expanded independent of the osteogenic vasculature in response to zoledronic acid, FASEB J. 33 (11) (2019) 12768–12779.
[47] G. Allocca, et al., The bone metastasis niche in breast cancer-potential overlap with the haematopoietic stem cell niche in vivo, J. Bone Oncol. 17 (2019) 100244.
[48] F.N. Soki, et al., The effects of zoledronic acid in the bone and vasculature support of hematopoietic stem cell niches, J. Cell. Biochem. 114 (1) (2013) 67–78.
[49] D. Santini, et al., Zoledronic acid induces significant and long-lasting modifications of circulating angiogenic factors in cancer patients, Clin. Cancer Res. 9 (8) (2003) 2893–2897.
[50] K. Miyamoto, et al., Osteoclasts are dispensable for hematopoietic stem cell maintenance and mobilization, J. Exp. Med. 208 (11) (2011) 2175–2181.
[51] T. Miyamoto, Role of osteoclasts in regulating hematopoietic stem and progenitor cells, World J. Orthop. 4 (4) (2013) 198–206.
[52] H.K. Brown, et al., A single administration of combination therapy inhibits breast tumour progression in bone and modifies both osteoblasts and osteoclasts, J. Bone Oncol. 1 (2) (2012) 47–56.
[53] M.T. Haider, et al., Modifying the osteoblastic niche with zoledronic acid in vivo-potential implications for breast cancer bone metastasis, Bone 66 (2014) 240–250.
[54] P.G. Bradford, et al., Estrogen regulation of apoptosis in osteoblasts, Physiol. Behav. 99 (2) (2010) 181–185.
[55] D.E. Hughes, et al., Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-beta, Nat. Med. 2 (10) (1996) 1132–1136.
[56] M. Martin-Millan, et al., The estrogen receptor-alpha in osteoclasts mediates the protective effects of estrogens on cancellous but not cortical bone, Mol. Endocrinol. 24 (2) (2010) 323–334.
[57] G.B. Di Gregorio, et al., Attenuation of the self-renewal of transit-amplifying osteoblast progenitors in the murine bone marrow by 17 beta-estradiol, J. Clin. Invest. 107 (7) (2001) 803–812.
[58] U.I. Modder, et al., Effects of estrogen on osteoprogenitor cells and cytokines/bone-regulatory factors in postmenopausal women, Bone 49 (2) (2011) 202–207.

[59] M. Almeida, et al., Estrogen receptor-alpha signaling in osteoblast progenitors stimulates cortical bone accrual, J. Clin. Invest. 123 (1) (2013) 394–404.
[60] C. Streicher, et al., Estrogen regulates bone turnover by targeting RANKL expression in bone lining cells, Sci. Rep. 7 (1) (2017) 6460.
[61] Early Breast Cancer Trialists' Collaborative, G., Adjuvant bisphosphonate treatment in early breast cancer: meta-analyses of individual patient data from randomised trials. Lancet, 2015; 386(10001): 1353–1361.
[62] R. Pio, L. Corrales, J.D. Lambris, The role of complement in tumor growth, Adv. Exp. Med. Biol. 772 (2014) 229–262.
[63] S.S. Pinho, C.A. Reis, Glycosylation in cancer: mechanisms and clinical implications, Nat. Rev. Cancer 15 (9) (2015) 540–555.
[64] A. Piroozmand, Z.M. Hassan, Evaluation of natural killer cell activity in pre and post treated breast cancer patients, J. Cancer Res. Ther. 6 (4) (2010) 478–481.
[65] J. Zekri, M. Mansour, S.M. Karim, The anti-tumour effects of zoledronic acid, J. Bone Oncol. 3 (1) (2014) 25–35.
[66] A. Sandstrom, et al., The intracellular B30.2 domain of butyrophilin 3A1 binds phosphoantigens to mediate activation of human Vgamma9Vdelta2 T cells, Immunity 40 (4) (2014) 490–500.
[67] M. Kondo, et al., Expansion of human peripheral blood gammadelta T cells using zoledronate, J. Vis. Exp. 55 (2011).
[68] K. Thompson, M.J. Rogers, Statins prevent bisphosphonate-induced gamma, delta-T-cell proliferation and activation in vitro, J. Bone Miner. Res. 19 (2) (2004) 278–288.
[69] A.J. Roelofs, et al., Peripheral blood monocytes are responsible for gammadelta T cell activation induced by zoledronic acid through accumulation of IPP/DMAPP, Br. J. Haematol. 144 (2) (2009) 245–250.
[70] D.W. Fowler, et al., Zoledronic acid causes gammadelta T cells to target monocytes and down-modulate inflammatory homing, Immunology 143 (4) (2014) 539–549.
[71] C. Fleming, et al., gammadelta T cells: unexpected regulators of cancer development and progression, Trends Cancer 3 (8) (2017) 561–570.
[72] M. Iwasaki, et al., Expression and function of PD-1 in human gammadelta T cells that recognize phosphoantigens, Eur. J. Immunol. 41 (2) (2011) 345–355.
[73] L.M. Francisco, et al., PD-L1 regulates the development, maintenance, and function of induced regulatory T cells, J. Exp. Med. 206 (13) (2009) 3015–3029.
[74] J.F. Jacobs, et al., Regulatory T cells and the PD-L1/PD-1 pathway mediate immune suppression in malignant human brain tumors, Neuro Oncology 11 (4) (2009) 394–402.
[75] H. Nishikawa, S. Sakaguchi, Regulatory T cells in tumor immunity, Int. J. Cancer 127 (4) (2010) 759–767.
[76] T. Kikuiri, et al., Cell-based immunotherapy with mesenchymal stem cells cures bisphosphonate-related osteonecrosis of the jaw-like disease in mice, J. Bone Miner. Res. 25 (7) (2010) 1668–1679.
[77] H. Liu, et al., Immune modulation of CD4(+)CD25(+) regulatory T cells by zoledronic acid, BMC Immunol. 17 (1) (2016) 45.
[78] M. Gobert, et al., Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome, Cancer Res. 69 (5) (2009) 2000–2009.
[79] H. Liu, et al., Zoledronic acid blocks the interaction between breast cancer cells and regulatory T-cells, BMC Cancer 19 (1) (2019) 176.
[80] N. Nilsson, H. Carlsten, Estrogen induces suppression of natural killer cell cytotoxicity and augmentation of polyclonal B cell activation, Cell. Immunol. 158 (1) (1994) 131–139.
[81] L. Xiang, D.M. Gilkes, The Contribution of the Immune System in Bone Metastasis Pathogenesis, Int. J. Mol. Sci. 20 (4) (2019).
[82] C.H. Lo, C.C. Lynch, Multifaceted roles for macrophages in prostate cancer skeletal metastasis, Front. Endocrinol. (Lausanne) 9 (2018) 247.
[83] S.K. Biswas, A. Mantovani, Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm, Nat. Immunol. 11 (10) (2010) 889–896.
[84] T.L. Rogers, et al., Macrophages as potential targets for zoledronic acid outside the skeleton-evidence from in vitro and in vivo models, Cell Oncol. (Dordr.) 36 (6) (2013) 505–514.
[85] M. Coscia, et al., Zoledronic acid repolarizes tumour-associated macrophages and inhibits mammary carcinogenesis by targeting the mevalonate pathway, J. Cell Mol. Med. 14 (12) (2010) 2803–2815.
[86] H.D. Foda, S. Zucker, Matrix metalloproteinases in cancer invasion, metastasis and angiogenesis, Drug Discovery Today 6 (9) (2001) 478–482.
[87] C. Melani, et al., Amino-biphosphonate-mediated MMP-9 inhibition breaks the tumor-bone marrow axis responsible for myeloid-derived suppressor cell expansion and macrophage infiltration in tumor stroma, Cancer Res. 67 (23) (2007) 11438–11446.
[88] P. Tsagozis, F. Eriksson, P. Pisa, Zoledronic acid modulates antitumoral responses of prostate cancer-tumor associated macrophages, Cancer Immunol. Immunother. 57 (10) (2008) 1451–1459.
[89] D.W. Fowler, et al., Zoledronic acid renders human M1 and M2 macrophages susceptible to Vdelta2(+) gammadelta T cell cytotoxicity in a perforin-dependent manner, Cancer Immunol. Immunother. 66 (9) (2017) 1205–1215.
[90] A. Diefenbach, et al., Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity, Nature 413 (6852) (2001) 165–171.
[91] O. Nussbaumer, et al., DC-like cell-dependent activation of human natural killer cells by the bisphosphonate zoledronic acid is regulated by gammadelta T lymphocytes, Blood 118 (10) (2011) 2743–2751.
[92] D. Sarhan, et al., Zoledronic acid inhibits NFAT and IL-2 signaling pathways in regulatory T cells and diminishes their suppressive function in patients with metastatic cancer, Oncoimmunology 6 (8) (2017) e1338238.
[93] S.K. Mueller, et al., Zoledronic acid negatively affects the expansion of in vitro activated human NK cells and their cytolytic interactions with Ewing sarcoma cells, Oncol. Rep. 29 (6) (2013) 2348–2354.
[94] L. Yang, et al., Posttranscriptional control of PD-L1 expression by 17beta-estradiol via PI3K/Akt signaling pathway in eralpha-positive cancer cell lines, Int. J. Gynecol. Cancer 27 (2) (2017) 196–205.
[95] M.J. Polanczyk, et al., Cutting edge: estrogen drives expansion of the CD4+CD25+ regulatory T cell compartment, J. Immunol. 173 (4) (2004) 2227–2230.
[96] C.Y. Luo, et al., Estrogen enhances the functions of CD4(+)CD25(+)Foxp3(+) regulatory T cells that suppress osteoclast differentiation and bone resorption in vitro, Cell. Mol. Immunol. 8 (1) (2011) 50–58.
[97] M. Yan, et al., Recruitment of regulatory T cells is correlated with hypoxia-induced CXCR4 expression, and is associated with poor prognosis in basal-like breast cancers, Breast Cancer Res. 13 (2) (2011) R47.
[98] W. Tan, et al., Tumour-infiltrating regulatory T cells stimulate mammary cancer metastasis through RANKL-RANK signalling, Nature 470 (7335) (2011) 548–553.
[99] M.J. Polanczyk, et al., Treg suppressive activity involves estrogen-dependent expression of programmed death-1 (PD-1), Int. Immunol. 19 (3) (2007) 337–343.
[100] Y. Eiraku, et al., Dendritic cells cross-talk with tumour antigen-specific CD8(+) T cells, Vgamma9gammadelta T cells and Valpha24NKT cells in patients with glioblastoma multiforme and in healthy donors, Clin. Exp. Immunol. 194 (1) (2018) 54–66.
[101] H.R. Ali, et al., Association between CD8+ T-cell infiltration and breast cancer survival in 12,439 patients, Ann. Oncol. 25 (8) (2014) 1536–1543.
[102] B.N. Bidwell, et al., Silencing of Irf7 pathways in breast cancer cells promotes bone metastasis through immune escape, Nat. Med. 18 (8) (2012) 1224–1231.
[103] V. Mendoza-Reinoso, L.K. McCauley, P.G.J. Fournier, Contribution of macrophages and T cells in skeletal metastasis, Cancers (Basel) 12 (4) (2020).
[104] A.G. Rijhsinghani, et al., Estrogen blocks early T cell development in the thymus, Am. J. Reprod. Immunol. 36 (5) (1996) 269–277.
[105] S.A. Okasha, et al., Evidence for estradiol-induced apoptosis and dysregulated T cell maturation in the thymus, Toxicology 163 (1) (2001) 49–62.
[106] A.L. Zoller, G.J. Kersh, Estrogen induces thymic atrophy by eliminating early thymic progenitors and inhibiting proliferation of beta-selected thymocytes, J. Immunol. 176 (12) (2006) 7371–7378.
[107] J. Lieberman, The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal, Nat. Rev. Immunol. 3 (5) (2003) 361–370.
[108] X. Jiang, et al., Interplay between the levels of estrogen and estrogen receptor controls the level of the granzyme inhibitor, proteinase inhibitor 9 and susceptibility to immune surveillance by natural killer cells, Oncogene 26 (28) (2007) 4106–4114.
[109] Y. Lin, J. Xu, H. Lan, Tumor-associated macrophages in tumor metastasis: biological roles and clinical therapeutic applications, J. Hematol. Oncol.. 12 (1) (2019) 76.
[110] A. Keselman, et al., Estrogen signaling contributes to sex differences in macrophage polarization during asthma, J. Immunol. 199 (5) (2017) 1573–1583.
[111] S. Kovats, Estrogen receptors regulate innate immune cells and signaling pathways, Cell. Immunol. 294 (2) (2015) 63–69.
[112] S. Svensson, et al., CCL2 and CCL5 are novel therapeutic targets for estrogen-dependent breast cancer, Clin. Cancer Res. 21 (16) (2015) 3794–3805.
[113] J. Hwang, et al., Role of annexin II in estrogen-induced macrophage matrix metalloproteinase-9 activity: the modulating effect of statins, Atherosclerosis 189 (1) (2006) 76–82.
[114] F. Garrido, T. Cabrera, N. Aptsiauri, "Hard" and "soft" lesions underlying the HLA class I alterations in cancer cells: implications for immunotherapy, Int. J. Cancer 127 (2) (2010) 249–256.
[115] N.F. Watson, et al., Immunosurveillance is active in colorectal cancer as down-regulation but not complete loss of MHC class I expression correlates with a poor prognosis, Int. J. Cancer 118 (1) (2006) 6–10.
[116] E.M. Curran, et al., Natural killer cells express estrogen receptor-alpha and estrogen receptor-beta and can respond to estrogen via a non-estrogen receptor-alpha-mediated pathway, Cell. Immunol. 214 (1) (2001) 12–20.
[117] E. Baral, E. Nagy, I. Berczi, Modulation of natural killer cell-mediated cytotoxicity by tamoxifen and estradiol, Cancer 75 (2) (1995) 591–599.
[118] A.E. Albrecht, et al., Effect of estrogen replacement therapy on natural killer cell activity in postmenopausal women, Maturitas 25 (3) (1996) 217–222.
[119] E.M. Curran, et al., Estrogenic regulation of host immunity against an estrogen receptor-negative human breast cancer, Clin. Cancer Res. 12 (19) (2006) 5641–5647.
[120] S. D'Oronzo, et al., Metastatic bone disease: Pathogenesis and therapeutic options: Up-date on bone metastasis management, J. Bone Oncol. 15 (2019) 4.
[121] P.A. van Dam, et al., RANK/RANKL signaling inhibition may improve the effectiveness of checkpoint blockade in cancer treatment, Crit. Rev. Oncol. Hematol. 133 (2019) 85–91.
[122] R. Coleman, et al., Effect of MAF amplification on treatment outcomes with adjuvant zoledronic acid in early breast cancer: a secondary analysis of the international, open-label, randomised, controlled, phase 3 AZURE (BIG 01/04) trial, Lancet Oncol. 18 (11) (2017) 1543–1552.
[123] J.A. Westbrook, et al., CAPG and GIPC1: breast cancer biomarkers for bone metastasis development and treatment, J. Natl. Cancer Inst. 108 (4) (2016).
[124] C. Tulotta, et al., Endogenous production of IL1B by breast cancer cells drives metastasis and colonization of the bone microenvironment, Clin. Cancer Res. 25 (9) (2019) 2769–2782.