Defining how oestrogen influences the anti-tumour effects of adjuvant bisphosphonates using in vivo models of breast cancer

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<tbody>
<tr>
<td>γδ T cell</td>
<td>Delta-gamma T cells</td>
</tr>
<tr>
<td>μCT</td>
<td>Micro-Computed tomography</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BV/TV</td>
<td>(Bone voxels/Total voxels) Bone volume fraction</td>
</tr>
<tr>
<td>CCD</td>
<td>Charged coupled device</td>
</tr>
<tr>
<td>CK5/6</td>
<td>Cytokeratin 5/6</td>
</tr>
<tr>
<td>CSCs</td>
<td>Cancer stem cells</td>
</tr>
<tr>
<td>CTC</td>
<td>Circulating tumour cells</td>
</tr>
<tr>
<td>CTIBL</td>
<td>Cancer Treatment–Induced Bone Loss</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma in situ</td>
</tr>
<tr>
<td>DFS</td>
<td>Disease-Free Survival</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DTC</td>
<td>Distant tumour cells</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Trypsin–ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to Mesenchymal Transition</td>
</tr>
<tr>
<td>ER (+ve; -ve)</td>
<td>Oestrogen receptor (positive; negative)</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FPPS</td>
<td>Farnesyl pyrophosphate synthase</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epithelial growth factor receptor 2</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard risk</td>
</tr>
<tr>
<td>HSC</td>
<td>Haematopoietic stem cell</td>
</tr>
<tr>
<td>IDC</td>
<td>Invasive ductal cancer</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL-β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IL-11</td>
<td>Interleukin 11</td>
</tr>
<tr>
<td>ILC</td>
<td>Invasive lobular cancer</td>
</tr>
<tr>
<td>IVIS</td>
<td>In-vivo imaging system</td>
</tr>
<tr>
<td>LCIS</td>
<td>Lobular carcinoma in situ.</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal to epithelial transition</td>
</tr>
<tr>
<td>METABRIC</td>
<td>Molecular Taxonomy of Breast Cancer International Consortium</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>MMP1</td>
<td>Matrix metalloproteinase-1</td>
</tr>
<tr>
<td>NCCN</td>
<td>National Comprehensive Cancer Network</td>
</tr>
<tr>
<td>N.Ob</td>
<td>Number of osteoblast</td>
</tr>
<tr>
<td>N.Oc</td>
<td>Number of cells osteoclast</td>
</tr>
<tr>
<td>NST</td>
<td>No Special/Specific Type</td>
</tr>
<tr>
<td>Ob.Pm.</td>
<td>Osteoblast perimeter in bone surface</td>
</tr>
<tr>
<td>Oc.Pm.</td>
<td>Osteoclast perimeter in bone surface</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>OVX</td>
<td>Ovariectomy</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PICP</td>
<td>Carboxy-terminal propeptide</td>
</tr>
<tr>
<td>PINP</td>
<td>Amino-terminal propeptide</td>
</tr>
<tr>
<td>PR (+ve ; -ve)</td>
<td>Progesterone receptor (postive; negative)</td>
</tr>
<tr>
<td>PTHrP</td>
<td>Parathyroid hormone-related peptide</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real time polymerase chain reaction (PCR)</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of nuclear factor kappa-β</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa-β ligand</td>
</tr>
<tr>
<td>R.C.F</td>
<td>Relative centrifugal force (times gravity)</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective oestrogen receptor modulators</td>
</tr>
<tr>
<td>SERD</td>
<td>Selective oestrogen receptor degraders</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF- α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>TRACP 5b</td>
<td>Tartrate-resistant acid phosphatase form 5b</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T lymphocyte</td>
</tr>
<tr>
<td>UTF</td>
<td>Tegafur/uracil; 1-[2-tetrahydro-furyl]-5-fluorouraci/uracil</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>Zol</td>
<td>Zoledronic acid / Zoledronate</td>
</tr>
</tbody>
</table>
Declaration

All work in this thesis is my own unless otherwise stated. Technical support was provided by Diane Lefley, Alyson Evans and Christopher George. I, the author, confirm that this Thesis is my own work. I am aware of the University’s Guidelines on the Use of Unfair Means. This work has not previously been presented for an award at this, or any other, University.
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Abstract

Bone is the most common site for breast cancer to metastasise to. Clinical trials have demonstrated that adding adjuvant zoledronic acid (Zol) to standard of care therapy reduces the risk of breast cancer relapse in bone and other organs in post-menopausal women. Whereas pre- and peri-menopausal women experience reduced bone metastasis but increased recurrence outside of bone. Serum analysis of the AZURE clinical trial has found a correlation between the concentration of oestradiol and the possible outcome following adjuvant therapy with Zol.

I hypothesise that Zol reduces bone metastasis via inhibition of bone resorption and further oestrogen driven changes to the bone microenvironment drives tumour cells to other sites. I aimed to establish the mechanism by which Zol interacts with oestrogen to affect tumour growth in bone and non-bone sites.

Methods: 12-week-old female BALB/c and BALB/c nude mice were ovariectomised (OVX) before receiving 0, 1.38 or 12.5 mg/L oestradiol to model pre-, peri- and post-menopausal conditions. All animals received 40ug/day Goserelin to prevent OVX induced FSH and animals were randomised to 100 µg/kg/week Zol or control. For tumour studies, MDA-MB-231-Luc2 or 4T1-Luc2 cells were administered via intra-cardiac injection, 3 and 4-days after OVX respectively for metastatic models or directly into the mammary fat pad for a model of spontaneous metastasis from the primary site. A clinically relevant dose of 100 µg/Kg Zol was administered weekly until end of experiment. Bone volume was measured by µCT, bone turnover and oestradiol/FSH were measured by ELISA. Histomorphometric analysis was carried out using Osteomeasure. Numbers of tumour and tumour growth was monitored by IVIS-Luc2 system and analysed using LUMINA II software.

Results/conclusions: Administration of 0, 1.38 or 12.5 mg/L oestradiol, to OVX mice, for 2-weeks resulted in serum oestradiol concentrations of 13±10 pg/ml, 49±18 pg/ml and 153±18 pg/ml, which mimic those found in pre-, peri- and post-menopausal women respectively. Oestradiol leads to increased bone volume, which is further increased in the presence of Zol (p=0.001 and p=0.036, respectively, observed in pre-menopausal concentration of oestradiol). In accordance with increased bone mass, increased numbers of osteoblasts and simultaneously decreased osteoclasts were observed. These effects were reversed when oestradiol levels were
decreased due to OVX; Effects of oestradiol on the bone microenvironment are strain-dependant. Similarly, Zol on osteoblasts varies between mouse model whereas it consistently decreases number and activity of osteoclasts across the different studies. Oestradiol did not alter the overall number of metastatic tumours in both immunocompromised and immunocompetent mouse model (p=0.595 & p=0.488, respectively), whereas Zol had a tendency towards a decrease in number of tumours in the immunocompromised model. In immunocompetent BALB/c mice, oestradiol did not alter tumour burden in the primary tumour model but in the metastatic model, post-menopausal concentrations of oestradiol increased tumour burden (p=0.012). Addition of Zol caused an increase of tumour burden in the primary tumour site under pre-menopausal concentrations of oestradiol (p=0.0002), and Zol decreased tumour burden in post-menopausal concentration of oestradiol in a metastatic model (p=0.080), indicating that the protective role of Zol is only observed under post-menopausal concentrations of oestradiol as observed in clinical studies.

Reduction of oestradiol as observed during the menopausal transition alters the bone cellular landscape by increasing bone resorption. Despite Zol affecting osteoclastic activity and cell number, bone mass did not return to pre-menopausal levels. Post-menopausal levels of oestradiol increased tumour burden in immunocompetent mice only. Zol reduced tumour burden in the post-menopausal group but not pre-menopausal group in the immunocompetent model. These results suggests that more complex cellular interactions apart from the osteoblast/osteoclast/tumour cells may be involved under the menopausal transition to produce the differential effect of Zol.
CHAPTER 1: INTRODUCTION
1.1 Introduction

Breast cancer is the leading type of cancer affecting women worldwide, accounting for 2.3 million new diagnosed cases in 2020 from which 685,000 resulted in death (WHO, 2023). Similarly, in the UK, breast cancer represents a 15% of all new cancer cases (CANCER UK, 2023), being an estimated of 55,000 new cases each year (CANCER UK, 2023), from which 11,400 cases resulted in death between 2017-2019.

The most common complication of breast cancer is the spreading to distant organs, also known as metastasis. Approximately 90% of the cancer-associated deaths are due to distant metastasis (Chaffer & Weinberg, 2011). The most common distant sites for breast cancer to metastasise are distant lymph-nodes, lungs, brain, liver and skeletal tissue (bone). From this list, skeletal tissue is the most commonly affected organ by metastasis, being observed in 70% of patients with late state breast cancer (Chaffer & Weinberg, 2011; Coleman & Rubens, 1987). To date, breast cancer metastasis in bone is an incurable condition, which tends to develop skeletal related complications. Metastatic breast cancer in bone can produce two main types of lesions, osteogenic lesions (inducing abnormal bone production) or, most commonly, osteolytic lesions (inducing bone degradation). Both type of lesions represents a problem to the general health of the patient as they often present skeletal related complications such as loss in the bone mass (mainly in osteolytic lesions), leaving the bone prone to fractures. Metastasis to bone can result in pain caused by nerve compression, especially in the spine cord. Hypercalcemia (high concentrations of calcium in the blood) is also observed as a result of an increased bone resorption. Increased levels of calcium can lead to generation of kidney stones, kidney failure, irregular heartbeat, and related neurological problems escalating from confusion to more severe conditions such as coma (O’Brien et al., 2010). Therefore, prevention and treatment of breast cancer metastatic to bone is urgent.

1.2 Risk associated with development of breast cancer

Breast cancer is a spontaneous disease from which half of the incidences have no specific attribution to a particular risk factor. However, there are several factors related to a higher risk of developing breast cancer. These factors are generally divided into two groups, endogenous and external factors. The endogenous factors are those which cannot be modified and are independent from the lifestyle of the individual, whereas external factors vary depending on
lifestyle. Within the endogenous factors, an increase in the incidence of breast cancer is strongly associated with age. Approximately a quarter of new incidences were observed in people aged 75 and older in the UK (Cancer Research UK, 2023). Although aging is a natural process and not a risk factor on its own, it is the accumulation of physiological and pathological events over time that increases the risk of developing breast cancer.

1.2.1 Endogenous factors

Sexual hormones (also referred as sex steroids), such as oestrogens (mainly oestradiol and oestriol), progesterone and testosterone, can be associated with a higher risk factor depending on their concentration during specific periods of a woman’s life. In post-menopausal women, the presence of relatively high concentrations of oestradiol (>9 pg/ml), oestriol, androstenedione and testosterone in serum is associated with a higher risk of breast cancer in comparison to women with half or lower concentrations of these hormones (Zhang et al., 2013). Conversely, in pre-menopausal women, the risk of developing breast cancer is not significantly associated with oestrogen levels but testosterone. Higher concentrations of testosterone in serum have been associated with a 56% higher risk, whilst other sex hormones show no clear association (Kaaks et al., 2014; Walker et al., 2011).

High breast density is another endogenous parameter which can be associated with a risk of breast cancer. Females with high breast density (accounting only for non-fatty breast tissue) have an increased risk, between 3 to 5 times higher, of developing breast cancer (McCormack & Silva, 2006; Pettersson et al., 2014) in comparison to lower breast density. Dense breasts are also associated with an increased risk of in situ breast carcinoma (Bertrand et al., 2015). However, evidence suggests that serum concentrations of oestrogens do not appear to have a significant correlation with breast density and the association of developing breast cancer (Schoemaker et al., 2014; Tamimi et al., 2007; Varghese et al., 2012).

Other endogenous factors such as family history and genetic make-up tend to indicate higher risk of breast cancer when present. Women with family history of one first-degree relative with breast cancer are twice more susceptible to develop breast cancer in comparison to women with no first-degree family history. This likelihood increases drastically with higher numbers of
affected family members or when relatives develop breast cancer under the age of 50 (Beral et al., 2001; Pharoah et al., 1997). Similarly, it has been proposed that Oestrogen Receptor-positive (ER+ve) and Oestrogen Receptor-negative (ER-ve) breast cancer subtypes are related to family history to a certain degree (Mavaddat et al., 2010). However, over 85% of women with a first-degree relative with breast cancer will unlikely develop breast cancer, whereas 87% of women with breast cancer have no first-degree relatives with the disease (Beral et al., 2001). In addition, women with mutations such as BRACA1 and BRACA2 are associated with a higher risk of developing breast cancer compared with non-carrier women (Lichtenstein et al., 2000). Women carrying either BRACA1 or BRACA2 mutations have a high risk of developing breast cancer by the age of 70 (Antoniou et al., 2003). This risk is further increased in BRACA2 carriers with previous cases of first-degree family members with breast cancer (Metcalfe et al., 2010). Although in the general population, the presence of BRACA1/2 mutations is relatively low (approximately 0.11% and 0.12% of the population, respectively), they account for a 2% of the cases of breast cancer overall and up to a 20% of the cases with family history with a first-degree relative with breast cancer (Peto et al., 1999; Turnbull & Rahman, 2008).

1.2.2 External factors

There are numerous external factors that can contribute to the development of breast cancer, from which, reproductive factors, body weight, breast feeding, consumption of oral contraceptives, alcohol and tabaco have a higher relevance.

An early menarche (first menstrual period) has been associated with an increase of 5% of developing breast cancer for each year younger at menarche. The associated types of breast cancer for this event are mainly hormone-receptor positive, oestrogen- or progesterone-receptor positive (ER +ve; PR+ve) (Beral et al., 2012; Bodicoat et al., 2014). On the other hand, having children during early adulthood has been associated with a lower risk in developing breast cancer. It has been observed that the risk decreases in an approximate 7% for each live birth (Ma et al., 2006). Also, the risk of developing either ER+ve or PR+ve is 25% lower in women who have had children in comparison to women who have not (Kim et al., 2012). Additionally, parity and the risk of breast cancer depends directly in the tumour type (Reeves et al., 2009). In contrary, it has been observed that having children at an older age is associated with a 15%
higher risk of developing breast cancer compared to women having children at a younger age, increasing a 3% for each year older. This correlation was found to be mostly relevant for ER +ve and PR +ve tumours, as human epidermal growth factor receptor 2 (HER2) positive and triple-negative tumours do not show any association with age at birth (Lambertini et al., 2016; Ma et al., 2006).

The lack of breastfeeding is also associated with a higher risk of breast cancer development. Approximately, a 5% of breast cancer cases are associated with the lack of breastfeeding (Brown et al., 2018), whereas breastfeeding lowers the risk of developing ER +ve or PR +ve tumours by approximately 23%, and triple-negative tumours by around 21% (Lambertini et al., 2016).

A high body weight may be associated with a higher risk of breast cancer, although in an indirect manner. A higher proportion of body fat in females tends to correlate with higher levels of steroid hormones, which in turn, might be associated with an increased risk of breast cancer (Key et al., 2003). However, evidence suggest that the gain of body weight is more critical at specific periods of a woman’s life, ant there is a higher association of developing breast cancer when body mass is gained after middle adulthood and specially after menopause (Gaudet et al., 2014). Similarly, exposure to a hormone replacement therapy increases the risk of breast cancer development during middle adulthood and after menopause, as previously mentioned (section 1.2.1). The impact of the hormonal replacement therapy depends in the type of hormone administered. An oestradiol-only replacement therapy is associated with a 37% higher risk of breast cancer, whereas a combined replacement therapy (oestrogen and progesterone) increases the risk to a 112% in comparison to women with no exposition to these therapies (Collaborative Group on Hormonal Factors in Breast Cancer, 2019).

The consumption of alcohol and tabaco may be associated with an increased risk of developing cancer. Diverse meta-analyses have demonstrated that both alcohol and tabaco are related with the development of diverse type of cancer (Bagnardi et al., 2014; Gaudet et al., 2013), an association with breast cancer development is noted in moderate and heavy consumers of alcohol in comparison to no consumers as well as current or former smokers in comparison to non-smokers.
1.3 Classification of Breast Cancer

The classification of breast cancer has evolved over the past decades. Initially, the classification of breast carcinoma was through histological analysis. This resulted in breast cancer being categorized into two main types, in situ, and invasive or infiltrating breast cancer. In situ carcinoma can be further sub-classified into ductal and lobular; ductal carcinoma in situ (DCIS) is considerably more frequent than the lobular carcinoma in situ (LCIS). DCIS is mostly referred as an abnormal growth of the resident epithelial cells contained within the breast’s ducts (Allred, 2010). Although its prognosis is highly favourable due to the metastasis-free survival rate of 99.1% after first 8 years upon diagnosis, it is the immediate precursor of invasive breast cancer. Before the introduction of mammography, early classification of DCIS was based on the presence of palpable masses or in the microscopic growth pattern and cellular features. DCIS is divided into comedo, cribriform, solid, micropapillary and papillary; nonetheless, a large proportion of DCIS shows complex mixtures of growth patterns, therefore, an additional classification using a numerical scoring and grading was adopted based on cellular differentiation and growth. This classification is ultimately recognised into 3 grades, corresponding to well (grade 1), moderately (grade 2), and poorly (grade 3) differentiated DCIS. When compared to previous classification, comedo DCIS is a poorly differentiated DCIS, whereas the rest types are variable (Shoker & Sloane, 1999). Current treatment for DCIS is surgery, according to the NICE guidelines (guideline NG101, N. I. C. E., 2018), preferably breast-conserving surgery over re-excision or mastectomy. The addition of adjuvant therapies, chemotherapy and/or radiotherapy is not necessary, however, it is suggested when there is evidence of invasive breast cancer.

LCIS, similarly to DCIS, is an abnormal growth of cells terminal ductal lobular unit of the breast which is not necessarily related with invasive breast cancer but is considered as a risk factor for the development of breast cancer. Unlike DCIS, diagnosis of LCIS is usually and incidental finding through histological examination as it is almost non-detectable on a mammography (prevalently classical LCIS) (Hande et al., 2021). LCIS is classified in classical and non-classical, being non-classical referring to pleomorphic-LCIS and LCIS with necrosis or florid LCIS. Due to its low risk of invasiveness and the lack of increased benefit after excision (KaKhlis et al., 2016), classical LCIS is normally non-treated, whereas non-classical LCIS tends to be managed with
surgical remotion in countries such as USA (The American society of breast surgeons, 2023); however, in the UK LCIS is not covered under the NICE guidelines for surgical management but with continuous follow ups (guideline NG101, N. I. C. E., 2018). Up to date, there is still discrepancy whether LCIS is a precursor of invasive lobular cancer (ILC) (Andrade et al., 2012; Lee et al., 2019).

Invasive breast carcinoma is divided into 6 subtypes. These are: invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC), mucinous (colloid), tubular, medullary and papillary carcinomas. The most prevalent invasive breast cancer is IDC, also refer as No Special/Specific Type (NST), which is present in between 70% to 80% of all invasive lesions (Li et al., 2005). IDC is also categorized into three different grades, well-differentiated (I), moderately differentiated (II), and poorly differentiated (III). This grading system, also known as the Nottingham Modification of the Bloom-Richardson system (Meyer et al., 2005), is based on the levels of nuclear pleomorphism, glandular/tubule formation and mitotic index (Dai et al., 2015). The grades can also give an insight of the prognosis of the disease. Grade I is normally associated with a better prognosis because of the high homology to the normal breast tissue. Grade II shows certain grade of differentiation and increased levels of nuclear pleomorphism. Grade III is poorly differentiated and presents a high level pleomorphism. These grades are associated with intermediate and poor prognosis respectively. However, grading alone is unable to predict the disease outcome, it must be analysed with the size of the tumour and the level of infiltration of the cancerous cells into the lymph-node (i.e., lymph-node involvement) (Pereira et al., 1995; Rakha et al., 2010). The combination of the histological tumour grading in association with the tumour type and immunohistochemistry observations of molecular changes such as ER/PR or HER2 amplification were the foundations of a more robust staging system, which could be applied to all subtypes of cancer and parameters of tumour, node, metastasis (TNM) (Brierley et al., 2017). Up to date, this staging system still represents the common language for evaluation and treatment of invasive breast cancer.

With the introduction of microarrays and gene expression profiling, it has become possible to implement molecular classifications of breast cancer. The analytical difference between the gene expressions in the tumour reflects the actual molecular differences in the distinct tumour phenotypes. Currently, there are 5 well accepted molecular subtypes of breast carcinoma which are robustly divided according to the presence of various biological markers (Hu et al.,
The intrinsic subtypes are the luminal A, luminal B, HER2 overexpression, basal-like, and normal breast tissue-like phenotype (Normal-like) (Weigelt et al., 2010). Examples of the markers used for the identification are hormone receptors, such as ER, PR, HER2, cell proliferation marker Ki67; cytokeratin 5/6 (CK5/6) and epidermal growth factor receptor (EGFR) (Chen et al., 2018) (Table 1).

Other classifications also recognise the presence of the 5 intrinsic breast cancer subtypes; however, they further subdivide a proposed subtype or incorporate new subtypes. An example of this is the identification of the interferon subtype (Hu et al., 2006), the molecular apocrine (Farmer et al., 2005) and the Claudin-low phenotype (Herschkowitz et al., 2007; Prat et al., 2010) (Table 1). The expansion of the breast cancer subtypes is mainly caused by new gene expression profiling studies and association of new gene clusters. It is also worth mentioning that the majority of these new profiling studies are not incorporated into the clinics due to their high costs. Therefore, several researchers and companies have narrowed down the number of gene signatures to be tested. This includes the use of assays such as quantitative real time PCR (qRT-PCR) in the case of the PAM50 (50-gene signature) and Oncotype DX (26-gene signature), also the use of diverse microarrays (Dai et al., 2015).

Currently, the use of high-throughput technology has incorporated a more complex and multi-level analysis of breast cancer tumours genomic, transcriptional, translational and epigenetic data. With these data, the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) study proposed a more refined breast cancer taxonomy where integrative clusters were created (Curtis et al., 2012). These clusters, named IntClust 1 to 10, incorporate the 5 intrinsic breast cancer subtypes where IntClusts 3, 7, 8 are primarily composed of luminal A tumours, IntClusts 1, 6, 9 are mainly luminal B cancers, IntClust 5 is composed by HER2 tumours, IntClust 10 contains the majority of basal tumors, IntClust 2 is enriched in luminal cancers but exhibits a high mortality rate, and IntClust 4 is composed of both ER+ and ER- tumors and varied intrinsic subtypes, and shares similar genomic features with IntClust 3.
### Table 1. Intrinsic subtyping classification.

<table>
<thead>
<tr>
<th>Molecular subtype</th>
<th>Biological marker</th>
<th>Additional biological marker</th>
<th>Grade classification</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Accepted intrinsic subtypes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal A</td>
<td>[ER+</td>
<td>PR+/−], HER2\text{low}, Ki67\text{low}</td>
<td>-</td>
<td>1,</td>
</tr>
<tr>
<td>Luminal B</td>
<td>[ER+</td>
<td>PR+/−], HER2\text{low}, Ki67\text{high}</td>
<td>-</td>
<td>2,</td>
</tr>
<tr>
<td>HER2 over-expression</td>
<td>[ER−</td>
<td>PR−], HER2\text{high}, CK5/6+, EGFR−</td>
<td>GRB7\text{high}, PGAP3\text{high}</td>
<td>2,</td>
</tr>
<tr>
<td>Basal-like</td>
<td>[ER−</td>
<td>PR−], HER2\text{low}, CK5/6+, EGFR+</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>Normal-like</td>
<td>[ER+</td>
<td>PR+], HER2\text{low}, Ki67\text{low}, CK5/6+, EGFR+</td>
<td>-</td>
<td>1,</td>
</tr>
<tr>
<td><strong>Supplementary subtypes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal-HER2</td>
<td>[ER+</td>
<td>PR+], HER2\text{high}, Ki67\text{high}</td>
<td>-</td>
<td>2,</td>
</tr>
<tr>
<td>Triple Negative non-basal</td>
<td>[ER−</td>
<td>PR−], HER2\text{low}, CK5/6−, EGFR−</td>
<td>P53 mutation\text{high}</td>
<td>3</td>
</tr>
<tr>
<td>Molecular apocrine</td>
<td>[ER−</td>
<td>PR−], HER2\text{high}, CK5/6+, EGFR−</td>
<td>AR+</td>
<td>2,</td>
</tr>
<tr>
<td>Claudin-low</td>
<td>[ER−</td>
<td>PR−], HER2\text{low}, CK5/6+, EGFR−</td>
<td>CDH1\text{low}, CLDN\text{low}</td>
<td>3</td>
</tr>
</tbody>
</table>

Adapted from Dai et al., 2015 and Chen et al., 2018.

**1.4 Classification and clinical features**

The luminal-like tumours have an expression profile of hormone receptors (as explained in table 1) and represents the most common subtype among breast cancer; nearly 65% of cases (O’Brien et al., 2010). From this, Luminal A is the most frequent subtype accounting for a 54.3% of all cancer cases. In general, luminal-like subtypes are related to a good prognosis because they tend to respond well to the therapy (Dai et al., 2015). However, Luminal B is always related to slightly worst prognosis due to its high proliferative phenotype. Luminal A tumours express ER +ve and PR +/- ve and HER2 negative (low expression), being highly dependent on hormones (oestradiol or progesterone) for its development. Therefore, use of endocrine therapy tends to be highly effective on this type of tumours. Treatment of hormone receptor positive tumours
can be treated by three major classes of endocrine therapies: Prevention of ovarian production of oestrogen (Goserelin), prevention of oestrogen production outside the ovary (Aromatase inhibitors, Letrozole), and blockage of the oestrogen receptor (Selective oestrogen receptor modulators [SERMs] and selective oestrogen receptor degraders [SERDs]). Although the main goal of these three therapies is to block ER signalling, their mechanism of actions differs between each other, as well as the strategy of use depending on the menopausal status of the patient (Dai et al., 2015; Patel & Bihani, 2018). The use of SERM, Tamoxifen, is one of the most common used endocrine therapies for the treatment of early ER +ve breast cancer as it can be used in pre-menopausal and post-menopausal women. Although Tamoxifen can still be used for the treatment of late stages of ER +ve breast cancer, other alternatives are preferred such as SERM, Toremifene (Mao et al., 2012), or Aromatase inhibitors (post-menopausal women) (Dowsett et al., 2010). Treatment in post-menopausal patients is mainly oriented towards a further reduction in the external production of oestradiol by use of Aromatase inhibitors (Letrozol), whereas in pre-menopausal patients, chemical induction of menopause (goserelin) causes the ovary to stop producing oestradiol. In cases where tumours have created resistance to the primary endocrine treatment, a different endocrine therapy or further combinations are proposed. The use of SERD, Fulvestrant, has shown positive activity on treatment-resistant breast cancer and represent a reliable alternative once previous endocrine therapies, such as Tamoxifen, had not caused a response on the tumours (Wardell, Marks, & McDonnell, 2011).

Unlike Luminal A, Luminal B tumours express ER +ve (PR +/-ve), but presence of a high expression of HER2 confers endocrine resistance, making the use of endocrine therapy only a challenge. Therefore, combination of endocrine therapy with anti-HER2 (lapatinib, trastuzumab) is preferred (Burstein et al., 2014), as well as endocrine therapy combined with chemotherapy (Dai et al., 2015).

HER2 over-expressing tumours are characterized by the lack of hormone receptors and it is normally associated with a moderate or a poor prognostic. Differently from the luminal subtypes, HER2 over-expression subtype does not benefit from endocrine therapy, therefore chemotherapy is normally the preferred therapy. In recent decades, the development of anti-HER2 therapies have yield the incorporation of monoclonal antibodies (margetuximab, pertuzumab, trastuzumab), antibody-drug conjugates (am-trastuzumab-deruxtecan-nxki; T-DM1 or ado-trastuzumab emtansine), Pan-HER inhibitor (neratinib), transduction and tyrosine...
kinase inhibitors (lapatinib and tucatinib, respectively) (Patel et al., 2020). Most of these therapies are used as complementation of primary chemotherapy or as a second line therapies after tumour resistance with positive results, including treatment of late stages of tumours and metastasis.

Basal tumours are identified for the ER, PR, and HER2 at low or no expression at all. This subtype accounts for 60% to 90% of the triple negative cases. Basal subtype is associated with poor prognosis due to the lack of standard target therapy and an alarming early relapse. Unlike the other subtypes, basal tumour affects young women, normally pre-menopausal (O’Brien et al., 2010). In recent years, new treatments such as PARP inhibitors (Olaparib, talazoparib, rucapabarib) have demonstrated a positive effect in managing primary and metastatic triple-negative breast cancer tumours. Combination with either radiotherapy or chemotherapy is always preferred as it maximises the benefits of the adjuvant therapy. Additionally, the introduction of immune checkpoints inhibitors (anti-PD-1 and PD-L1 blockage) have increased the management of these basal tumours. During tumour development, tumour cells evade immunological surveillance by disrupting either generation of activated tumour-directed T cells, infiltration of activated T cells into the tumour microenvironment, or killing of tumour cells by activated T cells. Immune checkpoints inhibitors prevent this ability from tumour cells to conditionate T cells via targeting PD1 and PD-L1 (Swoboda & Nanda, 2018). This therapy has not been limited to basal tumours only, but to all infiltrating breast cancer tumours, specially HER2 overexpression tumours.

1.5 Breast Cancer Metastasis

Cancer metastasis is a complex multistep process which starts with a small cell population, the metastasis-initiating cells, detaching from the primary tumour, followed by the intravasation to the blood or lymph circulatory systems, successful avoidance of the immune system, extravasation to adequate hosting tissue and proliferation.

In order to be able to break away from the tumour, the cancerous cell has to acquire an invasive and migratory phenotype of a mesenchymal cell. This morphological change is known as the Epithelial to Mesenchymal Transition (EMT) and is the result of molecular rearrangement influencing protein expression (Wang & Zhou, 2011). EMT has been observed in both normal
and pathological processes (Kalluri & Weinberg, 2010). Currently, EMT is divided into three types based on their biological role. Type 1, is observed in embryogenesis and histogenesis. Type 2, is involved in the non-neoplastic process and wound healing. Type 3, represents the cellular disorders such as cancer.

Although the trigger of type 3 EMT is unclear, there are several biomarkers that allows the identification between the epithelial and the mesenchymal-like phenotypes. Loss of E-cadherin, the molecule responsible for the cell-cell adhesion, has been accepted as a hallmark of EMT. Similar to E-cadherin, other cell-cell adhesion associated proteins are down-regulated such as claudin 3, 4 and 7; α-catenin; β-catenin and δ-catenin. On the other hand, vimentin, fibronectin, SLUG, and SNAIL (downstream of Transforming growth factor-β [TGF-β]) are upregulated during biomarkers in the EMT process (Wang & Zhou, 2011; Ottewell et al., 2015a).

Morphologically, the tumour cells undergoing EMT lose their cellular polarization and start showing a fibroblast-like elongation. It is worth mentioning that in EMT, the cancerous cells avoid anoikis (a programmed death cell occurring when there is no cell-cell or cell-cellular matrix contact) through a depletion of CDH1 caused by repressor factors such as TWIST (Onder et al., 2008). Acquiring a mesenchymal phenotype allows the cancerous cell to migrate from the primary tumour and further intravasation.

EMT, apart for being key in the onset of metastasis, is also vital for the cancerous cell survival in the bloodstream. EMT causes a detyrosination on the extracellular protein α-tubulin. This modification enables the production of tubulin microtentacles which provide the circulating tumour cells (CTC) the ability to attach to the endothelium and exit the bloodstream. Not only is the CTC able to intravasate, it can also increase its intravascular life from 3-5 minutes to 16 hours (Webster et al., 1987; Whipple et al., 2010).

Once in the bloodstream (or lymph), the CTCs have to home to and colonise distant organs that are permissible to allows their proliferation. Stephen Paget proposed “the seed and the soil” hypothesis to explain the invasion of cancerous cells to certain organs due to their compatible environment (Paget, 1889). To date, this hypothesis is well accepted, however, there are certain cellular conditions that the CTCs (seeds) have to fulfil in order to colonise distant organs (soil). Diverse investigations have demonstrated that not all the CTCs are able to create a
secondary tumour. They need to express a stem cell-like phenotype (Al-Hajj et al., 2003; Mirza et al., 2017). In breast cancer, markers such as CD44$$^{\text{high}}$$, CD24$$^{\text{low}}$$, EpCAM+ and CD47+ (the last two markers were confirmed in mice) are indicative of a stem cell-like phenotype and therefore indicates the potential to become a tumorigenic cell (Al-Hajj et al., 2003; Baccelli et al., 2013). In a study carried out by Morel et al. (2008), induction of EMT on normal-tissue-derived breast tumour cells in vitro enhanced the expression of CD44$$^{\text{high}}$$ and CD24$$^{\text{low}}$$, resulting in the acquisition of a stem cell-like phenotype. This finding suggested that stemness is acquired as a result of the activation of the mesenchymal phenotype which agrees with the findings that not all CTCs are tumour-forming cells. The metastatic niche also plays an important role in the CTC stemness and the proliferation to a secondary tumour. For instance, in bone, the haematopoietic niche is the main target of the CTCs, as this microenvironment is able to sustain the plasticity of the stem-like phenotypes (Plaks et al., 2015). Within the bone niche, the cancerous cells can either proliferate to overt metastasis or remain as a dormant cell. The mechanisms behind this cellular decision is still unclear. Dormancy also represents an obstacle for cancer therapies as this cell can be re-activated many years after treatment and cause relapse (Dittmer, 2017).

Although all the cancer stem cells (CSCs) have the potential to invade any organ in the body, evidence suggests that depending on the breast cancer subtype, there is an inclination to metastasise to specific organs rather than others (Chen et al., 2018). This section is covered further in this review.

To enable colonisation of the metastatic organ, the cancerous cell has to undergo further morphological reprogramming to re-acquire the epithelial phenotype again, through a process known as mesenchymal to epithelial transition (MET). The MET process is the reverse of EMT and is believed to be regulated by epigenetic changes rather than genetic alterations, since the same proteins and transcriptional factors axis are involved. Most of the regulations of the MET process occurs by MicroRNA targeting CDH1 suppressors (Ottewell et al., 2015; Takaishi et al., 2016).
1.6 Organotropism of breast cancer subtypes

Breast cancer metastasis has been observed as a non-random process. Retrospective investigations have shown certain molecular features of the intrinsic subtypes associated with site-specific metastasis (Kennecke et al., 2010; Smid et al., 2008). For instance, all subtypes are prone to bone metastasis, however, luminal HER2 subtype has a higher tendency to invade this site. HER2 over-expressing tumours are more likely to produce brain metastasis whereas those of luminal A and luminal B have a lower probability. Basal-like and triple negative subtypes are associated with a high rate of brain, lung, and distant lymph-node metastasis; however, basal-like subtype is specifically related to a reduced probability of bone and liver metastasis. Although the subtype has some tendencies to invade a specific organ, the metastatic organ requires specific microenvironment features to home the cancerous cells, which are summarised in figure 1 (Kennecke et al., 2010; Wu et al., 2017).
Figure 1. 1 Subtype-based organotropic metastasis. The most common sites for metastasis are bone, liver, lung, brain, and distant lymph-nodes. Each tissue is susceptible to be affected by distant metastasis according to the intrinsic tumour subtype as well as certain key molecules produced by the host tissue (Adapted from Chen et al., 2018).

**1.7 Attraction of breast cancer metastasis to bone**

Skeletal tissue is the most common site for breast cancer metastasis due to its dynamic biology of bone remodelling. In normal conditions, bone homeostasis is conserved by the balance of two processes: bone resorption (directed by osteoclasts), and bone formation (produced by osteoblast) (Tanaka et al., 2005). Osteoblasts are derived from mesenchymal progenitor cells committed to an osteoprogenitor phenotype. Osteoblasts use a vast molecular machinery including enzymes (such as alkaline phosphatase and collagenase), growth factors (Transforming growth factor β [TGF-β] and Insulin-like growth factor [IGF]), osteocalcin and type 1 collagen to produce new bone. Once the bone has been formed, osteoblasts can either undergo apoptosis or remain as lining cells at the bone surface. These cells will differentiate into osteocytes. Both lining osteoblast and osteocytes are important sources of receptor activator of nuclear factor kappa-B ligand (RANKL), which signals the maturation and activation...
of osteoclasts. Osteoclasts are multinuclear cells derived from a myeloid lineage. The osteoclast precursors express RANK and waits for activation through RANKL. Activated osteoclasts proceed to focally erode bone by an acidic and proteolytic mix of enzymes, osteoclast-derived cathepsin K, matrix metalloprotease MMP13 and tartrate-resistant acid phosphatase (TRAP). The bone degradation releases growth factors (TGF-β, IGF, Platelet derived growth factor [PDGF]) to allow stimulation of new osteoblast formation and the regeneration of the tissue (Figure 1.2). This bone cycle represents the perfect “soil” for metastatic cells by offering a variety of growth factors.

**Figure 1.2. The bone turnover process is maintained by the osteoclast and osteoblast.** Osteoblast maturation starts when growth factors stimulate the pre-osteoblast (derived from a mesenchymal stem cell (MSC)). Osteoblast, lining cells and osteocytes regulates the expression of RANKL, but osteoblast regulate this chemokine by osteoprotegerin (OPG), a decoy molecule of RANKL. RANKL will activate the pre-osteoclast (derived from a hematopoietic stem cell (HSC)) and gives rise to an osteoclast. The activated osteoclast will degrade the bone and release growth factors.

Other molecules also play an important role for metastatic cells homing to bone. OPG has been observed to protect breast cancer cells from TRAIL-induced apoptosis (Holen et al., 2005; Neville-Webbe et al., 2004). Integrins are adhesion proteins that allow cell-cell and/or cell-extracellular matrix interaction. In breast cancer cells, overexpression of integrin α5β3 facilitates bone metastasis since this integrin binds to the skeletal cellular matrix and other
structural proteins found in the bone tissue (Harms et al., 2004; Kwakwa & Sterling, 2017). Furthermore, integrin \( \alpha 4 \beta 1 \) has been proposed to be implicated in the transition of indolent bone micrometastasis to overt metastasis (Lu et al., 2011). Integrin \( \alpha 4 \beta 1 \) is expressed by different hematopoietic cells, including osteoclast progenitors and interacts with bone-homing dormant breast cancer cells by vascular cell adhesion molecule 1 (VCAM-1) to activate them (Lu et al., 2011).

Diverse cytokines, chemokines and other growth factors also play an important role in promoting bone metastasis. TGF-\( \beta \) is released from bone during osteoclastic bone resorption, which in combination with hypoxia, stimulates metastatic cells to express Interleukin 11 (IL-11), CXCR4 and MMP-1 (Kang et al., 2003). Commonly, overexpression of CXCR4 by the metastatic cells has been strongly associated with bone metastasis. This is due to the localized expression of the ligand CXCL12 in bone (also found in lymph-nodes, lung and liver) (Müller et al., 2001). However, CXCR4 should not be a universal marker for bone metastasis since bone-homing cell lines (MDA-MB-231) do not express this chemokine (Nutter et al., 2014). Additionally, IL-1\( \beta \) expression in breast cancer was found to be associated with bone metastasis. It is proposed that IL-1\( \beta \) promotes homing of tumour cells to bone and stimulates proliferation of dormant tumour cells into overt metastases in this organ (Nutter et al., 2014; Holen et al. 2016).

Distant tumour cells (DTCs) from breast cancer tend to home to the axial bones which contain mature bone marrow, evidencing the attraction of the DTCs to the mature stem cells niches (Coleman, 2006). Current understanding of bone microenvironment points out three primary niches within the bone marrow: the haematopoietic niche, the endosteal/mesenchymal niche and the vascular niche. The haematopoietic niche homes the well-defined hematopoietic stem cell (HSC), whereas the mesenchymal niche homes late mesenchymal stem cells (MSC) with cell lineage such as osteoblast, chondrocytes, fibroblasts and other stromal cells. Vascular niche, on the other hand, is well known to be highly dynamic due to the number of vessels and blood perfusion. Current knowledge point that the endothelial cells in the vascular niche are responsible to induce and maintain dormancy in metastatic breast cancer cells (Allocca et al., 2019; Ghajar et al., 2013). Similarly to the vascular niche, mesenchymal niche has an important role in the maintenance of tumour cell in a quiescent state. A study using bone anti-resorption agent, Zoledronic acid (Zol), showed that after a reduction in osteoblasts, tumour cells seek
regions dense in osteoblastic cells and stayed dormant (Haider et al., 2014). Finally, the haematopoietic niche has been well described to be highly dense in expression of CXCL12, thus attract tumour cells. At difference with the other niches, the haematopoietic niche is very competitive between tumour cells and HSC. Once the tumour cell has established in the niche, it proliferates to overt metastasis (Schuettpelz et al., 2011).
1.8 Bone physiology and microenvironment

The dynamics of bone tissue are established around balanced mechanisms of constant self-repair, involving reabsorption and cell renovation, in a process known as remodelling (Robling et al. 2006). The quantity and quality of this tissue renewal is determined by intrinsic and environmental factors, among others, mechanical loading, and the interaction with biomolecules such as cytokines, hormones, and other markers in its microenvironment (Teissier et al. 2022). Bone can be arranged as compact bone and spongy bone. It is surrounded by connective tissue called periosteum which has access to circulation (Robling et al. 2006).

Compact bone constitutes around 80% of the entire structure and its location is mainly cortical. It is shaped by bundles of tissue in a circular pattern called osteon (Isojima & Sims 2021), which is often described as the functional unit of bone and its arrangement is highly packed (Lopes et al. 2018). Each osteon has a central canal with blood and lymphatic vessels transporting nutrients and waste surrounded by lamellae, which is the rigid structural component of bone (Robling et al. 2006, Zhu et al. 2021). Embedded within the osteons are small pockets called lacunae in which cells are located (Aoki et al. 2021). In a similar manner between the lamellae, there are tinier channels known as canaliculi, which serve as communicating passages between cells (See figure 1.3).

The spongy bone takes more space and responds to stress more effectively, this type also contains layers of lamellae, however, these are arranged within big spaces called trabeculae. Since it is not as crowded as compact bone, nutrients can move around the extracellular matrix (ECM) more effectively by simple diffusion to transport molecules through (Callens et al. 2021).

Remodelling is happening at all times since bone serves as storage for calcium and other minerals, and this tissue respond to the stress placed on it (Robling et al. 2006, Hadjidakis et al. 2006). According to Wolff’s law, the change in bone metabolism occurs in response to altered loading patterns (Zhou et al. 2014). Thus, if we increase stress in bone, the mechanisms of osteogenesis will be activated, whereas if the stress is decreased, bone replacement will be slower (Sampath et al. 2015).
Figure 1. 3. Structural components of the skeletal tissue. Left to right, from macro to micro components of the bone is made up of compact tissue (the hard, outer layer) and cancellous tissue (the spongy, inner layer that contains red marrow [not shown]). Bone is composed from an array of organic (collagen) and non-organic (hydroxyapatite) structures. Bone tissue is maintained by bone-forming cells called osteoblasts and cells that break down bone called osteoclasts Adapted from Guanyin Zhu, 2021.

Other molecules like oestrogen, parathyroid hormone, vitamin D3, and calcitonin, have a direct influence in the remodelling process, altering speed and sites of remodelling. For instance, during menopause when the levels of oestrogen deficiency, that process of cleaning and clearing is outpaced by bone building (Weitzmann et al. 2006).

1.8.1 Extracellular matrix

Formed in its majority by collagen fibres (Mohamed 2008), the extracellular matrix (ECM) has among other functions to transport soluble and insoluble biomolecules from various cells. These includes cytokines, hormones, and other proteins, thus allowing the regulation of the microenvironment and the subsequent behaviour of cell homeostasis (Robling et al. 2006). Each collagen fibre is around 1µm in length and between 35-60 nm in diameter.
The ECM also contains a decent amount of non-collagenous proteins such as the adhesion protein fibronectin, osteonectin, osteocalcin, osteopontin, and proteoglycans (Liao et al. 2011). Even though the function of these is not structural, they play an important role in the cell fate and bone tissue regulation (Zhu et al. 2021, Mohammed 2008). The main inorganic component of the bone is hydroxyapatite. The mineralization of these crystals happens in between the collagen fibres in a regulated mechanism by over 200 different kinds of acid proteins (Zhu et al. 2021, Feng et al. 2021).

### 1.9 Cellular components of the bone

The bone tissue has its origin from distinct progenitor cell populations which undergo a variety of metabolic processes for reabsorption and repair. Around 90% of the cells in the bone are mature cells called osteocytes located in the lacunae (Aoki et al. 2021). These cells sense changes to stress placed on the bone so then can send chemical messengers to induce growth. This process requires the clearing of unwanted cells by degradation, a process done by cells called osteoclasts (Mohammed 2008); and rebuild, by which osteoblasts are the key cell (Ottewell et al. 2016).

Immune cells such as T cells, monocytes and macrophages are derived from hematopoietic stem cells (HSCs) within the bone. These will actively regulate the balance between the generation of new osteoclasts (osteoclastogenesis), and osteoblastogenesis (Zhu et al. 2021). Inflammatory cytokines are secreted by M1 and M2-type macrophages according to the microenvironment to aid bone tissue homeostasis and repair. For instance, Interferon-gamma (IFN-γ) and Toll-like receptor (TLR) ligands activate M1 macrophages to secrete interleukin-1 beta (IL-β), IL-12, 23 and tumour necrosis factor alpha (TNF-α). These inflammatory cytokines prevent the synthesis of ECM (Teissier et al. 2022). On the other hand, M2-macrophages secrete anti-inflammatory cytokines such as the receptor antagonist IL-1, IL-10, and tumour growth factor-beta (TGF-β) (Zhu et al. 2021), thus promoting bone repair through osteo and angiogenesis.
1.9.1 Osteoclast

Bone remodelling starts with the reabsorption phase, when osteoclasts start secreting enzymes and hydrogen ions to start dissolving the ECM of the bone which is mostly collagen and calcium. Osteoclasts are multi nuclear cells in charge of cleaning are active at the bone surface where they secrete acid and bone enzymes, eroding the minerals and matrix of the bone. Cavities are thus formed to cause bone degradation (Park 2019). A membrane is formed between the osteoclasts and the bone surface which is called the ruffled border. Osteoclast activity and reabsorption then end by programme cell death apoptosis (Jacome-Galarza et al. 2019).

1.9.2 Osteoblast

The bone tissue builders are the osteoclasts. Once the osteoclasts produce small scooped-out fragments, then cells around that area secret growth factor that signals the osteoblasts to start building. Osteoblasts deposit collagen and minerals and then follow one of 3 paths: either flatten out and lie in the surface of the bone, become an osteocyte or undergoes apoptosis (Robling et al. 2006, Ottewell et al. 2016). These cells fill in the bone cavities, multiply and form new bone. This process is stimulated by the secretion of pituitary and thyroid hormones such as oestrogens and androgens. Osteoblasts secrete a number of cytokines that promote the reabsorption of bone. The bone surface is covered by a protective layer of bone lining cells or inactive osteoblasts which serve as a barrier to certain destructive ions (Isojima & Sims 2021, Amarasekara et al. 2021).

1.9.3 Osteocytes

Osteocytes are responsible for conducting the mechanisms of either turning up or down bone remodelling. In other words, they have to turn mechanical stress into biochemical signals (Qin et al. 2020). These cells are surrounded by fluid in the lacunae and when the bone is under stress, the fluid flows through the cell membrane of the osteocytes which sense a force passing the cell (Creecy et al. 2021). Special mechanosensory receptors are embedded into the cell membrane. These receptors pick up in this movement of fluid and they work in different ways. Some are ion sensitive, when more ions than usual are moving it is probably due to fluid. Collectively these sensors activate as a consequence of changes in the calcium circulation and
are going to activate a chemical pathway that goes to the target cells for systemic bone-tissue homeostasis (Qin et al. 2020, Iandolo et al. 2021).

1.9.4 Bone niches

In the bone marrow (BM), the different microenvironments are classified in niches according to their location and specialization roles in the process of differentiation and self-renewal in the mature stem cells (Panvini et al. 2020). The main categories can be divided in: osteoblastic or endosteal, in charge of osteogenesis; vascular, and haematopoietic (Yin 2006). It has been showed from in vivo models that the interaction between these types of niches is of significant importance in cancer and might define whether tumours cell proliferate to bone metastasis, or become inactive or dormant (Ottewell et al. 2016).

1.9.4.1 Osteoblastic niche

Also known as endosteal niche, comprises the endocortical and trabecular bone surface (Callens et al. 2021, Sampath et al. 2015). It consists of active osteoblasts undergoing osteogenesis, and osteoclasts which are constantly clearing redundant bone tissue (Amarasekara et al. 2021,26). The origin of osteoblasts is highly regulated by a series of transcription factors from the mesenchymal stem cells (MSC) such as RUNX2 and osterix, which stimulate the development of matured osteoblasts (Amarasekara et al. 2021). These mature cells, secrete proteins such as collagen I, alkaline phosphatase and osteocalcin to form the ECM (Liao et al. 2011, Kolb et al. 2019). As previously stated, a second option is the adaptation of osteoblasts into a quiescent type to either form part of the structural bone lining or undergo apoptosis.

Current research suggests that osteoblasts perform different tasks in the natural disease history of bone cancer, and these subpopulations are recognized according to the expression of its different protein markers (Amarasekara et al. 2021, Kolb et al. 2019). Furthermore, is now known that some osteoblasts can be considered within the hematopoietic stem cells niche, aiding with the regulation of the quantity of HSCs (Yin 2006).

Zol has proven to re-locate bone tumour cells to areas with higher concentration of osteoblasts. The interaction of chemokines CXCR4 with CXCL12 shows a possible major role in the adhesion
of cancer cells to the bone metastatic niche. While prostate and breast cancer express CXCR4, osteoblasts express CXCL12 chemokine. Therefore, it is believed that this mechanism maintains the bone tumours in a quiescent state through the interaction of both chemokines (Ottewell et al. 2016).

1.9.4.2 Vascular Niche

Historically, the relevance of the vascular niche in bone cancer came from brain metastasis. Independent research of this niche has proven to be complex due to the closeness with the HSC and endosteal niches (Ottewell et al. 2016). It is known that in bone marrow, tumour cells in its dormant state are likely to be more abundant in the vascular niche. Within these cells there are oestrogen receptor-positive cells, identified as a type of metastases which develops after a long period of latency. Possibly, the depletion of oestrogen during menopause is responsible for the awakening of dormant oestrogen receptor positive tumour cells (Han et al. 2016).

1.9.4.3 Hematopoietic niche

Throughout life, the hematopoietic stem cells HSCs comprise the main force for the production of mature blood cells in a process known as haematopoiesis (Gomes et al. 2021). This niche is controlled by different mechanisms which include genetic and epigenetic process to balance the dynamic regulation of differentiation, quiescence and self-repair (Heo et al. 2015). Depending on their life cycle, HSCs can be classified in long-term (LT-HSCs) and short-term (ST-HSCs). LT-HSCs are capable of enduring haematopoiesis for months and are mainly kept as slow-cycling or even dormant, while ST-HSCs renewal ability is limited to weeks (Yin 2006).

Oestrogen plays a major role in many different signalling pathways including HSCs in the bone marrow, since this tissue does not display sex-specific morphologic differences (Heo et al. 2015, Nakada et al. 2014). The constant restoration of the haematopoietic niche enables the triggering of survival mechanisms such as continuous upkeep and protection against reactive oxygen species (ROS) or chemotherapy (Méndez-Ferrer et al. 2020).

1.10 Human menopause and its effect on skeletal tissue

Menopause is defined as the natural senescence of the ovarian functions followed by a permanent shift in the endocrine landscape. Prior to the onset of menopause, a menopausal
transition (also known as peri-menopause) occurs during mid to late adulthood (median age of 51 years). This transition is characterized by drastic fluctuation of hormones, such as oestrogens, follicle-stimulating hormone (FSH), inhibin A and B, principally, and tend to last between 4 up to 14 years before the final menstrual period (beginning of post-menopause) (Ji & Yu 2015). Clinically, menopause is confirmed 12 months after the last menstrual period, however, the variability in the length of the peri-menopausal period between individuals is significantly large, leaving the clinical diagnose of menopause through the last menstrual period relatively inaccurate. Therefore, an accurate diagnose of menopause is achieved by analysis of serum levels of oestradiol. Up to date, there are not specific values of oestradiol in the National Institute for Health and Care Excellence (United Kingdom) guidelines for the diagnose or classification of pre- and post-menopause (Lumsden, 2016); however, diverse cites agree that concentrations below 50 pg/mL (ideally, 30 pg/mL) is considered as post-menopausal (Depypere et al., 2015; University of Rochester medical center, 2023; Wilson et al., 2017).

In a normal pre-menopausal menstrual cycle, a tight regulation of hormones is present. The menstrual cycle commences with a rise in the concentrations of FSH as a result of pituitary stimulation caused by a decrease in oestrogens and progesterone concentrations. Increase in FSH stimulates the ovaries to develop several follicles which, in turn, will produce oestrogens, inhibin A and inhibin B, increasing their levels and slowly reducing FSH (Draper et al., 2018). During the menopausal transition, an overall decrease in the concentrations of inhibin B is one of the earliest markers, followed by a clear decrease in the concentrations of oestrogens (Davis et al., 2015). Clear decrement in the concentrations of oestrogens confirms the last stages in the menopausal transition (peri-menopause).

Oestrogens are a family of steroid hormones. In a woman, three types of oestrogen can be isolated: oestrone, oestriol and oestradiol, being this last one the hormone with the most abundancy found in the female body. Oestrogens have a wide effect over the body besides their role during the menstrual cycle. ERs are found in a variety of tissues across the body. In the bone, oestrogens act closely in the regulation of the remodelling process by adjusting the expression of RANKL in osteoblasts, affecting osteoclast mediated bone resorption (Streicher et al., 2017). The maintenance of osteoblast and osteoclast numbers and activity is essential during a healthy remodelling process, however, during menopause, a decrease in the
concentrations of oestrogens leads to an increase in osteoclast number and activity resulting in an increase bone resorption and further bone loss, also known as osteoporosis (Ji & Yu 2015). Similarly, ER are found in diverse immune cells affecting their activity depending on the immune cell type. Therefore, oestrogen variation confers a complex immune landscape in women (Irelli et al., 2020; Laffont et al., 2014; Phiel et al., 2005; Staples et al., 1999). Oestrogen is also an important hormone in the development and progression of breast cancer tumours. ER +ve tumours represent the majority of all breast cancer tumours (O’Brien et al., 2010), therefore, their proliferation and survival are in close relationship with this hormone.

1.10.1 Effect of oestrogens on the skeletal tissue cells

ERs are divided into two types, ERα and ERβ receptors, and their expression is ubiquitous throughout various tissues in the body. In the bone microenvironment, osteoblasts, osteoclasts and their respective precursor cells express either of these ERs which enable them to respond differently to variations in the levels of oestrogens. In osteoblastic cells and respective progenitors, physiological concentrations of oestrogens (pre-menopause) are evidenced to expand the lifespan of osteoblast through activation of ERβ (Bradford et al., 2010). Whereas in early osteoblast progenitors, similar concentrations of oestrogens cause this cell type to prevent its self-renewal mechanism, potentially by activation of ERα, and commit to differentiation (Di Gregorio et al., 2001; Mödder et al., 2011). Osteoblastic progenitors expressing osteoblast-specific transcription factor Osterix 1 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 (Almeida et al., 2013), instead they are prevented from undergoing apoptosis by inhibiting activation of caspase-3/7 (Bradford et al., 2010). Oestrogen also affects cellular mobility during osteoblast maturation. In pre-menopausal levels of oestrogens, early differentiated osteoblasts cells show and increase in the transcriptional levels of adhesion molecule N-cadherin. A higher expression of this molecule increases mobility of osteoblast towards zones of active resorption in the bone. A decrease in the levels of oestrogens is therefore linked with a lower cellular mobility (Mödder et al., 2011).

Osteoclasts and osteoclast lineage cells respond to oestrogen variations in directs and indirect manners, especially during maturation, activation, and their lifespan. For instance, high levels
of oestrogens (observed in pre-menopause) induce active osteoclasts to undergo apoptosis via TGF-β and activation of ERα (Hughes et al., 1996; Kameda et al., 1997) in both in vitro and in vivo (Martin-Millan et al., 2010). Also, high levels of oestrogens induce transcription of proapoptotic molecule Fas Ligand in osteoblasts via ERα. By cleaving Fas Ligand with MMP3, the soluble Fas Ligand acts directly on osteoclasts resulting in their apoptosis (Garcia et al., 2013). Conversely, an in vivo ERα knockout model of monocytes/macrophages demonstrated that in the absence of ERα an increase in the number of osteoclast and osteoclast progenitors was observed (Martin-Millan et al., 2010), confirming the importance of oestrogens as a direct resorption regulator.

Regulation in the activity of osteoclast by oestrogens occurs mainly by alterations of the microenvironment. In normal levels of oestrogens (pre-menopause), this hormone acts on haematopoietic and osteoblastic cells suppressing the release of osteoclastogenic factors such as IL-1, IL-6, TNFα, and macrophage colony-stimulating factor (M-CSF); however, under low levels of oestrogens (post-menopause), an 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 (Weitzmann et al., 2006). Additionally, oestrogens are involved in the valance of OPG/RANKL ration. Under the presence of oestrogens, a higher transcription of OPG is observed in osteoblastic cells (Bord et al., 2003) and affecting RANKL localization at the cell surface of osteoblasts (Martin et al., 2015).

Other cells are also affected directly or indirectly by oestrogens. For instance, oestrogens signals differentially affect HSCs and progenitor cells. In presence of oestrogen, HSC increase their self-renewal mechanisms (Nakada et al., 2014), whereas induces apoptosis in more mature HSC progenitor cells (Sanchez-Aguilera et al., 2014).

1.11 The “vicious cycle” of bone metastatic

Once metastatic breast cancer cells have lodged into the bone niche (as explained previously), it interferes with the bone turnover cycle. Tumour-derived factors such as osteopontin (OPN), parathyroid hormone-related peptide (PTHrP), heparanase, IL-1, IL-6, IL-11 and prostaglandin E2 (PGE2) prepare the bone microenvironment for the metastatic cells by increasing the activity of osteoclasts and bone resorption (Chen et al., 2018). In response to an increased rate
of bone resorption, bone-derived growth factors such as IGF1, TGF-β and PDGF, as well as calcium, are released and drive the metastatic tumour growth (figure 1.4). Although osteoblasts continue with their bone formation process, the level of repair is not enough to cope with the level of bone destruction (Lu et al., 2007). These conditions lead to osteolytic lesions and results in pathological complications (i.e. fractures, hypercalcemia as discussed previously).

**Figure 1.4. Schematic of the vicious cycle.** Metastatic breast cancer cells arrive to the bone niche through interaction with CXCR4/CXCL12 and stimulate the activation of osteoclasts by IL-6, PGE2, tumour necrosis factor and macrophage colony-stimulating factor. These cytokines in conjunction of the PTHrP increase the bone resorption and a predominance of RANLK against OPG. The increment of osteoclastic bone resorption releases growth factors which stimulates the metastatic cells and stimulates tumour growth.

### 1.12 Treatments for metastatic breast cancer

Despite the increasing progress in the understanding of bone metastasis during the past years, there are scarce approved options for the treatment of this disease.

Current therapeutic approaches for bone metastasis are focussed in three main principles (Gdowski et al. 2017; guideline CG81, N. I. C. E., 2009):

1. Tackling cancer cells is essential. Therapies have to abolish the proliferation of the cancer cells in order to generate a significant improvement in the metastatic lesion.
Failing to control cancer cell growth will result in a similar life expectancy as an untreated patient and/or generation of secondary sites of metastasis.

II. Altering the bone microenvironment is also considerably effective. As previously discussed, metastatic cells have a complex relationship with the bone microenvironment through the vicious cycle. Any alteration to this biological signalling can potentially affect the viability of the metastatic site for the cancer cells.

III. The use of palliative therapies to reduce the symptoms related to bone metastasis.

Due to the dissemination of the metastatic disease and the lack of certainty at locating possible metastatic sites, diverse systemic treatments are required. For instance, hormonal therapy, cytotoxic agents, targeted agents or a combination of above, are the first option to control the disease progression. The same guidelines used to determine the optimal therapy for the primary tumour of breast cancer are applied to metastatic breast cancer (guideline CG81, N. I. C. E., 2009). This means that hormonal therapies (i.e. tamoxifen and aromatase inhibitors) will be provided against ER +ve breast tumour/metastasis; Anti-HER2 therapies (i.e. trastuzumab, and pertuzumab) will be delivered to HER2 over-expressing tumour/metastasis, or chemotherapy that blocks cellular replication non-specifically (i.e. docetaxel, and cyclophosphamide) (guideline CG81, N. I. C. E., 2009; National Comprehensive Cancer Network [NCCN], 2023).

Although the idea of treating distant metastasis with the same therapy used for primary tumour is not incorrect, metastatic cells are molecularly different from their primary tumour at a certain level (Shah et al., 2009). Over time, the metastatic cells will adapt to the distant homing site and show a different dependence for other types of growth factors which were not present in the primary tumour. More specifically, in bone metastasis, the number of growth factors available from the bone matrix may compensate the cell proliferation and induce the metastatic cell to rely less on the ER. As a result, the metastatic cells will gradually show anti-ER therapy resistance, in spite of being susceptible at this treatment in the primary tumour (Amir et al., 2010). Therefore, the NCCN guidelines strongly suggest the use of adjuvant therapies to enhance treatment response (NCCN, 2023). Examples of adjuvant therapies are the bone-targeted agents such as bisphosphonates and the anti-RANK-L antibody denosumab. Several investigations have shown the effectiveness of bisphosphonates in combination with
chemotherapy and/or endocrinal therapy; however, there are certain consideration for the use of bisphosphonates as adjuvants (Coleman et al., 2018; Coleman et al., 2020; Early Breast Cancer Trialists’ Collaborative Group, 2015; Fick et al., 2015; Gnant et al. 2011; Gnant et al., 2019; Kuchuk et al., 2013).

It is also worth mentioning that, unlike localized tumours, metastatic lesions do not benefit from local treatments such as surgery and certain types of radiation therapy and interventional radiology (i.e. vertebroplasty, radiofrequency, cryoablation). Nonetheless, surgery and radiation therapy are accepted as palliatives alternatives for fractures/prosthesis placement and pain relief respectively (Dürr et al., 2002; Viganó et al., 2000).

1.13 The use of bisphosphonates in the clinics

Bisphosphonates are analogue molecules of pyrophosphate where the centre oxygen of the two phosphate group (P-O-P) is replaced by a germinal carbon (P-C-P) (Figure 1.5a). Due to its chemical structure, bisphosphonates bind quite avidly to the bone hydroxyapatite. The bisphosphonate stays in the bone surface until an osteoclast, by degrading the bone surface, releases and absorbs the bisphosphonate. Once the bisphosphonate enters the cells, it causes an interference in the Mevalonate pathway. Nitrogen containing bisphosphonates induce inhibition of farnesyl pyrophosphate synthase in osteoclasts, preventing the synthesis of FPP and geranylgeranyl diphosphate and then leading to reduced post-translational modification of GTPases, which are necessary for osteoclast function and survival (Figures 1.5 b & c) (Amin et al., 1992; Rondeau et al., 2006).

Due to the reduction in bone resorption, bisphosphonates were used as a treatment for bone degradation diseases such as osteoporosis and Paget’s disease. However, over the last decades, the interest in the use of bisphosphonates as an adjuvant therapy for prevention of bone metastasis has grown rapidly. The premise of using bisphosphonates to prevent metastatic lesions relies in the prevention and/or disruption of the vicious cycle by reducing osteoclastic bone resorption. Reduction in bone resorption would then directly impact the amount of nutrients, growth factors and chemokines released in the bone microenvironment, resulting in a less sustainable environment for the DTCs to home. Under this hypothesis, multiple pre-clinical and clinical research took place to evaluate the effectiveness of
bisphosphonates to prevent bone metastasis (Reviewed in Coleman, 2009; Early Breast Cancer Trialists' Collaborative Group [EBCTCG], 2015).

![Chemical structure and mechanism of action of bisphosphonates](image)

**Figure 1.5. Chemical structure and mechanism of action of bisphosphonates.** a) the chemical structure of the bisphosphonate. b) the different chain residuals that give rise to the different bisphosphonates. C) mechanism of action of the bisphosphonate (FPP, farnesyl diphosphate; GGPP, geranylgeranyl; farnesyl small G proteins) (Adapted from Neville-Webbe & Coleman, 2003).

Although all the vast majority of bisphosphonates are able to decrease bone resorption, only a few of these have been approved for their use in the clinical setting. Clodronate and Zol are examples of approved bisphosphonates for their use as adjuvants in combination of the standard of care therapy for treatment of early breast cancer patients (Coleman et al., 2014; Diel et al., 2008; Gnant et al., 2009; Powles et al., 2006).

Zol is regarded as the most potent bisphosphonate when comparing its anti-resorption effect. It has also been shown to elicit an anti-tumour effect over a range of cancer cells, especially breast cancer cells, under in vitro settings (Göbel et al., 2016; Jagdev et al., 2001, Oades et al., 2003). The combination of both clinical traits of Zol were used to target early stages of breast cancer with protection to the skeletal tissue. In the disease development, DTC can be found in early stages of breast cancer, which represents an increased risk for developing overt bone
metastasis. Even after primary tumour remotion, DTCs can be found in 30-40% of breast cancer patients (Braun et al., 2005). By introducing Zol into the bone microenvironment, changes in this site could affect the viability of these DTCs and prevent further metastasis development. Primary clinical observations of this showed promising results (Banys et al., 2013; Rack et al., 2010).

Analysis of a non-randomised phase 2 clinical trial where 31 women with early breast cancer and persisted DTCs in bone marrow receiving 4mg of Zol, 4 times per week for 6 weeks vs 141 control patients, showed that a significant reduction in the presence of DTC in bone marrow aspirates was observed in the Zol group compared to the control group (Rack et al., 2010). From this study, an improvement in the overall survival was noted in the patients with DTC-negative aspirates. A larger study evaluating the effect of Zol on DTCs recruited 3,141 patients with early breast cancer, from which, bone aspirates were performed to all of them, being 803 of these aspirates positive for DTCs. In this study, all patients received systemic treatment combined with adjuvant Zol (Hartkopf et al., 2014). Retrospective results showed that combined therapy with Zol in patients with positive DTCs had an increase in disease free survival (DFS) and a better overall survival. Interestingly, analysis of sub-grouped data demonstrated that post-menopausal patients had a better overall survival when compared to the pre-menopausal patients, leaving an open question to the influence of hormones and the protective effect of Zol.

Currently, the use of Zol has been approved as an adjuvant anti-cancer treatment for the elimination of micro metastasis. Various clinical studies evidencing the benefit of adjuvant Zol showed that a clear increase in DFS was observed in either post-menopausal patients with endocrine therapy (letrozole) (ZO-FAST study) (Coleman et al., 2013) or in pre-menopausal patients with endocrine therapies, rendering them in chemically-induced post-menopause (ABCSG-12 clinical trial) (Gnant et al., 2011). This last clinical trial, the Austrian Breast and Colorectal Cancer Study Group trial-12, evaluated the effect of treating 1803 pre-menopausal women with ER +ve breast cancer receiving adjuvant goserelin and endocrine treatment either with or without Zol (4mg every 6 months) (Gnant et al., 2011). Although the study reported a reduced risk of disease progression in patients receiving Zol, no differential effect of Zol was observed when comparing hormone variations as all patients had generally low levels of hormones as a result of the therapy.
In the AZURE (Adjuvant Zoledronic Acid to Reduce Recurrence) clinical trial, a total of 3360 women were randomised to standard adjuvant systemic treatment, with or without Zol with a treatment regimen of 4 mg of Zol 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 (figure 1.6). Unlike AGCSG-12 clinical trial, no discrimination was applied in the recruitment of any breast cancer subtype, neither specific menopausal status, meaning that the coverage of the study was more robust. Although initial results showed no particular benefit in overall survival or DFS between Zol-treated and no treated patients (Coleman et al., 2011), sub-grouped analysis evaluating the menopausal status evidenced that Zol had a differential effect at both ends of the menopausal statuses. Women who were 5 years or more into diagnosed menopause showed benefit with invasive DFS (iDFS) of 78.2% compared to control 71% (95% CI, P=0.02) and overall survival of 84.6% compared to control 78.7% after 5 years (95% CI, P=0.04). However, pre-menopausal woman did not experience all the benefits from adjuvant Zol as in the post-menopausal patients. A reduction in skeletal metastasis was noted but this was accompanied by an increased risk of extra skeletal recurrence (Coleman et al., 2014). These observations were further confirmed by a compelling meta-analysis of 26 randomised trials on adjuvant Zol in early breast cancer, encompassing 18,766 women (EBCTCG, 2015). Sub-group analysis showed that Zol had the most significant effects on bone recurrence, overall recurrence, distant recurrence, and mortality in post-menopausal women.
Diverse pre-clinical studies also confirmed the efficacy of Zol in combination with the standard therapy. In vivo mouse model of pre- and post-menopausal status proposed that the efficacy of the anti-tumour effect of Zol was due to induced osteoclast inhibition followed by a disruption of the vicious cycle (Ottewell et al., 2014). This finding was supported by the use of the anti-RANKL murine antibody to induce osteoclast inhibition, which also resulted in a reduction in bone metastasis (Ottewell et al., 2015b). However, subsequent clinical data studying the anti-RANKL antibody, denosumab, administration at pre- and post-menopausal women as adjuvant therapy did not result as observed in the pre-clinical scenario. According to the D-CARE clinical study, denosumab did not have any improvement in the DFS in both pre- and post-menopausal statuses (Coleman et al., 2018). It indicates that the relationship between Zol and menopausal status does not rely on osteoclast activity.
On the other hand, Wilson and collaborators (2017), by analysing randomised samples (n=865) from the AZURE trial, observed a correlation between the levels of ovarian hormones and breast cancer recurrence. Levels of oestradiol <50 pmol/L (post-menopausal) was associated with a reduction in DFS (Hazard risk: 1.36 95% CI: 1.05-1.78 p=0.022) in comparison to oestradiol ≥50 pmol/L (premenopausal). Additionally, the less desirable outcome of this adjuvant therapy was reflected in the increase of shorter invasiveness disease-free survival which was linked to premenopausal concentration of oestradiol. Interestingly, this invasion to distant organs different from bone was regardless of the primary tumour subtype, meaning that DTC prefer metastatic sites different from bone once a low level of oestradiol is sensed on this tissue. This observation can be proven by in vivo studies where Holen and collaborators (2016) showed that by a constant supplementation of oestradiol in a murine animal model, spontaneous metastasis to bone (from ER +ve cell line) was achieved. Therefore, high concentration of oestrogen can potentially be an attractive mechanism for DTC despite reduction in resorption caused by Zol. However, further research is required to elucidate the key mechanism that promotes anti-tumour activity of Zol.

**Project hypothesis and aims**

The project hypothesis is: Pre- and Peri-menopausal levels of oestrogens in combination of zoledronic acid impairs bone metastasis but influence the recurrence of breast cancer to other organs.

To test this hypothesis, three aims are proposed:

1. Establish how oestrogen and Zol interact to affect sites of metastatic spread of tumour cells
2. Determine how disseminated tumour cells in bone respond to Zol treatment under post-peri and pre-menopausal concentrations of oestrogen.
3. Evaluate cellular mechanisms of interaction between Zol and oestradiol in bone and non-bone metastatic sites.
CHAPTER 2: Methods
2.1 Cell culture

2.1.1 Cells

MDA-MB-231 and MDA-MB-231-GFP-LUC2

MDA-MB-231 are epithelial human breast cancer cells derived from a pleural effusion from a caucasian female with metastatic breast cancer. This cell line is a triple-negative breast cancer subtype with a high metastatic phenotype, also associated as high aggressiveness. Parental MDA-MB-231 (last authentication 03/2017) cells were purchased from European Collection of Authenticated Cell Cultures before stably transfecting with GFP and Luciferase to enable identification of tumour growth and metastasis by bioluminescence and fluorescence techniques (Holen, et al., 2016).

4T1-Luc2 and 4T1-Luc2-Bone-Retrieved

4T1-Luc2 is a triple-negative murine breast cancer cell line. This cell line was kindly donated by Dr. Amy Kwan, University of Sheffield, Sheffield, UK (Kwan, et al., 2021). 4T1-Luc2 were injected and recovered from bone-metastatic lesions from BALB/c mice as described in section 2.3. Recovered cell colonies were selected with 6-thyoguanine (Sigma Aldrich, UK) and bulked up to form 4T1-Luc2-Bone-Retrieved cells (Canuas-Landero, et al., 2021).

2.1.2 Maintenance of tissue culture

Cells were cultured and grown in a T75 tissue culture corning flask with Dulbecco’s modified Eagle’s medium (DMEM, Gibco, UK) with the addition of 10% of foetal bovine serum (FBS, Gibco, UK) at 37 °C and 5% of CO₂. No antibiotics were used. Cell monolayers were subcultured when they reached 70-80% of confluence. Continuation of cellular culture was carried with a proportion of 1:10 in a new T75 corning flask.
2.1.3 Cell passaging

Old DMEM was removed from the cell monolayer, followed by two consecutive washes of 5 mL of phosphate-buffered saline (PBS, Gibco, UK) at 37 °C. 1 mL of 0.25 % Trypsin – ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific) was added to the cell monolayer and incubated for 5 minutes at 37 °C to disassociate all cells (also referred as trypsinisation). 9 mL of pre-warmed DMEM + FBS was immediately added to the T75 flask, followed by 3 – 5 rinses with the same medium to the flask wall. The resulting 10 mL of cell suspension was then centrifuged at 400 R.C.F (x G) for 5 minutes to pellet the cells. After centrifugation, the supernatant was discarded and the resulting pellet was resuspended in 5 mL of media. 1/10 proportion of the cells were seeded in a new T75 containing 10 – 12 mL of DMEM + FBS.

2.1.4 In vitro growth curve

Cultured MDA-MB-231 were trypsinised as described in section 2.1.3. Cells were resuspended with 9 mL of pre-warmed DMEM + FBS. 10 µL from the cell suspension were loaded into a Haemocytometer (Neubauer-improved, Depth = 0.10 mm) to calculate cell density. The cell suspension was centrifuged at 500 R.F.C for 5 minutes to pellet the cells. Cell density was calculated by haemocytometer counting followed by the result of equation 1:

Equation 1: \[ \text{Cell density} = \frac{N\text{° of cells} \times 10,000 \times \text{suspension volume in ml}}{\text{number of squares read}} \]

The obtained number of cells was re-suspended in the resulting volume of DMEM + FBS from equation 2 to achieve a 1 x 10^6 cells/mL suspension.

Equation 2: \[ \text{Volume of diluen in ml} = \frac{\text{cell dencity}}{1 \times 10^6} \]

A second dilution was prepared to obtain a cell density of 1 x 10^4 cells/mL in DMEM + FBS. 12 wells of a 24-well plate were set with 1 mL of cell suspension for a 4-time-point growth curve (24, 48, 72 and 96 hours; n=3). The 24-well plate was incubated at 37 °C and 5% CO₂.

24 hours after incubation, old medium was removed from the respective wells (n=3). The wells were washed twice with pre-warmed PBS to remove medium remains and dead cells. 200 µL
of Trypsin-EDTA were added to each well followed by 5-minute incubation at 37 °C. 800 µL of PBS were added to each well and cell density was calculated using Haemocytometer followed by the result of equation 1. The same procedure was carried out for 48, 72 and 96-hour end point. Number of cells per time point were plotted in a graph. This process was carried out in triplicate giving three biological repeats and three technical repeats for each time point.

2.1.5 Identification of IC50 concentrations of Zol in vitro

MDA-MB-231 cells were trypsinised and re-suspended at 1 x 10⁶ cells/ml as described in section 2.1.4. 8, 27-mL sterile tubes were prepared with 15 mL of cell suspension at 1 x 10⁴ cells/ml in DMEM + FBS. 7, different volumes of Zol (supplied as disodium salt by Novartis) (1mM) were added to the cell suspension to obtain a final concentration of 5, 10, 15, 17.5, 20, 22.5, and 25 µM of Zol. Control treatment had PBS. Cells were seeded in a 24-well plate (n=3/treatment) and incubated at 37 °C with 5% CO₂ for 24, 48, 72, and 96 hours. 24 hours after incubation, cells were trypsinised and counted as described in section 2.1.4. The same procedure was carried out for 48, 72 and 96-hour end point. Each experiment was carried out in triplicate and total number of cells per treatment was normalized against control and plotted in a graph. IC50 was calculated as non-linear regression on GraphPad Prism 7.02 software.

2.1.6 Evaluation of the anti-tumour effect of Zol in presence of oestradiol in vitro

MDA-MB-231 cells were trypsinised and re-suspended at 1 x 10⁶ cells/ml as described in section 2.1.4 using Phenol-free MEM medium (Thermo Fisher Scientific, UK). 6 27-mL sterile tubes were prepared with 15 mL of cell suspension at 1 x 10⁴ cells/ml in phenol-free MEM + charcoal-stripped FBS (Gibco, UK). 150 µL of 17-β-oestradiol (Sigma Aldrich, UK) at 30.0 nM, 8.4 nM or PBS were added to 2 tubes to obtain a concentration of 300 pmol/L, 84 pmol/L, and 0 pmol/L of oestradiol, respectively. 375 µL of Zol at 1.0 mM were added to half of the different concentrations of oestradiol to obtain a concentration of 25 µM of Zol. Cells were plated in a 24-well plate (n=3/treatment) and incubated at 37 °C with 5% CO₂ for 24, 48, 72, and 96 hours. 24 hours after incubation, cells were trypsinised and counted as described in section 2.1.4. The same procedure was carried out for 48, 72 and 96-hour end points. Experiments were performed in triplicate and mean number of cells ± standard error of the mean per time point were plotted in graphical format.
2.1.7 Preparation of cells for intracardiac injection

Cultured MDA-MB-231-GFP-LUC2, 4T1-Luc2 or 4T1-Luc2-Bone retrieved cells were prepared in suspension as described in section 2.1.3, with the exception of using PBS + 10% of FBS instead of medium for resuspension after centrifugation. From the cell suspension, 10 µL were loaded into a Haemocytometer (Neubauer-improved, Depth = 0.10 mm). Number of cells was calculated by haemocytometer counting followed by the result of the equation 1. Obtained cell density was further diluted with the resulting volume of PBS + 10% of FBS using equation 2. The suspension was filtered by a 70 µm nylon mesh to avoid cellular agglomeration or clots and maintained on ice prior to intracardiac injection.

2.1.8 Preparation of cells for intra mammary gland injection

Cultured 4T1-Luc2-Bone retrieved cells were prepared in suspension as described in section 2.1.3. From cellular suspension, 10 µL were loaded into a Haemocytometer (Neubauer-improved, Depth = 0.10 mm). Number of cells was calculated by haemocytometer counting followed by the result of the equation 1. Obtained cell density was further diluted to achieve 1x10^5 cells /100 µL in a suspension of 33% matrigel (thawed and maintained at 4°C) (Sigma-Aldrich, UK): 66% PBS: 1% trypan blue. The new cellular suspension was maintained on ice at 4°C until injection.
2.2 *In vivo* studies

2.2.1 Animals

12-week-old female BALB/c mice (cAnNCrl) and BALB/c nude (cOlHsd-Foxn1*nu/nu*) were obtained from Charles River, UK. Upon arrival, all mice were acclimatised for at least one week in the Biological Services Unit, Royal Hallamshire Hospital. The room conditions were: Temperature: 19-23 °C; Relative humidity: 45-55; Illumination: 350-400 lux in a period of 12 h light/dark and food/water was freely available. All studies were carried out in accordance with local guidelines and with Home Office approval under project licences 70/8964 and P99922A2E, University of Sheffield, UK.

2.2.2 Ovariectomy and oestrogen replacement

Ovariectomy (OVX) is a surgical procedure for the bilateral removal of the ovaries. This procedure has been used in different nonhuman primates and rodent animal models to cause a deprivation of ovarian hormones such as oestrogen (Brinton, 2012). The procedure in mice consists of accessing the visceral cavity from the lumbar area of the mouse, followed by surgical resection of the ovaries. The OVXs were performed by Dr Penelope Ottewell, Mrs. Diane Lefley, Mr. Christopher George and Mr. Victor Canuas-Landero.

Prior to surgery, 12-week-old female mice were anaesthetised with isoflurane (VetPharma) (2.5% vaporisation rate and oxygen flow of 1.5-2 L/min) followed by a subcutaneous injection of analgesic (buprenorphine: Vetergesic, CEVA Animal Health) (3 µg/25 g) and a dose of broad-spectrum antibiotic (enrofloxacin: Baytril, Bayer) (250 µg/25 g). The lower back was cleared of hair using clippers and sanitised with iodine. A longitudinal incision was performed from the mid to the lower back (lumbar area) following the spinal cord using a scalpel blade. The skin was subsequently detached from the muscle wall on both sides of the back by gentle blunt dissection. With a needle-point tweezers, an incision was made through the muscle wall at approximately 1 cm away from the spinal cord. Cat-spay forceps were used to grab the top of the womb, the ovary was surgically removed by blunt dissection. Once the ovary was successfully removed, the connecting tissue was placed back into the visceral cavity. The same procedure was repeated on the other side to remove the second ovary. When both ovaries
were successfully removed, the skin was sutured by simple interrupted technique, using a polypropylene monofilament suture.

After the procedure, all mice were placed into a clean incubator at 30 °C until fully recovered from anaesthesia. All mice were closely monitored until the incision on the back was fully healed. A second dose of analgesic and antibiotic was applied 24 hours after OVX.

After fully recovery, normal drinking water was given to mice with no oestrogen replacement. Drinking water with 1.38 mg/L or 12.5 mg/L of 17-β-oestradiol (Sigma-Aldrich, UK) was given to mice with low or high oestrogen replacement respectively (BALB/c n=5/group; BALB/c nude with MDA-MB-231-GFP-LUC2 n=10/group & BALB/c with 4T1-Luc2-Bone retrieved n=10/group).

Immediately following OVX, daily administration of goserelin (40 µg/Kg/day as goserelin acetate, Sigma Aldrich, UK) was given to all mice via subcutaneous injection. Combination of goserelin and letrozole (FEMARA, Novartis) (1 mg/kg every 2 days) was administered via subcutaneous injection. Administration of Zol (Novartis) or PBS occurred at day 7 after OVX and continued on a weekly basis until the end of the procedure (BALB/c n=5/group; BALB/c nude with MDA-MB-231-GFP-LUC2 n=10/group & BALB/c with 4T1-Luc2-Bone retrieved n=10/group).

2.2.4 Intracardiac injection

The delivery of cancer cells via injection into the left cardiac ventricle is one of the most commonly used in vivo techniques for the study of bone metastasis (Brown et al., 2012; Haider et al., 2014; Ottewell et al., 2014). Although the injection of cells directly into the blood stream negates important features of the metastatic process such as tissue invasion and intravasation, it does allow the study of latest stages of metastasis such as arrival to distant organs, extravasation and tumour proliferation (Campbell, et al., 2012). Also, by inoculating cancer cells into the circulation, the observation of metastasis in specific organs, such as skeletal tissue, are highly increased in comparison to those seen from the primary tumour (Campbell, et al. 2012).

12-week-old female BALB/c nude mice were anaesthetised in an induction chamber with isoflurane at 2.5% of vaporisation rate and oxygen flow of 1.5-2 L/min. All mice were positioned straight and facing up on a heating pad, then the sternum was exposed by applying gentle bi-
lateral pressure to the rib cage. A syringe with a needle of 27G calibre, previously loaded with 100µL of cell suspension (prepared as described on section 2.1.7), was introduced approximately 2mm away from the right side of the sternum. The needle penetrated the chest cavity until it reached no further than half the size of the sternum (see figure 2.1). A correct insertion of the needle into left cardiac ventricle was verified by slightly pulling the plunger and observe the presence of bright red (oxygenated) blood. 100µL of cell suspension was injected gently preventing any movement of the needle and or syringe.

After the injection, all mice were placed into a clean incubator until a full recovery from anaesthesia. The mice were closely monitored for 12 h post injection.

Figure 2. Position of the hearth in a nude mouse. The heart is located at approximately midway of the sternum bone (dotted line). Left ventricle is located slightly on the right side (Operator view) of the sternum as pointed with the black dot.
2.2.5 Intra mammary gland injection

Orthotropic delivery of tumour cell in the mouse mammary gland has been performed for the study of both, primary tumour development and spontaneous metastasis from primary tumour (Hiraga et al., 2004; Lefley et al., 2019; Lelekakis et al., 1999). This last feature depends strictly on the cellular model used. For this project, intra mammary gland injection was performed in syngeneic BALB/c and 4T1-Luc2 mouse model to produce spontaneous metastasis.

12-week-old female BALB/c nude mice were anaesthetised in an induction chamber with isoflurane at 2.5% of vaporisation rate and oxygen flow of 1.5-2 L/min. Prior injection, all mice were treated with an application of hair removal cream (Nair, commercial cream) in the abdominal area (at the level of the 4th nipple) to removed hair. After 1-minute application, cream and removed hair were cleaned with distilled water followed by disinfection of the area using iodine. All mice were positioned straight and facing up on a heating pad. Pre-loaded syringes containing 50 µL of cellular suspension prepared as described in section 2.1.8 were placed on ice to prevent solidification of matrigel. The injection was performed by gently graving the 4th nipple with forceps followed by the introduction of a 27G-needle syringe in a 90° angle relative to the nipple and slowly introduction of the 50-µL cellular suspension (total of 5x10^4 cells). Same procedure was carried out in the contralateral nipple.

After the injection, all mice were placed into a clean incubator until a full recovery from anaesthesia. The mice were closely monitored for 12 h post injection.

2.2.6 In vivo imaging

Luciferase emits a luminescent signal at 562nm in presence of adenosine triphosphate (ATP). Its application in in-vivo studies has determined the use of luciferase as a high-efficiency gene reporter (Nguyen et al., 1988). The bioluminescence produced by the luciferase-luciferin complex can be detected through soft tissue by charged coupled device (CCD) cameras, which translate the number of photons into electron signal (Sadikot & Blackwell, 2005).

For this experiment, tumour burden was monitored weekly after intracardiac injection of MDA-MB-231-GFP-LUC2 and 4T1-Luc2 cells, or twice a week after primary tumour injection with 4T1-
Luc2 cells, using the *in-vivo* imagine system (IVIS) LUMINA II (Caliper Life Sciences, UK). 100 µL of D-luciferin (30 mg/kg, Invitrogen, UK) was injected subcutaneously. Single image of the dorsal side of mice were taken 4 minutes post injection. Single image of the ventral side of mice were taken 6 minutes post injection.

Images were analysed on Living Image software version 4.1 (Caliper Life Science, UK). All images were analysed by creating a region of interest (ROI) around the tumour, tumour signal was acquired in radiance (photons/second). Percentage of mice with tumours was calculated based on data collected from images, as well as number of tumour and localization of tumour per mouse.

### 2.2.7 Primary tumour measurement

During the experiment analysing the effect of different concentrations of oestradiol on BALB/c model with 4T1-Luc2 bone retrieved primary tumour, the primary tumour was monitored constantly. Tumour growth was recorded using callipers and by bioluminescence using the IVIS-Luc2 imaging system.

Measurement of tumour volume by callipers is still regarded as robust tool during the analysis of tumour progression (Faustino-Rocha *et al.*, 2013). Primary tumours were measured once a palpable tumour of a minimum of 1.0 mm was reach in any width or length. Both tumours (right and left) were measured equally.

Tumour volume was calculated by the following formula:

\[
Volume (\text{mm}^3) = \frac{(W(\text{mm})^2 \times L(\text{mm}))}{2}
\]

where \(W\) is tumour width and \(L\) is tumour length.

Tumour measurement by bioluminescence was performed as described in 2.2.6.
2.2.8 Sample collection for ex-vivo analysis

At the end of each experiment, whole blood was obtained by cardiac puncture following anaesthesia induced by pentobarbital. Blood was left to clot at room temperature followed by centrifugation at 4000 r.p.m for 10 minutes. Serum was collected and stored at -80 °C for downstream biochemical analysis.

On termination of the experiment, both legs were dissected removing the soft tissue from the bones and separate tibia from femur. The skeletal tissue was fixed into 4% paraformaldehyde (PFA, Fisher Scientific, UK) solution for 72 hours followed by decalcification with EDTA (Sigma, UK) at 0.5M.

Other organs such as lung, liver, and spleen were also dissected and conserved into FBS + 10% of dimethyl sulfoxide (DMSO, Sigma Aldrich, UK) stored a -80 °C for future downstream analysis.

2.3 Generation of 4T1-Luc2 Bone retrieved cell line

4T1-Luc2-bone retrieved cell line was generated by adapted methodology described in Lelekakis et al. (1999). 5 x 10^4 4T1-Luc2 cells were prepared as described in 2.1.7 and injected in the left cardiac ventricle of 12-week-old female BALB/c mice (n=5) (Charles River, UK) as described in 2.2.4. Tumour growth was monitored every 3 days after tumour cell injection by IVIS Lumina II system (Calliper Life Sciences, UK). Upon metastasis confirmation by bioluminescence, tumours were left to reach maximum size (approximately 2 weeks after injection of tumour cells). After reaching humane end-point, mice were culled and sanitized for further dissection in and sterile environment. Based on in vivo bioluminescence, mice were dissected and all tumour-bearing bones were isolated by removing muscular, fatty and connective tissue in a sterile environment. Bones tissue was then disassociated using a tumour dissociation kit under manufacturer’s indications (Miltenyi Biotech, Germany); bones were broken and all fragments as well as bone marrow were incubated with manufacturer’s enzymatic cocktail to achieve enzymatic degradation of the extracellular matrix. After enzymatic digestion, cellular suspension was filtered to remove unwanted material (bone mainly) followed by a series of washes to neutralized and remove enzymes. Resulting single cell suspension containing 4T1-Luc2 cells were then cultured in 24-well-plate using DMEM media. 24h, 48h and 72h after first incubation, non-adherent and dead cells were remove from the
wells and new media was added. 94h after incubation all cells were trypsinised and transferred to a 6-well-plate where cell selection was performed. Selection of 4T1-luc2 bone retrieved cells was carried out by addition of 60 μmol/L 6-thioguanine to the culturing media. Cultured colonies were grown and used for further in vivo experiments.

2.4 μComputed tomography imaging

μcomputed tomography (μCT) is a technology that allows the scanning and reconstruction of bones with a micrometric resolution. The μCT scanner, uses the same principle of a CT scanner, it irradiates a bone with X-rays beams at different angles to the sample, generating single images for each angle. All single images are then intersected to create a 3D reconstruction of the sample (Bouxsein, et al., 2010).

In this project, bone scanning was carried out using a Skyscan 1172 X-ray-computed microtomography scanner (Skyscan, Aartselaar, Belgium) equipped with an x-ray tube (voltage, 49kV; current 200μA) and a 0.5 mm aluminium filter. The scanner resolution was 4.3 μm with a rotation.

Pre fixed tibias were wrapped with cling film to prevent drying out and ensure steadiness before being mounted on the rotator base. Rotation was set at 180° (images every 0.66°). After scanning, bones were reconstructed using NRecon software (Brunker microCT), setting the threshold at 0 – 0.16. Trabecular analysis was carried out using the CTan software (Brunker microCT). The region of interest (ROI) was set up 0.200 mm after first broken bridge of the growth plate, and a height of 0.500 mm was imaged (see figure 2.2). Trabecular bone volume fraction (BV/TV) was analysed.
Figure 2. Images from reconstructed tibias observed by sections on CTan software. A) Representation of the broken bridge of the growth plate, also observed as spongy bone. B) ROI (red area) drawn in the trabecular section of the tibia. ROIs are drawn away from the endosteum to avoid inclusion of non-trabecular bone.
2.5 Biochemical Analysis

2.5.1 N-terminal propeptide of type I procollagen (P1NP)

Bone matrix is composed by 70-90% of mineral components and the rest of the organic material is mainly composed by type I collagen. During collagen synthesis, both amino-terminal propeptide (PINP) and the carboxy-terminal propeptide (PICP) parts are cleaved off and released from the procollagen molecule. These molecules are secreted to the blood circulation and processed by liver endothelial cells. The serum levels of PINP and PICP can be detected by commercial immunoassay kits. Currently, PINP is commonly used as a biomarker for bone formation (Melkko et al., 1996; Seibel, 2005). For the purposes of this study we have used P1NP as a surrogate for osteoblast activity.

For my experiments, a Rat/mouse PINP enzyme immunoassay kit was used in accordance with manufacturer’s instructions (Immunodiagnostic System Limited): 50 µL of calibrators, controls and sample (5 µL diluted with 45 µL of sample diluent) were added to a polyclonal rabbit anti-PINP antibody coated 96-well plate. Biotin was added to the 96-well plate to label the PINP and is incubated for 1 hour at room temperature. After incubation, liquid was removed, and wells washed 3 times with washing buffer 1X. Horseradish peroxidase labelled avidin was added and binds selectively to the biotin complex. After incubation for 30 minutes at room temperature, and a washing step, colour was developed by adding a chromogenic substrate (TMB). The generation of colour was stopped with hydrochloric acid and the absorbance of the reaction was read at 450 nm using a SpectraMax 5Me plate reader. Results were calculated based on the obtained calibration curve.

2.5.2 Tartrate-resistant acid phosphatase form 5b (TRACP 5b)

TRAP is a group of enzymes synthesised by osteoclasts, macrophages and dendritic cells in various organs from which, bone, spleen, and liver have the highest activity of this enzyme. The subform b of the isoform 5 is the most specific for osteoclasts (Haymand, et al., 2000). Osteoclast secrete TRACP 5b into the blood circulation as an active enzyme that is inactivated and degraded to fragments before it is removed from the circulation. Levels of TRACP 5b in
serum have been correlated with the activity of osteoclasts, also as selective marker of bone resorption (Alatalo, et al., 2003).

For my experiments, a MouseTRAP™ (TRAcP 5b) ELISA kit was used in accordance with manufacturer’s instructions (Immunodiagnostic System Limited). Polyclonal antibody against recombinant mouse TRAcP 5b was added to an anti-rabbit IgG-coated 96-well plate. After incubation and washing steps, 100 µL of standards, control, and samples (25 µL of serum with 75 µL of 0.9% NaCl) were incubated in the wells, and bound TRAcP 5b activity was determined with a chromogenic substrate to develop colour. The reaction was stopped using sodium hydroxide, and the absorbance of the reaction was read at 405 nm using a SpectraMax 5Me plate reader. Results were calculated based on the obtained calibration curve.

2.5.3 Follicle stimulating hormone (FSH)

Mouse FSH ELISA kit was used in accordance with manufacturer’s instructions (Elabscience). 100 µL of calibrators, controls and serum were added to an anti-mouse FSH antibody coated well plate followed by an incubation. A biotinylated detection antibody specific for mouse FSH was added straight after incubation. After a washing step, Avidin-horseradish peroxidase conjugate was added to the well plated and incubated and followed by a washing step. Substrate is added to the conjugated to develop colour and stopped after an incubation period. Absorbance of the reaction was read at 450 nm using a SpectraMax 5Me plate reader. Results were calculated based on the obtained calibration curve.

2.5.4 Oestradiol

Mouse/Rat oestradiol ELISA kit was used in accordance with manufacturer’s instructions (Calbiotech). 25 µL of calibrators, controls and serum were added to an anti-oestradiol polyclonal antibody coated well plate, followed by the addition of oestradiol enzyme conjugate and further incubation. During incubation, horseradish peroxidase-labelled oestradiol competes with endogenous oestradiol in calibrators, controls and serum. After the incubation a washing step was performed. A solution of TMB Reagent was added to generate colour. After incubation colour generation was stopped. Absorbance of the reaction was read at 450 nm using a SpectraMax 5Me plate reader. Results were calculated based on the obtained calibration curve.
2.6 Analysis of histological samples

2.6.1 Tibiae sections

Mouse tibiae were fixed in 4% of Paraformaldehyde (PFA) (Merk) for 72 h. After fixation and uCT analysis (see section 2.4), tibiae were decalcified with EDTA solution at 0.4 M (pH adjusted at 7.50 ± 0.5). The EDTA solution was changed every 3rd day for 3 weeks or until fully decalcification was confirmed by µCT scanner. Once the tibiae were decalcified, they were placed in histology cassettes and dehydrated through increasing concentrations of ethanol before being embedded in paraffin wax. Embedded tibiae were mounted into cassette blocks and cooled down for sectioning. Using a RM2265 microtome (Leica), tibias were trimmed (10µm section) until the tibia head was revealed, thereupon, 3 µm sections were collected and left to stretch in a water bath at 45°C. When sections were totally flat and with no wrinkles, they were picked up using positively charged slides (Superfrost Plus Slides). Samples were dried on a hot plate followed by overnight drying in an oven at 37°C. Sections were stored at room temperature until use.

2.6.2 TRAP Staining for osteoclasts

The principle of the method is the reaction of the enzyme with a naphthol-AS-BI phosphate substrate. The resulting product of this reaction is further treated with a diazonium salt (hexazotised pararosaniline), giving a red precipitate that can be visualised under light microscopy (Janckila et al., 2001; Galvao et al., 2011).

The preparation of acetate-tartrate buffer consisted in adding sodium tartrate into acetate buffer. The buffer was left to warm up in an incubator at 37 °C for 2-3 hours prior staining. Bone section obtained as described on section 2.6.1 were dewaxed and placed in acetate-tartrate buffer for 5 minutes. Solution A was then prepared by mixing 50 mL of Acetate-tartrate buffer to 1mL naphtol/dimethylformamide solution, previously prepared with naphthol AS-BI phosphate into 1mL dimethylformamide. Bone sections were incubated for 30 minutes at 37 °C in solution A. Solution B was prepared by adding 2 mL pararosaniline stock at 50mg/mL to 2 mL of 4 %sodium nitrate solution and let it stand for 5 minutes. 2.5 mL of the hexazotised solution were added to 50 mL Acetate-tartrate buffer. Bone sections were incubated in solution...
B for 15 minutes and washed twice through distilled water. Sections were counterstained in Gill’s II haematoxylin for 20 seconds. Sections were dehydrated and mounted with DPX mounting medium.

2.6.3 Numeration of osteoclasts and osteoblasts by osteomeasure

After TRAP staining, osteoclasts are stained bright red and osteoblast acquire a blue colour as seen in figure 2.3.

Figure 2. 3. Example of bone section stained with TRAP staining. Osteoclasts acquire bright red colour and are large multinucleated cells (black arrow) and osteoblasts stain with blue/purple colour and are columnar cells that form in chains (white arrows) (Cropped image from a 20x magnification).

Two parameters were analysed: number of cells osteoclast (N.Oc.) and number of osteoblast (N.Ob.), and the parameter that each type of cell is in contact within the bone surface (Oc.Pm. & Ob.Pm.). Scoring was performed in the metaphysis of the tibia. Both measurements were scored in two different regions of the tibia, the trabecular area and the cortical area.

Data was collected using Leica RMRB upright microscope and OsteoMeasure software (Osteometrics Inc.) and Open-Source Software for Bone Histomorphometry, free Jpeg extension (The University of Liverpool, UK).
2.7 Statistical analysis

Stated values are presented as mean ± standard deviation. Graphs values are presented as mean ± standard deviation (SD). Statistical analysis was performed using unpaired Student’s t-Test, 1-way ANOVA and 2 ways ANOVA followed by Tukey’s post-hoc test on GraphPad Prism versions 7.02 and 8.0. Exclusion of outliers was carried out using ROUT method with a maximum false discovery rate (Q) of 1.0 %. This procedure was applied in specified sections only. P values are presented as, * p≤0.05; ** p≤0.01, ***p≤0.001, ****p≤0.0001 and no significance: ns.
CHAPTER 3: Establishing a mouse model for the study of pre-, peri- and post-menopausal concentrations of oestradiol +/- Zol and their effects on the bone microenvironment
3.1 Introduction

Bisphosphonates are a family of molecules with a chemical structure similar to a pyrophosphate. Due to this similarity, bisphosphonates exhibit a high affinity to the inorganic component of the bone tissue. Once bisphosphonate molecules reach the bone, they cause a decrease in the number of osteoclasts by inducing apoptosis (see section 1.13). The use of bisphosphonates has been targeted mainly to prevent events of bone loss caused by excessive osteoclastic resorption, such as those observed in tumour-related bone loss (Coleman *et al.*, 2013; McClung *et al.*, 2007). Preclinical studies focused on the effect of bisphosphonates on tumour cells have demonstrated that these molecules can elicit a direct anti-tumour effect on different cancer cell types *in vitro* (Göbel *et al.*, 2016; Jagdev *et al.*, 2001, Oades *et al.*, 2003)

Preclinical and clinical studies have demonstrated that incorporation of Zol in tumour-bearing bone microenvironment have a positive effect decreasing tumour growth (Coleman *et al.*, 2018; Gnant *et al.*, 2011; Göbel *et al.*, 2016; Jagdev *et al.*, 2001; Ottewell *et al.*, 2014; Yoneda *et al.*, 2000); however, the premise of whether it is a direct effect of Zol on tumour cells has not yet been answered. Interestingly, diverse clinical trials have demonstrated that the anti-tumour action of Zol could depend on external factors which regulates the bone microenvironment. An example of this observation was reported in the AZURE and ABCSG12 clinical trials where the protection of bone tissue after use of adjuvant Zol for prevention of bone metastatic was noted. Moreover, in the AZURE clinical trial, a further benefit was observed in the population of patients who had been clinically define as post-menopausal. This extended benefit was demonstrated as an increase in DFS, including distant soft tissue, in comparison to the other patient groups (Coleman *et al.*, 2018). A post-analysis of the AZURE clinical data found a correlation between the concentration of hormones involved in the menopausal transition, FSH and oestradiol, and the possible outcome of the adjuvant therapy (Wilson *et al.*, 2017). Both hormones play important physiological roles in the homeostasis of diverse organs, especially the bone tissue. It is in bone that oestradiol has a protective function decreasing bone resorption and increases longevity in bone building cells (osteoblasts), among other maintenance functions (Bradford *et al.*, 2010; Hughes *et al.*, 1996; Martin-
Millan et al., 2010); however, these functions are compromised with the transition and complete establishment of menopause.

Human menopause is a complex hormone deregulation caused by a physiological ovarian senescence (Koebele & Bimonte-Nelson, 2016). One of the most noticeable changes during the menopausal transition is the gradual decrease of ovarian hormones such as oestrogens (mainly oestradiol) and inhibins. The concentration of these hormones continues to drop until non-detectable concentrations when establishment of menopause is clinically defined. In contrast, FSH shows an increase as a physiological response from the pituitary hormone when low concentrations of oestrogens are sensed. Both ovarian and FSH hormones have an important impact in the bone microenvironment as well as in tumour growth (Wilson et al., 2017).

Since in vitro assays lack the complexity of bone and tumour microenvironment, it was imperative to establish an in vivo model where hormone interaction as seen in menopause can be analysed; however, menopause is not naturally seen in laboratory animals as observed in humans. Additionally, small rodents such as mice produce lower circulating concentrations of oestradiol in pre-menopausal healthy status when compared to humans (Koebele & Bimonte-Nelson, 2016). One of the most widely used methods to model human menopause in rodents is the removal of the ovaries. Therefore, to establish the menopausal transition as seen in humans, we assessed the effect of OVX in combination with oestrogen replacement in a mouse model via drinking water.

To understand the mechanisms of how oestrogen and Zol interact in bone tissue, we have developed an in vivo model simulating the transition to menopause. However, to solely investigate the effect of oestradiol in the microenvironment, production of FSH had to be impaired with the gonadotropin-releasing hormone agonist, goserelin. Restriction of FSH production was done in order to prevent interference of this hormone on the data generated. The proposed concentration of oestradiol in the post-menopausal status was <10 pmol/L which correlates to proposed concentrations observed in clinical observations (Wilson et al., 2017). This concentration of oestradiol can be achieved by OVX; however, aromatase inhibitor Letrozole was tested in order to achieve a complete depletion in the production of oestradiol from extra-gonadal sources.
3.2 Results

3.2.1 Establishing a mouse model for accurate representation of post-menopausal concentration of oestradiol and suppression of FSH

Based on previous in vivo experiments performed in the research group, in order to observe statistical significance effects in bone microenvironment as well as the assessment of bone biomarkers, 5 animals per group was used (Ottewell et al., 2014).

For this experiment 12-week-old BALB/c mice were used (N=40; n=5 per group). Sham-operated mice were used to investigate a murine physiological concentration of oestradiol and FSH. No additional modifications were performed to this group (no oestrous synchronization). To investigate oestradiol depletion and FSH increase and depletion, groups underwent OVX followed by administration of daily Goserelin (40µg/Kg) with Letrozole (1 mg/kg every 2 days), Goserelin (40µg/Kg) only and no additional treatment (figure 3.1). These groups represented surgical and chemical removal of oestradiol and FSH depletion; surgical removal of oestradiol and FSH depletion; and surgical removal of oestradiol only, respectively. 7 days after surgical procedure, groups were administered saline or Zol (100 µg/Kg). 14 days after surgical procedure, mice were culled and samples were collected (bone and blood).
Figure 3.1 Experimental outline of in vivo establishment of post-menopausal concentration of oestradiol model and FSH baseline concentrations.

12-week-old female BALB/c mice underwent surgical procedures (N=40; n=5 per group). Mice were divided by sham (no oestrous synchronisation), OVX only, OVX + goserelin and OVX + goserelin + Letrozole. Goserelin was administered daily and Letrozole was administered every other day. 7 days after surgical procedure a single dose of Zol or saline was administered. 14 days after surgical procedure the experiment reached the endpoint and all animals were culled and samples were collected.

Serum concentrations of oestradiol in the sham-operated group (control, n=5), showed an average of 68.44 ± 31.17 pg/mL. This variability was not normalized or correlated to the oestrous cycle of the animal at the day of culling. A decrease in the concentration of oestradiol was observed in the mice were OVX was performed, averaging 26.72 ± 3.23 pg/mL; however, this decrease was no statistically significant (ANOVA, p=0.066). Concentrations of oestradiol were further reduced when OVX was combined with the administrations of goserelin (ANOVA, p=0.033). These values were below the limit of detection of the ELISA (< 3 pg/mL) indicating a successful reduction in the internal synthesis of oestradiol. When OVX mice were administered a combination of goserelin and letrozole 13.9 ± 10.5 pg/mL of oestradiol was detected in the serum; however, no difference was observed when compared with Sham or OVX-only concentrations of oestradiol (ANOVA, p=0.121) (figure 3.2).

Administration of Zol elicited a significant increase in the concentration of oestradiol of OVX + goserelin + letrozole group (ANOVA, p=0.006). This increase represented at least a 2.5-fold of increase compared with the other groups. However, Zol had no significant effect on circulating oestradiol concentrations in Sham operated or mice recieving OVX-only.
Although Zol showed an increase in the concentration of oestradiol in OVX with goserelin (t-Test, $p<0.001$), this concentration remained within the range accepted as post-menopausal concentration of oestradiol. The combination of OVX + goserelin + letrozole on the other hand had an obvious increase of oestradiol and, therefore, was excluded for further experiments.

Analysis of FSH in serum showed no difference in between the different groups (ANOVA, $p=0.615$). This remained unaltered when Zol was administered to the respective groups (ANOVA, $p=0.603$).
Figure 3.2. Evaluation of the concentrations of oestradiol and FSH in Sham, OVX, OVX with Goserelin (Gos) and OVX with Goserelin & Letrozole (Gos+Let) mice.

14 days after surgical procedure and respective treatment (single dose of Zol or saline at day 7), serum was collected and quantified for concentrations of oestradiol A) and FSH B) by ELISA. Data from groups is presented as Mean ± SD. 1 way ANOVA was performed for groups under treatment (Zol or saline) (solid comparison line), whereas t-Test was performed between same procedure group (Sham, OVX, OVX+Gos & OVX+Gos+Let) to compare Zol vs saline (dotted comparison line). Statistical significances are represented as * = P < 0.05, ** = P < 0.01 and no significance: ns.
3.2.2 Modelling of pre- peri- and post-menopausal serum concentrations of oestrogen +/- Zol in a mouse model.

To model clinically relevant concentrations of oestrogen that are found in women during the different stages of the menopause, 12-week-old BALB/c mice (n=30) underwent OVX followed by supplementation of oestradiol through their drinking water (n=5 per group). Due to the reduce information on oestradiol replacement via drinking water, an adjusted concentration from solid-intake administration (Strom et al., 2012) was applied. To mimic pre-, peri- and post-menopausal concentration of oestradiol 12.5 mg/L, 1.38mg/L or 0mg/L 17-β-oestradiol were supplemented to the drinking water. Daily dosage of goserelin (40µg/Kg) was administered. 7 days after OVX a single dose of 100 µg/Kg Zol was administered. 14 days after OVX all mice were culled, and samples were collected (figure 3.3).

Figure 3.3. Experimental outline of in vivo establishment of pre-, peri- and post-menopausal concentration of oestradiol model in a BALB/c.

12-week-old female BALB/c mice (N=30; n=5 per group) were OVX followed by oestradiol replacement using 12.5 mg/L, 1.38 mg/L or 0 mg/L of oestradiol added to drinking water. Daily administration of goserelin (40µg/Kg) was given. At day 7 after OVX, a single dose of Zol (100µg/Kg) was administered. 14 days after OVX all animals were culled and samples were taken for downstream analysis.
2 weeks after OVX, mice who received 12.5 mg/L oestradiol exhibited serum concentrations of oestradiol of 153.0 ± 18.1 pg/mL which is representative of concentrations anticipated in a pre-menopausal woman. Mice supplemented with 1.38 mg/L oestradiol showed serum concentrations of 48.6 ± 18.4 pg/mL, and mice receiving no addition oestradiol showed serum concentration 2 ± 0.447 pg/mL of oestradiol (figure 3.4a). These concentrations were significantly different between each other and were considered to model the variation of oestradiol found in the three menopausal conditions (ANOVA, p<0.001).

Importantly, FSH concentrations remained at very low concentration in all groups (pre-menopausal group; 0.86 ± 0.80 ng/mL; peri-menopausal group: 4.71 ± 3.89 ng/mL; and post-menopausal group: 3.52 ± 2.13 ng/mL) and no significantly differences were observed (ANOVA, p=0.615). Therefore, these models were considered to be suitable for investigating the effects of Zol under different menopausal concentrations of oestradiol without the interference of FSH (figure 3.4b).

Administration of single dose of Zol did not alter the concentration pattern of oestradiol observed in the control groups. Hormone replacement of 12.5 mg/L, 1.38 mg/L and 0mg/L of oestradiol resulted in serum concentrations of 100.0 ± 18.2 pg/mL, 26.3 ± 3.76 pg/mL and 18.3 ± 2.74 pg/mL respectively. These concentrations were markedly different between each other (ANOVA, p=0.001). More importantly, Zol did not have any interference in the serum levels of pre-menopausal and peri-menopausal concentrations of oestradiol (t-Test, p= 0.104, p=0.281, respectively). Although in the post-menopausal concentrations of oestradiol the addition of Zol was observed with an increase in the levels of oestradiol (t-Test, p<0.001). Nonetheless, these values can still be representative of post-menopausal concentrations of oestradiol.

Zol did not increased the concentrations of FHS which remained at minimal levels (ANOVA, p=0.603)
Proposed menopausal status was established by OVX + oestradiol replacement (day 0). Pre-menopausal group (n=5) received 12.5 mg/L of oestradiol in their drinking water, whilst Peri-menopausal (n=5) and post-menopausal (n=5) groups received 1.38 mg/L and 0 mg/L of oestradiol respectively. Groups received a single dose of Zol or saline at day 7. 14 days after menopausal status establishment, serum samples were collected and analysed for oestradiol (A) and FSH (B) by ELISA. Data from groups is presented as Mean ± SD. 1 way ANOVA was performed for groups under treatment (Zol or saline) (solid comparison line), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group) to compare Zol vs saline (dotted comparison line). Statistical significance is represented as, ** = P < 0.01, *** = P < 0.001, and no significance: ns.
3.2.2.1 The effects of Zol on the bone microenvironment under pre-post and peri-menopausal concentrations of oestradiol.

Bone is a dynamic tissue maintained by continuous bone remodelling. During the menopausal transition, elevated concentrations of FSH and low concentrations of oestradiol produce an inflammatory environment in the bone which in turn shift the balance of remodelling towards an increased bone resorbing phenotype (Manolagas, 2000). Previous studies have addressed the differences in bone structure and microenvironment in mouse models using sham or oestradiol supplemented mice vs OVX mice to model pre- and post-menopause respectively (Holen et al., 2016; Ottewell et al., 2014); however, the pre-menopausal status group was not accurately represented as the concentrations of oestradiol either overpassed or underreach the biologically relevant concentration. In addition, control over FSH is crucial when modelling low and limited concentrations of oestradiol as seen in the peri- and post-menopausal status. Therefore, with the hormonal replacement approached on this mouse model, investigation of bone remodelling and how it is affected under pre-, peri and post-menopausal concentrations of oestradiol in the absence of FSH, as well as how these parameters were altered following administration of Zol, was possible.

3.2.2.2 Effect of Zol in the bone structure under pre-, peri-, and post-menopausal concentrations of oestradiol.

Both tibiae from all mice (modelling pre- peri- and post-menopausal concentrations of oestradiol) were used to study how oestrogen and Zol affects bone architecture and histomorphometry. As both oestrogen and Zol have previously been shown to exert changes on trabecular bone (Holen et al., 2016; Ottewell et al., 2014), we first investigated architectural changes in this area by measuring bone volume (BV/TV) using MicroCT technology.

Mice in the pre-menopausal group had significantly increased bone volume (BV/TV % = 16.0 ± 0.9) in comparison to the peri- (BV/TV % = 16.0 ± 0.9), and post-menopausal (BV/TV % = 12.0 ± 0.6) groups (ANOVA, p<0.001 and P<0.001 respectively). Peri-menopausal levels of oestradiol also showed an increase in bone volume in comparison to the post-menopausal group (ANOVA, p=0.049). Therefore, the response of skeletal tissue, in terms
of bone volume, is proportional to concentrations of oestradiol (ANOVA, p<0.001) (figure 3.5a).

Similarly to the control group, a marked difference was observed between the three menopausal groups in the Zol treated mice (ANOVA, p<0.001). The same proportional increment in bone volume was observed with increasing concentrations of oestradiol in Zol treated mice, however, a greater bone volume was observed in the Zol treated group in comparison to the control groups. This difference accounted for an approximated 2% more bone volume in all three groups (t-Test, pre-, p=0.036; peri-, p=0.024; and post-menopause, p=0.042) (figure 3.5b).
Figure 3.5. Analysis of bone volume from pre-, peri-, and post-menopause status mice after a single dose of Zol using μCT analysis.

Menopausal status establishment was performed on mice at day 0 (N=30) as referred previously. Premenopausal, peri-menopausal, and post-menopausal status groups (n=5 per group) were given a single dose of Zol or saline at day 7. Tibiae were collected at day 14 after menopausal status establishment and μCT scanned within 72h after sample collection. Analysis of Bone volume was performed 0.2mm above growth plate from the proximal head of tibiae and a region of interest was then drawn covering 0.5mm of bone tissue (A). A representative image of the trabecular area from the ROI is presented (B). Data from groups is presented as Mean ± SD. 1-way ANOVA was performed for groups under treatment (Zol or saline) (solid comparison line), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group) to compare Zol vs saline (dotted comparison line). Statistical significance is represented as * = P < 0.05, and *** = P < 0.001.
3.2.2.3 The effect of Zol in the bone microenvironment under pre- peri- and post-menopausal concentrations of oestradiol.

To study the effects of different concentrations of oestradiol at a cellular level, bone resorption biomarkers PINP for osteoblast activity, and TRAcP 5b for osteoclast activity were quantified. The numbers of cells were subsequently analysed by histomorphometry using tibiae sections stained for TRAP.

In the control group, bone building biomarkers demonstrated a clear difference in the activity of osteoblasts (PINP). In pre-menopausal concentration of oestradiol group, the concentration of PINP was 20.7 ± 1.30 ng/mL. This value was significantly lower in comparison to the peri-menopausal group where 31.30 ± 4.44 ng/mL were observed (ANOVA, p=0.017). Similarly, the concentration of PINP in the pre-menopausal group was significantly lower in comparison to the post-menopausal group (33.01 ± 6.40 ng/mL) (ANOVA, p=0.007) (figure 3.6a).

Osteoclast activity (TRAcP 5b) varied strongly over the different menopausal groups (ANOVA, p<0.001). At high levels of oestriadiol (pre-menopausal concentrations) osteoclast activity was 5.81 ± 0.95 U/L which increased significantly to 10.51 ± 1.41 u/L in mice with peri-menopausal levels of oestradiol (ANOVA, p<0.001). A similar increase was also observed in the post-menopausal concentrations of oestradiol where TRAcP 5b levels reached 8.79 ± 0.56 U/L (ANOVA, p=0.003), indicating an increase activity in osteoclast as the circulating concentrations of oestradiol were decreased (figure 3.6b).

Osteoblast activity by PINP measurement revealed no significant differences between menopausal groups after Zol treatment (t-Test, p=0.668) (figure 3.6a). A comparison with the control groups showed that pre-menopausal group had significantly increased levels of PINP after treatment with Zol (t-Test, p=0.008). Nonetheless, peri- and post-menopausal groups did not show any significant difference in levels of PINP after Zol treatment in comparison to the control groups (t-Test, p=0.901, and p=0.229, respectively) (figure 3.6a).

The administration of Zol affected the significatively the concentrations of TRAcP 5b in the three menopausal concentrations of oestradiol. Interestingly, neither a high
concentration of oestradiol nor a minimal concentration of the hormone had a significant
difference between each other (<0.3 U/L and 0.95 ± 2.07 U/L of TRAcP 5b for pre-
menopause group and post-menopause group, respectively) (ANOVA, p=0.587). The peri-
menopausal concentration of oestradiol, on the other hand, showed a significant increase
in osteoclast activity when compared to both extremes (3.78 ± 1.20 u/L) (ANOVA,
p=0.001 and p=0.006 when compared to pre- and post-menopausal groups, respectively)
(figure 3.6b). However, when comparing Zol-treated groups against control groups, a
marked decrease in the levels of TRAcP was observed in all Zol-treated groups (p<0.001)
(figure 3.6b). Overall, these results agree with previous studies carried out in similar
conditions (Ottewell et al., 2014), indicating that Zol decreases importantly the activity of
osteoclasts despite the menopausal status.
Figure 3. Effect of pre-, peri- and post-menopausal concentrations of oestradiol in bone turn over biomarkers after a single dose of Zol.

Menopausal status establishment was performed on mice at day 0 (N=30) as referred previously. Pre-, peri- and post-menopausal status groups (n=5 per group) were given a single dose of Zol or saline at day 7. Serum samples were collected at day 14 and analysed for bone osteoblastic marker PINP (A) and bone osteoclast activity marker TRAcP 5b (B) by ELISA. Data from groups is presented as Mean ± SD. 1-way ANOVA was performed for groups under treatment (Zol or saline) (solid comparison line), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group) to compare Zol vs saline (dotted comparison line). Statistical significance is represented as * = P < 0.05, ** = P < 0.01 and *** = P < 0.001.
In the control groups, there were no significant differences in the number of osteoblasts throughout the different menopausal groups (ANOVA, p=0.136). However, there is a trend towards a reduction in the number of osteoblast when concentrations of oestradiol are less available as in the case of peri- and post-menopausal groups. This phenotype was more evident in the Zol treated groups, mice with pre-menopausal concentrations of oestradiol had a significantly increased osteoblast numbers in comparison to the post-menopausal group (ANOVA, p=0.047). Despite not being significant (ANOVA, p=0.052), a gradual reduction in the numbers of osteoblast was observed when the levels of oestradiol were reduced. Osteoblast number was not significantly altered after Zol administration in comparison to non-treated groups (ANOVA, p=0.733; p=0.890; and p=0.079, for the pre-, peri-, and post-menopausal group, respectively; figure 3.7a).

In contrast, osteoclast numbers were reduced in the pre-menopausal group in comparison to the peri- and post-menopausal groups (ANOVA, p=0.005 and p=0.016, respectively). However, there was no difference in the number of osteoclasts between the peri- and post-menopausal levels of oestradiol (ANOVA, p=0.740). These results support the common knowledge where in less availability of oestradiol, the bone microenvironment turns into a more resorbing phenotype increasing the activity and number of active osteoclasts. However, there was a significant reduction in number of osteoclasts in the post-menopausal group treated with Zol in comparison to the control group (t-Test, p=0.023). Similar results were observed in the peri-menopausal model, where the Zol-treated group showed a tendency towards reduction in osteoblasts numbers in comparison to the control group (t-Test, p=0.052). No significant variation was seen in both Zol-treated and control pre-menopausal groups (t-Test, p=0.348) (figure 3.7b). (No significant differences in the number of osteoclasts were observed between any of the groups after a single dose of Zol; t-Test, p=0.303).

An example of the cell differences in the histomorphometry of the three menopausal models is provided in figure 3.8.
Figure 3. 7. Histomorphometry analysis of tibiae samples from pre-, peri- and post-menopausal concentrations of oestradiol in tibiae after a single dose of Zol.

Menopausal establishment was performed at day 0 on BALB/c mice (N=30), as previously described. A single dose of Zol or saline was given at day 7, and tibiae samples were collected at day 14. After fixation, μCT scanning and decalcification, tibiae were processed and sectioned for histomorphometry analysis using TRAP staining. Osteoblasts (A) and osteoclasts (B) were scored in trabecular and cortical regions of tibiae. Data from groups is presented as Mean ± SD. 1-way ANOVA was performed for groups under treatment (Zol or saline) (solid comparison line), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group) to compare Zol vs saline (dotted comparison line). Statistical significance is represented as * = P < 0.05 and ** = P < 0.01.
Figure 3. TRAP stained bone sections of mice tibiae from the three menopausal states.

Osteoclasts acquire a red colour making their identification easier. Osteoblasts are stained with blue/purple colours. Osteoblasts are laid next to the bone surface forming long chains of cells. Morphologically, osteoblasts tend to have a square shape.
3.3 Discussion

3.3.1 Establishment of a mouse model representative of pre-, peri-, and post-menopausal concentrations of oestradiol.

OVX has been widely used in several animal models to cause a deprivation of ovarian hormones inducing a menopause-like state. In mouse models, the use of OVX has been established as the gold standard in menopause-related research (Koebele & Bimonte-Nelson, 2016). In the current study, mouse models of the menopausal transition (pre-, peri-, and post-menopause) were achieved. The establishment of the post-menopause mouse model was achieved by performing OVX with administration of the FSH-inhibitor goserelin. This model represents a baseline for oestradiol replacement at 2 different concentrations simulating the concentrations of this hormone observed in pre-, and peri-menopausal women. Although the use of OVX has been criticised for not being an ideal model for the study of peri-menopause due to an abrupt increase/decrease in FSH/oestradiol (Brooks et al., 2014). This sudden decrease in oestradiol is prevented by the administration of a representative concentration of oestradiol through drinking water, while FSH is downregulated using daily doses of goserelin.

OVX alone caused a reduction in the concentration of oestradiol by almost 50% in comparison to sham mice (no oestrous synchronised). These concentrations of oestradiol did not meet the ranges of our proposed model to mimic the concentration of oestradiol observed in post-menopausal women. Interestingly, this result agrees with a similar study carried out by Wright et al., (2008) where OVX decreased the circulating concentration of oestradiol but did not achieve a complete reduction of oestradiol. Therefore, the use of the anti-hormone therapy, goserelin, was needed to achieve very low concentrations of oestradiol required for this study. The combination of OVX with goserelin accomplished reductions in the concentrations of oestradiol similar to those seen in post-menopausal women, thus, this group meet the requirements to represent the post-menopausal population.

It is important to establish that sham-operated mice were not treated to compensate for any hormonal variation due to the oestrous cycle. In rodents, the oestrous cycle can be compared to the human reproductive cycle, also known as menstrual cycle. The oestrous cycle is divided into 4 stages: pro-oestrous, oestrous, metouestrus and dioestrous. These stages of the cycle are
coupled by a marked variation of hormones such as progesterone and oestrogens (mainly oestradiol) (Wood et al., 2007). In mice, ovulation occurs at oestrous and is when the concentration of oestradiol in serum reaches its peak. This period is known to last between 12 to 48 hours. After this time, oestradiol decreases gradually and progesterone increases, then a decline is observed at pro-oestrous. Inversely, oestradiol starts to increase at pro-oestrous until a new oestrous occurs (Fata et al., 2001; Walmer et al., 1992). The fluctuation of these hormones occurs in a window of hours which represents a significant challenge during the sample collection. This hormonal fluctuation can be observed in the variability of oestradiol concentrations from the sham-operated group receiving saline. Interestingly, no reports have shown any correlation between the administration of Zol and a reduction of oestradiol fluctuation. A possible alternative to prevent future increase in hormonal variation is the induction and synchronisation of the oestrus cycle. This synchronisation has been demonstrated to be effective and can be performed through different methods from which, external hormone-like substances, such as cloprostenol and progesterone, yield a reliable induction of oestrous cycle (Pallares & Gonzalez-Bulnes, 2009; Wei et al., 2015).

Drinking water supplemented with two concentrations of oestradiol was an adequate method of administering oestradiol for the replacement of this hormone in the mouse models used in this study. Mice who underwent OVX in combination with goserelin and received either high or low oestradiol through drinking water had concentrations of oestradiol similar to those proposed to represent women at the pre-, and peri-menopause respectively. This method resulted in circulating oestradiol concentrations that were more representative of those found in women than previous studies where oestradiol replacement was via subcutaneous implant resulting in excessively high concentrations of oestradiol that were unsuitable for study (Holen et al. 2016; Ottewell et al., 2014; Ottewell et al., 2015). Nonetheless, a possible limitation to this method could be encountered by a disproportional individual consumption of liquid.

Although direct administration of oestradiol via intraperitoneal or subcutaneous injection has demonstrated to produce reliable results in oestradiol replacement after OVX (Raafat et al., 1999), early reports have demonstrated that there is no significant physiological difference in the administration of oestradiol either orally or subcutaneously (Gordong et al., 1986). In addition, administration of oestradiol through drinking water is more humane and has the advantage of being in parallel with the circadian patterns of drinking.
Taken together, data shows that the models of the menopausal transition produced in this study enable the identification of how oestradiol causes significant changes in parameters associated with the bone microenvironment in the absence of other reproductive hormones.

### 3.3.2 Effect of OVX in serum concentration of FSH

The concentrations of FSH in both, control and OVX treated mice, were either very low or under the detection limit of the quantification method used. In sham-operated mice, the concentration of FSH fluctuated at 0.63 ± 0.53 ng/mL. This concentration is approximately 10 times lower in comparison to the concentrations reported in BALB/c mice under similar conditions (Rodin et al., 1990). In addition, normal concentrations of FSH in serum tend to be consistent throughout different mouse strains at similar age (~10 ng/mL) (Lohff et al., 2006; Mayer et al., 2004), which indicates that very low concentrations of FSH in untreated mice are rare to occur.

This inconsistency in the concentration of FSH becomes more evident in the OVX only group where values of FSH were undetectable. Diverse studies carried out in mice with impaired ovarian functionality by either OVX or chemically-induced ovarian failure (Lohff et al., 2006; Mayer et al., 2004; Rodin et al., 1990) have demonstrated that, similarly to humans, concentrations of FSH increase drastically when there is an absence of ovarian hormones. In mice, concentration of FSH after ovarian failure reach levels equal or higher than 30 ng/mL. Therefore, the concentrations of FSH obtained in this study by the ELISA quantification method are not reliable since they do not correlate with expected tendencies previously established.

In support to this, Tan et al., (2018) commented that despite the specificity of ELISA for the quantification of mouse FSH, the structural complexity of the mouse reaction lowers the efficacy of commercial ELISA kits to produce accurate results in comparison to other techniques such as radioimmunoassay (RIA).

The use of goserelin has been reported to be a potent inhibitor of FSH production in mice. Diverse studies have shown that goserelin and similar gonadotropin-releasing hormone agonists cause a suppression in the production of FSH by reducing pituitary gonadotrophin secretion through receptor down-regulation (Blaakaer et al., 1995; Detti et al., 2014; Meirow et al., 2004). Thus, any possible increase of FSH should have been prevented by daily administration of goserelin.
3.3.3 Concentration-dependant effect of oestradiol on bone formation and resorption in the bone microenvironment

The established models of the menopausal transition in combination with the use of µ-CT technology evidenced significant changes in the bone structure. These changes correlate with those occurring in women under the different menopausal stages. It is widely accepted that under normal hormonal conditions, as found in pre-menopause women, the maintenance of bone turnover is balanced between osteoclasts resorption and bone formation by osteoblasts (Manolagas, 2000), however, in the late pre-menopause there is an increase in the bone turnover process (Ebeling et al., 1996). This increased turnover has been demonstrated to be triggered when the levels of inhibin A and B begin to drop, even under normal levels of oestradiol (Perrien et al., 2006). In peri-menopausal women, the increase of bone turnover is more evident since the levels of oestradiol decrease gradually followed by a slow increase in FSH until reach the post-menopausal stage, where the development of a pathological osteoporosis is observed (Perrien et al., 2006).

In this current study, the pre-menopause model had the highest percentage of trabecular bone volume. This percentage decreased gradually as the concentration of oestradiol in circulation were lower, as seen in the model of peri-menopause. And finally, the lowest percentage of bone volume were observed when there was a complete deprivation of oestradiol. This progressive decrease in bone volume reflected completely with what occurs in women during menopause, giving more evidence that the established mouse model is representative of the menopausal transition.

Analysis of the bone microenvironment revealed that there was a decrease in the activity of osteoblasts when high concentrations of oestradiol were present. However, the histomorphometry indicated that there was a tendency towards an increase in the number of osteoblasts under high concentrations of oestradiol (p=0.122). Despite the apparent contradictory of this data, evidence suggest that high concentrations of oestradiol are a strong anabolic stimulus that increase the number of osteoblasts rapidly (Bord et al., 2002). Although in the aforementioned research they did not evaluate the osteoblast activity, these data suggest that a proportional activity should be seen. Additionally, it has been shown that oestradiol plays an important role in the osteoblast lifespan. Oestradiol inhibits the promoter
of type 1 InsP3R, an intracellular calcium release channel that binds cytochrome c and sensitizes cells to apoptotic stimuli in osteoblasts (Bradford et al., 2010). Thus, low concentrations of oestradiol result in a shorter lifespan of osteoblastic cells. In contrast, high levels of oestradiol reduce osteoclast activity by either increasing production of TGF-β and inducing apoptosis or by direct downregulation of osteoclast activity via activation of the oestrogen receptor (Bord et al., 2002; Kameda et al., 1997) as observed in our current pre-menopausal model where the activity and number of osteoclasts was significantly reduced in comparison to the peri-, and post-menopausal groups.

Overall, high concentrations of oestradiol result in a shift in the bone remodelling cycle into a pro-bone formation environment, whereas low and limited concentrations of oestradiol promote an increase bone resorption.

### 3.3.4 Zol modifies osteoclasts and osteoblasts in the bone microenvironment.

Studies carried out in BALB/c mice have demonstrated that a single dose of Zol generates changes in bone microenvironment as soon as 3 days after administration (Haider, et al., 2014). However, these changes did not take into a consideration the menopausal transition and the possible variation that Zol might have under different concentrations of hormones. In this current study, the changes that Zol cause to the bone microenvironment were analysed in relation to the hormonal levels of oestradiol.

Zol did not alter the decreasing pattern in the percentage of bone volume observed in the pre-, peri-, and post-menopausal concentrations of oestradiol. However, Zol significantly increased the percentage of bone volume in all menopausal groups equally (p<0.001). These results agree with previous experiments where increase in the bone volume was the result of decreased resorption activity from osteoclasts (Haider, et al., 2014; Ottewell et al., 2014).

In the bone microenvironment, osteoblast activity did not undergo any alterations after a single dose of Zol. However, the number of osteoblastic cells showed a significant decrease at reduced concentrations of oestradiol whereas at concentrations of oestradiol equally or above peri-menopause the tendency to increase remained similar to the untreated groups. The diminution in the number of osteoblasts after Zol has been already observed in different studies (Brown et al., 2012; Haider, et al., 2014). Although there is no proposed mechanism for
the decrease on osteoblasts in these studies, the evidence that at high concentrations of oestradiol the number of osteoblasts was not affected suggests that osteoblasts with limited oestradiol are more susceptible to apoptotic stimuli as demonstrated by Bradford, et al. (2010). Under low concentrations of oestradiol, type 1 InsP3R is expressed in the osteoblasts making the osteoblastic cell more responsive to undergo apoptosis under the stimuli of TNFα and IL-1β (Bord et al., 2001). Similarly, activity of osteoclasts and number were drastically decreased as observed in diverse studies (Brown et al., 2012; Haider, et al., 2014; Ottewell et al., 2014; Ottewell et al., 2015).

Overall, these results fully agree with the action of bisphosphonates drugs, such a Zol, which cause a decrease in bone resorption by inducing apoptosis in active osteoclasts. Therefore, a decrease in the number of active osteoclasts is related to a decrease in bone resorption biomarker TRAcP 5b. As a result of decreased bone resorption, bone volume (% BV/TV) was higher in Zol treated mice.
CHAPTER 4: Effects of pre-, peri-, and post-menopausal concentrations of oestradiol on Zol induced changes to the bone microenvironment and metastatic outgrowth of human breast cancer cells in an immune-compromised mouse model
4.1. Introduction

Dissemination of metastatic tumour cells to distant organs has been suggested to take place early in the development of the primary tumour, even before clinical detection of the primary tumour (Hosseini et al., 2016). A limited subpopulation of cells capable of detaching from the primary site and migrating through the vascular system towards distant organs are key for the metastatic process. These disseminated tumour cells (DTCs) are largely eliminated during the migration process, mainly by the immune system. It is estimated that as little as a 0.02% of DTCs can successfully reach an adequate second site to form metastasis (Brechbuhl, et al., 2020). Although the ability of DTCs to outgrow and form secondary tumours depends on multiple variables, it is well known that the local microenvironment plays an important role (Dasgupta, et al., 2017). Bone tissue is one of the most affected sites for metastatic outgrowth, reaching approximately 70% or more in positive cases of metastasis (Chaffer & Weinberg, 2011; Coleman & Rubens, 1987). Bone is also a ‘fertile soil’ for DTCs in order for ‘seed’ to proliferate due to its biological homeostasis (highly remodelling phenotype) and the complex communication between the different cell types and niches (refer to section 1.9). Interestingly, in bone, diverse bone marrow niches can play protecting roles of DTC’s, this by inducing a state of cell dormancy on the DTCs, allowing DTCs to avoid immune surveillance (Alloca et al., 2019; Brechbuhl, et al., 2020; Dasgupta, et al., 2017; Kang & Pantel, 2013). Changes in the bone microenvironment, such as hormone deprivation or overstimulation, alter the interactions between niche-resident cell and DTCs, which ultimately results in the stimulation of DTCs to grow into overt metastasis. Unfortunately, this event can occur at unpredicted periods of time, being years, after first diagnose of cancer (Braun et al., 2005; Shiozawa et al., 2011).

Recent studies have shown that breast cancer subtypes expressing hormone receptors have a higher percentage to metastasise to bone in comparison to non –hormone receptors expressing subtypes (Yue et al., 2017; Wu et al., 2017). Nonetheless, bone continues to be the most frequent site of metastasis regardless of the molecular subtype, or the site with major tumour incidence when metastasis occurs in multiple organs (Parkes et al., 2018; Schröder et al., 2017). In addition, evidence suggests that menopausal transition also plays a role in the incidence of certain breast cancer subtypes over others. Luminal A (ER+) is the most prevalent subtype throughout the menopausal transition, followed by Triple Negative (TN) subtype
TNBC has two main incident peaks, one at early ages <35 years (predominately pre-menopausal status) and a second at ages between 51 to 65 years (mainly post-menopausal status). In the AZURE clinical trial, results showed an increase in DFS in the bone tissue in Zol treated population, with an expanded benefit on distant soft tissue in the post-menopausal subpopulation only. This result was irrespective of the breast cancer subtype despite ER +ve subtype being the most predominant type of cancer observed in the study (Coleman et al., 2014). Subclinical data has also confirmed that the protective effect of Zol is due to alterations to the bone microenvironment, independently from cancer subtype (Holen et al., 2016; Ottewell, et al., 2014). Despite ER +ve breast cancer subtype being the most prevalent in the AZURE trial, certain limitations arise when working with human ER +ve breast cancer subtypes in in vivo models. Unlike ER -ve human breast cancer cell lines, ER +ve cell lines require a sustained administration of oestradiol in order to grow and metastasise to the skeletal tissue. This cellular requirement from ER +ve subtype prevents the development of an accurate post-menopausal concentration of oestrogen model. In order to overcome this challenge, a human TN breast cancer cell line with high bone tropism was used (MDA-MB-231). As evidenced by the current literature and previous experiments (Chapter 3), variation in the concentration of oestradiol has a profound effect on the bone microenvironment, as well as the addition of Zol. In addition, and in order to evidence whether a possible direct effect from oestradiol on the activity of Zol in tumour cells was present, an in vitro assay was carried out evaluating representative concentrations of oestradiol on the MDA-MB-231 tumour cells and addition of Zol.

To better understand possible changes in bone homeostasis caused by oestradiol and/or addition of Zol affect the breast cancer metastatic process, a murine immunocompromised model was implemented. 3 menopausal concentrations of oestradiol were established and tested (pre-, peri- and post-menopausal levels of oestradiol) as described in previous chapter. Inoculation of human MDA-MB-231 tumour cells was performed after menopausal establishment. Tumour growth in bone tissue was monitored and analysed after sample collection as well as analysis of bone tissue.
4.2 Results

4.2.1 Analysis of the direct effect of pre-, peri- and post-menopausal concentrations of oestradiol +/- Zol on tumour cell viability \textit{in vitro}.

Zol is a third generation, nitrogen-containing bisphosphonate, with a reported anti-tumour activity when exposed to diverse tumour cells \textit{in vitro} (Göbel \textit{et al.}, 2016; Jagdev \textit{et al.}, 2001, Oades \textit{et al.}, 2003). The mechanism by which Zol acts on tumour cells is the same as observed in osteoclast, being farnesyl pyrophosphate synthase (FPPS) enzyme the target molecule of the drug (Jagdev \textit{et al.}, 2001). Although this mechanism of action is independent of the concentration oestradiol in the environment, there is not enough evidence to assess the anti-tumour effect of Zol at different concentrations of oestradiol.

To investigate the direct anti-tumour effect of Zol at different concentrations of oestradiol, an \textit{in vitro} experiment was carried out where representative concentration of oestradiol for pre-, peri-, and post-menopause were introduced in the \textit{in vitro} environment. Determination of the representative values of oestradiol were based in previous reports from clinical data where pre-menopausal levels of oestradiol fluctuate between 350 pmol/L – 2000 pmol/L and post-menopause is determined at \leq 50 pmol/L (Depypere \textit{et al.}, 2015; Wilson \textit{et al.}, 2017). It is important to mention that, up to date, there are not specific values of oestradiol in the National Institute for Health and Care Excellence (United Kingdom) guidelines for the diagnose or classification of pre- and post-menopause (Lumsden, 2016). Therefore, concentrations of 300 pmol/L, 84 pmol/L, and 0 pmol/L of 17-β-oestradiol were assigned for pre-, peri- and post-menopause status respectively.

A non-oestrogen responsive breast cancer cell line, MDA-MB-231, was used to determine direct interactions between oestrogen and Zol on tumour cell viability \textit{in vitro}. Cells were seeded and left to adhere for 24h prior Zol addition. 5 increasing concentrations of Zol (15μM, 17.5μM, 20μM, 22.5μM and 25μM) or PBS (n=3 per treatment) were used to produce a half maximal inhibitory concentration (IC50) normal condition media (figure 4.1a). During the first 48 hours, no significant changes were observed throughout the different groups (2-way ANOVA, p>0.999). 72 hours after continuous exposure to Zol, a
significant difference was observed between 25.0µM, 22.5µM, 20.0µM of Zol and the control group (2-way ANOVA, p<0.001) (figures 4.1a). The IC50 at 72h was calculated to be 28.1 ± 4.57 µM of Zol (figure 4.1h). At 96 h the difference between the control and all the concentrations of Zol was notorious (p<0.0001) (figures 4.1a), the IC50 at 92h dropped to 17.75 ± 1.3 µM of Zol (figure 4.1j). All treatments groups and control showed an increase in the number of cells over time (2-way ANOVA, p<0.0001) (figures 4.1b-g); however, treatment groups showed a slower rate in number of cells growth in comparison to the control group. In all Zol treated groups, a significant difference was only observed 72h after exposure to Zol (2-way ANOVA, p=0.0257, p=0.0002, p=0.0024, p=0.0054 for 15µM, 17.5µM, 20µM and 22.5µM, respectively), at the exception of 25µM of Zol, where a significant increase in number of cells was only seen after 96h after Zol exposure (2-way ANOVA, p=0.0039).

These results agree with previous data (Neville-Webbe et al., 2005; Senaratne et al., 2000) where the effect of Zol is concentration-dependant and exposure-time-dependant in various breast cancer cell lines. For further experiments, the concentration of 25 µM of Zol at 72h was therefore selected.
Figure 4.1. Evaluation of MDA-MB-231 cell viability in a 96h exposure of increasing concentrations of Zol in normal media.

MDA-MB-231 breast cancer cells were seeded and left to adhere for 24h. 5 different concentrations of Zol or PBS (Control) (n=3 per treatment) were added to the media followed by cell counting at 24h, 48h, 72h and 96h (A). The treatments were recorded as Control (B), 15µM (C), 17.5µM (D), 20µM (E), 22.5µM (F) and 25µM of Zol (G). Respective IC50 were then produced at 72h and 96h after exposure of Zol. Data from groups is presented as Mean ± SD. A 2-way ANOVA analysis was carried to compare all treatments and time of exposure. Statistical significances are represented as * = P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001 and no significance: ns.
To determine the effects of decreasing concentrations of oestradiol, as observed in the menopausal transition, on the ability of Zol to reduce cell viability, MDA-MB-231 cells were cultured in oestradiol-free media supplemented with 300 pmol/L, 84 pmol/L and 0 pmol/L of oestradiol, representing pre-, peri-, and post-menopausal concentrations of this hormone. 24 hours after cellular seeding and adhesion, cells were administered with 25μM of Zol or PBS (n=3 per treatment). Cell number was determined every 24h until 96h. Oestradiol alone had no effect on viability of MDA-MB-231 cells irrespective of the concentration administered (2-way ANOVA, p=0.612). Whereas addition of Zol caused a significant decrease in the number of viable cells after 24 hours (2-way ANOVA, p=0.030) in all three oestradiol concentrations when compared with PBS groups (figure 4.2). This difference was increased after 72 hours (2-way ANOVA, p<0.001) and reaching almost total cell ablation at 96 hours (2-way ANOVA, p<0.001). Oestradiol had no effect on the ability of Zol to reduce viability of MDA-MB-231 cells (2-way ANOVA, p=0.536), indicating that the direct anti-tumour effect of Zol is not altered by the variations in oestradiol levels in the cellular environment. Therefore, future experiments focussed on how oestrogen and Zol affect the tumour microenvironment are required to determine if changes on the microenvironment are responsible for increased DFS observed in post-menopausal women treated with Zol.
Figure 4. Evaluation of cellular growth of MDA-MB-231 in oestradiol-free medium supplemented by 300 pmol/L, 84 pmol/L and 0 pmol/L of oestradiol in presence of Zol.

MDA-MB-231 breast cancer cells were seeded and left to adhere for 24h in oestradiol-free media supplemented with 3 different concentrations of oestradiol. 25 µM of Zol or PBS (control) were added to the corresponding treatments (n=3 per treatment), followed by cell counting at 24h, 48h, 72h and 96h. Data from treatments is presented as Mean ± SD. A 2-way ANOVA analysis was carried to compare all treatments and time of exposure. Statistical significances are represented as * = P < 0.05, *** = P < 0.001, and no significance: ns.
4.2.2 Effects of pre-, peri- and post-menopausal concentrations +/- Zol on total tumour number and intensity.

To investigate the effect of pre-, peri- and post-menopausal concentrations of oestradiol in combination with Zol-induced effects in breast cancer metastatic progression, an immunocompetent BABL/c \textit{fox/}\- nude mouse model was used. Based on previous studies performed in the research group (previously calculated statistical power analysis), a total number of 60 mice was evaluated, being a total of \( n=10 \) per group (Ottewell et al., 2014) (figure 4.3). 12-week-old female BALB/c nude mice underwent OVX followed by supplementation with different concentrations of oestradiol representative of pre-, peri- and post-menopausal status as observed in humans (12.5 mg/L, 1.38 mg/L and 0.0mg/L of 17-\( \beta \)-oestradiol, respectively) via their drinking water. A daily injection of 40\( \mu \)g/Kg goserelin was administered to prevent OVX-induced increase of FSH. 4-days after OVX, \( 1 \times 10^5 \) MDA-MB-231-GFP-Luc2 cells were delivered via intracardiac injection. 7 days after OVX, mice were randomised for administration of saline or Zol (100mg/Kg). Injection of saline or Zol was repeated weekly until end of the experiment, for a total of 4 doses. Number of tumours and tumour burden were monitored using IVIS-Luc2 system on weekly basis. At day 28, all animals were culled, bones and blood were collected for downstream analysis.
Figure 4.3 Experimental outline for the development of a metastatic model of human breast cancer in BALB/c-nude supplemented with pre-, peri-, and post-menopausal concentrations of oestradiol and +/- Zol.

12-week-old female BALB/c-nude (N=60, n=10 per group) underwent OVX followed by oestradiol replacement via drinking water to achieve pre-, peri-, and post-menopausal concentrations of oestradiol. All mice received a daily dose of 40µg/Kg of goserelin. 4 days after OVX, 1x10^5 MDA-MB-231-GFP-Luc2 cells were injected via intracardiac injection. 7 days after OVX, saline or 100 µg/Kg of Zol was administered with a consecutive dose at days 12, 19 and 26 after OVX. At day 28 after OVX, all animals were culled followed by sample collection for downstream analysis.

4.2.2 Confirmation of relevant oestradiol concentration replacement for the establishment of pre-, peri- and post-menopausal status in BALB/c-nude metastatic model +/- Zol.

To assess the accuracy of the conditions established in previous mouse model (BALB/c immunocompetent) (refer to chapter 3, section 3.2.2), serum from all groups was collected at experiment endpoint (day 28). Analysis of the concentration of oestradiol carried out by ELISA did not yield accurate readings (Data not shown). Therefore, an alternative method for oestradiol replacement was implemented.

Uterine size has been used as direct indicator of the menopausal status and as an indirect predictor of oestrogen levels. This is also applicable to rodents, such as mice (Merz et la, 1995; Orimo et al., 1999). Decreasing concentration of oestrogens (mainly oestradiol) leads to a constant reduction and atrophy of the uterine tissue, reflected in decreased volume and weight. Therefore, confirmation of adequate hormone replacement was performed by analysing uterine volume.

Dissected uterus from all groups were weighed and analysed against the total weight of the mouse before culling (tissue weight/total weight). Variation in the concentration of
oestradiol available through drinking water showed a significant difference between the proposed menopausal status groups (ANOVA, p=0.004) (figure 4.4). Mice receiving 12.5 mg/L of oestradiol by drinking water had 1.11 ± 0.54 % of tissue weight/total weight. The percentage of uterine tissue decreased as the concentration of oestradiol was reduced. In the peri-menopausal status group (receiving 1.38 mg/L of oestradiol), the percentage of uterine tissue weight was reduced to 0.54 ± 0.34 %. A similar reduction was observed in the post-menopausal status group (receiving no oestradiol) resulting in 0.64 ± 0.23 % of tissue weight/total weight.

Similar results were observed in the Zol treated groups. Mice receiving 12.5 mg/L of oestradiol (pre-menopausal status) showed a larger percentage of uterine tissue (1.12 ± 0.22 % of tissue weight/total weight) in comparison to the groups receiving 1.38 mg/L (0.57 ± 0.21 % of tissue weight/total weight) and 0 mg/L of oestradiol (0.63 ± 0.24 % of tissue weight/total weight) (peri- and post-menopausal status, respectively) (ANOVA, p<0.001) (figure 4.4).

The difference in uterine tissue weight from respective menopausal status groups was not altered after continuous dose of Zol (t-Test, p=0.905, p=0.808, p=0.983). These results showed a functional replacement of oestradiol for the proposed menopausal status, particularly in the pre- and post-menopausal settings.

It is important to mention that during the animal study, an intermittent lack of eating and drinking was detected in diverse animals across all groups. Upon discovery of sudden weight lost or lethargic behaviour, mice were supplemented with mashed food supplemented with water containing the same concentration of oestradiol as present in their respective drinking water.
Figure 4. Analysis of uterine tissue weight vs total mouse weight for assessment of oestradiol replacement.

12-week-old female BALB/c-nude (N=60, n=10 per group) underwent OVX followed by oestradiol replacement via drinking water to achieve pre-, peri-, and post-menopausal concentrations of oestradiol. After experimental study (figure 4.3) competition (day 28 after OVX), uterus from all animals were dissected and weighed. Uterine tissue weight was recorded and compared to total weight of the animal at culling. Serum concentration of oestradiol are linked uterine mass and volume. Data from groups is presented as Mean ± SD. 1 way ANOVA was performed for groups under treatment (Zol or saline) (solid comparison line), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group) to compare Zol vs saline. Statistical significance is represented as * = P < 0.05, ** = P < 0.01 and *** = P < 0.001.

4.2.2.2 Scoring and classification of tumour number and affected tissue site in pre-, peri-, and post-menopausal BALB/c-nude models of human metastasis +/- Zol

In order to understand the effect that different concentrations of oestrogen play in the metastatic pattern of MDA-MB-231 cells directly injected in blood stream, the total number tumours per mouse was analysed (N=60, n=10 per group). Tumour was monitored by IVIS-Luc2 imaging system. Mice showing a bioluminescence signal in the chest only were excluded from the analysis due to unsuccessful injection of cells. Similarly, tumours present in the jaw were excluded from the total count of tumours due to the high rate of remodelling of the maxilla and mandible bones in rodents. The physiology of these bones is adapted for rodents to allow continue growth of their teeth as they have an open-rooted dentition. In addition, continues dosing of Zol have been demonstrated to cause osteonecrosis of the jaw in murine models.
(Kikuiri et al., 2010; Park et al., 2015), which interfere with both, tumour growth and maintenance of the animal wellbeing. Number of tumours was counted from bioluminescence analysis using IVIS-Luc2 imaging system in live animals and confirmation with ex-vivo imaging (IVIS-Luc2 system) at the experiment endpoint (Figures 4.5a & b). After confirmation of tumours in the ex-vivo imaging, a percentage of mice with tumour was reported, as well as the tissue where the respective tumours were located (figure 4.6a &b).
Figure 4.5 Representative images from ex-vivo tumour imaging to confirm tumour location from MDA-MB-231-GFP-Luc2 cells injected via cardiac injection on BALB/c-nude mice supplemented with pre-, peri- and post-menopausal concentrations of oestradiol +/- Zol. 12-week-old female BALB/c-nude were established for pre-, peri- and post-menopausal concentration of oestradiol (N=60, n=10 per group), followed by MDA-MB-231-GFP-Luc2 cells inoculation via intracardiac injection and a weekly dose of Zol or saline, as described in figure 4.3. Tumours were monitored on a weekly basis using IVIS-Luc2 imaging system. A representative image of final IVIS imaging is presented (A).
Figure 4.5. Representative images from ex-vivo tumour imaging to confirm tumour location from MDA-MB-231-GFP-Luc2 cells injected via cardiac injection on BALB/c-nude mice supplemented with pre-, peri- and post-menopausal concentrations of oestradiol +/- Zol. (CONTINUATION).

(B) Confirmation of tracked tumours occurred in an ex-vivo imaging after final dose of luciferin followed by dissection. Hind limbs, soft tissue organs and suspected tumour-bearing bones were placed for imaging. Tumour was counted as positive when confirmed with ex-vivo imaging.
The percentage of mice with developed metastasis was between 70% for the pre-menopausal with and without Zol groups and the peri-menopausal group to 80% in the peri-menopausal with Zol, and post-menopausal with and without Zol groups (figure 4.6a). The remaining percent of mice were excluded for either developing tumour in the chest cavity only (unsuccessful delivery of tumour cells by intracardiac injection) or not developing metastatic tumours (possible clearance of tumour cells or reduced viability of tumour initiating cells). Initial signs of tumour development by tumour bioluminescence were observed at day 20 after OVX.

From the mice with positive metastatic tumours, a further classification was performed. Tumours were allocated depending of their affecting site, primarily hind limbs and skull. Other bone affected sites were pooled together (spine, ribs, axial bones) as well as soft tissue organs.

A reduced number of tumours affecting the hind limbs was noted. In most groups, mice with tumours in the hind limbs did not overpass the 50%. Only in the pre-menopausal concentration of oestradiol with and without Zol the percentage of mice with tumours in the hind limb were 57.1% and 85.7%, respectively (figure 4.6b). This observation had an important impact in the analysis of bone microstructure (µCT analysis), as it is performed on tibiae.
As previously described in figure 4.3, 12-week-old BALB/c-nude mice (N=60, n=10 per group) underwent OVX with immediate oestradiol replacement for pre-, peri-, and post-menopausal concentrations of the hormone. MDA-MB-231-GFP-Luc2 cells were inoculated followed by weekly dose of Zol or saline. At day 28, metastatic tumours were recorded by bioluminescence in-vivo (IVIS-Luc2 imaging system) and confirmed by ex-vivo imaging after dissection. Mice with tumours were registered, excluding mice with chest-only bioluminescence signal (A). Then, further classification was performed dividing tumours by their location in the skeleton (hind limbs, skull, or other bone tissue) or in soft tissue. Tumours in the jaw were excluded due to the physiology of this specific bone in rodents (B).
4.2.2.3 Analysis of total tumour number and tumour burden in a pre-, peri- and post-menopausal concentrations of oestradiol +/- Zol in a BALB/c-nude model of human breast cancer metastasis.

Variation in the concentration of oestradiol, from high (pre-menopausal) to low (peri-menopausal) and limited (post-menopausal) had no impact on the total number of developed tumours from MDA-MB-231 cell line (ANOVA, p=0.595) (figure 4.7a). Administration of Zol to the respective groups had no effect on the number of tumours between groups neither (ANOVA, p=0.204). However, indication of a tendency towards a reduction was observed in the mouse model of post-menopausal concentrations of oestradiol. The number of tumours in the control group with limited concentrations of oestradiol was 2.88 ± 2.10 tumours/mouse, which was reduced to 1.33 ± 0.707 tumours/mouse after a continuous administration of Zol. Due to the number of mice expressing tumours, this reduction was not statistically significant (t-Test, p=0.055); however, it gives a strong correlation with the data observed in the clinics. In the clinical trials AZURE and ABCSG-12, the correlation to an increase DFS was observed in patients with an established post-menopausal status (Coleman et al., 2018; Gnant et al., 2011; Holen et al., 2016). Equally important, no benefit in reduced number of tumours was observed in the groups where concentrations of oestradiol were high or low (pre- and peri-menopause; t-Test, p=0.384 and p=0.866, respectively).

Analysis of tumour bioluminescence evidenced a large variation of tumour intensity between single groups, therefore, a test for the exclusion of outliers was performed prior to further analysis (ROUT test, Q=1%).

Variation in the concentration of oestradiol had no statistical effect in the tumour burden of tumours measured as bioluminescence (ANOVA, p=0.901). This was also unaltered by the administration of Zol (ANOVA, p=0.225) (figure 4.7b). Unlike the number of tumours in models with limited concentration of oestradiol (post-menopausal status), tumour burden was not decreased when Zol was administered (t-Test, p=0.267). Interestingly, in conditions of low concentration of oestradiol (peri-menopausal status), the observed tumour burden was partially reduced when Zol was administered; however, no statistical difference was noted despite having similar tumour counts per mouse (t-Test, p=0.210). An opposite observation was
made when Zol was administered in the mouse model with high concentrations of oestradiol (pre-menopausal). At this pre-menopausal status model, tumour burden appeared to increase after a continuous dose of Zol when compared with control tumours, but no statistical significance was reached (t-Test, p=0.927).

These results show indications that the anti-tumour effect of Zol is potentially exerted under limited concentrations of oestradiol only. However, it only affects the number of the sites affected but does not alter tumour burden.
**Figure 4. 7 Analysis of total tumour number and intensity in a BALB/c-nude for human metastasis model expressing peri- and post-menopausal concentrations of oestradiol +/- Zol.**

Immunocompromised BALB/c-nude mice with established menopausal concentration of oestradiol (N=60, n=10 per group) were injected with MDA-MB-231-GFP-Luc2 cells via intracardiac injection, followed by weekly dose of Zol or saline, as described previously (figure 4.3). At experiment endpoint (day 28), final number of tumours was recorded based on bioluminescence emission (IVIS-Luc2 system) and confirmed with ex-vivo imaging (excluding chest-only signal mice, and jaw tumours) (A). Similarly, confirmed tumours and their respective tumour sites were measured individually for total bioluminescence emission (total FLUX; photons/second) with further exclusion of outliers (B). Data from groups is presented as Mean ± SD. 1 way ANOVA was performed for groups under treatment (Zol or saline), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group) to compare Zol vs saline. Statistical significance is represented as: no significance: ns.
4.2.3 Effects of pre-, peri- and post-menopausal concentrations of oestradiol +/- Zol on tumour number and intensity in bone tissue of a BALB/c-nude model of human breast cancer metastasis.

To further understand the effect that Zol might have at the different site of metastasis under different menopausal concentrations of oestradiol, tumours were identified as either ‘bone tissue’ or ‘non-bone/soft tissue’ dependent on the location of their Luc-2 signal via IVIS. Then, for analysis, we only considered the ‘bone tissue’ sub-group as this mouse model was primarily focussed on bone tumour metastasis development (figure 4.8).

Oestradiol did not alter the number of tumours affecting bone tissue (ANOVA, p=0.876). When Zol was added, no alteration in the number of tumours was also noted (ANOVA, p=0.421). This observation was further confirmed when groups representing different menopausal concentrations of oestradiol showed no difference between control and Zol treated groups (t-Test, p=0.356, p=0.723, and p=0.253; for pre-, peri-, and post-menopause concentrations of oestradiol, respectively) (figure 4.8a). Therefore, an analysis of tumour burden was required to determine any possible differences in tumour size between treatments.

Similarly to the analysis of total tumour bioluminescence previously described, a large variation of tumour intensity between single groups was observed, therefore, a test for the exclusion of outliers was performed prior further analysis (ROUT test, Q=1%).

After exclusion of outliers, analysis of tumour bioluminescence evidence that tumour burden tends to be similar in the pre- and peri-menopausal concentration of oestradiol groups, and show a tendency towards a decrease in environments where no oestradiol was present (post-menopausal group); however, no statistical difference was observed when compared these three mouse models (ANOVA, p=0.112). Interestingly, when Zol was administered to the corresponding groups, a mild decrease in the total mean of the tumour burden was observed for the peri-menopausal group, which reached a similarity in tumour burden to the one observed in the post-menopausal group; nonetheless, no statistical significance was observed when comparing the three groups (ANOVA, p=0.194).

It is important to mention that addition of Zol in the pre-menopausal group increased the variation of tumour burden to the one observed in the control group. Both groups remain
without statistical difference (t-Test, p=0.750). In the peri-menopausal concentration of oestradiol setting, Zol did not elicit a statistically significant reduction (t-Test, p=0.131), however, the intensity of Zol treated tumours showed a reducing trend. Interestingly, Zol had no effect on tumour burden in the post-menopausal concentration of oestradiol environment (t-Test, p=0.990), indicating that tumour development remained unaltered (figure 4.8b). Taken together, these results vary from those observed in previous clinical and preclinical studies (Coleman et al., 2014; Ottewell et al., 2014). Therefore, a further understanding of the events happening at the tumour microenvironment in the bone was required to propose a possible explanation to the variation of the current results. Thus, investigation of bone microenvironment was carried out in the different murine model of menopause.
Figure 4. Analysis of the effect of pre-, peri- and post-menopausal concentrations of oestradiol on Zol-induced changes in skeletal tissue tumours in a BALB/c-nude mouse model with MDA-MB-231-GFP-Luc2 cells.

12-week-old BALB/c-nude mice with established menopausal concentration of oestradiol (N=60, n=10 per group) were injected with MDA-MB-231-GFP-Luc2 cells via intracardiac injection, followed by weekly dose of Zol or saline, as described previously (figure 4.3). At experimental endpoint (day 28), final number of tumours was recorded based on bioluminescence emission (IVIS-Luc2 system) and confirmed with ex-vivo imaging (excluding chest-only signal mice, and jaw tumours). Confirmed tumour affecting bone tissue were analysed (A). From confirmed tumours affecting bone tissue, individual tumours were measured for total bioluminescence emission (total FLUX; photons/second) with further exclusion of outliers (B). Data from groups is presented as Mean ± SD. 1 way ANOVA was performed for groups under treatment (Zol or saline), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group) to compare Zol vs saline. Statistical significance is represented as: no significance: ns.
4.2.4. Zol induced changes to the bone structure and composition under pre-, peri- and post-menopausal concentrations of oestradiol in immunocompromised mice.

4.2.4.1 Effects of oestradiol on Zol induced changes to bone volume in a BALB/c-nude mouse model of human breast cancer metastasis.

To study the alterations in the bone structure, tibiae were collected and scanned using μCT scanner followed by digital reconstruction (1 tibia per animal). Tumour bearing and non-tumour bearing tibiae were included in the digital reconstruction. Bone volume fraction analysis was performed by measuring the percentage of mineralized bone volume compared with total tissue volume. After 28 days of oestradiol replacement, bone volume was significantly increased in the tibiae of mice supplemented with pre-menopausal concentrations of oestradiol compared with peri-or post-menopausal (ANOVA, p<0.001) but no differences were observed between mice that received peri-menopausal concentrations of oestradiol and post-menopausal concentration of oestradiol group (ANOVA, p=0.571) (Fig 4.9a). Bone volume reached 20.4 ± 3.0 % of BV/TV, following supplementation with pre-menopausal concentrations of oestradiol whereas in the group with post-menopausal concentrations of oestradiol, bone volume fraction was 8.08 ± 1.71 % of BV/TV. Interestingly, supplementation with peri-menopausal concentrations of oestradiol, showed comparable bone volume fraction to those supplemented with post-menopausal concentrations of oestradiol, being of 6.83 ± 1.18 % of BV/TV (ANOVA, p=0.571). Nonetheless, these values were significantly lower from those of the pre-menopausal group (ANOVA, p<0.001), indicating a marked gap between the effects of hormone replacement with high, medium or very low concentrations of oestrogen on trabecular bone volume.

Oestradiol had similar effects on bone volume in Zol treated animals and non-Zol treated animals. Mice administered pre-menopausal concentrations of oestradiol had significantly higher percentage of bone volume (25.6 ± 3.78 % BV/TV) compared with those given premenopausal and the post-menopausal concentrations (8.10 ± 1.37 % BV/TV and 9.96 ± 1.57 % BV/TV respectively; ANOVA, p<0.001). Whereas the comparison between the perimenopausal group and the post-menopausal group was not statistically significant, similarly seen in the control groups (ANOVA, p=0.353).
Importantly, when we looked at the effects of Zol on bone volume fraction in the mice representing pre-, peri- and post-menopausal oestradiol environment, Zol was able to significantly increase bone volume fraction under pre-menopausal concentrations of oestradiol only, but not under peri- or post-menopausal concentrations of this hormone (figure 4.9a). Zol treated animals had an increase in total bone volume of 5.21 (± 3.78) % BV/TV on average in comparison to the control groups (t-test, p=0.009). These changes in bone volume suggest that despite being a potent anti-resorptive agent, Zol is not able to rescue bone degradation in conditions where limited concentrations of oestradiol are available in the immunocompromise bone environment.

Due to the reduced number of tumours found in tibiae bones, not enough data was collected to present an analysis of bone volume fraction with tumour-bearing tibiae only. Therefore, a comparative analysis was performed between data from all tibiae per group and data were tumour-bearing tibiae were excluded. The analysis revealed that removal of tumour-bearing tibiae from the groups did not affect significantly the mean from each group, however, an increase in the SD was observed in the non-tumour-bearing tibiae as a result of a decrease in total number of data (t-test: p=0.661, p=0.693, p=0.975, p>0.999, p=0.798, and p<0.999; for pre-, peri-, post-, pre- + Zol, peri- + Zol, and post- + Zol, respectively). Therefore, subsequent analyses were performed in using both, tumour-bearing and non-tumour-bearing tibiae (figure 4.9b).
Figure 4.9 Effect of Zol and oestrogen replacement on bone volume from BALB/c-nude mice injected with MDA-MB-231 cells.

BALB/c-nude mice with established menopausal concentration of oestradiol (N=60, n=10 per group) were injected with MDA-MB-231-GFP-Luc2 cells via intracardiac injection, followed by weekly dose of Zol or saline, as described previously (figure 4.3). Within 72 h after experimental endpoint, tibiae were μCT scanned and reconstructed. Analysis of Bone volume was performed 0.2mm above the growth plate from the proximal head of the tibia and a region of interest was then drawn covering 0.5mm of bone tissue. All tibiae were analysed (tumour and non-tumour-bearing) (A). Due to a low number of tumour-bearing tibiae across all groups, a comparison between groups presenting all data from non-tumour-bearing and mixed tibiae was produced to evidence any possible difference. Data from groups is presented as Mean ± SD. 1-way ANOVA was performed for groups under treatment (Zol or saline) (solid and dotted comparison lines), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group) to compare Zol vs saline and w/o tumour-bearing vs tumour-bearing data. Statistical significances are represented as * = P < 0.05, ** = P < 0.01, *** = P < 0.001, and no significance: ns.
4.2.4.2 Analysis of bone cellular composition of osteoblasts and osteoclasts by histomorphometry

To further correlate bone remodelling with oestradiol concentrations and Zoledronic acid, bone histomorphometry was carried out in the trabecular and high trabecular region of the tibiae to investigate the number of osteoblasts as well as osteoclasts.

The analysis of osteoblast number in the trabecular area of the control group evidenced that the cell numbers were similar throughout the three menopausal groups (ANOVA, p=0.244). Cell numbers did not show any particular trend or inclinations based on the hormone concentration status as observed in the mean of number of osteoblasts per millimetre (pre-menopausal status, 8.87 ± 3.96 cells/mm; peri-menopausal status, 10.0 ± 3.30 cells/mm; and post-menopausal status 11.50 ± 6.15 cells/mm) (Figure 4.10).

Similarly to the control groups, Zol-treated groups showed that the number of osteoblasts were completely invariable independently of the hormonal status (ANOVA, p=0.762).

When compared with the number of osteoblasts of the control group against the Zol treated group, data show that Zol significantly affects the number of osteoblasts. In condition of high concentration of oestradiol (pre-menopausal), the number of osteoblasts was reduced from 8.87 ± 3.96 cells/mm to 5.74 ± 2.47 cells/mm after administration of Zol, however, this reduction did not reach statistical significance (t-Test, p=0.079). In conditions with low concentration of oestradiol, Zol had a clear reduction in number of osteoblasts, going from 10.0 ± 3.30 cells/mm to 5.30 ± 2.24 cells/mm (t-Test, p=0.005). Similarly, a significant reduction was observed in the post-menopausal concentration of oestradiol group where the number of osteoblasts went from 11.50 ± 6.15 cells/mm to 4.83 ± 2.37 cells/mm (t-Test, p=0.025). This data differs from previous observations in an immunocompetent mouse model (figure 3.7a), where the number of osteoblasts showed a reduction as the circulating concentrations of oestradiol were reduced and Zol did not affect the number of osteoblasts. However, both mouse models are immunologically independent as well as the Zol dose regiment was different.
Figure 4. Effect of oestradiol and Zol in the number of osteoblast cells in the trabecular region of BALB/c-nude tibia.

As previously described (figure 4.3), BALB/c-nude mice with established menopausal concentration of oestradiol (N=60, n=10 per group) were injected with MDA-MB-231-GFP-Luc2 cells via intracardiac injection, followed by weekly dose of Zol or saline. After fixation, μCT scanning and decalcification, tibiae were processed and sectioned for histomorphometry analysis using TRAP staining. Osteoblast cells were scored in trabecular regions of tibiae. Data from groups is presented as Mean ± SD. 1-way ANOVA was performed for groups under treatment (Zol or saline), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group) to compare Zol vs saline (dotted comparison line). Statistical significance is represented as * = P < 0.05, ** = P < 0.01 and ns: no significance.

Osteoclast count of the control groups showed that there was no overall difference between them (ANOVA, p=0.385) (Figure 4.11). In the pre-menopausal environment, the count of osteoclasts was 2.81 ± 0.90 cells/mm being the lowest between the control groups. However, the high concentration of oestrogen did not affect the increase or decrease of this number when compared to the peri-menopausal group (3.56 ± 1.44 cells/mm) or the post-menopausal group (3.04 ± 0.70 cells/mm).

Similarly, the count of osteoclast in the Zol treated group showed no differences overall (ANOVA, p=0.953). With all three groups representing pre- post- and peri-menopausal oestradiol showing similar osteoclast count. The pre-menopausal group had an average of 1.81
± 0.91 cells/mm, peri-menopausal group 1.91 ± 0.95 cells/mm, and post-menopausal 1.78 ± 0.73 cells/mm. These data correlate with those observed in the control groups, indicating that oestradiol has no affected osteoclast number in immunocompromised mice. However, when comparing the control group with Zol treated mice, significant differences were observed in all menopausal groups (t-Test; pre-menopausal group, p=0.038; peri-menopausal group, p=0.017; and post-menopausal group, p=0.009, respectively). These data indicate that oestradiol does not affect the ability of zol to inhibit activity of osteoclasts. Although, differently from the immunocompetent mouse model (figure 3.7b), a major difference is observed in the effect of Zol over osteoclasts. After continuous doses of Zol, the number of osteoclasts in the bone microenvironment decreases in a direct relationship.
Figure 4.11 Effect of oestradiol and Zol in the number of osteoblast cells in the trabecular region of BALB/c-nude tibia.

As previously described (figure 4.3), BALB/c-nude mice with established menopausal concentration of oestradiol (N=60, n=10 per group) were injected with MDA-MB-231-GFP-Luc2 cells via intracardiac injection, followed by weekly dose of Zol or saline. After fixation, µCT scanning and decalcification, tibiae were processed and sectioned for histomorphometry analysis using TRAP staining. Osteoblast cells were scored in trabecular regions of tibiae. Data from groups is presented as Mean ± SD. 1-way ANOVA was performed for groups under treatment (Zol or saline), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group) to compare Zol vs saline (dotted comparison line). Statistical significance is represented as * = P < 0.05 and ** = P < 0.01.
As observed in the bone volume analysis, the replication of the menopausal concentrations of oestradiol in the murine model led to marked structural changes in the bone. Although these changes evidence a directly proportional effect of oestrogen concentration in bone production, it is not possible to measure the rate of bone building or bone resorption activity. In addition, the presence of Zol in the bone microenvironment has previously been shown to primarily affect osteoclast cells, causing a reduction in both number and activity, and, as consequence, decreasing bone resorption process. Moreover, the number and activity of osteoblasts are also affected by Zol, although less severe than osteoclasts. However, repeated dosing with Zol could gradually expand the effect of this bisphosphonate on both bone cells affecting at a larger scale their rate of bone remodelling.

Another factor affecting the bone remodelling rate as well as changes in the bone microenvironment is the presence of bone metastases, therefore, to further investigate the changes in the bone microenvironment caused by oestrogen replacement as well as repeated dosing with Zol in combination with the introduction of metastatic cancer cells, an analysis of bone remodelling biomarkers was carried out. Cellular activity of osteoblast and osteoclast were measured by serum concentration of P1NP and TRAcP 5b biomarkers, respectively.

Concentrations of P1NP showed that after a 28-day oestradiol replacement treatment there was no significant effect on the overall activity of osteoblasts (ANOVA, p=0.224) (figure 4.12a). When the groups were exposed to 4 doses of Zol, no major changes in the concentration of P1NP were observed (ANOVA, p=0.110). This result indicates that variation in concentration of oestradiol did not alter osteoblast activity despite the time of exposure to either very limited concentration of oestradiol or high concentrations of the hormone.

When the comparing the control groups against the zol treated groups under conditions of high and low concentration of oestradiol, a clear reduction on osteoblast activity was noted in both Zol treated groups (t-Test, p=0.001 and p=0.012, respectively). Interestingly, under post-menopausal concentration of oestradiol, the concentration of P1NP between control and Zol treated groups was similar (p=0.457). This observation suggests that in presence of oestradiol,
Zol tends to affect more the activity of osteoblast than when there is limited circulation of oestradiol.

The analysis of osteoclast biomarker TRAcP 5b by ELISA quantification showed that no major changes were seen in the overall osteoclast activity at the different concentrations of oestradiol (ANOVA, p=0.757) (figure 4.12b). This result shows some disparity with previous models as osteoclast activity is increased by the lack of oestradiol, meaning that higher volume in osteoclast activity was expected in the post-menopausal concentration model. Similarly, the administration of Zol had a minimal effect in the concentration of TRAcP 5b at the different concentrations of oestradiol (ANOVA, p=0.757). However, when comparing the control menopausal concentrations of oestradiol against the zol treated group, a clear reduction in osteoclast activity was achieved. In the pre-menopausal status group, the concentration of TRAcP 5b was reduced from 7.06 ± 4.03 U/L to 3.10 ± 1.03 U/L after administration of Zol (t-Test, p=0.017). In the peri-menopausal concentration of oestradiol the concentration of TRAcP 5b went from 6.73 ± 1.09 U/L to 4.51 ± 1.91 U/L after administration of Zol (t-Test, p=0.017). Similarly, a reduction was observed in the post-menopause status going from 7.846 ± 2.27 U/L to 4.43 ± 0.84 U/L after Zol administration. Despite the lack of increment in osteoclast activity as the oestradiol concentrations are decreasing, the presence of Zol agrees with the results seen previously in immunocompetent model of BALB/c mice.
Figure 4.12 Effect of the variation on oestradiol concentration +/- Zol on the activity of osteoblasts and osteoclasts.

As previously described (figure 4.3), BALB/c-nude mice with established menopausal concentration of oestradiol (N=60, n=10 per group) were injected with MDA-MB-231-GFP-Luc2 cells via intracardiac injection, followed by weekly dose of Zol or saline. After study endpoint, serum samples were collected and analysed for bone osteoblastic marker PINP (A) and bone osteoclast activity marker TRAcP 5b (B) by ELISA. Data from groups is presented as Mean ± SD. 1-way ANOVA was performed for groups under treatment (Zol or saline), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group) to compare Zol vs saline (dotted comparison line). Statistical significance is represented as * = P < 0.05 and ** = P < 0.01.
The use of bisphosphonates as an adjuvant therapy for the prevention of breast cancer recurrence have demonstrated to elicit a protective effect on skeletal tissue. Independent clinical trials have shown that use of Zol in combination of standard of care increases DFS in bone, with an expanded benefit in clinically or chemically post-menopausal women (Coleman et al., 2014; Gnant et al., 2011). However, a comprehensive mechanism in how Zol prevents the relapse of bone metastasis in the presence or absence of menopausal hormones is yet to be elucidated.

As observed by Banys et al., (2013) and Rack et al., (2010), administration of Zol decreased the number of DTC in bone marrow aspirated regardless of the menopausal status. This observation suggested that Zol-treated bone became less permissive for DTC to home, therefore a less chance to develop into overt metastasis. Pre-clinical studies also showed a similar tendency (Holen et al., 2016; Ottewell et al., 2008a; Ottewell et al., 2008b; Ottewell et al., 2014) where Zol treated animal models under post-menopausal conditions exerted a reduced bone metastasis when compared with control groups. The hypothesised mechanism by which a DFS in the bone was observed involved the inactivation and apoptosis of osteoclasts, thus, reduced bone resorption. This condition in combination with the limited circulating concentration of oestradiol represented a less attractive environment for DTC forcing them to seek another niche. However, these studies were limited at the analysis of the post-menopausal status only, as no appropriate model of pre-menopause was implemented. Additionally, there was no emphasis on isolation of either oestradiol or FSH, which represents a possible combined effort.

In this study, we have modelled three clinically relevant concentration of oestradiol comparable to those seen in pre-, peri- and post-menopausal women with control over FSH-induced effects on the bone microenvironment. A bone tropic, oestrogen independent, breast cancer cell line was used to model a metastatic process, and a confirmation of the direct effect of Zol on breast cancer cell line in representative menopausal concentrations of oestradiol in vitro.
4.3.1 Analysis of the direct effect of Zol under pre-, peri- and post-menopausal concentrations of oestradiol on tumour cell viability in vitro.

Zol is a third generation, nitrogen-containing bisphosphonate that has being regarded as the most potent bisphosphonate in bone resorption. The mechanism of action by which Zol reduces bone resorption is through the induction of apoptosis in osteoclasts (Hughes et al., 1995, Luckman et al., 2008). Apoptosis is induced by inhibiting enzymes of the mevalonate pathway which prevents the isoprenylation of small GTP-binding proteins such as Ras and Rho (Luckman et al., 2008). Since the mevalonate pathway is a ubiquitous pathway in several types of cells, including tumour cell, Zol has been reported to have a direct effect as anti-tumour drug (Göbel et al., 2016; Jagdev et al., 2001).

In an unaltered in vitro environment using commercial medium, MDA-MB-231 were susceptible to Zol at an IC50 of 28.1 which decreased over time. This result agrees with previous studies on the same cell line where the IC50 showed the same decrement pattern over time (Jagdev et al., 2001). This also correlates with the fact that Zol is not metabolized in the body, therefore, it gets released again after apoptosis (Hughes et al., 1995).

When the in vitro conditions changed to an oestradiol-concentration-controlled environment, MDA-MB-231 showed a decrease in the growth curve, which indicates that the change in medium composition have an effect in the cellular growth. However, this decrease in the growth rate was independent of the concentrations of oestradiol, corroborating that MDA-MB-231 is an ER-independent cell line. When the IC50 concentration of zol was present in this oestradiol-concentration-controlled medium, the MDA-MB-231 showed an increased rate of cell death, which is associated with decreased cellular growth, meaning that the cell line became more susceptible to Zol. Similarly, this increased cell death was not dependant of the Oestradiol.

This data suggests that the anti-tumour effect of Zol is independent of the levels of oestradiol in a ER-independent cell line. However, the effect of Zol depended completely on the concentration at which this drug is administered.
4.3.2 Corelation of uterine tissue with oestradiol hormone replacement for pre-, peri-, and post-menopausal status in BABL/c-nude mice.

Establishment of the three proposed menopausal concentration of oestradiol in a mouse model after OVX has been successfully achieved in previous experiments (chapter 3). Confirmation of oestradiol replacement was obtained by direct quantification of this hormone in serum using commercial ELISA kit. However, in this experiment, inaccurate ELISA values for oestradiol resulted in the restriction to directly confirm hormonal replacement. Therefore, an indirect alternative method to confirm correct oestradiol replacement was implemented.

Oestrogens are a master regulator of multiple functions, mostly of those protective, in different tissues, being the gonads the main target of these hormones (Hewitt et al., 2020; Merz et al., 1995). In murine female gonads, oestrogens help in the development of the luminal epithelium of the endometrium and maintenance of the uterus for oocyte housing during the oestrous cycle (Hewitt et al., 2020). Lack of oestrogen, on the other hand, is associated with uterine tissue atrophy (Orimo et al., 1999). In this study, uterine tissue weight was used as an indirect confirmation of circulating levels of oestradiol. The combination of OVX and post-menopausal concentration of oestradiol showed a negative effect on uterine weight percentage, as this tissue evidenced a clear reduction in mass and volume. The opposite effect was observed in the combination of OVX and supplementation of pre-menopausal concentration of oestradiol, where the uterine tissue retained most of its integrity. These results fully agree with the literature (Hewitt et al., 2020; Orimo et al., 1999) where re-incorporation of oestradiol (externally administered) after OVX, rescued tissue functionality of the uterus, indicating that current methodology of oestradiol delivery in this experiment (through drinking water) not only increased circulating concentration of oestradiol (not confirmed), but physiologically affected target organs.

Despite presenting an indirect confirmation of the oestradiol concentrations in the pre- and post-menopausal concentration of oestradiol, confirmation of peri-menopausal concentration of oestradiol remained inconclusive. Nonetheless, confirmation of this group is difficult to achieve without direct analysis of oestradiol in serum. Similarly, the continuous administration of Zol did not affect uterine tissue weight, which can correlate as a no effect in the final
establishment of the three menopausal statuses; however, this statement can only be confirmed by direct measurement of oestradiol concentration in serum.

In addition, the sporadic periods of dehydration observed in some animals during the length of the study did not have an impact in the establishment of the different menopausal status. This observation can be observed in the consistency of the data acquired during the confirmation of oestradiol replacement.

4.3.3 Effects of pre- post and peri-menopausal concentrations +/- Zol on total tumour number and growth.

The development of a metastatic model of human breast cancer in a BABL/c Fox/- nude immunocompromised mouse model yielded relevant data which can be compared with pre-clinical studies. However, due to the different settings proposed in this experiment, such as establishment of pre-, peri- and post-menopausal concentrations of oestradiol without the influence of FSH, this represented an important variable that has not been addressed fully by similar studies (Holen et al., 2016; Ottewell et al., 2008, Ottewell et al., 2014; Ottewell et al., 2015).

Using a BABL/c Fox/- nude immunocompromised model with a MDA-MB-231 (oestrogen independent) cell line, we identified that different concentrations of oestradiol had a minimal impact in the total number of tumours. Similarly, the presence of Zol had no effect in the total number of tumours across the different concentration of oestradiol. The identification of tumours was carried out meticulously. Comparison of in-vivo and ex-vivo tumour imaging data allowed a clear discrimination between tumour location and tumour burden. Although tumours the majority of animals developed relevant tumours, not all of these were consistent across all the groups, meaning that tumour comparison could be affected. Previous studies have shown that location of metastatic tumours cells is crucial for their development onto overt metastasis (Brown et al., 2012; Allocca et al., 2019). One of the places where a major development of tumour cells is observed is in the long bones from the hind limbs. Unfortunately, in the current BALB/c-nude with human breast cancer cells MDA-MB-231, the number of tumours affecting these sites was reduced. In addition, bones are a direct target of oestradiol concentration in both humans and mouse models (Holen et al., 2016; Manolagas et al., 2013; Ohlsson et al., 2020; Ottewell et al., 2014), especially long bones, as those in the hind
limbs; therefore, modification of these sites can have alterations to the microenvironment beyond those analysed in this study.

These results evidence that oestradiol alone has a more complex role in the development of bone metastasis. A similar study carried out by Ottewell and collaborators (2014), demonstrated that OVX mice (representing a post-menopausal status) had an increase in tumour number as the concentration of oestradiol was limited. In addition, the presence of Zol altered this phenomenon. However, this animal model did not have control over the levels of FSH as no goserelin administration was provided. FSH has an important role in the bone microenvironment and has been pointed out as another possible factor for the protective effect of Zol when used as adjuvant (Wilson et al., 2017). Therefore, a lack of response in the number of tumours seen in this experiment could be attributed at oestradiol being a lesser factor for the overall tumour formation.

Interestingly, in post-menopausal concentration of oestradiol, the addition of Zol had a tendency towards a reduction in the total number of tumours. This result agrees to certain extents with the study abovementioned (Ottewell, et al., 2014). This tendency also agrees that an extended benefit in DFS can be observed when Zol is added in the post-menopausal population only. Nevertheless, tumour intensity showed that no benefit was gain throughout the different menopausal concentration of oestradiol, with and without Zol. This observation reiterates that in this specific model, the role of oestradiol could not be strong enough to elicit a more obvious effect of Zol once this drug is applied.

When the analysis of tumours affecting the skeletal tissue only was performed, no statistical significance was observed. However, due to the relative lower amount of this tumours as well as the increase variation between the number of these inside of the different menopausal groups, reduced the statistical power to observe possible changes. As a result, both tumour number and tumour burden were comparable across all three proposed menopausal status with no observable effect after continuous dose of Zol. These results differ from studies performed with similar settings (Holen et al., 2016; Ottewell et al., 2014).

Additionally, relevant studies analysing the effect of Zol on tumour metastasis in bone showed tumour reduction and prevention in the bone after Zol treatment (Hiraga et al., 2004; Yoneda et al., 2000). The proven mechanism of action is through direct apoptosis of osteoclast in the
tumour microenvironment. Also, previous administration of Zol to the bone microenvironment prevents further colonization of tumour cells but increases the possibility of visceral metastasis.

4.3.4 Effect of oestradiol with and without Zol in the bone microenvironment of tumour bearing bones.

Oestradiol is one of the major anabolic hormones in the bone structure and microenvironment (Bradford et al., 2010; Hughes et al., 1996; Manolagas et al., 2013; Martin-Millan et al., 2010; Ohlsson et al., 2020). In this experiment, hormonal replacement was established after OVX with pre-, peri- and post-menopausal concentration of oestradiol. Although direct concentrations of oestradiol were not confirmed after experimental endpoint, an indirect confirmation of the pre- and post-menopausal concentration of oestradiol was carried out. Similarly, skeletal tissue is a direct target organ for circulating oestradiol, meaning that a direct correlation with the concentrations of oestradiol can be assessed.

The hormone replacement process evidenced that high concentrations of oestradiol were correlated with an increase in bone volume and an expected decrease of the same parameter as the concentrations of oestradiol were lower and limited. Although the oestradiol replacement showed in the pre-menopausal a potent anabolic effect which was further increased by the addition of Zol, the same effect was not seen in the peri- and post-menopausal group. This observation differs from previous model establishment (see chapter 3) where a significant decrease in bone volume fraction was observed from the per-menopausal model to the post-menopausal mouse model. A similar observation was noted in the examination of the percentage of uterine tissue, where no difference was found between peri- and post-menopausal concentration of oestradiol. These results suggest that, perhaps, a proper representation of the peri-menopausal group was not achieved as seen in previous mouse-model (chapter 3). However, it is important to point out that despite oestradiol having an important role in bone development across different systems (Manolagas, 2000), changes in the mouse strain, immunological component of the environment and presence of foreign cells, such as tumour cells, also play an important role in the results obtained.

Due to the relative lower number of tumours affecting the hind limbs, as previously discussed, it was difficult to establish a comparison of tumour-bearing vs non-tumour-bearing tibiae.
Tumour-bearing bones in humans and animal models tend to show a strong decrease in bone tissue due to an increase in bone resorption (Lu et al., 2007; Tulotta et al., 2019); however, factors such as metastatic tumour location as well as time of tumour development were limiting factors, among others, in this BALB/c-nude metastatic human breast cancer model. As previously described, initial signs of tumour development were observed at day 20 after OVX. This tumour incubation period was comparable to previous studies using similar cell line (Ottewell et al., 2008; Ottewell et al., 2014; Ottewell et al., 2015); however, the time for tumour development on the cited studies had a larger window in comparison to this study, where experimental endpoint occurred at day 28, only 8 days after first insights of tumour development. Therefore, due to the reduced period of time allocated to the tumour development, most tumour did not reach an important bone lesion in the respective µCT scans as observed previously. Furthermore, evidence of tumour size at histological level explored in previous studies (Yoneda et al., 2000) suggests that despite MDA-MB-231 cells being relatively larger in comparison to syngeneic cell in the same environment, the tumours from the human cell line tend to be smaller within the same window of time, accrediting then, a minor bone lesion.

4.3.5 Effect of oestradiol with and without Zol in the bone cellular component

The menopausal transition has an important impact in the cellular component of the bone, being oestrogen one of the most representative hormones in the regulation and maintenance of different niches (Bradford et al., 2010; Hughes et al., 1996; Manolagas et al., 2013; Martin-Millan et al., 2010). A decrease concentration of oestradiol causes a deterioration and imbalance of bone building cells, osteoblasts, and bone resorbing cells, osteoclast. In this immunocompromised model, the number of osteoblasts showed an unexpected increased number of cells as the concentration of oestradiol were reduced. This result was partially corroborated by the osteoblast activity measured as concentrations of P1NP. A possible explanation to this effect could potentially be a compensation mechanism caused by an acute increase in bone resorption. However, previous investigations have pointed out that presence of tumour cells in bone are associated with a decrease in osteoblasts (Haider et al., 2014; Hughes et al., 2019; Ottewell et al., 2008). Moreover, in previous analysis (see chapter 3), number of osteoblasts tended to decrease as the concentrations of oestradiol decreased. Interestingly, osteoblast activity on this previous analysis also showed an increment from high
concentrations of oestradiol to limited concentration of oestradiol. However, the presence of Zol decreased the number of osteoblasts, especially in low and limited concentrations of oestradiol. As previously reported (Brown et al., 2012), a continuous administration of Zol causes osteoblast reduction which is maintained even after a complete stop of the drug. Therefore, these results agree with the expected activity of Zol.

The number and activity of osteoclasts were not altered by the lack of oestradiol, as it was expected. A decrease in the concentration of oestradiol is associated with an increase in bone resorption, as previously discussed (chapter 3). However, presence of tumours, even at distant sites, has been widely reported to influence bone resorption, especially MDA-MB-231 cell lines (Guo et al., 2019, Yao et al., 2022). The administration of Zol reduced both number and activity of osteoclasts regardless the concentration of oestradiol. The reduction was even throughout the groups meaning that a basal number of osteoclasts was still maintained. The activity of Zol on osteoclast activity has been widely reported, therefore, cellular reduction evidenced a positive effect in the bone microenvironment.

Taken all this information together suggests that the effect of oestradiol on the bone microenvironment of an immunocompromised mouse might not be enough trigger to cause a difference in the metastatic pattern of an ER-ve breast cancer cell line. When compare these results with those of similar premise (Ottewell et al., 2014), it suggests that a possible interaction of oestradiol and FSH could play an important role in allowing DTC to prefer bone tissue in limited concentrations of oestradiol. By allowing an established homing of DTC into bone, the effect of Zol on this environment can therefore be evaluated. Nevertheless, this evaluation cannot be fully tested when the bone microenvironment is not as attractive to DTC.
CHAPTER 5: Evaluation of an immunocompetent BALB/c syngeneic model using 4T1-Luc2 cell line for the development of spontaneous metastasis in pre-, peri- and post-menopausal concentrations of oestradiol +/- Zol.
5.1. Introduction

Development of breast cancer is a complex multistage pathological process. Most of the stages observed in the primary tumour environment, such as evasion of immune surveillance, alteration of signalling pathways, angiogenesis, development of the tumour microenvironment, and the carcinogenic characteristics of these cells, among others, are processes that can only be assessed in relevant *in vivo* models (Ottewell *et al.*, 2006; Tulotta *et al.*, 2019). Currently, there are a number of animal models capable of rendering most of the complexity of the tumour development; however, syngeneic murine models of breast cancer allow a greater similarity to the process observed in late stages of primary breast cancer (stage IV) towards a spontaneous metastasis, such as the case of the 4T1 murine breast cancer model (Lelekakis *et al.*, 1999; Yoneda *et al.*, 2000). The use of this murine syngeneic model as a preclinical tool for spontaneous metastasis has shown that alterations in the target metastatic organs by Zol interfere with the metastatic pattern of this model (Baklaushev *et al.*, 2016; Hiraga *et al.*, 2003; Yoneda *et al.*, 2010). This data has closely represented the results observed in different clinical trials where treatment of primary tumours with standard of care therapy in combination with adjuvant Zol resulted in observations of soft tissue metastatic events after treatment (Coleman *et al.*, 2014; Coleman *et al.*, 2018; Gnant *et al.*, 2011). Although this syngeneic model has provided first evidence in the mechanism of actions of Zol on the target metastasis microenvironment (primarily bone microenvironment) and how alters its metastatic pattern, more data from interactions in the target metastatic microenvironment is required to propose a robust mechanism.

In the AZURE clinical trial (Coleman *et al.*, 2018), a positive reduction of bone metastatic events after treatment with standard of care coupled and adjuvant Zol was observed across all patients, while an increase in DFS was only observed in the post-menopausal patient cohort. Similar clinical trials assessing the effect of Zol in post-menopausal patients, naturally of chemically induced (Bundred *et al.*, 2008; Gnant *et al.*, 2015), also reported a decrease in the number of metastatic events in bone after therapy. However, patients deemed as pre-menopausal at the time of Zol adjuvant treatment experienced a significant reduction in DFS in comparison to the post-menopausal cohort. The suggested underlying cause for these split results between cohorts was the relationship with hormonal levels and the current
physiological condition of relevant tissues at the moment of the treatment. To assess whether a particular hormone from the menopausal transition could affect the anti-tumour effect of Zol outside the bone microenvironment, an analysis of serum samples from AZURE patients was carried out (Wilson et al., 2017). Subsequent results pointed out that either FSH or oestradiol were the hormones with a major effect on the anti-tumour effect of Zol, as inhibin A (hormone involved in the menopausal transition) did not affect the metastatic pattern of breast cancer tumour cells in bone and outside bone in an in vivo model (Wilson et al., 2016).

Based in the observation from pre-clinical data, deprivation of oestradiol in a mouse model of human breast cancer metastasis (Ottewell, et al., 2014; Ottewell, et al., 2015) resulted in an increase of development of bone metastasis, whereas supplementation of oestradiol resulted in spontaneous metastasis to bone in an ER +ve cell line (Holen et al., 2016). In both studies, Zol inhibited the development of tumours in the bone tissue in the absence of chemotherapy. Taking all the information together, data indicates that pre-menopausal concentrations of oestradiol favours bone metastasis and possible dormancy of tumour cells, as well as allowing their spread to soft tissue organs after Zol administrations (observed in Yoneda et al., 2000).

Therefore, to further understand the mechanism by which oestradiol conditions the bone microenvironment in combination with Zol before the metastatic process occurs, we proposed a syngeneic breast cancer model of spontaneous metastasis. Using a BALB/c model with syngeneic primary tumour of 4T1 breast cancer cells, we established a physiologically relevant pre-, peri- and post-menopausal concentrations of oestradiol with a controlled inhibition of FSH to prevent the interference of this hormone over possible oestradiol-exclusive changes.
5.2 Results

5.2.1 Analysis of the direct effect of pre-, peri- and post-menopausal concentrations of oestradiol +/- Zol on primary tumour development in a BALB/c syngeneic model.

To study the effect of Zol on primary tumour development under pre-, peri- and post-menopausal status and the presence of tumours in a fully immunocompetent model, a syngeneic mouse model using BALB/c mice was established.

Based on previous in vivo experiments performed in the research group, in order to observe statistical significance effects in bone microenvironment as well as the assessment of bone biomarkers, 10 animals per group was used (Ottewell et al., 2014). 12-week-old female mice (N=60) underwent OVX followed by immediate oestradiol replacement via drinking water to achieve pre-, peri- and post-menopausal concentrations of oestradiol (12.5mg/L, 1.375mg/L and 0mg/L of oestradiol accordingly) (n=10 per group). Daily dose of goserelin (40 µg/Kg) was administered to all animals to prevent increased serum levels of FSH. 7 days after menopausal status establishment, 5x10⁴ 4T1-Luc2-bone-retreived cells were inoculated into the mammary gland through the 4th nipples, in order to establish a primary tumour. 5 days after cell injection, a dose of Zol (100µg/Kg) or saline was injected, followed by a continuous dose on a weekly basis until the end of the experiment. Tumour burden was monitored using IVIS-Luc2 system twice a week until the end of the experiment. Tumour size (volume) was monitored using calipers. At day 36 after menopause establishment (OVX) all mice were culled. Primary tumours were dissected, and blood was collected for downstream analysis (figure 5.1).
12-week-old female BALB/c mice (N=60; n=10 per group) were OVX followed by oestradiol replacement using 12.5 mg/L, 1.38 mg/L or 0 mg/L of oestradiol added to drinking water. Daily administration of goserelin (40µg/Kg) was given. 7 days after OVX, 5x10⁴ 4T1-Luc2 bone retrieved cells were injected via intra-nipple injection. 12, 19, 26, and 33 days after OVX, saline or 100 µg/Kg of Zol was administered and IVIS-imaging was performed. At day 36 after OVX, all animals were culled followed by sample collection for downstream analysis.

5.2.2 Confirmation of the establishment of pre-, peri- and post-menopausal status +/- Zol on primary tumour development in a BALB/c syngeneic model.

Delivery of oestradiol to the animals through drinking water has been successfully established in previous experiments (chapters 3 and 4), however, during the development of this experiment, an infectious outbreak occurred in the animal facility preventing a normal continuation of study protocol.

36 days after menopausal establishment the concentration of oestradiol in serum were analysed using ELISA. For the pre-menopausal concentration of oestradiol group, the replacement of oestradiol via drinking water (12.5mg/L) achieved levels of 44 pg/mL ± 33.6 pg/mL of oestradiol in serum. For the group representative of peri-menopausal concentration of oestradiol, the serum levels of oestradiol decreased to 20.4 pg/mL ± 12.8 pg/mL, however, was not significantly different from the pre-menopausal group (ANOVA, p=0.168). The concentration of oestradiol showed a significant decline in the post-menopausal group, where no supplementation of oestradiol was given (ANOVA, p=0.039). In this group, the serum
concentration of oestradiol was 10.7 pg/mL ± 6.66 pg/mL, which is still representative of the post-menopausal status (figure 5.2).

A similar trend was observed when Zol was administered to the three menopausal groups (pre-, peri-, and post-menopause). For the pre-menopausal concentration of oestradiol group, the concentration of oestradiol in serum was 37.7 ± 40.3 pg/mL. In the peri-menopausal concentrations of oestradiol the observed values in serum were 21.7 pg/mL ± 14.6 pg/mL, which were not statistically significant from the previous group (ANOVA, p=0.505). In the post-menopausal group, where no oestradiol was replaced via drinking water, the concentration of oestradiol on serum showed a trend towards a reduction with levels of 2.33 pg/mL ± 3.34 pg/mL (ANOVA, p=0.073) (figure 5.2).

The addition of Zol did not alter the serum concentration of oestradiol observed in the pre-menopausal or the peri-menopausal groups (t-test: p=0.741 and p=0.873 respectively). However, a reduction in oestradiol was observed when mice were administered the post-menopausal concentration of oestradiol group when Zol was added (t-test: p=0.021).

These results showed a similar correlation with previous establishment of menopause transition in mouse models, however, distressing events such as underlying infections or borderline limits of tumour volume prevent animals to achieve a correct maintenance of their wellbeing.
12-week-old female BALB/c (N=60, n=10 per group) underwent OVX followed by oestradiol replacement via drinking water to achieve pre-, peri-, and post-menopausal concentrations of oestradiol, as described (figure 5.1). After 4T1-Luc2 bone retrieved tumour implantation and continuous dose of Zol, mice were culled (day 36). Serum samples were collected and analysed for oestradiol by ELISA. Data from groups is presented as Mean ± SD. 1 way ANOVA was performed for groups under treatment (Zol or saline) (solid comparison line), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group) to compare Zol vs saline (dotted comparison line). Statistical significance is represented as, * = P < 0.05.
5.2.3 Analysis of primary tumour growth of pre-, peri- and post-menopausal status +/- Zol in a BALB/c syngeneic model of 4T1-Luc2 murine breast cancer.

5.2.3.1 Evaluation of tumour growth by calliper from the menopausal transition.

In this experiment, 7 days after menopause establishment, 4T1-Luc2 bone retrieved cells were injected into the mammary gland. Tumour growth was monitored by calliper measurements as well as in vivo imaging of tumour burden via IVIS-Luc2 imaging system. 5 days after cellular injection, a tumour signal was recorded using IVIS-Luc2 image system; however, no physical sign of the tumour was recorded with the calliper.

Measurement of tumour volume by the use of callipers is still regarded as robust tool during the analysis of tumour progression (Faustino-Rocha et al., 2013). However, obvious limitations occur when tumours are not palpable. Tumour volume was calculated by the following formula: ‘Volume = (W^2 x L)/2’; where W is tumour width and L is tumour length (mm^3).

Tumour volume showed a continuous tumour growth independently of the concentration of oestradiol across the three menopausal status (2-way ANOVA, p<0.0001) (figure 5.3a). In all three menopausal status models, a significant increase in tumour volume was observed at day 28 after OVX when compared with the first tumour volume read (day 15 after OVX). At this day, most tumours were averaging 500 mm^3. After 34 days post OVX, the tumour volume had increase considerably in comparison to day 28 (2-way ANOVA, p<0.001) reaching the maximum tumour size proposed for the study (≤1000mm^3).

An identical trend was observed in the tumour volume of the pre-, peri- and post-menopausal groups treated with Zol. Tumour volume increased between the three groups a similar manner, independently the concentration of oestradiol (2-way ANOVA, p<0.0001) (figure 5.3b). Similarly, tumour volume showed a significant increase at day 28 when compared with the first read at day 15 after OVX (2-way ANOVA, p<0.0001). Tumour volume reached the maximum allowed volume at day 34 after OVX.

Tumour volume was not affected by the addition of Zol in any of the menopausal concentration of oestradiol group when compared with their respective control (2-way ANOVA, p=0.757).
It is important to mention that at day 34 after OVX, some tumour started to develop signs of internal necrosis, which developed ulcers by the time of culling. Also, due to the maximum volume reach by most the tumour, most mice started to show discomfort by the time of culling.

A) 

![Graph A](image1)

B) 

![Graph B](image2)

**Figure 5. 3. Analysis of primary tumour volume of a BALB/c syngeneic model with 4T1-Luc2 murine breast cancer cells.**

12-week-old female BALB/c (N=60, n=10 per group) underwent OVX followed by pre-, peri-, and post-menopausal status establishment, as previously described (figure 5.1). Tumour volume was measured upon palpability (day 15) using callipers. Continuous measurements were taken at days 23, 28 and 34 after OVX. Data from groups is presented as Mean ± SD. 2-way ANOVA was performed for all groups. Statistical significance is represented as, **** = P < 0.0001.
5.2.3.2 Evaluation of tumour burden by IVIS imaging from the representative menopausal status BALB/c syngeneic models +/- Zol.

Analysis of tumour growth using IVIS-Luc2 imaging system provided a more accurate and representative evolution of the tumour development in comparison to the measurement of tumour volume by calliper (figure 5.4). In addition, examination of tumour bioluminescence allows the tracking of instant metastasis as was expected from this 4T1-Luc2 bone retrieved model. However, data from tumour burden showed a considerable variation in tumour bioluminescence between mice of a menopausal status group (figure 5.5a & b). Due to this variation, tumour bioluminescence was normalized against the first reading of the tumour. First tumour read also coincided with the first dose of Zol (Zol injection was given after IVIS imaging), meaning that the tumour burden obtained was still naïve to Zol treatment.

Normalized data from tumour burden showed no significant increment in tumour growth from all menopausal groups throughout the experiment (2-way ANOVA; p=0.805, p=0.577 and p=0.0912, for pre-, peri- and post-menopausal groups respectively). Comparison in the growth of tumour burden between the three menopausal groups also showed no statistical difference (2-way ANOVA, p=0.117) (figure 5.5c).

Administration of Zol showed similar tendency in tumour growth as observed in the control group. Tumour burden showed a plateau upon detection (day 12) until day 26 after menopausal establishment. During this period, no significant difference was observed in growth neither between treatments. After day 26, tumour burden showed an exponential increase in all groups, but being only statistically significant in the pre- and peri-menopausal concentration of oestradiol groups (2-way ANOVA, p<0.0001). By day 35 all three menopausal concentrations of oestradiol showed a significant difference in tumour burden when compared to previous days (2-way ANOVA, p<0.0001). Nonetheless, no statistical significance was observed when comparing the tumour growth between the three concentrations of oestradiol at any time point (1-way ANOVA, p=0.073) (figure 5.5d).

Although the addition of Zol was observed with a tendency towards a larger tumour burden in the pre-menopausal concentration of oestradiol group after day 31, this difference did not reach statistical significance (1-way ANOVA, p=0.143 and p=0.440, for days 31 and 35, respectively) (figure 5.5e). Similarly, the addition of Zol in the peri- and post-menopausal
groups did not affect tumour burden when compared to the respective control group (1-way ANOVA, \( p > 0.999 \) and \( p > 0.999 \) for peri- and post-menopausal group, respectively) (figures 5.5f & g).

Taken together, this evidence suggest that Zol might affect primary tumour site by increasing tumour burden in condition of high concentration of oestriadiol, however, a longer study and/or an increase in the number of animals per group is required to validate these findings.

Unfortunately, due to diverse circumstances such as infectious outbreak, compromised wellbeing of the animals, maximum allowed tumour sizes, and restriction in the duration of daily injections (Project Licence Restriction), the BALB/c syngeneic model reached experimental endpoint without the presence of spontaneous metastasis in any of the menopausal concentrations of oestradiol models with or without Zol.
Figure 5.4. Primary tumour progression recorded by bioluminescence (IVIS-Luc2 system) of a BALB/c syngeneic model with 4T1-Luc2 murine breast cancer cells.

12-week-old female BALB/c (N=60, n=10 per group) underwent OVX followed by pre-, peri-, and post-menopausal status establishment, as previously described (figure 5.1). Tumour burden was recorded using IVIS-Luc2 imaging system twice per week. Representative images of tumour progression in animals are presented. Bioluminescent images were taken at day 12, 14, 19, 21, 26, 31 and 34 after menopausal establishment (OVX).
Figure 5. Analysis of primary tumour burden by bioluminescence of a BALB/c syngeneic model with 4T1-Luc2 murine breast cancer cells.

12-week-old female BALB/c (N=60, n=10 per group) underwent OVX followed by pre-, peri-, and post-menopausal status establishment, as previously described (figure 5.1). Tumour burden was measure using IVIS-Luc2 imaging system after day 12 post-OVX. In vivo imaging was performed twice a week. Total tumour burden as bioluminescence is showed for Control groups (A) & Zol treated groups (B). All data analysis was carried out in normalized data against the first tumour reading (C) & (D). Data from groups is presented as Mean ± SD. A 2-way ANOVA analysis was performed followed by Tukey’s post hoc test for comparison of tumour growth in both Total Flux and normalized tumour burden. Statistical significance is represented as, ns: no significant.
Figure 5.5. Analysis of primary tumour burden by bioluminescence of a BALB/c syngeneic model with 4T1-Luc2 murine breast cancer cells (Continuation).

Individual comparison between control and Zol treated groups of pre-menopause E), peri-menopause F) and post-menopause G) are presented. Data from groups is presented as Mean ± SD. 1-way ANOVA was performed followed by Tukey’s post hoc test for comparison of normalized tumour burden per time point. Statistical significance is represented as ns: no significant.
5.3 Discussion

The importance of choosing a clinically relevant in vivo models for specific steps of breast cancer development is crucial. Currently, one of the most relevant models to study late stages of primary breast cancer with spontaneous metastasis is the 4T1 murine breast cancer model. This cellular model of murine spontaneous metastasis is the result of a selective sub cloning of a spontaneous carcinoma from the BALB/cfC3H mouse model (Aslakson & Miller, 1992; Lelekakis et al., 1999). Early studies using the 4T1 primary breast cancer model, in which tumour cells spontaneously metastasise from the primary site to bone and soft tissues demonstrated that Zol alone prevents the development of metastatic tumours in bone but not in soft tissues. Combining chemotherapy agents with Zol (i.e. doxorubicin or tegafur/uracil [UFT]) evidenced that metastasis in both bone and soft tissue organs were reduced, inferring that the anti-tumour role of Zol in soft tissue environments was minimal (Hiraga et al., 2004; Yoneda et al., 2000). However, during the same study carried out by Yoneda et al. (2000), by increasing the dosing regimen of Zol (4 µg of Zol/animal daily for 4 weeks) before and during the presence of bone metastasis in a human breast cancer mouse model (MDA-231F9AD/Luc), led to an increase of soft tissue metastasis, indicating a possible differential effect of Zol which could be dependent on either the concentration of Zol or the cell line tested.

Although these metastatic models gave primary insight of a differential effect of Zol by decreasing tumour burden in bone, driven primarily by osteoclast inhibition, but an increased tumour burden in soft tissues, complementing pre-clinical data had pointed out that hormones involved during the menopausal transition play important roles in the development of tumour metastasis and the potential fate of DTC during metastasis development (Ottewell, et al., 2014; Holen et al., 2016).

In this study we have proposed an experiment model where generation of 4T1-Luc2 spontaneous metastasis from primary tumour on a BALB/c mouse model would be assessed under clinically relevant murine models of pre-, peri- and post-menopausal concentrations of oestradiol as well as the addition of Zol. However, due to environmental factors as well as protocol limitations, final objectives were not reached.
5.3.1 Establishment of pre-, peri- and post-menopausal concentration of oestradiol on BALB/c primary tumour model +/- Zol

Establishment of the three concentration of oestradiol representative of the human menopause by a combination of OVX and oestrogen supplementation via drinking water as proven to be a successful methodology across different mouse models (Chapters 3 and for; Canuas-Landro et al., 2021). However, important limitations, such as food and water intake, can prevent a full hormone replacement during the development of the animal model, especially where a high concentration of oestradiol needed to be maintained (pre-menopausal status). During the course of this animal study, there was a sporadic outbreak of mouse herpes symplex virus in the Biological Service Unit where all mice were kept. This virus significantly impacted on the growth of primary tumours (an observation made in my experiment and the experiments of others in the Department [data not shown]). In other experiments performed within my research team, the 4T1 model develops tumours of ~1cm³ within 21 days and within this timeframe tumours have produces large metastases in the lungs and bone (Tulotta et al. 2021). It is possible that exposure to this virus may also have impacted on the amount of water that animals drank as the drinking water with supplemented oestradiol was refilled less frequently in this experiment than previously. A sub-optimal hormone replacement by drinking water in combination may have prevented a successfully establishment of the menopausal transition as observed in the data shown.

A high variation in the serum concentrations of oestradiol in the pre-menopausal settings prevented this group to reach representative level of oestradiol as seen previously the pre-menopausal status of other mouse models (chapter 3). Although a significant difference was observed between this group and the post-menopausal concentration of oestradiol group, the values of oestradiol do not resemble those observed in previous model (chapter 3), where marked difference between all menopausal status groups was established. Nonetheless, this model was able to produce significant difference in the development of primary tumours which are consistent with other similar models with externals supplementation of oestradiol and tumour development of 4T1 cell-line (Ouyang et al., 2016; Yang et al., 2013). Importantly, addition of Zol to the pre- and peri-menopausal status models continue to show no difference in the oestradiol concentration, whereas in the post-menopausal setting, it decreased further.
the concentration of this hormone. A possible explanation to this isolated effect could be dependent on the caloric intake of these two different groups (Baer et al., 2004; Heard et al., 2016); however, no evidence was collected to determine whether food intake had a positive contribution in the concentrations of oestradiol.

5.3.2 Effect of pre-, peri- and post-menopausal concentration of oestradiol on 4T1-Luc2 bone retrieved primary tumour development +/- Zol

Upon establishment of the three menopausal status groups, the analysis of tumour progression was performed by two methodologies. Tumour measurement by the use of callipers is considered to be a gold standard for tumour monitoring as it provides an accurate reading with a minimal equipment (Faustino-Rocha et al., 2013). However, obvious limitations are encountered when the primary tumour is either not palpable or the growth tends to prefer internalization to the surrounding tissue or the formation of it is completely irregular. In this experiment, the 4T1-Luc2 bone retrieved orthotopically injected tumour cells produced a spherical shaped tumour, which was easily detectable and measurable after 15 days post establishment of menopause. One obvious disadvantage of tumour measuring by callipers was the low sensitivity of the readings to small increments of tumour volume, as well as the challenge faced by measuring individual tumour shapes. Nonetheless, bioluminescence analysis provided a more sensitive method for tumour measuring where a greater tumour burden was observed.

The development of the 4T1-Luc2 primary tumour in this experiment showed preliminary data in the assessment of the possible anti-tumour effect of Zol on tumour microenvironment. In this 4T1-Luc2 mouse model, tumour burden was not influenced by pre-, peri- or post-menopausal concentrations of oestradiol. Similarly, the addition of Zol did not alter tumour burden in pre-, peri- and post-menopausal concentration of oestradiol groups, however, it was observed that under high concentrations of oestradiol (pre-menopausal group), Zol had a tendency towards increasing tumour burden. This observation requires further confirmation in order to investigate the action of Zol in primary tumour under pre- and post-menopausal concentration of oestradiol. These result of Zol not altering tumour burden under pre-, peri- and post-menopausal concentration of oestradiol partially agrees with previous reports by Hiraga et al., and Yoneda et al, where Zol did not interfere in primary tumour development and
tumour burden (Hiraga et al., 2004; Yoneda et al., 2000). However, the beforementioned studies did not investigated the effect of external factors, such as hormonal levels, in the development of primary tumour, leaving an open gap for further investigation.

Current hypothesis involves the effect immune cells in the tumour microenvironment as well as possible direct effect of oestradiol over 4T1-Luc2 cells tumour development *in vivo*. The role of immune cells in the tumour development is largely complex as multiple variables are involved. Example of this is found in the recruitment and conditioning of immune and stromal cells in primary tumour followed by adaptation and preparation of the metastatic site during the migration process (Monteran et al., 2020; Ouyang et al., 2016). In addition, increased concentrations of oestradiol, as proposed in the pre-menopausal model, have shown to increase 4T1-Luc2 tumour growth and metastasis by directly tumour microenvironment stimulation and by increasing tumour-associated stroma cells and immune-suppressor myeloid cells (Ouyang et al., 2016; Yang et al., 2013).

Although the 4T1-Luc2 murine breast cancer model has been reported to spontaneously metastasise from primary sites (time-dependant), during the course of this study, no positive metastasis was observed after primary tumour implantation. In comparison to other studies, including those previously carried out in my laboratory using the same 4T1 cells that I used in my experiments, spontaneous metastasis was observed as early as 2 weeks after primary tumour establishment (Hiraga et al., 2004; Yang et al., 2013; Yoneda et al., 2000, Tulotta et al. 2021, Kwan, et al., 2021), whereas in the current animal study, no metastatic signal was observed within 4 weeks after primary tumour establishment. Possible explanation of this phenomenon could yet again include the presence of immune cells. During the course of this animal study, a viral outbreak was reported and affected multiple animals within the study. This infection could have increased an immunity surveillance, resulting in a potential clearance of DCT from the 4T1-Luc2 tumour as suggested by Boutte et al., (2011) in a similar controlled scenario. Nonetheless, this assumption cannot be confirmed as adequate samples were not collected and analysis of this data would not be the scope of this project.
CHAPTER 6: The effect of Zol under pre-, peri-, and post-menopausal concentration of oestradiol in a syngeneic model of murine metastatic breast cancer
6.1 Introduction

The cytotoxic effect of Zol over breast cancer cells has been reported to be independent of the cancer subtype in in-vitro studies (Göbel et al., 2016; Jagdev et al., 2001, Oades et al., 2003). In both, clinical and pre-clinical studies, evidence has shown that anti-tumour effects of Zol can be achieved in diverse breast cancer subtypes including murine models, however, it is still controversial as to whether clinically relevant doses of zol can exert anti-tumour effects alone or they need to be combined with other treatments for benefits outside of bone to be observed (Coleman et al., 2014; Gnant et al., 2011; Hiraga et al., 2003; Hiraga et al., 2004; Holen et al., 2016; Ottewell et al., 2014; Yoneda et al., 2000). The protective effect of Zol on bone DFS has also been documented to be general across the different menopausal status patients. Nevertheless, patients under post-menopausal status have shown an extended DFS whereas pre-menopausal patients do not or have an increased risk of recurrence in distant organs. This phenomenon has also been independent of the breast cancer subtype. The link between a successful adjuvant therapy is therefore with the microenvironment. Interestingly, previous experiments (chapter 4) investigating the bone microenvironment with the variation of oestradiol only did not show conclusive evidence.

The role of a competent immune system during the metastatic process is quite complex and is not only limited to the elimination of tumour cells. Recent studies have demonstrated that immune cells are key elements during the conditioning and further homing of tumour cell into the skeletal tissue in a syngeneic model (Monteran et al., 2020; Reviewed in George et al., 2020). In an immunocompromised model such as BALB/c foxn1 nude mice, a repression in Lymphocytes T cells creates relatively permissive environment for human metastatic cell lines to develop (chapter 4). Nonetheless, this permissive environment also prevents any further conditioning of T cells by tumours and therefore any further possible interaction favourable for the development of tumour cells. Although the process for a DTC to outgrow into overt metastasis depends on a considerable number of different factors, the role of the immune system in bone metastasis has become more apparent in last decade. Additionally, both Zol and oestradiol have significant effect on this system, mostly antagonising the immune cells (Kondo et al., 2011; Luo et al., 2011; Polanczyk et al., 2004; Roelofs et al., 2009; Thompson et al., 2004).
Previous experiment (chapter 4) showed that an immunocompromised model using human breast cancer cells and isolation of the concentration of oestradiol did not mimic entirely the results observed in the clinical and pre-clinical scenario when Zol is used as adjuvant therapy, especially in pre- and post-menopausal patients. A suggestion of oestradiol being a lesser effector hormone in the bone metastasis process could be debated, however, oestradiol could also indirectly elicit a bone metastatic effect (pro-tumour immune cell environment) by acting outside the bone microenvironment in an immunocompetent system as proposed by Yang et al., (2017). To understand the effect of high, low, and limited concentration of oestradiol in combination of Zol in an immunocompetent mouse model, a syngeneic animal model was implemented. Immunocompetent BALB/c mice were OVX followed by oestradiol replacement and FSH inhibition. Inoculation of 4T1-bone retrieved cells was performed via intra-cardiac injection followed by administration of Zol to observe Zol-affected changes in the bone microenvironment. Tumour growth was monitored, and the samples were collected at the end of the experiment for further analysis.
6.2 Results

6.2.1 Analysis of the direct effect of pre-, peri- and post-menopausal concentrations of oestradiol +/- Zol on 4T1 tumour cell line in vitro.

Zol has a direct anti-tumour effect on multiple types of tumour cells, including murine breast cancer 4T1 cell line (Hiraga et al., 2003; Hiraga et al., 2004; Yoneda et al., 2000). The confirmed mechanism of action by which Zol exert this anti-tumour effect has been determined to be through interruption of the mevalonate pathway, as observed in osteoclasts (Hiraga et al., 2004). In previous in vitro experiments (chapter 4), oestradiol has been proven to not affect the direct anti-tumour effect of Zol on human breast cancer cells MDA-MB-231; however, similar setting has not been used to evidence either a direct cellular response to oestradiol or a cellular protection from Zol in murine 4T1 cell line.

To investigate the direct anti-tumour effect of Zol at different concentrations of oestradiol as performed previously (chapter 4), an in vitro experiment was carried out where representative concentration of oestradiol for pre-, peri-, and post-menopause (300 pmol/L, 84 pmol/L, and 0 pmol/L of 17-ß-oestradiol, respectively) were introduced in the in vitro environment. The IC50 concentration for Zol in the 4T1-Luc2 cell line was 40 µM (experiment data kindly provided by George, 2022).

4T1-Luc2 cells were seeded in an oestradiol free media, supplemented with 300 pmol/L, 84 pmol/L, and 0 pmol/L of oestradiol to emulate the proposed menopausal levels of oestradiol. 24 hours after cellular seeding and adhesion, cells were administered with 40 µM of Zol or PBS (n=3 per treatment). Cell number was determined at 48h and 72h.

After 48h, oestradiol alone had no effect on viability of 4T1-Luc2 cells irrespective of the concentration administered (2-way ANOVA, p=0.927). However, addition of Zol showed a strong trend towards a reduction in all groups (2-way ANOVA, p=0.061), especially in the post-menopausal concentration of oestradiol (t-Test, p=0.035). No difference was observed in all Zol treated groups (2-way ANOVA, p=0.998). At 72h, the difference in cellular number between Control and Zol treated was evident across all menopausal concentrations of oestradiol groups (2-way ANOVA, p<0.001). Individual treatments (Control and Zol-treated) showed no statistical difference between groups (2-way ANOVA, p=0.863 and p=0.669, respectively). These results
agreed with previous experiments carried out on human breast cancer cells MDA-MB-231 cell line, indicating that oestradiol does not affect the direct anti-tumour effect of Zol on murine breast cancer cell 4T1-Luc2. In addition, cellular growth of 4T1-Luc2 in independent of oestradiol concentration.

**Figure 6.1 Evaluation of cellular growth of 4T1-Luc2 in oestradiol-free medium supplemented by 300 pmol/L, 84 pmol/L and 0 pmol/L of oestradiol in presence of Zol.**

4T1-Luc2 murine breast cancer cells were seeded and left to adhere for 24h in oestradiol-free media supplemented with 3 different concentrations of oestradiol. 40 µM of Zol or PBS (control) were added to the corresponding treatments (n=3 per treatment), followed by cell counting at 48h and 72h. Data from treatments is presented as Mean ± SD. A 2-way ANOVA analysis was carried to compare all treatments and time of exposure (solid comparison line), and t-Test comparison (dotted comparison line). Statistical significances are represented as * = P < 0.05, ** = P < 0.01 and no significance: ns. (experiment data kindly provided by George, 2022)
6.2.2 Effects of pre-, peri-, and post-menopausal concentrations of oestradiol +/- Zol on bone turnover from 4T1 cells in a syngeneic BALB/c murine model.

To study the effect of Zol on the bone architecture under pre-, peri- and post-menopausal status and the presence of tumours in a fully immunocompetent model, a syngeneic mouse model using BALB/c mice was established (figure 6.2). As previously described, pre-, peri- and post-menopausal status were established by OVX followed by oestradiol replacement via drinking water (12.5mg/L, 1.375mg/L and 0mg/L of oestradiol accordingly). Daily dose of goserelin (40 µg/Kg) was administered to prevent increased serum levels of FSH. 6 days after menopausal status establishment, a metastatic model of murine breast cancer 4T1-Luc2-Bone Retrieved cell line was inoculated via intracardiac injection. Mice were randomized for administration of saline or Zol (100µg/Kg) at days 9 and 14 after OVX. Number of tumours and tumour burden were monitored using IVIS-Luc2 system. At day 15, all animals were culled, and internal organs, bones and blood were collected for downstream analysis.

![Experimental outline of in vivo syngeneic model for BALB/c mice using 4T1 cells.](image)

12-week-old female BALB/c (N=72, n=12 per group) underwent OVX followed by oestradiol replacement via drinking water to achieve pre-, peri- and post-menopausal concentrations of oestradiol. All mice received a daily dose goserelin at 40µg/Kg. 6 days after OVX, 5x10^4 4T1-Luc2 bone retrieved cells were injected via intracardiac injection. 9 and 14 days after OVX, saline or 100 µg/Kg of Zol was administered. At day 15 after OVX, all animals were culled followed by sample collection for downstream analysis.
6.2.2.1 Confirmation of relevant oestradiol concentration replacement for the establishment of pre-, peri- and post-menopausal status in BALB/c syngeneic metastatic model +/- Zol.

To assess and confirm the accurate modelling from proposed oestradiol concentrations of the menopause transition, an analysis of the uterine tissue was carried out as shown previously (section 4.2.2.1). Circulating concentration of oestradiol have a direct effect on the maintenance of the uterine tissue in murine model, this as observed in tissue volume and mass (Hewitt et al., 2020; Orimo et al., 1999). At experiment endpoint (day 15), uterus from all animals were dissected and measured for tissue surface area (figure 6.3a). Uterus were placed on a millimetric paper for further measurement of tissue area (mm²) using imaging software (image-J).

Variation in the concentration of oestradiol available through drinking water showed a significant difference between the pre-, peri- and post-menopausal status groups (ANOVA, p<0.001) (figure 6.3b). Mice receiving 12.5mg/L of oestradiol by drinking water had a tissue area of 109 ± 29.4 mm², whereas mice receiving 1.38 mg/L of oestradiol had a tissue area of 96.1 ± 25.8 mm². Both menopausal status groups had not statistical difference in relation of their uterine tissue area (ANOVA, p=0.509); however, uterine tissue area showed a significant decrease to 52.5 ± 17.4 mm² in mice with no supplementation of oestradiol when compared to pre- and peri-menopausal status group (ANOVA, p<0.001 and p=0.001, respectively).

Similar results were observed in the Zol treated groups (ANOVA, p<0.001). Mice receiving 12.5 mg/L of oestradiol (pre-menopausal status) had a uterine tissue area of 106 ± 16.2 mm², which had tendencies to be a larger area in comparison to the tissue area of mice receiving 1.38 mg/L of oestradiol (88.4 ± 15.5 mm²) (ANOVA, p=0.084). The tissue area observed in mice with no supplementation of oestradiol through their drinking water was statistically smaller when compared with the pre- and peri-menopausal status groups (62.2 ± 21.8 mm²) (ANOVA, p<0.001 and p=0.006, respectively).

Addition of Zol did not alter the uterine tissue area when compared to the control groups (t-Test, p=0.814, p=0.419, and p=0.262, for pre-, peri- and post-menopausal concentration of oestradiol, respectively).
Taking all the data together, these results showed a functional replacement of oestradiol for the proposed menopausal status, particularly in the pre- and post-menopausal settings.

Figure 6. Analysis of uterus surface area for the confirmation of pre-, peri-, and post-menopausal concentrations of oestradiol in a BALB/c syngeneic model of metastatic breast cancer.

12-week-old female BALB/c (N=72, n=12 per group) underwent OVX followed by oestradiol replacement via drinking water to achieve pre-, peri- and post-menopausal concentrations of oestradiol, as presented before (figure 6.2). After study endpoint (day 15 after OVX), uterus from all animals were dissected and placed on a millimetric paper for future analysis of area using imaging software Image-J. Serum concentration of oestradiol are linked uterine mass and volume. Data from groups is presented as Mean ± SD. 1 way ANOVA was performed for groups under treatment (Zol or saline) (solid comparison line), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group) to compare Zol vs saline. Statistical significance is represented as, ** = P < 0.01 and *** = P < 0.001.
6.2.2.2 Analysis of bone volume fraction of pre-, peri- and post-menopausal concentration of oestradiol in a BALB/c syngeneic 4T1 metastatic model +/- Zol.

To analyse bone structure in the BALB/c mouse model, tibiae were collected and scanned using µCT scanner followed by digital reconstruction. Tumour bearing and non-tumour bearing tibiae were included in the digital reconstruction. Bone volume was analysed by measuring the percentage of trabecular bone fraction (% of BV/TV) in the metaphysis of the tibia, as described in the materials and methods section. Tumour bearing and non-tumour bearing samples were used in the analysis.

In the control group, the µCT analysis showed a clear difference between the three menopausal concentration of oestradiol models after a 15-day treatment of oestradiol hormone replacement (figure 6.4a). Bone volume percentages decreased with decreasing concentration of oestradiol (ANOVA, p<0.001). Trabecular bone volume fraction (BV/TV) decreased from 21.4 ± 2.33 % in mice with premenopausal concentrations of oestradiol to 16.1 ± 2.93 % of BV/TV in mice with perimenopausal concentrations of oestradiol (ANOVA, p<0.001). A further decrease to 13.1 ± 1.36 % of BV/TV was obtain in mice with post-menopausal concentration of oestradiol (ANOVA, p<0.001). The addition of Zol did not change the decreasing pattern of bone volume fraction, as observed in the control group. In mice with premenopausal concentration of oestradiol and Zol, percentages of BV/TV were 21.2 ± 1.97 %, which decreased to 18.1 ± 1.94 % BV/TV in mice with perimenopausal concentrations of oestradiol and Zol (ANOVA, p=0.002) and to 13.4 ± 1.97 % BV/TV in mice with post-menopausal concentrations of oestradiol and Zol (ANOVA, p<0.001).

Unlike previous BALB/c mouse model tested (see chapter 3), Zol did not elicit a significant increase in trabecular bone volume under any representative menopausal concentrations of oestradiol of this model; however, a tendency towards an increase in bone volume fraction after Zol administration was noted in the peri-menopausal concentration of oestradiol group (t-Test, p=0.073). In addition, previous model (BALB/c immunocompetent, chapter 3) were not exposed to any metastatic tumour cells, meaning that integrity of bone tissue was observer, whereas in the current experiment, BALB/c mice have been exposed to a syngeneic metastatic model.
A) uCT analysis of bone volume fraction (% of BV/TV) for Pre-Menopausal, Peri-Menopausal, and Post-Menopausal groups. Data from groups is presented as Mean ± SD. 1-way ANOVA was performed for groups under treatment (Zol or saline) (solid and dotted comparison lines), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group) to compare Zol vs saline. Statistical significance are represented as ** = P < 0.01 and *** = P < 0.001.

B) Figure 6.4 Bone volume analysis of tibia from BABL/c syngeneic animal model.

12-week-old female BALB/c (N=72, n=12 per group) underwent OVX followed by oestradiol replacement via drinking water to achieve pre-, peri- and post-menopausal concentrations of oestradiol. 6 days after OVX, 4T1-Luc2-Bone-retrieved cells were delivered via intracardiac injection, followed by a dose of Zol at days 9 and 14 after OVX, as presented previously (figure 6.2). Within 72 h after experimental endpoint, tibiae were µCT-scanned and reconstructed. Analysis of Bone volume was performed 0.2mm above growth plate from the proximal head of tibiae and a region of interest was then drawn covering 0.5mm of bone tissue. All tibiae were analysed (tumour and non-tumour-bearing) (A). Data from groups is presented as Mean ± SD. 1-way ANOVA was performed for groups under treatment (Zol or saline) (solid and dotted comparison lines), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group) to compare Zol vs saline. Statistical significance are represented as ** = P < 0.01 and *** = P < 0.001.
6.2.3 Effects of oestradiol +/- zol on bone cell activity in tumour bearing BALB/c mice.

6.2.3.1 Analysis of osteoblast and osteoclast activity by serum biomarkers P1NP and TRAcP 5b.

Assessing bone remodelling by the quantification of biochemical biomarkers such as P1NP and TRAcP 5b, provides insight of the cellular landscape inside of the bone microenvironment. Concentrations of both biomarker P1NP and TRAcP 5b are directly proportional to the cellular activity of osteoblast and osteoclast activity, respectively.

In this study, the values of P1NP via ELISA showed that decreasing concentration of oestradiol from pre-menopausal to post-menopausal concentration of oestradiol did not have a significant impact on osteoblast activity, as observed previous models. Values of 15.8 ± 4.94 ng/mL were observed in premenopausal concentration of oestradiol, which then reached 18.8 ± 6.79 ng/mL and 21.5 ± 6.85 ng/mL in the peri-menopausal and post-menopausal concentration of oestradiol groups, respectively. Nonetheless, these changes were not statistically significant (ANOVA, p=0.113) (Figure 6.5a).

Administration of Zol, however, elicited a marked decrease in serum concentration of P1NP in both pre- and peri-menopausal concentration of oestradiol (ANOVA, p=0.001), but not in post-menopausal concentrations of oestradiol, where the values of P1NP remained at a high concentration (20.0 ± 7.96 ng/mL). Compared with the respective non-treated control groups, Zol decreased osteoblast activity in pre- and peri-menopausal concentrations of oestradiol (t-Test, p=0.021 and p=0.002, respectively). Interestingly, in the post-menopausal environment, Zol had no effect on osteoblast activity in comparison to the control group (t-Test, p=0.62).

The evaluation of bone resorption was carried out by analysing the serum concentrations of TRAcP 5b via ELISA. Osteoclast activity increased with decreasing circulating concentrations of oestradiol (ANOVA, p=0.003) (figure 6.5b). Oestradiol activity increased from 3.1 ± 0.71 U/L in mice with pre-menopausal concentrations of oestradiol to 3.8 ± 1.08 U/L in mice with peri-menopausal concentrations of oestradiol and to 4.66 ± 1.20 U/L in mice with pre-menopausal concentrations of oestradiol (ANOVA, p=0.246 and p=0.002, respectively).

Administration of Zol reduced osteoclast activity throughout the three menopausal concentrations of oestradiol independently of the concentration of the hormone. The concentration of TRAcP 5b remained similar between all groups (ANOVA, p=0.764). When
compared against the non-treated group, Zol had a significant reduction effect in TRAcP 5b observed in peri-menopausal concentrations of oestradiol (t-Test, p=0.012) and post-menopausal concentration of oestradiol (t-Test, p=0.001).

![Graph A](A) PINP (ng/ml)

![Graph B](B) TRAcP 5b (U/L)

Figure 6. 5. Analysis of PINP and TRAcP 5b biomarkers for bone remodelling assessment under pre-, peri- and post-menopausal oestradiol concentrations +/- Zol on a BALB/c 4T1 syngeneic of metastasis.

As previously described (figure 6.2), 12-week-old female BALB/c (N=72, n=12 per group) were established representative pre-, peri- and post-menopausal concentrations of oestradiol. Inoculation of 4T1-Luc2-bone-retrieved murine tumour cells via intracardiac injection was followed by 2 doses of Zol (1 dose/week). At experimental endpoint (day 15), serum samples were collected and analysed for bone osteoblastic marker PINP (A) and bone osteoclast activity marker TRAcP 5b (B) by ELISA. Data from groups is presented as Mean ± SD. 1-way ANOVA was performed for groups under treatment (Zol or saline), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group) to compare Zol vs saline (dotted comparison line). Statistical significance is represented as * = P < 0.05 and ** = P < 0.01, and ***= p<0.001.
6.2.3.2 Analysis of osteoblast and osteoclast cellular number by bone histomorphometry.

Tibiae from all groups (n=12 per group) were collected at experimental endpoint, fixed, decalcified and further processed for histological analysis. A single representative section was selected and stained for TRAP staining. The section was then scanned with a high-resolution microscope at a 20X magnification. Cellular identification and scoring of the sections were carried out manually and semiautomatic, respectively.

Decreased concentrations of oestradiol were directly proportional with a decrease in the number of bone building cells, osteoblasts (ANOVA, p=0.026) (figure 6.6a). The number of osteoblasts decreased from 5.7 ± 1.63 cells/mm in pre-menopausal concentration of oestradiol to 2.8 ± 0.60 cells/mm in post-menopausal concentration of oestradiol (ANOVA, p=0.048).

Administration of Zol had no significant effect in the number of osteoblasts throughout the different concentration of oestradiol (ANOVA, p=0.473). However, a clear reduction in the number of this cells type was observed in conditions of high concentrations of oestradiol (pre-menopause) (t-Test, p=0.006) when compared with the untreated group. This result agrees with previous studies and models explained on chapters 3 and 4, showing that Zol not only affects osteoclast, but also osteoblast cells.

The analysis of bone resorbing cells, osteoclast, evidenced an increase in the number of cells as the oestradiol concentrations were decreasing (ANOVA, p=0.024). Although the difference in number of osteoclasts between pre-menopausal and peri-menopausal concentration of oestradiol was minimal (ANOVA, p=0.993), when the concentration of oestradiol dropped to post-menopausal levels, the increase in number of osteoclasts was significantly evident (ANOVA, p=0.022) (figure 6.6b).

Addition of Zol resulted in a reduction of the number of osteoclasts (ANOVA, p=0.021), however, this reduction was only significant at peri-menopausal concentration of oestradiol (t-Test, p=0.019) and not in all groups as was previously expected. Nevertheless, when comparing the Zol treated peri-menopausal and post-menopausal groups against their respective untreated groups, the reduction of osteoclast achieved by Zol was evident (t-Test, p=0.009 and p<0.001, respectively).
Figure 6. Analysis of osteoblasts and osteoclasts cells in tibiae under pre-, peri- and post-menopausal oestradiol concentrations +/- Zol on a BALB/c 4T1 syngeneic of metastasis.

As previously described (figure 6.2), 12-week-old female BALB/c (N=72, n=12 per group) were established representative pre-, peri- and post-menopausal concentrations of oestradiol. Inoculation of 4T1-Luc2-bone-retrieved murine tumour cells via intracardiac injection was followed by 2 doses of Zol (1 dose/week). After µCT scanning, fixation, and bone processing, tibiae were sectioned for histomorphometry analysis using TRAP staining. Osteoblast cells were scored in trabecular regions of tibiae. Data from groups is presented as Mean ± SD. 1-way ANOVA was performed for groups under treatment (Zol or saline), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group) to compare Zol vs saline (dotted comparison line). Statistical significance is represented as * = P < 0.05.
6.2.4 Effects of pre-, peri-, and post-menopausal concentrations of oestradiol +/- Zol on metastatic outgrowth of 4T1 cells in a syngeneic BALB/c murine model.

In the AZURE clinical study, the use of Zol as adjuvant therapy showed a decrease in bone metastases as a first DFS recurrence, this independently of the menopausal status (Coleman et al., 2018). However, an increase in metastasis to distant organ was also observed with correlation to pre-menopausal patients. In order to investigate the role of the different concentration of oestradiol and the administration of Zol on tumour dissemination and metastasis outgrowth, analysis of live tumour imaging was performed. Murine breast cancer 4T1 cells expressing Luc2 and retrieved from bone of an in vivo passage, were injected via intracardiac injection followed by IVIS imaging system to monitor tumour location and development.

6.2.4.1 Effects of pre-, peri- and post-menopausal concentration of oestradiol with or without Zol on overall tumour burden.

Tumour scoring and confirmation of tumour sites was performed as explained in chapter 4 (section 4.2.2.2), where tumours were divided in skeletal and soft tissue. In this experiment, all mice developed tumours in both relevant tissue sites.

Analysis of tumour number showed that oestradiol had no direct effect on the total number of tumours affecting both, bone and soft tissue organs in the BALB/c syngeneic model (ANOVA, p=0.488). This observation remained unaltered following administration of Zol (ANOVA, p=0.790) (figure 6.7a), indicating that the number of tumours affecting bone and soft tissue organs was independent from possible changes seen by Zol. However, tendency towards a decrease in the total number of tumours was observed in after Zol administration in high concentrations of oestradiol when compared to the control group (t-Test, p=0.080).

In accordance with data published from clinical and preclinical studies, tumours were more aggressive in post-menopausal concentration of oestradiol compared with mice receiving pre-menopausal concentrations of oestradiol. Tumour burden of mice with pre-menopausal concentration of oestradiol went from $5.92 \times 10^6 \pm 3.54 \times 10^6$ p/s to $1.48 \times 10^7 \pm 6.63 \times 10^6$ p/s in mice with post-menopausal concentration of oestradiol. This difference in tumour burden
based on tumour bioluminescence indicates a statistically significant increase (ANOVA, p=0.012) between the two menopausal conditions (figure 6.7b).

Importantly, administration of Zol reduced tumour burden in mice receiving post-menopausal concentration of oestradiol when compared with control mice (t-Test, p=0.080). These data consistently agree with the reported anti-tumour effect of adjuvant administration of Zol in patient populations with confirmed post-menopausal status. Although not significant variations were observed in the total tumour burden affecting pre- and peri-menopausal mouse models bone and soft tissues (t-Test, p=0.642 and p=0.183, respectively), a potential trend could be observed when isolating tumours affecting specific tissue sites.
Figure 6. 7. Analysis of tumour number and tumour burden in a BALB/c syngeneic metastatic model with established pre-, peri- and post-menopausal concentration of oestradiol +/- Zol.

12-week-old female BALB/c (N=72, n=12 per group) were established representative pre-, peri- and post-menopausal concentrations of oestradiol. Inoculation of 4T1-Luc2-bone-retrieved murine tumour cells via intracardiac injection was followed by 2 doses of Zol (1 dose/week), as described previously (figure 6.2). At experiment endpoint (day 15), final number of tumours was recorded based on bioluminescence emission (IVIS-Luc2 system) and confirmed with ex-vivo imaging (A). Similarly, confirmed tumours and their respective tumour sites were measured individually for total bioluminescence emission (total FLUX; photons/second) with further exclusion of outliers (B). A representative image of tumour bioluminescence is presented (C). Data from groups is presented as Mean ± SD. 1 way ANOVA was performed for groups under treatment (Zol or saline), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group) to compare Zol vs saline. Statistical significance is represented as * = P < 0.05, and no significant= ns.
6.2.4.2 Effects of pre-, peri-, and post-menopausal concentrations of oestradiol +/- Zol on BALB/c with syngeneic tumours affecting bone tissue.

The different concentration of oestradiol had no effect on the number of tumours affecting skeletal tissue in BALB/c mice (ANOVA, p=0.830). This result remained unaltered when Zol was administered throughout the groups (ANOVA, p=0.239) (figure 6.8a). However, an increase in the aggressiveness of the tumours at post-menopausal concentration of oestradiol was observed when compared to pre-menopausal and peri-menopausal concentrations of oestradiol (ANOVA, p=0.005 and p=0.010, respectively) (figure 6.8b). This increase in tumour burden in the pre-menopausal and peri-menopausal concentration of oestradiol group went from $6.17 \times 10^5 \pm 4.30 \times 10^5$ p/s and $9.43 \times 10^5 \pm 4.30 \times 10^5$ p/s, respectively, to $3.18 \times 10^6 \pm 1.90 \times 10^6$ p/s in the post-menopausal concentrations of oestradiol. These results correlate with clinical data where tumours often increase growth rates in conditions of limited concentrations of oestradiol. However, these results differ from early studies using a human breast cancer cell line (MDA-MD-231) as the human tumour cell line showed a minimal tendency towards a decrease in the tumour burden as the concentrations of oestradiol were decreasing (section 4.2.3). This effect can be a result of different cellular aggressiveness profiles as well as the effect of the mouse model strain where a immunocompetent BALB/c model expresses a different bone microenvironment when compares to a immunocompromised BALB/c-nude model.

Administration of Zol also gave indications of a tendency towards a reduction in tumour burden in the post-menopausal group, indicating that the lack of oestradiol in the bone microenvironment is preferred for Zol to elicit a protective effect (t-Test, p=0.071), as reported in pre-clinical and clinical data. However, no further tumour burden reduction was observed in conditions of high or low concentrations of oestradiol (t-Test, p=0.755 and p=0.678, for pre- and peri-menopausal status, respectively).

All this evidence suggests that, in order for adjuvant Zol to elicit a reduction over tumour growth, a bone environment lacking oestradiol is preferred for Zol to show a protective effect, also agreeing with clinical observations where patients clinically confirmed as post-menopausal show a protective effect from Zol (Coleman et al., 2014; Coleman et al., 2015).
Figure 6.8. Effect of pre-, peri- and post-menopausal concentration of oestradiol +/- Zol on bone metastasis affecting bone tissue.

12-week-old female BALB/c (N=72, n=12 per group) were established representative pre-, peri- and post-menopausal concentrations of oestradiol. Inoculation of 4T1-Luc2-bone-retrieved murine tumour cells via intracardiac injection was followed by 2 doses of Zol (1 dose/week), as described previously (figure 6.2). At experiment endpoint (day 15), final number of tumours was recorded based on bioluminescence emission (IVIS-Luc2 system) and confirmed with ex-vivo imaging. Tumours affecting only bone tissue were selected (A). From confirmed tumours affecting bone tissue, individual tumours were measured for total bioluminescence emission (total FLUX; photons/second) with further exclusion of outliers (B). Data from groups is presented as Mean ± SD. 1 way ANOVA was performed for groups under treatment (Zol or saline; solid line), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group; dotted line) to compare Zol vs saline. Statistical significance is represented as * = P < 0.05, ** = P < 0.01, and no significant= ns.
6.2.4.3 Effects of pre-, peri-, and post-menopausal concentrations of oestradiol +/- Zol on BALB/c with syngeneic tumours affecting soft tissue.

Different concentrations of oestradiol had no impact on the total number of developed tumours affecting soft tissues in BALB/c mice (ANOVA, p=0.095). This remained unaltered after administration of Zol (ANOVA, p=0.863) (figure 6.9a).

Similarly to previous observations, a decrease in the concentration of oestradiol was correlated with a tendency towards an increase in the tumour burden affecting soft tissues (ANOVA, p=0.053). Tumour burden increased from 5.04x10^6 ± 3.07x10^6 p/s in pre-menopausal concentrations of oestradiol to 1.18x10^7 ± 6.67x10^6 p/s in post-menopausal concentrations of oestradiol (ANOVA, p=0.046). These results closely mirror data published from clinical and preclinical studies where subjects with established post-menopausal status increase the risk of tumour development in soft tissue (figure 6.9b).

Administration of Zol on the three menopausal concentration of oestradiol groups showed that Zol had no effect on tumour burden in any of the treated groups (ANOVA, p=0.806). However, when the Zol group was compared to the Control group, a tendency towards a decrease in tumour burden was observed in the post-menopausal concentration of oestradiol model treated with Zol (t-Test, p=0.073), where tumour bioluminescence decreased from 1.18x10^7 ± 6.67x10^6 p/s to 5.67x10^6 ± 4.40x10^6 p/s (non-treated and Zol treated, respectively). Although this data does not reach statistical significance, it follows the previously observed trend where Zol exert an anti-tumour effect in post-menopausal concentrations of oestradiol. No further tendencies were observed when comparing Zol and control from pre- and peri-menopausal concentration of oestradiol groups (t-Test, p=0.755 and p=0.678, respectively).
Figure 6. 9. Effect of pre-, peri- and post-menopausal concentration of oestradiol +/- Zol on bone metastasis affecting soft tissue.

12-week-old female BALB/c (N=72, n=12 per group) were established representative pre-, peri- and post-menopausal concentrations of oestradiol. Inoculation of 4T1-Luc2-bone-retrieved murine tumour cells via intracardiac injection was followed by 2 doses of Zol (1 dose/week), as described previously (figure 6.2). At experiment endpoint (day 15), final number of tumours was recorded based on bioluminescence emission (IVIS-Luc2 system) and confirmed with ex-vivo imaging. Tumours affecting only bone tissue were selected (A). From confirmed tumours affecting bone tissue, individual tumours were measured for total bioluminescence emission (total FLUX; photons/second) with further exclusion of outliers (B). Data from groups is presented as Mean ± SD. 1 way ANOVA was performed for groups under treatment (Zol or saline; solid line), whereas t-Test was performed between same procedure group (pre-, peri- & post-menopausal group; dotted line) to compare Zol vs saline. Statistical significance is represented as * = P < 0.05, and no significant= ns.
6.3 Discussion

The use of bisphosphonates as an adjuvant therapy has been evaluated in diverse clinical studies demonstrating an increase in DFS in bone, preferably used in post-menopausal patients (Bergh et al., 2015; Gnant et al., 2011). However, the AZURE clinical trial reported that women in premenopausal status showed an increased tendency to relapse, mainly in distant soft tissues, meaning a decrease in DFS (Coleman et al., 2014). The concrete mechanism of this phenomenon is still unknown; however, it has been linked to variation in FSH and/or oestradiol (Wilson et al., 2017). Previous analysis of the bone microenvironment under high, low and limited concentrations of oestradiol in combination with Zol, did not revealed a conclusive answer when using an immunocompromised mouse model (Chapter 4). In addition, the immunocompromised model did not incorporate the analysis of soft tissue as it was mainly a bone tropic model. In the current experiment, the implementation of a BALB/c syngeneic mouse model of murine breast cancer metastasis allowed the study of a relevant aspects of the metastatic process, such as invasion of both bone and soft tissue. Not only is the study of both metastatic sites relevant, but the incorporation of immunological features (adaptive white blood cells/ T cells) which play important roles during the metastatic process (Monteran et al., 2020; Ouyang et al., 2016)

As previously assessed, the direct anti-tumour effect of Zol on the 4T1-Luc2 cell line was tested in an in vitro experiment. By exposing 4T1-Luc2 tumour cells to the representative concentrations of oestradiol seen in the menopausal transition, it was possible to confirm that this cell line grows independently of oestradiol concentrations. In addition, direct anti-tumour effect was indented of oestradiol concentrations in the environment. Also, a higher concentration of Zol was required to create a representative damage to the cells in comparison to previous MDA-MB-231 cell line. This result fully agrees with early observations of 4T1 cell line exposed to increasing concentrations of Zol (Hiraga et al., 2004).
6.3.1 Effects of pre-, peri-, and post-menopausal concentrations of oestradiol +/- Zol on bone turnover in presence of 4T1 tumour cells

In this study, a BALB/c immunocompetent mouse model was successfully established with the three proposed menopausal status by the delivery of this hormone through drinking water. This was confirmed by the measurement of direct response of target organs from oestradiol: the uterine and skeletal tissue. As previously presented, physiological maintenance of the uterus relies in the production of oestradiol during the oestrous cycle in a mouse model (as observed in humans during the menstrual cycle) (Hewitt et al., 2020; Orimo et al., 1999). A loss of volume and mass in this tissue is observed in the absence of oestradiol, as seen in this study. By assessing the surface of the uterine tissue, it was possible to evaluate the concentrations of oestradiol in the animal’s circulation and confirm the 3 representative menopausal concentrations of oestradiol as established in the previous mouse models (Chapter 3 and 4).

Complementing these data, results from the bone volume analysis carried out by µCT scanning demonstrate that pre-menopausal concentrations of oestradiol exerted an increase in the bone volume fraction which decreased directly as concentrations of oestradiol were reduced in the peri-menopausal mouse mode up to a total reduction as observed in the post-menopausal mouse model. These data correlates completely with previous animal studies (chapter 3 and 4). Similarly to the previous study (chapter 4), the presence of tumour-bearing tibiae did not influenced a statistically significance between the groups due to the lower number count of tumours affecting this specific bone. However, a mixed analysis of different tumour-bearing bones would produce more representative data regarding effect of tumour growth and bone lesions.

The administration of 2-weekly injections of Zol had no effect on the bone volume fraction in experiments carried out on BALB/c mice following intra-cardiac injection of 4T1 cells (figure 6.4), despite having significant effects on reducing osteoclast numbers and activity (figures 6.5 and 6.6). Although this finding does not compare with first establishment of the experimental model (chapter 3), where a single dose of Zol increased bone volume in bone, the presence of tumours was not accounted for and it may be that tumour induced lesions in mice that had received tumour cells are masking the bone preserving effects of Zol in this model. Additionally,
comparable models of bone metastasis have reported differences between bone volume of control vs Zol treated mice with presence of tumours in a larger period of time (35 days) (Ottewell et al., 2014), whereas in this study, tumours were only grown for 8 days as well as first and second dose of Zol was provided at 1 and 7 days before the experimental endpoint.

Despite these differences with other studies (Hiraga et al., 2003; Hiraga et al., 2004; Holen et al., 2016; Ottewell et al., 2014; Yoneda et al., 2000), the conditions representing the pre-, peri-, and post-menopausal concentrations of oestradiol were successfully achieved to provide reliable data from both tumour metastasis and bone microenvironment.

6.3.2 Effects of pre-, peri- and post-menopausal concentrations of oestradiol +/- Zol on bone cells in tumour bearing BALB/c mice

The bone microenvironment is a highly active place for homeostasis maintenance (Manolagas, 2000). Large number of cell interaction in between the different bone niches, internal and external factors such as hormones, play important role in the regulation of the bone integrity, especially under disease or biological stress (Wilson et al., 2016). In this study, the evaluation of different concentrations of oestradiol and inoculation of metastatic 4T1-Luc2 bone retrieved tumour cells impacted the bone microenvironment, mostly in a negative manner.

The reduction of oestradiol caused a direct reduction in bone volume as previously discussed. At cellular level, oestradiol plays a protective role on osteoblasts (Bradford et al., 2010; Martin-Millan et al., 2010) allowing them to extend their lifespan, meaning that reduced circulating level of oestradiol is directly seen on osteoblast number. This effect was observed in the current animal model of BALB/c mice, where a clear reduction of osteoblasts cells was seen from the pre-menopausal concentration of oestradiol group to the post-menopausal concentration of oestradiol group. Similarly, this result agrees with previous trend observed in the establishment of the menopausal model (chapter 3) where no tumour cells were introduced to the environment. Interestingly, the addition of Zol in the current model had a negative effect on the osteoblasts count in the high concentrations of oestradiol only, whereas the peri- and post-concentrations of oestradiol remained unaltered. This unaltered characteristic in the peri- and post-menopausal group was previously observed in the BALB/c
naïve model, however, in the current model BALB/c with 4T1 cells model, Zol had an enhanced
effect on the osteoblasts in high concentrations of oestradiol which contradict previous
observations (chapter 3 model). Although oestradiol is an osteoblast protective hormone
(Bradford et al., 2010; Martin-Millsn et al., 2010), it has been reported that the presence of
tumour as well as a single dose of Zol is enough stimuli to reduce number of osteoblasts in a
mouse model (Brown et al., 2012; Haider et al., 2014).

The effect of the pre- and post-menopausal concentration of oestradiol was also observed in
the activity of osteoblasts. In lower concentrations of oestradiol, the remaining osteoblast cells
appeared to have increased their activity (measured as PINP) when compared to the pre-
menopausal concentrations of oestradiol osteoblasts. This observation agrees with previous
naïve model (chapter 3) and has been proposed to be a mechanism of compensation for an
increased bone resorption. In addition, the exposure of osteoblast with Zol at high and low
concentrations of oestradiol showed a decrease in the activity in both groups. Although these
results mirror the reduction in cell number, they do not follow earlier model observations of a
naïve mouse model (Chapter 3), but agrees when presence of tumours are found in an
immunocompromised model (chapter 4). Therefore, the presence of tumours can explain the
reduction of osteoblast activity as observed in the pre-menopausal BALB/c model with 4T1-
Luc2 metastasis.

The analysis on the activity and number of osteoclasts reassures the phenotype of an increase
bone resorption as the concentrations of oestradiol are reduced. The amount of bone
resorption increased markedly from a pre-menopausal concentration of oestradiol group to a
post-menopausal group, as observed in previous models (Chapter 3 and 4). The administration
of Zol, on the other hand, had an impact on osteoclast numbers and activity as predicted.
However, the reduction in osteoclast by Zol was not enough to show significant bone increase
when compared to the control group. It is important to mention that there was no
discrimination of tumour-bearing and no-tumour-bearing mice when doing the analysis of cell
number and activity by biomarker in serum, therefore, these results have similar tumour
presence/absence variable.

Similarly to the observation in bone volume, a greater effect in bone resorption could have
been increased further with a longer period of study. Nonetheless, important changes in the
bone microenvironment were observed. Further benefit could be obtained by investigation of bone niches, such as the osteoblastic niche, due to its importance in tumour development (Allocca et al., 2019; Brown et al., 2012; Hughes et al., 2019)

6.3.3 Effect of oestradiol +/- Zol on 4T1 tumour number and growth

The reduction of oestradiol during the menopausal transition in the immunocompetent BALB/c with 4T1 metastatic mouse model showed that there was no difference in the total number of tumours affecting the different menopausal groups. Administration of Zol did not alter this trend. Although this observation can be correlated to a previous immunocompromised mouse model (chapter 4), it does not agree with previous published studies observations (Hiraga et al., 2003; Hiraga et al., 2004; Yoneda et al., 2000), where administration of Zol reduced the number of tumours overall.

The analysis of tumour burden, on the other hand, showed that oestradiol had an effect on tumour size. Tumours from the pre-menopausal concentration of oestradiol were significantly smaller (calculated by bioluminescence) when compared to the tumours from the post-menopausal group. This primarily finding shows strong correlation with the trend observed in previous studies where deprivation of oestradiol increases the outgrowth of metastasis in a (Ottewell et al., 2014; Ottewell, et al., 2015), however, these studies used the human MDA-MB-231 breast cancer cell line with an immunocompromised model, whereas a syngeneic 4T1 cell line was used with the immunocompetent mouse model in this study. Conflicting evidence shows that high concentrations of oestradiol can increase the metastatic outgrowth of 4T1 cell lines (Yang et al., 2013), despite this cell line being ER -ve and oestradiol independent, as previously demonstrated in vitro. The reason for an increase in growth has been proposed to be due to the direct effect of oestradiol over the microenvironment rather than the effect of this hormone on the 4T1 cell line. Nonetheless, the results from this study do not show that tendency. As widely described in the literature, post-menopause and aging are one of the risk factors in order to develop tumours (refer to section 1.2). Therefore, the increase in tumour burden in the post-menopausal status model has most resemblance with data observed from the clinic, giving it a stronger relevance.
The administration of Zol, however, had a reduction in the total tumour burden of the post-menopausal group (figure 6.7). More specifically, when looking directly at the tumour burden affecting bone tissue, post-menopausal concentrations of oestradiol increased tumour size whereas administration of Zol showed a tendency towards a reduction of the tumour size in the bone (figure 6.8). Although no benefit was observed in the other menopausal groups, this result reflects to certain degree clinical data where post-menopausal patients where the most benefited group after receiving Zol (Bergh et al., 2015; Coleman et al., 2018; Gnant et al., 2011). Similarly, a pre-clinical study carried out in immunocompromised BALB/c mice demonstrated that Zol decreases metastatic tumour burden in bone tissue in mice were OVX was performed (comparable to post-menopausal setting) but not in sham groups (comparable to a peri-menopausal status) (Ottewell, et al., 2014), stating that oestradiol has an important role in the anti-tumour effect of Zol. Other mouse models where metastatic 4T1 cells were injected into BALB/c mice (Hiraga et al., 2003; Hiraga et al., 2004; Yoneda et al., 2000) reported that Zol had a clear effect reducing tumour number and tumour burden; however, the longer duration of those studies and higher number of Zol doses administered to the animals failed to represent clinically relevant scenarios. In addition, the results from these studies are limited to a single menopausal status and does not incorporate the variation of the hormonal landscape observed during the menopausal transition. This limitation creates a difficulty to compare the results from these studies to those obtained in my study.

The observed increase in tumour burden affecting soft tissue in the post-menopausal status group suggests that a low concentration of oestradiol might produce a more desirable environment for metastatic cells to proliferate. This result also agrees with the risk factors for breast cancer development (see section 1.2.1) where post-menopausal women have an increased risk of developing tumours (Walker et al., 2011). In the current analysis, the pre-menopausal group showed tumours of smaller size when compared with the post-menopausal group. The administration of Zol on the post-menopausal group had a tendency towards a reduction in the tumour burden affecting soft tissue. This tendency could be produced by the documented anti-tumour effect of Zol in clinical and preclinical data where Zol has a beneficial effect only in the post-menopausal environment (Coleman et al., 2014 and 2018; Ottewell et al., 2014). When compared with previous studies, the reduction in tumour burden of a metastatic 4T1 model after the administration of Zol has already been documented (Hiraga et
al., 2003, Hiraga et al., 2004), however, does not take into account conditions of high or low concentrations of oestradiol (pre- and post-menopausal status, respectively) and, therefore, ignored the possibility of a differential effect of Zol. Interestingly, Zol did not affect primary tumour growth or tumour burden in either pre- or post-menopausal concentrations of oestradiol in a syngeneic BALB/c 4T1 model (chapter 5). However, this syngeneic model requires further confirmation as a tendency towards an increase in tumour burden after addition of Zol in the pre-menopausal model was noted, but other environmental factors could have caused the beforementioned tendency.

Both metastatic and primary tumour models represent two different stages of the disease. It is noted that Zol might have a differential effect in both stages by direct and indirect mechanisms (i.e. altering bone resorption in the bone and systemically alter immune cells, respectively) (Rogers et al., 2020). In addition to this, both high and low concentrations of oestradiol, Zol has showed opposing tendencies in the metastatic (tendency towards a reduction in tumour burden in post-menopause model) and syngeneic model (tendency towards an increase in tumour burden in the pre-menopausal model) meaning that external factors from the bone tissue, which are oestradiol modulated, are also implicated in the differential effect of Zol at pre- and post-menopausal concentrations of oestradiol. Evidence of this has been the proposed interaction of oestradiol and Zol with the immune system in both myeloid and adaptive linages (Kondo et al., 2011; Luo et al., 2011; Ouyang et al., 2016; Polanczyk et al., 2004; Roelofs et al., 2009; Thompson et al., 2004; Yang et al., 2013)

Taken together, oestradiol has a potent effect in a syngeneic immunocompetent mouse model. Decrease of this hormone alter the homeostasis of the bone cellular components, decreasing osteoblast and increasing osteoclasts. Although an increased osteoclast activity promotes a high release of nutrients and growth factors to the environment, the remaining osteoblasts increase their activity to compensate the imbalance produced by an excessive resorption. Despite this imbalance being more attractive for the tumour cells to extensively grow in the bone microenvironment, other factors need to be also considered as is the case of the immune system.
Chapter 7: General discussion
Bisphosphonates have been used as a treatment for bone loss for many decades. This class of drug is a Ca\textsuperscript{2+} chelator and therefore has high affinity to the inorganic fraction of bone. During the process of bone resorption osteoclasts take up the bone bound bisphosphonate which, in turn, inhibits the mevalonate pathway within osteoclasts leading to loss of the ruffled boarder, loss of contact with bone and apoptosis. This combination of disrupted osteoclast activity and induction of apoptosis results in reduced bone resorption and prevention of bone loss (Neville-Webbe & Coleman, 2003, Coxon et al., 2008).

The mevalonate pathway is ubiquitous to all mammalian cells, therefore it was hypothesised that bisphosphonates may be able to induce apoptosis in cancer cells as well as osteoclasts. Thus, during the early 2000, a significant amount of research was undertaken to determine whether these drugs could show promise as anti-cancer agents in their own right. This work led to a number of publications demonstrating significant anti-tumour effects in a broad number of cancer types affecting bone, including breast cancer, prostate cancer, lung cancer, multiple myeloma, among other (Coleman et al., 2013; McClung et al., 2007; Rosen et al., 2001; Rosen et al., 2004; Saad et al., 2004). More specifically, Zol, a third generation, nitrogen-containing bisphosphonate was demonstrated to directly show anti-tumour effects by inducing cell apoptosis, reduced cellular proliferation, reduced migration and invasion on breast cancer ER +ve and Triple Negative models \textit{in vitro} (Coleman et al., 2018; Gnant et al., 2011; Göbel et al., 2016; Jagdev et al., 2001; Yoneda et al., 2000). However, when administered to mouse models, clinically relevant doses of Zol exerted few to no anti-tumour effects and it was generally thought that this lack of anti-tumour activity may be due to its short half-life in the blood (~2.5h) (Ottewell, et al., 2008a; 2008b; 2010). Interestingly, when given in combination with chemotherapy (doxorubicin) Zol synergistically increased the anti-tumour effects of the chemotherapy alone and increased inhibition of the mevalonate pathway were observed suggesting that chemotherapy may increase the uptake of Zol into the tumour microenvironment (Ottewell, et al., 2008a; 2008b; 2010). The mechanism by which this occurs remains to be established but evidence suggests that Zol may be delivered to tumours via macrophages and therefore therapies that stimulate immunogenic cell death such as, doxorubicin, may promote the appropriate conditions for increased exposure to Zol (Rogers et al., 2020).
Simultaneous to pre-clinical studies that were designed to identify the potential anti-tumour mechanisms of bisphosphonates, clinical trials assessing the effects of Zol in combination with standard of care as an adjuvant therapy for the treatment of breast cancer and prevention of bone metastasis took place (Coleman et al., 2013; Coleman et al., 2015; Coleman et al., 2018; Gnant et al., 2011). The first clinical data evidenced that Zol may have anticancer effects, reducing relative risk of disease recurrence, as observed in the ABCSG12. (Gnant et al., 2011). However, in the AZURE clinical trial, when Zol was given in combination with standard of care only, no increase in DFS or overall survival was noted (Coleman et al., 2018). The major differences in these trials were that in the ABCSG12 trial all patients were ER +ve and received goserelin in addition to further endocrine therapies, rendering them chemically post-menopausal whereas in the AZURE trial the patient population were a mixture of pre- and post-menopausal women with different breast cancer subtypes receiving neoadjuvant treatments that were dependent on their tumour subtype. Further analysis of the AZURE trial demonstrated that only the post-menopausal subgroup benefited from adding Zol to adjuvant therapy in terms of increased disease free and increased overall survival, in contrast the pre-menopausal subgroup appeared to be adversely affected when given the same treatment. Interestingly, it was also noted that both the pre- and post-menopausal women benefitted from reduced recurrence in bone when Zo was added to adjuvant therapy. Because the obvious difference between pre- and post-menopausal women are their circulating levels of steroid, reproductive hormones, Wilson et al (2017) carried out additional analysis on serum samples from patients who had enrolled in the AZURE study during which she demonstrated a clear correlation between circulating levels of oestradiol and anti-tumour response to adjuvant Zol. To further confirm the importance of this hormone above the other reproductive hormones (inhibin A and FSH) mouse model experiments demonstrated no correlation between inhibin A and anti-tumour response to Zol (Wilson et al., 2017) whereas in contract removal of oestrogen with OVX increased anti-tumour response to Zol in similar mouse models, however, as removal of the ovaries results in an increase of FSH as well as a decrease in oestrogen this study could not discount the role of FSH in anti-tumour response to Zol (Ottewell et al., 2014).

The aforementioned studies, led to the hypothesis that oestradiol is the hormone responsible to preventing Zol from exerting anti-tumour effects outside of bone and/or that oestradiol may lead to changes in the bone microenvironment which cause tumour cells to disseminate.
to other organs following administration of Zol. To test this hypothesis for my project I generated mouse models that represented circulating oestrogen concentrations at the different stages of human menopausal transition (pre-, peri- and post-menopausal). I then investigated how concentrations of oestradiol in combination with Zol affected the bone microenvironment and how these changes altered metastatic outgrow of human and syngeneic breast cancer cell lines in bone and soft tissues of mice.

7.1 Establishment of mouse models representing human pre-, peri- and post-menopausal concentrations of oestradiol and evaluation of effects of Zol in the bone microenvironment.

Menopause is a physiological deterioration of the ovarian tissue until its failure and is defined as the end of the reproductive cycle. In mammals, most species experience menopause between mid- to late-life. However, and contrary to humans, these other species’ lifespans do not far surpass their reproductive years (Koebele & Bimonte-Nelson, 2016). Due to this limitation, the implementation of an artificial menopausal models is required to study and replicate physiological or disease development features of human menopause in animal models.

Evidence from independent clinical trials has demonstrated that early breast cancer patients with no signs of metastasis experience a differential benefit from adjuvant Zol depending on their menopausal status (Coleman et al., 2018; Gnant et al., 2011). While multiple hormones are involved during the transition from pre-menopause to post-menopause, FSH and oestradiol are the hormones with a strong correlation to the observed differential effect (Wilson et al., 2017). Therefore, in this project, I first developed mouse models representative of the human menopause through surgical removal of the ovaries (OVX) followed by replacement of oestradiol at pre-, peri- or post-menopausal concentrations in combination with the administration of an FSH inhibitor, goserelin.

Initial experiments demonstrated that oestrogen deprivation by OVX alone did not achieve the concentrations of oestradiol representative of the post-menopausal status, however, when OVX was coupled with a gonadotropin-releasing hormone agonist, goserelin (to prevent OVX
induced FSH), the levels of oestradiol were comparable to those seen in post-menopausal concentration.

Hormone replacement has been proposed to be carried out by diverse methodologies. These include supplementation of oestradiol in food, directly injection of the hormone into the circulation or implantation of a slow-release oestradiol pellet (Holen et al., 2016; Strom et al., 2012). Although these techniques have been shown to achieve success for the proposed objectives related to their respective experiments, these methodologies come with associated issues: For the for the length and sample size of the current study, injecting oestradiol daily in addition to, the already intensive treatment schedule, would come with ethical issues and, in our experience, implanting oestradiol pellets into mice has been associated with tumour cells metastasising to the pellet. Therefore, for my studies I implemented oestradiol supplementation through drinking water to yield the circulating concentrations of oestradiol that were comparable to those found in pre-, peri- and post-menopausal women. Additionally, the implementation of drinking water as administration route was convenient for large studies where 60 or 72 mice were used (Chapters 4, 5 and 6), as single dose of these animals via food, direct injection or by individual implantation of oestradiol pellets represented a significant cost and technical challenge.

By supplementing oestradiol via the drinking water, not only did the model successfully replicate clinically representative levels of oestradiol in serum (directly analysed), but also physiological effects of the supplemented hormone were as expected. Oestradiol is a key hormone in the maintenance of the bone microenvironment (Manolagas, 2000) as well as in the maintenance of the uterine tissue (Merz et al., 1995; Orimo et al., 1999). In both organs, the representative concentrations of oestradiol seen in the pre-, peri-, and post-menopausal groups directly correlated with bone volume (seen as BV/TV) and uterine tissue mass and volume throughout the different mouse models implemented in the present study (chapters 3, 4, 5 and 6). This is strong evidence supporting the efficacy of the developed methodology, where modelling the menopausal concentrations of oestradiol in mice is not only reached, but also physiologically representative. This is in contrast to previously published studies where the development of representative pre-menopausal status model was either over stimulated by oestradiol through the use of oestradiol pellets or underrepresented oestradiol
concentrations that are found in women, as in the case of using sham operated mice (Holen et al., 2016; Ottewell et al., 2014).

In the current thesis, I have shown that circulating concentrations of oestradiol have direct effects in bone. In accordance with previously published literature, bone turnover was directly correlated to oestradiol concentration. (Bradford et al., 2010; Martin-Millan et al., 2010). In mice, high concentrations of oestradiol led to an imbalance of bone turnover where osteoblast activity was increased in comparison to osteoclast activity, this ultimately resulted in mice supplemented with concentrations of oestradiol that were representative of those found in human pre-menopausal women having excessively high bone volume. The likely reason for this is that female mice naturally have much lower concentrations of oestradiol compared to human women therefore higher than normal concentrations affect the normal bone physiology though activating oestrogen receptors on osteoblasts (Di Grigorio et al., 2001; Mödder et al., 2011). Under conditions of low or extremely low concentrations of oestradiol (representative of human peri- and post-menopausal concentrations), an increase in the osteoclast resorption was significantly seen. This pattern of bone remodelling, under reduced oestradiol, mimics similarly most of the features present during the human menopause, where a high rate of bone turnover occurs in a response to a decrease circulating concentration of oestradiol (Manolagas et al., 2013; Noirrit-Esclassan et al., 2021). Importantly, addition of Zol had a significant effect on the macro structure of the bone increasing bone volume, as well as the cellular level, where a single dose of Zol was enough to exert changes in the cellular count and activity. Although the primary target of Zol, as with other third generation, N-containing bisphosphonates, is reduced bone resorption via osteoclast inhibition, loss of the ruffled boarder and apoptosis through interruption of the mevalonate pathway, an effect on osteoblast was also observed. Similar studies looking at the effect of Zol on different cell niches in the bone microenvironment have reported similar findings (Brown et al., 2012; Haider et al., 2014; Hughes et al., 2019) where osteoblast, blood vessels and osteoblast progenitors are affected in a dose-depending manner. Interestingly, during the course of the different studies carried out during the development of this thesis, the effect of Zol on osteoblasts varied across the proposed mouse models, whereas a consistent decrease in osteoclast number and activity was observed overall following administration of this bisphosphonate.
It is important to mention that previous studies have found a tendency of a stabilization period in bone remodelling biomarkers after induction of menopause by OVX when compared to sham-operated mice (Ottewell et al., 2014). Also, an increase in the bone formation biomarker PINP was observed over time in the OVX group, presumably as a compensation mechanism for an excessive bone resorption (Ottewell et al., 2014). In this current study, biomarkers for bone cell activity were only measured at the endpoint of the experiment, therefore we do not know how activity changed over time under pre-, peri- and post-menopausal conditions. However, at the experimental endpoint, PINP was generally higher in the pre-menopausal group compared with the peri- or post-. This trend towards increased osteoclast activity was noted in both control and Zol groups suggesting that oestradiol stimulated osteoclast activity even in the presence of Zol.

Overall, the development of a clinically relevant mouse model for investigating the of oestradiol at pre-, peri- and post-menopausal conditions in the bone microenvironment was successfully achieved.

7.2 The effect of pre-, peri- and post-menopausal concentrations of oestradiol +/- Zol on human metastatic breast cancer cells.

Following confirmation that human breast cancer cell line MDA-MB-231 did not respond to different concentrations of oestradiol as observed in vitro, in terms of altered proliferation of acquisition of resistance mechanisms against direct anti-tumour effect of Zol (chapter 4), I tested the hypothesis that oestradiol-driven changes in the bone microenvironment were responsible for the differential anti-tumour effect of Zol observed under pre- and post-menopausal conditions. To test this A BALB/c Fox−/− nude model was used to enable the successful xenotransplantation of human, bone trophic, MBA-MB-231 breast cancer cells into mice with established oestradiol concentrations that were representative of the three menopausal conditions. The use of bone tropic MDA-MB-231 cell line was preferred to increase the number of metastatic tumours in bone. Although metastatic tumours showed a preference towards bone tissue, no difference were observed in numbers of metastases at other sites when comparing all three menopausal status groups. Differently from previous
observations in similar models (Ottewell et al., 2014; Ottewell et al., 2015), low concentration of oestradiol (post-menopause [OVX]) did not increase the overall number of tumours. This observation was also noted following weekly administration of Zol, where no alteration to the total number of tumours was seen. Similarly, the anti-tumour effect of Zol on MDA-MB-231 cell line had been previously reported in numerous studies (Ottewell et al., 2008a; Ottewell et al., 2014; Ottewell et al., 2015; Yoneda et al., 2000), indicating that some aspects of the proposed mouse model has not been replicated. Further analysis of tumour affecting skeletal tissue only confirmed that the number of tumours affecting this tissue was not different between groups, although a tendency toward a reduction in the number of tumours was observed after weekly injection of Zol under post-menopausal concentrations of oestradiol. Interestingly, although numbers of tumours were not changed, there was a trend towards reduction in tumour burden (determined by bioluminescence) under conditions of low concentrations of oestradiol (post-menopause). These findings differ from previous reported studies where low concentrations of oestradiol increase bone metastasis, primarily caused by an increased bone resorption (Ottewell et al., 2014). Reduction in the circulating concentrations of oestradiol results in an increased bone turnover caused mainly by overstimulation of osteoclasts cells (see section 1.10.1). In order for metastatic tumour cells to outgrow into overt metastasis, certain conditions in the bone microenvironment require to be present. The proposed mechanism to stimulate metastatic outgrow includes the presence of increased numbers of active osteoclasts (Ottewell et al., 2014; Yoneda et al., 2000). This condition was not observed when comparing the number and activity of osteoclasts between the different menopausal concentrations of oestradiol of the current BALB/c nude model. In addition, the location of metastasis also impacts the number and size of the tumours (Brown et al., 2012; Allocca et al., 2019), with the long bones being the preferred site for tumour development in this model. However, in this current study, the number of metastases was low compared with previously published studies, making it difficult to make sensible comparisons to previous reported studies. Other important factor during the metastasis development is the time of tumour growth. During the course of the BALB/c nude study, the presence of tumours was observed at similar period of time as previous reported studies (Holen et al., 2016; Ottewell et al., 2014); however, due to restriction in the project licence for animal experimentation, the duration of the animal study was reduced in comparison to previous
studies. This reduction in the length of the study could have impacted the course of tumour development and preventing possible changes only observed at late stages. Nonetheless, a tendency towards a reduction in tumour number after weekly Zol exposure was observed under post-menopausal concentrations of oestradiol, agreeing with clinical and pre-clinical data where low concentrations of oestradiol are seen with the extended anti-tumour effect of Zol (Coleman et al., 2018; Ottewell et al., 2014). On the other hand, high concentrations of oestradiol cause anabolic changes in the bone tissue, however, in the BALB/c nude model, Zol did not contributed to additional changes neither tumour reduction. It is possible that other interactions between oestradiol and the bone microenvironment are required for Zol to act as an anti-tumour drug which are less present in the immunocompromised mouse background.

7.3 Oestradiol alters the anti-tumour effects of Zol in a syngeneic model of metastatic breast cancer

Although the development of a human metastatic breast cancer model with an environment representative different menopausal concentration of oestradiol is a relevant pre-clinical model, some important features of the metastatic process cannot be studied in immunocompromised mice. These limitations include the loss of metastatic interactions with the immune system as BALB/c nude mice lack adaptive immune cells. In order to compare the effects of menopausal concentrations of oestradiol +/- Zol on metastasis in an immune competent model and to assess effects on a different breast cancer cell line I next developed a complementary mouse model in immune competent BALB/c mice

To study the hypothesis that pre-, peri- and post-menopausal concentration of oestradiol affect the bone microenvironment and the development of metastatic tumour from a primary site after Zol administration, a syngeneic BALB/c model injected with murine breast cancer 4T1-Luc2 cell was used. In this thesis, two types of tumour progression were established, a primary tumour and a metastatic model. Analysis of tumour burden from the primary tumour model showed that the different concentrations of oestradiol did not affect tumour growth in the primary site. However, weekly administration of Zol show tendencies towards an increased tumour burden when high concentrations of oestradiol were present in the circulation (pre-menopausal model, chapter 5). Although primary evidence of the effect of Zol on 4T1 primary
tumour cells indicate that exposure to this drug decreases tumour burden (Hiraga et al., 2004), the evaluation of the anti-tumour effect of Zol was not observed under different concentration of oestradiol. As previously presented, oestradiol is a key hormone in diverse physiological processes, such as reproductive cycle, maintenance of a healthy bone turnover and regulation of immune cells, therefore, its incorporation to investigate tumour progression is important.

High concentrations of oestradiol have been shown to induce an immunosuppressive phenotype by decreasing T cell development and cytotoxicity by natural killer T cells (Baral et al., 1995; Okasha et al., 2001). Also, oestradiol has been reported to influence polarization of macrophages towards a pro-tumour phenotype (Svensson et al., 2015). Contrary to the effect of oestradiol in the immune system, exposure to Zol causes an activation of anti-tumour phenotypes in immune cells such as macrophages (Coscia et al., 2010), as well as an increased activity of gamma-delta T (γδ-T) cells, mediator T cells during the immune response against tumour progression (Kondo et al., 2011), and a decrease in the regulatory T lymphocytic (Treg) cells activation, which in turn disinhibit anti-tumour immunity preventing tumour progression (Liu et al., 2016). Due to these contrasting effects of Zol and high concentrations of oestradiol, a possible mechanism for altered anti-tumour effects of Zol under different menopausal conditions could be noted. Whilst in low concentrations of oestradiol and exposure to Zol, a synergic effect of tumour suppression is observed. Unfortunately, spontaneous metastasis was not observed in this mouse model, which prevented the confirmation of the observed pattern of tumour progression in the metastatic environment. Previous studies have evidenced a prevention in bone metastasis but a tendency to an increase in soft tissue metastasis from primary tumour cells after exposure to Zol (Yoneda et al., 2000). However, no discrimination in the menopausal status was carried out as the aforementioned animal model had no hormonal manipulation.

In the current study presented on this thesis, no analysis of the immunological landscape in the primary tumour was carried out to confirm presence of pro-tumour immune cells under high conditions of oestradiol and Zol and/or the lack of those cells under post-menopausal concentrations of oestradiol as this work was the focus of a different PhD project in the lab. Because of the technical difficulties experienced working with the syngeneic model of spontaneous metastasis (including difficulties in visualising metastases in live animals and
factors beyond my control such as the BSU being infected with a mouse virus) future experiments were carried out using, a second BALB/c mouse model of 4T1 metastasis. The use of a syngeneic metastatic model allowed the study of both bone and soft-organ metastasis as the 4T1 cell line metastasise equally to these tissues (Yoneda et al., 2000; Hiraga et al., 2004; Monteran et al., 2020). Nonetheless, oestradiol derived changes in the different environments did not affect the metastatic pattern of this cell line as reflected in the similar number of tumours observed throughout the three menopausal groups. Although the present observation agrees with the previous orthotopic model where primary tumour progression did not change under different oestradiol concentrations, it also differs partially from the proposed mechanism of metastasis where conditions of increased bone resorption create a better environment for higher number of metastatic outgrowths (Ottewell et al., 2014; Ottewell et al., 2015). Nonetheless, in the post-menopausal concentration of oestradiol model, a clear increase in tumour burden was observed when compared with the pre-menopausal concentration of oestradiol group, which follows the mechanism reported by Ottewell et al., (Ottewell et al., 2014; Ottewell et al., 2015). In addition, it was noted that weekly injections of Zol did not change the number of tumours colonising bone or soft tissue under any of the three menopausal concentrations of oestradiol. This result in an agreement with some previous studies and in contrast with others. Ottewell et al., has previously shown that clinically relevant doses of Zol (as used in the current study) has no direct anti-tumour effects in bone, when given without chemotherapy (Ottewell, et al., 2008), whereas others have used higher doses of this bisphosphonate to show direct effects of Zol on tumour development, especially in the skeletal tissue (Hiraga et al., 2003; Hiraga et al., 2004; Yoneda et al., 2000). Despite replicating similar tumour reduction on an ER +ve and ER -ve human breast cancer models, no assessment on the effect of hormonal fluctuation over tumour progression was performed on the studies using the 4T1 cell line by Hiraga et al., and Yoneda et al., leaving an open gap on the role of oestradiol on tumour development (Hiraga et al., 2003; Hiraga et al., 2004; Yoneda et al., 2000).

In the current metastatic study, the anti-tumour effect of Zol was possibly observed under post-menopausal concentrations of oestradiol, where a reduction in the total tumour burden (tumour bioluminescence of both soft tissue organs and bone) showed a tendency towards a
reduction when compared to control. This finding shows close similarities with clinical and pre-clinical data, where Zol exert an anti-tumour effect in post-menopausal groups (Coleman et al., 2018; Gnant et al., 2015; Ottewell et al., 2014), however, the BALB/c 4T1 models fails to replicate the increase in visceral/soft tissue tumours under pre-menopausal concentrations of oestradiol that were observed in the AZURE clinical trial (Coleman et al., 2018). Nevertheless, the possible anti-tumour effect of Zol observed as a tendency towards a reduction in tumour burden in the post-menopausal concentration of oestradiol in skeletal and in soft tissue (figures 6.8 and 6.9) suggests a possible synergic mechanism in the bone microenvironment and the metastatic environment outside bone.

In the bone microenvironment, the depletion of oestradiol naturally increases the population and activation of osteoclasts, as observed across all experimental models in this thesis. Proposed mechanisms have pointed out that abundance of osteoclast number and activity create a metastasis-supporting environment thanks to the continuous bone resorption (Ottewell et al., 2014; Yoneda et al., 2000). Under pre-menopausal conditions, high concentrations of oestradiol prevent excessive osteoclast activation, meaning that established metastatic foci in bone may not be exposed to optimal sustainable conditions, which may be the cause of reduced tumour growth observed through the low tumour bioluminescence reading of this group. The presence of Zol results in an efficient reduction of osteoclasts and bone resorption, and this in itself may have a direct impact on the sustenance of metastases, observed as a decreased tumour bioluminescence. This is mostly evident in the post-menopausal model, probably because of the ability of Zol to reduce the increased bone turnover seen with reduced oestrogen. Because increased osteoclast activity has been associated with increased metastatic outgrowth in bone, previous research has been carried out to establish the effects of alternative therapies that prevent osteoclast activation by directly blockage of RANKL such as Denosumab on tumour growth in bone have emerged. The conflicting evidence from mouse and human studies using RANKL blockers (Coleman et al., 2020; Gnant et al., 2019; Ottewell et al., 2015) have left an open question in whether osteoclasts are the dominant cellular component dictating the fate to an overt metastasis. Furthermore, current evidence has demonstrated that other cellular interaction from the osteoblastic and the haematopoietic niches, as well as immune cells (polarised macrophages, γδ-T, Treg cells, among others), play an important role in the establishment and maintenance
of metastatic cells (Allocca et al., 2019; Canuas-Landero et al., 2021, Hughes et al., 2019). Interestingly, these different cells are also affected by Zol, which has been shown to decrease their tumour-supporting phenotype. Although these niches were not evaluated in the current experiments, it might be possible that interactions other than osteoclast cells are relevant as no benefit from the anti-tumour effect of Zol was observed in the bone tumour burden from the pre- and peri-menopausal groups.

When investigating tumours affecting soft tissue, an identical trend to tumours affecting bone tissue, where a tendency towards a reduction in tumour burden was observed but only in post-menopausal concentrations of oestradiol. However, from previously published clinical and pre-clinical evidence (Coleman et al., 2018; Yoneda et al., 2000), a tendency towards an increase in the tumour burden affecting soft tissues was expected in the pre-menopausal concentration of oestradiol model, but it was never observed. Zol induced a tendency towards a reduction in tumour burden under post-menopausal concentrations of oestradiol but did not alter tumour burden under pre- and peri-menopausal concentrations of oestradiol. Interestingly, in the studies carried out by Hiraga et al., and Yoneda et al., Zol did not exert anti-tumour effects in soft tissue metastasis when clinically relevant doses were used. However, when excessive, non-clinically relevant, doses of Zol were administered before tumour inoculation, soft tissue tumours were decreased. On the other hand, in previous studies using 4T1 tumour cells, neither time of treatment or concentration of Zol had any effect on tumour burden in the soft tissue (Hiraga et al., 2004; Yoneda et al., 2000). Unlike previous observation, in my experiments using 4T1 cells as a primary tumour model Zol increased tumour burden under high concentrations of oestradiol, but this finding was not replicated in my 4T1 metastatic model as no changes were observed following administration of Zol in soft tissue. One of the major differences between the two 4T1 models that I used was the time of tumour progression. In the primary tumour model, no spontaneous metastasis was observed in a course of 4 weeks and mice were culled because primary tumours were growing significantly slower than is normal for this model possibly due to the mice contracting mouse-HPV virus which was endemic in the BSU at the time of this experiment. Despite these possible negative interaction from external factors, overall survival was significantly longer in comparison to the metastatic model. In the metastatic model, mice were sacrificed 9 days after tumour injections due to the presence of different tumour-related issues, such as general distress, lethargic behaviour and
breathing problems. The evolution of metastatic tumours was more rapid in comparison to the orthotopic tumour, resulting in the mice receiving fewer doses of Zol. If this experiment was to be repeated in the future, I would suggest injecting fewer tumour cells into the metastatic model to prolong the study and delay the humane endpoint of the study, allowing to observe a possible difference between the control and Zol treated group from the pre-menopausal concentration of oestradiol model. Additionally, a slower tumour progression could give more insights in whether immune cells under the different concentrations of oestradiol respond similarly to the tumour microenvironment in soft tissue and if this behaviour is altered with the presence of Zol, as proposed in the syngeneic model (chapter 5). However, the current results show that high concentrations of oestradiol do not interfere with Zol to elicit a differential effect in soft tissue metastasis of the pre-menopausal when compared to the post-menopausal status model.

Overall, this syngeneic metastatic mouse model provides results which agree with the data from clinical trials (ABCSG-12 and AZURE) where extended benefits with the administration of Zol were observed in the post-menopausal patients. Under these circumstances, lack of oestradiol allows Zol to exert an anti-tumour effect by decreasing bone resorption and possible modify the interactions of surrounding cells such as osteoblastic progenitors, haematopoietic cells, and immune cells. Nonetheless, the effects that oestradiol may have on the immune cells (influencing the activity of dendritic cells, macrophages, neutrophils, natural killer cells, B cells, CD4+ T cells, and regulatory Tregs) are profound and constantly opposing to the ones elicit by Zol (altering macrophage polarisation in the tumour microenvironment, γδT-cell activation, NK cell activity, Treg activation and infiltration, and T-cell function), proposing that anti-tumoral effect of Zol in soft tissue metastasis may be closely regulated by immune mechanisms and other mechanisms not yet elucidated.

7.4 Future directions

In this study, a successful modelling of the concentrations of oestradiol observed in the human menopausal transition was achieved in different mouse models. This developed model allowed the study of the isolated effects of oestradiol in the skeletal tissue, yielding physiologically
relevant changes in the bone microenvironment. Although these oestradiol driven changes in the bone microenvironment showed a high level of resemblance with results from clinical trials in the post-menopausal setting, it has also left more unanswered questions regarding other cellular interactions in the bone microenvironment apart from the bone resorption unit (osteoblasts and osteoclasts) during the development of metastasis. Despite having obtained relevant data from the different mouse models used, external limitations prevented a successful competition of some experimental objectives, such as development of spontaneous metastasis or a higher number of metastatic events occurring on hind limb bones. An increase in the numbers of mice included in each experiment is, therefore, recommended.

To elucidate other interactions from the tumour cells with the bone microenvironment, and how oestradiol driven chances +/- Zol affect these interactions, a closer look to the cellular niches in the bone is recommended. Thanks to the development of a high-resolution 3D confocal imaging methodology (Kusumbe et al., 2015), works from close research groups have demonstrated the effect of Zol on osteoblastic niches as well as the important relationship of the haematopoietic niches in metastasis progression (Allocca et al., 2019; Hughes et al., 2019). Thus, an evaluation to these bone niches under the concentrations of oestradiol observed during the human menopausal transition would produce translationally relevant data. In addition, a paralleled in vitro assay co-culturing tumour cells with bone niche cells could give insights of primary mechanistic pathways (use of qPCR/Nanostring analysis for gene expression), in how interactions between these cells can affect towards a differential effect of Zol in the pre- and post-menopausal setting (evaluation of tumour migration and invasion).

Although the presented effects of Zol under pre-, peri- and post-menopausal concentrations of oestradiol in the bone microenvironment have shown insights towards a differential effect, the incorporation of standard of care for tumour treatment could enhance possible observations of differential effect of Zol as noted in the AZURE trial. Therefore, a pilot study assessing the presence of chemotherapy (i.e. doxorubicin) under pre- and post-menopausal concentrations of oestradiol +/- Zol is recommended.
7.5 Concluding remarks

The clinical use of adjuvant Zol is the standard for the prevention of bone metastasis events in patients with early breast cancer but only after a confirmed post-menopausal status. The AZURE clinical trial has evidenced that pre-menopausal patients show a benefit in the prevention of bone metastasis, however, an increase in soft tissue metastasis was observed resulting in a decrease in DFS and a lower overall benefit. This differential effect of Zol raised the question of whether hormones could affect the final outcome of the adjuvant therapy in pre-menopausal patients. This project aimed to understand the mechanisms by which a differential of Zol was observed and hypothesise that oestradiol driven changes in the bone microenvironment were responsible for creating a less permissive environment for the tumour cells to grow, forcing them to home and outgrow in soft tissues. Thus, mouse models of the menopausal concentrations of oestradiol observed in humans were set up. Characterization of the bone microenvironment with and without the presence of Zol were successfully noted (table 2.1). Incorporation of human and syngeneic breast cancer cells provided evidence with potential clinical translation where post-menopausal concentrations of oestradiol responded positively to Zol treatment in both skeletal and soft tissues, as observed in clinical trials. However, under the current mouse models proposed (BALB/c and BALB/c nude), high concentrations of oestradiol did not elicit the expected differential effect in soft tissue metastasis after Zol treatment.
<table>
<thead>
<tr>
<th>Type of model</th>
<th>Study duration</th>
<th>No. of Zol doses</th>
<th>High concentration of oestradiol (Pre-menopausal)</th>
<th>Low concentration of oestradiol (Pre-menopausal)</th>
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<tr>
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<td>Study duration</td>
<td>No. of Zol doses</td>
<td>Bone volume fraction compared to control</td>
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<td>Immunocompetent</td>
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<td>1</td>
<td>↑ Overall Bone volume fraction</td>
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<tr>
<td>BALB/c</td>
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<td>↓ Overall Bone volume fraction</td>
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<td></td>
<td>Primary 36</td>
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<td>↑ Overall Bone volume fraction</td>
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<td>4T1</td>
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*↑* = Increase; *˅* = tendency towards increase; *↓* = Decrease; *˄* = tendency towards a decrease, and *ND* = no difference


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