

Faculdade de Engenharia da Universidade do Porto



**Sympathetic innervation in Breast cancer bone
metastasis:
exploring the role of alpha2C-adrenergic signalling
pathway in the bone metastatic niche**

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Resumo

De acordo com Organização Mundial de Saúde, 2.3 milhões de mulheres foram diagnosticadas com Cancro da Mama em 2020, tendo sido o cancro com mais incidência neste grupo nos últimos 5 anos. Pacientes com Cancro da mama apresentam um risco elevado de desenvolver metástases ósseas, o que afeta negativamente a qualidade e esperança média de vida. Apesar da grande atenção que o Cancro da mama tem atraído, as interações estabelecidas entre o ambiente metastático e as células tumorais ainda são pouco compreendidas. Esta falta de conhecimento é um obstáculo para o desenvolvimento de estratégias terapêuticas que permitam colmatar esta doença.

O Sistema Nervoso Simpático tem sido estudado como uma nova componente nas vias de sinalização que estão envolvidas no desenvolvimento de metástases ósseas, devido à expressão, tanto pelas células do cancro como pelas células residentes no osso dos recetores adrenérgicos α e β para as catecolaminas libertadas pelas fibras nervosas simpáticas. Além disso, as vias de sinalização pelos recetores adrenérgicos β , particularmente os recetores adrenérgicos β_2 , estão associadas a uma maior incidência de metástases ósseas e a um efeito negativo na densidade óssea, como consequência de um aumento de reabsorção óssea. Sendo assim, o Sistema Nervoso Simpático apresenta uma potencial ação no comportamento e estabelecimento da metástase óssea relacionada com o Cancro da mama. Contudo, nesta interação ainda existe uma lacuna no conhecimento referente às vias de sinalização pelos recetores adrenérgicos α_2 , que controlam a libertação das catecolaminas pelas fibras nervosas. Além disso, o papel do osteoclasto, a célula responsável pela reabsorção da matriz óssea, é ainda muito pouco conhecido tanto na intervenção do Sistema Nervoso Simpático na remodelação óssea como na metástase óssea derivada do cancro da mama, uma vez que a maioria dos estudos se focam no papel da célula responsável pela formação de matriz óssea, o osteoblasto.

A relação do Sistema Nervoso Simpático na metástase óssea do cancro da mama apresenta então uma lacuna no conhecimento, apesar do aumento da compreensão em relação às vias moleculares e celulares envolvidas nesta interação. Sendo assim, este projeto tem como objetivo dissecar o papel modulatório da via de sinalização do recetor α_{2C} no nicho metastático ósseo do cancro da mama. Para tal, três objetivos foram delineados: i) Avaliação *in vitro* do papel do Sistema Nervoso Simpático no nicho ósseo; ii) Avaliação *in vitro* do papel do Sistema Nervoso Simpático no nicho metastático ósseo; iii) Determinação do papel do Sistema Nervoso Simpático

num *modelo in vivo* da metástase óssea do cancro da mama, abordando a expressão das fibras nervosas simpáticas e do recetor adrenérgico α_{2C} .

Para tal usou-se uma monocultura de osteoclastos e uma co-cultura de osteoclastos e osteoblasto de origem animal (murganhos) para avaliar a influencia do Sistema Nervoso Simpático, pela via de sinalização do α_{2C} , no nicho ósseo. Para tal foi usada noradrenalina como um agonista adrenérgico e JP1302 como um antagonista específico do recetor adrenérgico α_{2C} , sendo avaliada a diferenciação e atividade do osteoclasto. Durante o bloqueio desta via de sinalização a função do osteoclasto pareceu ser prejudicada, tal como a sua morfologia, principalmente quando foi também adicionado a concentração de $10^{-9}M$ noradrenalina. Na co-cultura, o aparente aumento da expressão do RANKL pelos osteoblastos durante o bloqueio da via de sinalização do α_{2C} não foi suficiente para contrabalançar o efeito desta via de sinalização no osteoclasto. Como não se verificou toxicidade associada ao uso do antagonista no osteoclasto, estes resultados apoiam uma possível função da via de sinalização do α_{2C} na formação e função dos osteoclastos.

O efeito do Sistema Nervoso Simpático pela via de sinalização do α_{2C} na metástase óssea foi também avaliado, através do tratamento de uma linha celular do cancro da mama com tropismo para o osso (4T1.2), com os mesmos agonista e antagonista. Primeiramente, foi avaliado o impacto direto nas células cancerígenas na sua capacidade proliferativa e de migração. O bloqueio da via de sinalização do α_{2C} diminui o número de células após 24 horas, no entanto o índice de proliferação ki67 manteve-se constante. Como as células do cancro apresentam uma alta capacidade metabólica, o efeito do bloqueio pode ter ocorrido mais cedo. No que concerne à capacidade de migração, esta foi avaliada pelo uso de um dispositivo de microfluídica. No entanto não foi possível retirar conclusões depois de 72 horas, visto que a linha celular apresentou uma pequena capacidade de migração em todas as condições. Uma melhor abordagem seria reaplicar o estímulo. Em segundo lugar, ambas as culturas previamente referidas foram estimuladas com o meio condicionado proveniente da estimulação/bloqueio da via de sinalização do α_{2C} nas células do cancro com tropismo para o osso. Como sucedeu no efeito direto desta via de sinalização, a atividade do osteoclasto pareceu negativamente afetada. Todavia, o efeito na expressão do RANKL pelos osteoblastos foi contrário, tendo sido reduzida após o bloqueio da via de sinalização do α_{2C} nas células cancerígenas. Deste modo o uso de drogas que permitam o bloqueio da via de sinalização do α_{2C} pode ser benéfico no tratamento da metástase óssea em pacientes com cancro da mama.

Para aumentar o conhecimento desta via de sinalização na metástase óssea do cancro da mama foi utilizado um modelo *in vivo* da metástase óssea no cancro da mama. Sendo avaliado a presença de fibras nervosas simpáticas e a expressão do recetor adrenérgico α_{2C} . Apesar de não terem sido obtidas diferenças significativas entre o grupo da metástase óssea e o grupo de controlo, foi possível verificar que a presença de fibras simpáticas parece só acontecer após proliferação e crescimento da metástase óssea. A presença do recetor adrenérgico do α_{2C} e de fibras simpáticas ao dia 14, suporta um potencial papel do Sistema Nervoso Simpático durante a metástase óssea.

Os resultados obtidos durante esta dissertação são preliminares, no entanto sugerem que a metástase óssea provocada pelo cancro da mama está sobre a influencia do Sistema Nervoso Simpático, particularmente pela via de sinalização do α_{2C} . Contudo mais estudos são necessários para aumentar o tamanho da amostra e conseguir atingir diferenças significativas, confirmando assim estas observações.

Abstract

According to the World Health Organization, 2.3 millions of women were diagnosed with Breast Cancer in 2020, being the most prevalent cancer in the last 5 years. Breast cancer patients have an elevated risk of suffering from bone metastasis, which leads to a poor outcome in patient health and survival. Despite the great focus being drawn to the Breast cancer field, the complex interactions that are established between the metastatic environment and the tumour cells are still not completely understood. Thus, the lack of knowledge is a major obstacle for the development of adequate strategies and treatments to tackle this disease.

The sympathetic nervous system (SNS) is being studied as a new player in the intricate signalling pathways that drive bone metastasis, as Breast cancer cells and the bone resident cells express specific α - and β -adrenoreceptors (ADR) for the catecholamines released from the sympathetic nerve fibers. Furthermore, the β -adrenergic pathways, specifically the β_2 -ADR, is connected to a higher incidence of bone metastasis and a deleterious effect in bone mass density, due to an increase in the bone resorption. Therefore, the SNS might have a crucial role in the metastatic behaviour and establishment of Breast cancer in bone. However, there is still a need to investigate the α_2 -adrenergic pathways, as they control catecholamine release from the nerve terminals. In addition, the role of osteoclasts, the bone resorbing cell, is also poorly understood as most studies limit their focus on the role of the osteoblasts, the bone forming cell, both in the impact of SNS in the bone niche and in the Breast cancer bone metastasis.

Despite the increasing knowledge obtained on the molecular and cellular pathways involved in Breast cancer bone metastasis, the relationship with SNS still presents fundamental gaps. Thus, this project aims to dissect the modulatory role of the α_{2C} -adrenergic pathway in the bone metastatic niche. As such three objectives have been outlined: i) Evaluate in vitro the role of SNS on the bone niche; ii) Assess in vitro the role of SNS on breast cancer bone metastatic niche; iii) Determine in vivo the role of SNS on breast cancer bone metastatic niche by addressing the expression profile of TH-positive fibres and α_{2C} -ADR of a mouse model of breast cancer bone metastasis.

As such, a monoculture of mouse osteoclasts and a co-culture of mouse osteoclast/osteoblast was established to study the influence of SNS, through the α_{2C} -adrenergic pathway, in the bone niche. The cultures were treated with norepinephrine (NE), a broad ADR-agonist, and with the α_{2C} -adrenergic blocker JP1302, and it was evaluated the osteoclast differentiation and resorptive activity. During α_{2C} -adrenergic blockage the osteoclast differentiation and the resorptive activity

seemed to be impaired, and its morphology was also modified, especially in the case where it was also added a concentration of 10^{-9} M NE. In the co-culture, the apparent increased expression of the nuclear factor- κ B ligand (RANKL) by the osteoblasts during the α_{2C} -adrenergic blockage was not sufficient to counteract the effect of this ADR directly in the osteoclast activity. As no toxicity of the α_{2C} -adrenergic blocker was observed in the osteoclast culture, this results indicate a role for the α_{2C} -adrenergic pathway in osteoclast formation and function.

The effect of SNS, through the α_{2C} -adrenergic pathway, in the bone metastatic niche was also evaluated by treating the bone tropic Breast cancer (4T1.2) cells with the same agonist and antagonist. Firstly, the impact of the treatment on the proliferative and migration potential of the bone tropic cell line was assessed. The α_{2C} -adrenergic blockage decreased cell numbers after 24h, however the proliferative ki67 index remained unaltered. As cancer cells present a high metabolic activity, the effect of the blockage might have occurred at an earlier time point. Regarding migration, it was assessed in a microfluidic device, though no conclusive results were observed after 72h, since 4T1.2 cells seem to be able to migrate in all conditions tested. As stimulus was only applied after seeding, and as previously hypothesised stimulus might act in a short period of time, there is a need to reapply stimulus every 24h. Secondly, the mouse osteoclast monoculture and the co-culture were treated with the condition media (CM) retrieved from bone tropic Breast cancer cells after stimulus/blockage of the α_{2C} -signalling pathway. As in the direct effect of the α_{2C} -adrenergic pathway, osteoclast differentiation and activity appeared to be hindered after α_{2C} -adrenergic blockage in the 4T1.2 cells. Contrary to the direct effect on osteoblast, RANKL expression seemed to be reduced when α_{2C} -adrenergic blockage of Breast cancer cells was performed. Thus, α_{2C} -adrenergic blockage might have beneficial outcomes as a therapy in bone metastasis.

To better understand this effect in vivo, a mouse model of Breast cancer bone metastasis was used to evaluate the sympathetic nerve profile and α_{2C} -ADR expression in the bone metastatic niche. Although no significant differences were found between the bone metastasis (BM) group and the sham-operated (SO) group, it allowed to assess that sympathetic innervation in the tumour is only present after its' proliferation and growth, as sympathetic nerve fibers were only observed after day 7 in the tumour surroundings and centre. At day 14, α_{2C} -ADR expression was evaluated. It was confirmed its presence in the tumour which further supports a role for this pathway in the breast cancer bone metastasis.

The preliminary data obtained in the scope of this dissertation suggests that breast cancer bone metastasis is under the influence of the SNS, in particular by α_{2C} -adrenergic signalling pathway. However, there is a need to perform more studies to increase the sample size, to achieve powerful statistical significance and confirm these observations.

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Abbreviations and acronyms

List of abbreviations and acronyms (sorted alphabetically)

2D	2 Dimensional
3D	3 Dimensional
ADR	Adrenoreceptors
ATF4	Activating transcription factor 4
BM	Bone metastasis
BMP	Bone morphogenetic proteins
BMU	Basic multicellular unit
cAMP	Cyclic adenosine monophosphate
CTSK	Cathepsin K
CM	Condition medium
CNS	Central Nervous System
CREB	cAMP response element-binding protein
DBH	Dopamine β -hydroxylase
EMT	Epithelial to mesenchymal transition
Epi	Epinephrine
ER	Oestrogen responsive
FOXP3	Forkhead box P3
HER2	Human Epidermal growth factor Receptor 2
IGF	Insulin growth factors
IL	Interleukin
ISO	Isoproterenol
M-CSF	Macrophage colony-stimulating factor
MMP	Matrix metalloproteases
MSC	Mesenchymal Stem cells
NE	Norepinephrine
OPG	Osteoprotegerin
PD-1	Programmed death 1
PD-L1	Programmed death-ligand 1

PEG2	Prostaglandin E2
PR	Progesterone responsive
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RLU	Relative light unit
Runx2	Runt-related transcription factor 2
SCG	Superior cervical ganglions
SNS	Sympathetic Nervous System
SO	Sham operated
TGF- β	Tumour Growth factor beta
TH	Tyrosine hydroxylase
TNF- α	Tumour necrosis factor- α
TRAP	Tartrate-resistant acid phosphatase
VEGF	Vascular endothelial growth factor
α -ADR	Alfa-adrenergic receptors
β -ADR	beta-adrenergic receptors

Chapter 1

Motivation and Aims

1.1 - Motivation

Cancer is a highly prevalent disease worldwide and unfortunately its incidence is expected to increase. Breast cancer is one of the most common cancers with a high incidence in developed countries, although mortality is higher in developing ones [1]. Also, breast cancer is the most diagnosed cancer in women being in second place as cause of cancer related deaths [1, 2]. The problem of breast cancer gets even worse when patients develop bone metastasis, as current treatments only mitigate the skeletal-related complications without improving life expectancy and overall survival. Both the inefficiency of current treatments of bone metastasis as well as the expected increase in healthcare costs brings a need to develop new therapeutic strategies [3-5].

Worst clinical prognosis, reduced survival, high rate of recurrence and increasing metastatic tumours are associated with high sympathetic activity in breast cancer patients [6-8]. Also, β -blockage is being associated with better outcomes in breast cancer patients [9-12]. Therefore, sympathetic nervous system (SNS) might be implicated in the process of breast cancer development and metastasis in a deleterious way for patient health. Moreover, SNS is being extensively studied in the process of bone remodelling. In fact, the β_2 -adrenergic stimulation is predominantly linked to a low bone mass density, specifically by acting on the osteoblast [13]. Wherever not much information is still gathered from the effect of the α -adrenergic stimulation, specifically of the α_2 -adrenergic stimulation. As α_{2C} -adrenergic receptors (ADR) are autoreceptores that control sympathetic activity [14] and worst prognosis are linked to sympathetic hyperactivation, understanding its effect on breast cancer bone metastasis could open up new strategic therapies.

Therefore, the motivation of this work is to study the role of the α_{2C} adrenergic pathway, since it is the predominant autoreceptor in nerve endings, in the breast cancer bone metastasis and its effect on the bone metastatic niche, specifically in the osteoclast activity. Furthermore,

this work intends to assess the sympathetic nerves profile and α_{2C} -ADR expression in an *in vivo* model of bone metastasis.

1.2 - Aims

The aim of this dissertation is to evaluate, the involvement of the sympathetic nervous system, through the α_{2C} -ADR signalling pathway, in the development of bone metastasis induced by breast cancer using both an *in vivo* and an *in vitro* approach.

For that purpose, this work will consist on:

1. Assess *in vitro* the role of SNS on the bone niche, by determining:
 - a. the role of α_{2C} -signalling pathway on the osteoclast activity;
2. Assess *in vitro* the role of SNS on breast cancer bone metastatic niche, by determining:
 - a. the role of α_{2C} -signalling pathway on bone-tropic breast cancer mouse cells activity (namely proliferation and migration); and
 - b. how breast cancer cells under the control of α_{2C} -signalling affect osteoclast resorptive capacity;
3. Evaluation of the expression profile of Tyrosine hydroxylase (TH) -positive fibres and α_{2C} -ADR on bone tissues retrieved from an *in vivo* mouse model of breast cancer bone metastasis.

Chapter 2

Introduction

Breast cancer and Bone Metastasis

1.1-Breast cancer

Breast cancer is the most diagnosed cancer in women, being in second place as cause of death [1, 2]. So, understanding its mechanisms and the development of highly effective therapies are a major necessity. Breast cancer is a heterogeneous disease, being composed of multiple subtypes with distinct characteristics leading to different responsive patterns to treatments and clinical outcomes (Table 1.1) [15-17]. Breast cancer can be classified based on histology and molecular markers. Histologically breast cancer can be classified into *in situ* carcinoma and invasive carcinoma. Further subdivision is applied in the *in situ* group as either ductal or lobular [15]. Through the genetic expression assays three different Breast cancer groups were identified: luminal, HER2-enriched and triple-negative. The luminal group presents genes that encode typical proteins of luminal cells and cells are oestrogen (ER) or progesterone (PR) positive. This group is further subdivided in luminal A and luminal B. Luminal A presents higher incidence and better prognostic, whereas luminal B presents a higher expression of proliferation related genes and a small percentage is HER2-positive which leads to a more aggressive phenotype, higher relapse rate and consequent worse prognostic. The HER2-enriched group is also more aggressive since this pathway activates genes involved in cell proliferation, angiogenesis, invasion and metastasis. The last group presents the lowest incidence, however present high mitotic and proliferative indices leading to worse clinical outcomes. This cancer cells are ER, PR- negative and HER2-negative. A further subtype of the triple-negative cancers was characterized by low expression of genes involved in cell-to-cell interactions - being designed as claudin-low [16, 17].

The categorization of the different breast cancer subtypes through their molecular markers lead to an improvement and specificity of treatments. In the case of the luminal A endocrine therapy presents high success rate, while luminal B requires a conjugation with

chemotherapy. Whereas HER2-enriched have high expression of the HER2 receptor, and as such a monoclonal antibody to the HER2 (trastuzumab) is frequently used. In the case of the triple-negative group conventional chemotherapy with anthracycline and taxane is applied, however further therapeutic options are being studied [15, 16, 18].

Besides the importance observed in the specificity of the clinical therapy, evidence for a preferential metastatic site to each Breast cancer subtype was demonstrated [19-21]. The process of metastasis is complex and highly selective (Figure 1.1). Metastasis is a multi-step process comprised of an invasion step, where the epithelial to mesenchymal transition (EMT) is an essential step, followed by intravasation to the blood circulation, where cells need to survive in the blood environment. The final steps are the arrest and extravasation from the blood to the distant organ leading to the colonization [22]. The site of colonization is not random as Paget proposed in the “seed and soil” hypothesis [23]. While breast cancer luminal tumours preferentially develop metastasis in bone, HER2-enriched and triple-negative tumours develop metastasis in the brain or visceral organs, as liver and lungs [19-21].

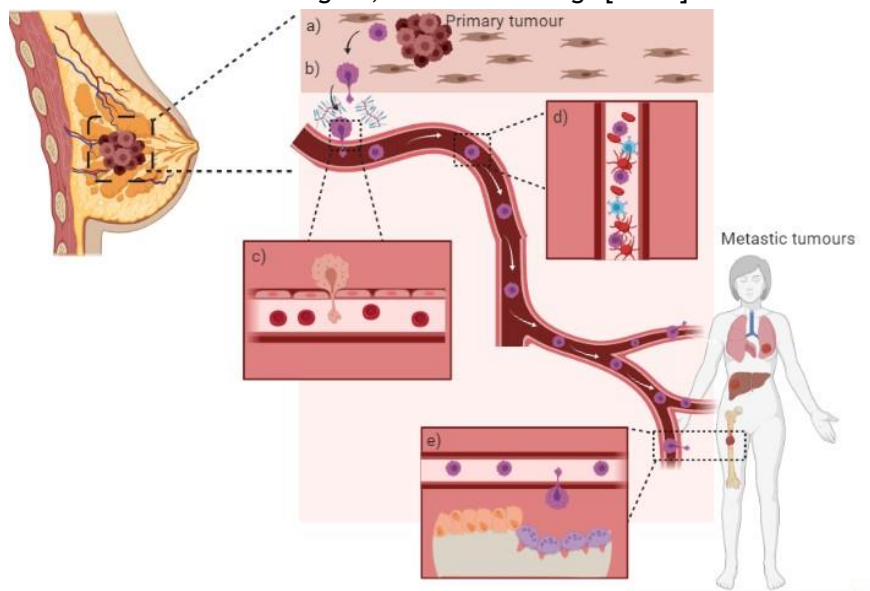


Figure 2.1- Schematic representation of the metastatic cascade and the common sites of Breast cancer metastasis. a) The epithelial to mesenchymal transition (EMT) is an important process that enables the metastasis to distant places. During these process cells acquire properties that are fundamental to the invasion and metastatic dissemination. These properties are motility and capacity to degrade extracellular matrix. b) After EMT, cancer cells are able to migrate leading to the invasion to other tissues. c) Intravasation to the blood or lymph circulation allows dissemination to distant sites. d) After entering in circulation tumour cells are referred as circulating tumour cells and they encounter an inhospitable environment due to a lack of a substrate to attached, hydrodynamic conditions, shear stress and are more vulnerable to the immune cells attack. To overcome these drawbacks cancer cells associate themselves with other cell types as monocytes/macrophages, platelets, neutrophils or endothelial cells. This process is highly selective and only a small percentage of cells survive. e) The final steps of the metastatic process consist in extravasation, which is the contrary of intravasation, and the colonization to a new tissue. The homing to a new tissue is also high discriminating as cells are usually eliminated or lack the capacity to proliferate. Most cells enter in a dormant state resulting in a no net increase in the size of the metastatic clusters. Later cancer cells become active and start to proliferate. Adapted from [24].

Table 2.1- Characteristics of Breast cancer subtypes. Adapted from [16, 18-21].

	Expression Profile	Characteristics	Current treatments	Metastatic behaviour
Luminal	Luminal A PR ⁺ or ER ⁺ HER2 ⁻ low Ki67 index	Higher incidence (50-60%) Higher expression of ER-related genes Lower expression of proliferative genes Good prognosis Low histological grade Low mitotic activity Low relapse rate Expression patterns of 'normal luminal epithelial cells' of the mammary gland	Hormone therapy	Low frequency of metastasis Bone Less than 10% in liver, long, CNS
	Luminal B PR ⁺ or ER ⁺ HER2 ⁻ High Ki67 index Small percentage PR ⁺ or ER ⁺ HER2 ⁺	15-20% incidence Higher grade than luminal A Lower expression of ER-related genes Higher expression of proliferative genes Good prognosis (slightly lower than luminal A) Higher recurrence FGFR1 amplification show Expression patterns of 'normal luminal epithelial cells' of the mammary gland	Hormone therapy and chemotherapy	Bone Less than 10% in liver, long, CNS
HER2-enriched	HER2 ⁺ PR ⁻ ER ⁻ HER2 ⁺	15-20% incidence High proliferative High expression of HER2 genes and amplification High histological grade 40% p53 mutations Higher relapse	Transtuzumab Sensitive to doxorubicin Anthracycline and taxane based chemotherapies	Brain and visceral organs specially liver Early metastasis to liver
Triple-negative	Basal like PR ⁻ ER ⁻ HER2 ⁻ High levels of basal myepithelial markers	High histological grade High mitotic and proliferative indices Mutation on p53 gene High risk of relapse	Anthracycline and taxane based chemotherapies Deficiency in BRCA1 gene allow response to PARP therapeutic regime	Brain and visceral organs specially lung
	Claudin-low PR ⁻ ER ⁻ HER2 ⁻ High levels of basal myepithelial markers Low claudin 3,4 and 7 expression	High histological grade High mitotic and proliferative indices Mutation on p53 gene High risk of relapse High expression of epithelial to mesenchymal transition genes	Same as basal-like	Brain and visceral organs

1.2- Breast Cancer Bone metastasis

1.2.1 - Bone microenvironment

Bone is a dynamic tissue with capacity for self-renew. It is mostly (~90%) composed by matrix of which 65% is inorganic or mineral, 20% organic (collagen type I, proteoglycans, glycoproteins and growth factors), lipids and water [25]. Due to its unique composition bone is a reservoir of minerals (Ca^{2+} , PO_4^{4-} , Mg^{2+} and Na^+), being involved in the homeostasis of the human body. In addition, bone provides a niche for the bone marrow and haematopoiesis.

1.2.1.1- Bone structure and remodelling

Bone is composed by cortical and trabecular bone. The cortical is dense, with 5% porosity, surrounding the marrow space. It is composed by Harversian systems that consist in a central Harversian channel surrounded by concentric osteons. It provides compressive strength and mechanical resistance. Trabecular bone presents a higher porosity, and its formed by concentric lamellae with semilunar shape called packets. It permits elasticity and absorption of loads. It also presents the higher metabolic activity. The periosteum is a fibrous connective tissue that ensheats the surface of the bone. It provides a local to tendon and ligament attachment and contributes to the blood supply [25, 26].

Bone cells - osteoblasts, osteoclasts, osteocytes and bone lining cells that form the basic multicellular unit (BMU) - are responsible for maintaining bone structure, growth, remodelling and repair. Osteoblasts are derived from mesenchymal stem cells (MSC) from the bone marrow stroma. The activation of Runx2 gene is a key step for the expression of osteoblastic differentiation markers as alkaline phosphatase (ALP), collagen type I, osteopontin, osteocalcin and bone sialoprotein 2 [26, 27]. These cells are responsible for bone formation. After which osteoblasts can: (1) become inactive/quiescent - the bone-lining cells, (2) undergo programmed cell death, or (3) become osteocytes as they are embedded in the secreted matrix [28]. Osteocytes are the major cell type (~90%) present in bone. They present cytoplasmatic processes that enable communication between osteocytes, osteoblasts and bone lining cells. Osteocytes are involved in the regulation of bone formation and bone resorption [27, 28]. They are a source of plasm calcium and are mechanosensory cells playing a role in the anabolic response of bone to mechanical stimuli [27]. The bone lining cells functions are not clearly understood, however these cells inhibit the direct interaction between osteoclast and bone matrix, preventing bone resorption [27]. Finally, the osteoclast, a multinucleated giant cell of hematopoietic origin, is responsible for bone resorption [26]. During bone resorption the osteoclast acquires a polarized membrane that can be divided in four regions: clear zone, ruffled border, basolateral membrane and functional secretory domain. The formation of an actin ring is closely linked with the resorptive activity of the osteoclast. The formation of the resorption lacuna/ Howship lacuna is the result of this membrane polarization where there is the acidification and consequent degradation of the bone matrix. Important hydrolases for the degradation of bone are the cysteine protease cathepsin K (CTSK), the tartrate resistant acid phosphatase (TRAP) and the metalloproteinases (as MMP-9 and MMP-2) [29].

The process of bone remodelling can then be divided in three parts: (1) resorption of bone by the osteoclasts, (2) a reversal phase that consists in the transition from osteoclast to osteoblast activity and (3) the bone formation by the osteoblasts [30]. This process is tightly regulated by local and systemic factors. An imbalance leads to deregulation of bone homeostasis and pathologies[31]. The first step is initiated by osteoclastogenesis. For that there are two fundamental cytokines: macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor kappa B (NF- κ B) (RANK) ligand (RANKL) that are expressed by osteoblasts, while osteocytes only express RANKL [32]. M-CSF is important for survival and proliferation of osteoclasts precursors. Whereas, RANKL leads to commitment to the osteoclast phenotype (TRAP-positive, calcitonin receptor and CTSK positive cells) and to activation of mature osteoclasts [31]. Contrary to RANKL, osteoprotegerin OPG is a soluble decoy receptor

for RANK, that inhibits osteoclastogenesis and osteoclast function by sequestering the RANKL [31]. The ratio of RANKL/OPG is influenced by multiple factors whose majority act on the osteoblasts. Examples are: parathyroid hormone (PTH), parathyroid hormone-related protein (PTHrP), 1,25-dihydroxy vitamin D₃ (1,25(OH)₂D₃) [33], prostaglandin E₂ (PGE₂), and pro-inflammatory cytokines including tumour necrosis factor (TNF- α), interleukin (IL-1, IL-6, and IL-17) that upregulate RANKL production [34, 35]. Osteocytes can also be influenced to increase production of RANKL by TNF- α [36] and IL-6 [33]. Furthermore, stimulation of osteoclastogenesis and osteoclast activity can occur independently of RANKL/RANK signalling. Cytokines as TNF- α , IL-1, IL-6, IL-11 and transforming growth factor beta (TGF- β) act on osteoclast precursors or osteoclasts stimulating differentiation and maturation [37].

During bone resorption factors previously entrapped in the matrix are liberated and may have an impact in the regulation of bone formation by the osteoblasts. Some of these factors are bone morphogenetic proteins (BMPs), insulin growth factors (IGFs) I and II and TGF- β . Also osteoclasts secrete factors that directly affect osteoblast survival and activity [29]. As osteoblasts are activated they start producing matrix - collagen, non-collagenous proteins (osteocalcin, bone sialoprotein, osteopontin and osteonectin).

1.2.2 - Bone metastasis in Breast Cancer

Bone is a favourable place to metastasis due to its properties as being highly vascularized and a reservoir of multiple nutrients. Metastasis to the skeleton is a frequent consequence of Breast cancer which leads to a decrease in overall survival of the patient [3-5]. Bone metastasis result from a disequilibrium in bone remodelling that promotes two types of lesions. Osteolytic lesions that consist in an overall decrease of bone mass that is a result of high osteoclast activity, while there is an impairment in osteoblast activity. In contrary osteoblastic lesions are a result of an increase of disorganized bone formation, with reduced bone resorption that leads to a high bone mass phenotype. Breast cancer bone metastasis are predominantly osteolytic. In consequence patients present elevated bone pain, pathological fractures, nerve compression and metabolic disturbances as hypercalcemia or acid/base imbalance [38, 39].

Breast cancer cells are capable to home, adhere, survive and proliferate in the bone environment. These can be in part for their osteomimicry as they express osteopontin, osteocalcin, osteonectin, bone sialoprotein and RANK and due to the expression of Runx2 that is the major regulator of osteoblast differentiation [38, 40].

When Breast cancer cells enter the bone environment they impact the cell-cell interactions with consequent disruption of the balance in bone remodelling. This process is referred as “vicious cycle” since the effect of the Breast cancer cells in the bone cells, will have influence in their responses to the bone environment as well. A simple description of this “vicious cycle” is that Breast cancer cell release cytokines that will influence osteoblasts to increase the production of osteoclastogenic factors (as RANKL, IL-6), as described in Figure 2.2. These factors will in their turn increase osteoclastogenesis and osteoclast activity/resorption, that will liberate the cytokines entrapped in bone matrix that are known influencers of cancer cell activity. In this model the osteoclast plays a central role having a rise of its function by the

effect not only of osteoblast products but also from cytokines release from the Breast cancer cells. While in their turn osteoblast have an impairment of their bone formation function.

Current treatments of bone metastasis target the osteoclast activity with the use of bisphosphates or the disruption of the RANK/RANKL pathway through the use of the human antibody denosumab [40].

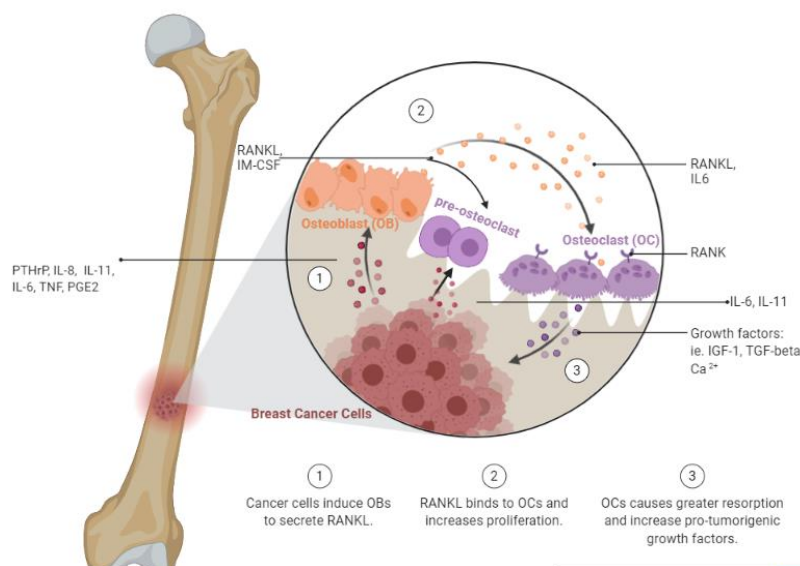


Figure 2.2 - Breast cancer bone metastatic vicious cycle in osteolytic lesions. After homing in the bone environment Breast cancer cells can enter a latent/quiescent stage resisting to conventional therapy. Bone metastasis can star to develop years after primary tumour. When Breast cancer cells escape from this dormant state they start to actively proliferate. To that end breast cancer cells start to secrete osteolytic factors such as PTHrP, inflammatory cytokines IL-8, IL-11, TNF- α and PGE2 that influence the production of pro-osteoclastic factors (RANKL and IL-6) on osteoblasts. Consequently, there is an increase in osteoclastogenesis and osteoclast activity as RANKL binds to its receptor. This effect is further boosted by the release of inflammatory cytokines as IL-6, IL-11 by breast cancer cells that act directly in osteoclast precursors. As bone resorption is elevated cytokines previous entrapped in the bone matrix as IGF-1, TGF-B and Ca^{2+} are release and stimulate Breast cancer cells activity. At the same time bone formation is impaired as osteoblast activity is reduced. A positive feedback loop is set perpetuating this cycle, forming a osteolytic lesion. Adapted from [38, 40].

Sympathetic Nervous System and Breast Cancer

2.1 - Sympathetic Nervous System (SNS)

The sympathetic nervous system is associated with the “fight or flight” response of the central nervous system, which translates the adaptive response of the individual to the environmental conditions. It is a branch of the autonomic nervous system that is structurally and functionally positioned as an interface between the external and internal environment, coordinating bodily functions to ensure homeostasis and adaptation to stress situations. Environmental and psychosocial factors such as depression, anxiety and acute or chronic stress trigger this pathway leading to the activation of the SNS and consequent release of catecholamines, such as norepinephrine (NE) and epinephrine (Epi). The prolonged exposure to catecholamines such as in case of chronic situations negatively affects biological functions, with deleterious consequences in health [13, 41, 42].

NE, the major neurotransmitter released, is released from sympathetic nerve fibers that directly innervate the organs, whereas Epi is distributed vascularly after release from the adrenal gland [43]. These catecholamines bind to different adrenergic receptors (ADRs) subtypes which are G-protein-coupled receptors. The two main classes of ADRs are the alpha (α -ADR) and the beta (β -ADR). These can be further subdivided into 9 subtypes: α_1 ADR (α_{1A} , α_{1B} , and α_{1D}), α_2 ADR (α_{2A} , α_{2B} , and α_{2C}) and β -ADR (β_1 , β_2 , β_3). The α_1 - and β -ADRs are excitatory receptors promoting the release of catecholamines, while the α_2 -ADRs regulate and inhibit catecholamine release in a negative feedback loop.

The α_2 -ADRs located presynaptically regulate the release of NE and transmitters such as dopamine, serotonin. When located postsynaptically they modulate neuronal excitability by regulation of ion channels [44]. α_{2A} and α_{2C} -ADRs are the ones involved in the presynaptic inhibition of NE. The NE presents a higher affinity and potency for the α_{2C} -ADR, which leads to an inhibition of NE release at lower NE frequencies [14]. The α_{2C} -ADRs also present slower deactivation upon removal of NE. Furthermore, the α_{2C} -ADRs produce a limited inhibition of NE release in the brain (maximum 20-30% in hippocampal tissue), since they are prominent in peripheral sympathetic nerve endings and tissues [45] and are not involved in cardiovascular regulation [44], as is the case of the α_{2A} -AR. The α_{2C} -ADRs are linked to changes in behaviour,

its' impairment may lead to a better modulation of cognition as activation of these receptor inhibit the processing of sensory information and impaired spatial and nonspatial water test in mice. In addition, mice deficient in α_{2C} -ADR had enhanced startle responses, diminished repulse inhibition, and shortened attack latency in the isolation aggression test, making it valuable for treatment of disorders as schizophrenia [14]. Furthermore, an association between depression and the α_{2C} -adrenergic pathway is being uncover, as mice lacking this receptor had decrease immobility in the forced swim test (FST) and brain function in the pathogenesis of major depressive disorder is modulated by the α_{2C} -signalling pathway [44, 46, 47].

2.2 - SNS in Bone

2.2.1- Bone innervation

Bone is an highly innervated tissue [13, 48]. Sensory nerve fibers (marked for α -calcitonin gene-related peptide, vasointestinal peptide P, or substance P) are present in the periosteum and trabecular bone. They are associated with pain and are sensitive to mechanical stimuli [48]. The majority of the sympathetic nerve fibres (expressing for tyrosine hydroxylase-TH, dopamine β -hydroxylase-D β H or neuropeptide Y) are associated with blood vessels in the compact bone and bone marrow and have a spiral-like morphology. However, in the periosteum they assume a more linear bifurcated shape [13]. The areas that receive the highest mechanical stimuli, present higher metabolic activity and vascularization are the ones with higher sensory and sympathetic fiber density [13]. Due to its incidence in the skeleton, a role for the nervous system in bone homeostasis is being considered. During fracture healing, reinnervation was reported and absence of the receptor Y_1 for the neuropeptide Y delay the fracture healing process by impairing the initial phases of the process of callus formation [49]. Moreover, reinnervation was shown to have an impact on the mechanical characteristics and structure of the new bone formed, as Tac1-deficient (lacking substance P) and sympathectomised mice present lower mechanical properties as resistance to torque and mechanical stiffness and reduced bone mass, density and trabecular connectivity [50, 51].

Furthermore, multiple studies point to an association between depression and lower bone mineral density (BMD) [52-54]. A mouse chronic mild stress model was developed to infer whether the effect of depression on the BMD was due to endocrine factors (as sexual hormones), or corticosteroids, or inflammatory cytokines (IL-1 or IL-6) or to an increase on the sympathetic activity. In this model NE contend was remarkably increase, and the use of propranolol (an β -ADR antagonist) reduced the effects of stress on the bone mass and structure of the animals [55]. Sympathectomised mice or FoxO1_{dbh-/-} mice models, presenting low catecholamine synthesis since FoxO1 is a regulator of Dbh that is an important enzyme in neurotransmitter synthesis, are shown to present higher bone mass phenotype through an increase in osteoblast number and formation rate, with a decrease in osteoclast activity [56, 57]. Also, leptin control of bone remodelling was shown to be dependent of sympathetic signalling as Dbh-deficiency mice present a high bone mass phenotype even after leptin infusion [58]. These evidences bring to the table a potential role of the sympathetic system in the bone remodelling process.

2.2.2- SNS in bone remodelling

The influence of β -blockers in fracture risk or as osteoporosis treatment are being explored as β - stimulation, specifically β_2 , appears to have an important role in bone remodelling. *In vivo* studies of mice treated with nonselective β -ADR agonist isoproterenol (ISO) or β_2 -ADR agonists presented a decrease in bone parameters (as mechanical resistance, bone volume/tissue volume) and in bone formation parameters (bone mineral apposition rate, mineral surface/bone surface) [59-61]. In addition, genetic deletion of β_2 -ADRs on osteoblasts of mice lead to a high bone mass phenotype [62] and transplantation of *Adrb2*^{-/-} bone marrow cells into wild type mice hampered the effect on bone of the ISO stimulation [63]. In fact β_2 -ADR is the main receptor express in osteoblasts and osteocytes both having weaker expression of β_1 -ADR [58, 63-66]. *In vitro* studies showed that β_2 -adrenergic stimulation increases RANKL production in both cell types [66-68]. Furthermore, β_2 -adrenergic stimulation reduces osteoblast activity through the decrease of oxytocin and BMP2 production, important factors for osteoblast differentiation and bone mineralization [69, 70]. The conjugation of these effects leads both to a decrease in bone formation due to the impaired osteoblast activity, and an increase in bone resorption, that consequently leads to a decrease in bone mass. So the use of an β_2 -ADR blocker might potential mitigate these effects, however β_2 -ADR stimulation controls RANKL expression through the ATF4 pathway, whereas osteoblast proliferation is dependent on the CREB pathway which explains why mice lacking one of those receptors, still present low bone mass after leptin infusion [63, 68, 71]. Regarding the osteocyte it was observed that stimulation by ISO lead to an increase in RANKL expression, which was maintained during β_1 -ADR blockage but not β_2 -ADR blockage [66]. This result showed a potential opposite effect of β -ADR signalling on bone remodelling, with β_2 -ADR having a catabolic stimulus while β_1 -ADR has an anabolic stimulus. In fact, mice models of β_1 -, β_2 - and β_1/β_2 -ADR deletion showed the expected increase in BMD in the *Adrb2*^{-/-} mice. While *Adrb1*^{-/-} and *Adrb1b2*^{-/-} mice present a low bone mass phenotype, though vertical cortical bone recover with aging. However, in these mice IGF1 levels, related to bone growth and peak bone mass acquisition, were markedly reduced during growth which might confound interpretation of the results [72]. Another study where the three β -ADRs were deleted the increase in bone parameters was associated with obesity. This general ablation increase complexity of the model as there is an increase in mechanical loading due to obesity and altered expression of endocrine factors that influence bone processes [62]. So the specific role of β_1 -ADR pathway was to be carefully analysed.

The osteoclast is not only an indirect target of β -ADR stimulation, but presents β_2 -ADR which makes them susceptible to direct stimulation [67, 73]. *In vitro* stimulation of osteoclast cultures with ISO lead to an increase in the expression of osteoclast specific genes, resorptive activity and promote osteoclastogenesis [67, 73-75]. So the use of β -ADR blockers might impair the SNS repercussions in bone, by disrupting the increase of osteoclast activity that is directly or indirectly provoked and the impairment of bone formation by osteoblasts. However, in one study treatment with propranolol, a non-selective β antagonist, alone lead to an increase in bone mass of wild type mice [58]. Despite that human evidence of the preventive use of β -ADR blockers in risk fracture is still unclear, but a tendency to a protective effect is seen [76].

In the case of α -ADRs stimulation, different outcomes are obtained depending on the α -ADR family. α_1 -ADRs are expressed in osteoblasts and osteoclasts [73, 77]. During *in vitro* experiments the use of cirazoline or phenylephrine, both α_1 -ADRs agonists, in human osteoblasts induce higher proliferation rates, increase OPG and RANKL expression, especially

the α_{1B} -receptor [77, 78]. Despite increase RANKL expression, the overall effect of α_1 -ADR activation is anabolic as systemic administration of a specific blocker or α_{1B} -ADR ablation in mice decrease bone mass in consequence of impaired osteoblast proliferation [79]. Although pointing to an anabolic effect of α_1 -ADR stimulus in mature osteoblasts, α_1 -ADR blockage was shown to increase osteoblastic stem cell differentiation [80].

In the case of α_2 -ADR, their stimulation is catabolic. Double disruption of α_{2A} -ADR and α_{2C} -ADR lead to a high bone mass phenotype in female mice, which was unexpected as mice presented sympathetic hyperactivity - elevated NE plasma levels. This phenotype could be explained by the increase osteoclast formation (higher number of osteoclast) and activity when bone marrow cells were culture with clonidine, an α_2 -ADR agonist [81]. Also, in a sympathectomised mice model of fracture healing the new bone formed presented lower bone parameters as there has an increase in TRAP positive cells. As NE and Epi contend is 80% reduced and at low concentrations these catecholamine preferentially bind to α_2 -ADR receptors, the authors suggested that the phenotype was the result of α_2 -ADR stimulation [50]. Despite the high bone mass phenotype in the double disruption, when only ablation of the α_{2C} -ADR receptor was performed, mice presented a heterogenic phenotype with increase cancellous bone mass in the vertebrae and lower in the femurs [82]. In humans a single dose of clonidine, an α_2 -ADR agonist, increase bone resorption marker CTx. To infer if clonidine directly targets the osteoclast, an *in vitro* culture of human osteoclasts was performed in presence of the agonist. No significant variation on TRAP positive cells or percentage of reabsorbed area was observed, so the effects of α_2 -ADR signalling may be directed by the osteoblasts or osteocytes [73, 83].

A role for sympathetic nervous system in bone remodelling is being dissected (Table 3.1), nevertheless further studies are needed to clarify it.

Table 2.2 - Summary of the effects of SNS in bone remodelling.

		Condition	Results
B- ADR stimulation	In vitro	β_2 -ADR stimulation of osteoblast	Decrease oxytocin Decrease BMP2 Increase RANKL
		β_2 -ADR stimulation of osteocyte	Increase RANKL
		β_2 -ADR stimulation of osteoclast	Increase expression of osteoclast specific genes Increase resorptive activity Increase osteoclastogenesis
	In vivo	DBH deficient mice	High bone mass phenotype and Reduced body fat when leptin was perfused
		ISO treatment	Decrease in bone parameters
		Salbutamol and clenbuterol treatment	Inhibit bone formation
		<i>Adrb2</i> ^{-/-} ob mice	High Bone Mass
		<i>Adrb2</i> ^{-/-} mice	High Bone Mass
		<i>Adrb1</i> ^{-/-} mice	Low bone mass
		<i>Adrb1b2</i> ^{-/-} mice	Decrease in IGF1
		B-ADR ablation	High Bone Mass Obesity
α -ADR stimulation	In vitro	Cirazoline Phenylephrine	Increase OPG expression Higher proliferation rates
		Clonidine	No significant variation on TRAP positive cells No significant variation of reabsorbed area
	In vivo	α_{1B} -ADR ablation in mice	Decrease bone mass in consequence of impaired osteoblast proliferation
		α_{2A} -ADR and α_{2C} -ADR ablation in mice	High bone mass
		α_{2C} -ADR ablation in mice	Increase cancellous bone mass in the vertebrae lower in the femurs

2.2 - SNS in Breast Cancer

Depression and anxiety have been linked to poor prognosis and reduced survival of breast cancer patients [6-8]. Evidence for a role of the sympathetic nervous system in breast cancer is being uncovered as TH positive fibers were found in human Breast cancer tumour biopsies with increased expression in patients with recurrence [84] and in mice tumours xenografts [84, 85]. Local release of NE is thought to be main source of catecholamine in tumour environment, so the presence of nerve fibers in the tumour is necessary to SNS involvement [84-86]. Being so, sympathetic denervation of breast tumours in mice lead to a decreased in tumour volume and tumour associated macrophages and suppression of immune checkpoints (PD-1, PD-L1, and FOXP3), promoting an immunosuppressive environment [84, 85]. Social isolation, a milder form of stress, performed in mice after tumour inoculation, showed a transiently increase in tumour growth and an increase of the tumour associated macrophage population [87]. The same effect on the macrophage population has observed by *Sloan et al*, as chronic stress induced an increase in metastasis through the recruitment of tumour macrophages that present a M2 phenotype [88]. Chronic stress was also associated with the regulation of the lymphatic remodelling in cancer, since it increases lymphatic vasculature in the primary tumour, promoting increase lymph node metastasis and metastasis escape [89]. However a recent report associated chronic stress, to a reduction of primary tumour growth with no impact on metastasis [90].

Breast cancer cells functionally express receptors for sympathetic stimulation [91, 92], making them susceptible to adrenergic stimulation. In fact, a correlation between the expression of the ADRs and the Breast cancer subtypes was demonstrated. Immunohistochemistry of Breast cancer tumours showed higher expression of β_2 -ADR receptors in luminal tumours [93, 94] and improved disease free-survival during hormonal therapy [93, 95]. However, in human Breast cancer cell lines higher β_2 -ADR was associated with the MDA-MB-231, a triple-negative cell line, and in the ER non-responsive MDA-453 in relation to MCF7, an ER-positive cell line [91, 96]. Furthermore, an association between HER2 expression and β_2 -ADR was observed when the cell line MCF7 was transfected to overexpress HER2. In this case HER2 lead to an autocrine release of Epi, that induced an up-regulation of the β_2 -ADR, increasing the proliferation and secretion of IL-8 and VEGF (important cytokines in metastasis), which might increase tumour aggressiveness [96]. For the α -ADRs, the presence of α_{2C} -ADR and α_{1B} -ADR was associated with more aggressive tumours (triple negative and HER2 enriched) in tissue samples [93, 94]. While α_{2A} -ADR was present in ER-positive and HER2-negative Breast cancer tumours [95]. In Breast cancer cell lines α_{2C} -ADR and α_{2B} -ADR was found in the MCF7, whereas α_{2B} -ADR was the only adrenergic receptor expressed by the MDA-MB-231 [92].

Contradicting effects of the sympathetic nervous system in Breast cancer are reported. Regarding β -adrenergic stimulation, β_2 -ADR is the most express β -ADR which indicates its importance on the SNS impact on Breast cancer. Cakir *et al* reported different responses to *in vitro* β_2 -ADR stimulation by different Breast cancer cell subtype, which might imply different responses in tumour subtypes. In their study only non-oestrogen responsive cells increase proliferation when treated with ISO, however treatment with an β_2 -ADR antagonist inhibit DNA synthesis in all cell lines tested [97]. The same proliferative effect was visualized on triple-negative Breast cancer cell line MDA-MB-231 by an increase in the expression of ki-67 marker [98], increase invasion capacity [99] which might be explained by enhanced epithelial-mesenchymal transition and increased expression of MMP2 [100]. In other study β_2 -ADR stimulation increase IL-6 production and VEGF production in a brain seeking variant of the MDA-MB-231, with no effect on cell proliferation [101]. The use of propranolol, an β -ADR antagonist, lead to a decrease in invasion [102]. Nevertheless, contrary effects of the β -ADR in Breast cancer were observed decrease on cell proliferation [103], increase apoptosis [104, 105] and decrease of invasion as a consequence of the decrease expression of CXCR4 [106].

In vivo evidence in mouse models are also conflicting. Chronic stress, obtained by restraint stress or social isolation, increase metastasis through activation of the β_2 -ADR stimulation of the stromal cells [88, 107] or the Breast cancer cell line, as β_2 -ADR knockdown of the Breast cancer cells abrogated the effect of chronic stress on tumour metastasis [100]. Other report indicated that β_2 -ADR stimulation decrease primary tumour growth that was eliminated by the use of propranolol (β -ADR antagonist). However treatment with the antagonist alone had an similar effect of the agonist on tumour growth [103]. This effect of propranolol was also seen in one patient with late stage Breast cancer hormone responsive tumour. A decrease in proliferation rates and increase in apoptotic markers where obtained in the biopsy tissue after

25 days of treatment with propranolol. It is in accordance with the effect in the MDA-MB-231 cell line when treated with propranolol [98]. In fact the use of beta-blockers is being linked with increase survival of breast cancer patients, since there is a reduction of metastatic burden and tumour recurrence [9-12] despite the lack of evidence in some studies[108].

In relation to α -ADR stimulation, the α_1 -ADR pathway seems to have no impact on Breast cancer [109]. In the other hand, α_2 -ADR stimulation increases [^3H]-thymidine incorporation in oestrogen and triple negative cell lines associated with a decrease in cAMP levels, which indicates a proliferative effect of this receptor. α_2 -ADR stimulation is also associated with an increase migration and invasiveness of breast cancer cells[110]. Moreover, tumorigenic breast cell lines are more sensitive to α_2 -ADR stimulation than non-tumour breast cell lines [92, 103, 111]. In one study serum from Breast cancer patients that receive dexmedetomidine (DEX - selective α_2 -ADR agonist) before surgery was collect 24 hours after and used to treated the MCF-7 cell line. As the outcome the MCF-7 cell line present a more aggressive phenotype due to increase proliferation, migration and invasion when compared to cells that were treated with serum retrieved from patients before DEX treatment and serum collected from patients that received a saline solution [112]. The effect of α_2 -ADR stimulation in Breast cancer pathogenesis is not only linked to the tumour cells. Spzunar *et al* inject 4T1 cells in female BALBc/cByl mice and observed and increase metastasis to the lungs when the animals were treated with DEX. As 4T1 cells do not response to sympathetic stimulation, the result was a consequence of the response of the stromal cells [113]. The same hypothesis was purposed by Lamkin *et al*, as the cancer cell line MDA-MB31HM is irresponsive to Epi or NE *in vitro*, despite that there was an increase of tumour growth when mice were subjected to chronic stress that was obviated by treatment with an α_2 -blocker [114]. Bruzzzone *et al* [115] demonstrated that the cancer associated fibroblasts are one of the cells responsible for this response through α_{2C} -ADR and α_{2B} -ADR. These point out to a stimulatory effect of α_2 -ADR in Breast cancer, that can be counterbalanced by the use of α_2 -ADR blockers. However, the α_2 - blockers need to be carefully investigated as in a mouse model of Breast cancer the use of efaroxan increase tumour growth and metastasis in normal conditions despite inhibiting the effect of chronic stress [114]. However, rauwolscine other α_2 -blocker seems to have no effect alone [103].

The effects of SNS in Breast cancer are still unclear. As various Breast cancer subtypes exist with different responses, conclusions need to be done carefully.

Table 2.3- Summary of the effects of SNS in Breast cancer.

	Cell line	Condition	Result
B- stimulation	In vitro	MCF7, ZR75 MDA-MB-361 MDA-453, MDA-435 MDA-468	β_2 -stimulation Increase DNA synthesis (Non estrogen lines MDA-453, MDA-435)
		MDA-MB-231	β_2 -ADR blockage Decrease DNA synthesis in all cell lines
			β_2 stimulation Increase of ki-67 marker Enhanced epithelial-mesenchymal transition Increased expression of MMP2 Increase invasion capacity Decrease invasion by the Nav1.5
		β_2 -ADR blockage (propranolol)	
		a brain seeking variant of the MDA-MB-231	β_2 -stimulation Increase IL-6 production and VEGF
		MDA-MB-231	β_2 -stimulation - ISO Decrease cell number Inhibition of DNA synthesis Decrease invasion by decrease in CXC4 expression
			β_2 -stimulation - NE
		MCF-10A	β_2 -stimulation - EPI decreasing cell proliferation and migration
			Sympathetic denervation of breast tumours in mice Decrease in tumour volume Decrease in tumour associated macrophages Suppression of immune checkpoints (PD-1, PD-L1, and FOXP3)
	In vivo		Social isolation Increase in tumour growth and an Increase of the tumour associated macrophage population M2 Increases lymphatic vasculature in the primary tumour, promoting increase lymph node metastasis and metastasis escape
α -stimulation		β_2 -KO MDA-MB-231	Chronic stress β_2 -KO Abrogated the effect of chronic stress
			β_2 -ADR blockage - Propranolol Decrease tumour size
	In vitro	MCF7	α_2 - stimulation - clonidine Increase cell proliferation Increase migration
		MDA-MB-231	Increase in cell migration
	In vivo	4T1	α_2 - stimulation -DEX Increase metastasis to lung
		MDA-MB31HM	Chronic stress with α_2 -ADR blockage Decrease tumour metastasis
			α_2 -ADR blockade - efaroxan Increase primary tumour size and metastasis
		MDA-MB-231	α_2 -ADR blockade- rauwolcisne Decrease tumour growth

2.3 - SNS in Breast Cancer Bone metastasis

As discussed above the sympathetic nervous system has a role in both bone remodelling and breast cancer cell metastatic capacity. Therefore, this raises the possibility that the sympathetic nervous system may have also a role in Breast cancer metastasis to bone. As the metastatic process is a multistep process, the SNS might have influence in the different stages. In light of that studies exploring this pathway showed that it is the influence of the SNS on stromal cells of the bone, specifically the osteoblast, that promotes the development of the

bone metastasis [116-118]. This increase metastatic potential, translated in an increase of the extravasation and homing of the breast cancer cells in the bone.

As previously discussed β_2 -ADR stimulation is associated with increased RANKL expression in the osteoblast. Furthermore, RANK/RANKL pathway is also interconnected with the migratory capacity of Breast cancer cell and invasion to bone tissues, increasing its metastatic burden in the bone niche [119]. Campbell *et al* [117] reported that chronic stress or ISO treated mice that were intracardiacally inject with MDA-MB-231VU, a bone tropic cell line, developed higher tumour burden in bone and presented increased osteolytic lesions. However, *in vitro* stimulation with ISO of MDA-MB-231VU decrease cell growth. In addition, subcutaneously inoculation or inoculation in the mammary fat pad decrease tumour volume, when mice were treated with ISO. This implied that the effect was on the colonization to bone of the Breast cancer cells and not in its capacity to proliferate and migrate from the primary site, and that it may be regulated by other cell types. As treatment with ISO before inoculation, increase the number, volume and area of the bone lesions whereas ISO treatment after inoculation only showed an increase in the size of the osteolytic lesions the effect of sympathetic stimulation would be in the stromal cells of bone, and the consequent homing of the Breast cancer cells. From a transwell assay the migratory capacity of the Breast cancer cells during ISO treatment was only increase when osteoblasts were present. This effect was abrogated with the use of OPG or when silencing the expression of the RANK receptor in the Breast cancer cells was applied. As β -stimulation is associated with an increase in the RANKL expression of osteoblasts, and the RANK/RANKL pathway is associated with increase breast cancer bone metastasis, these results imply that the RANK/RANKL pathway regulates the SNS effect of bone metastasis, specifically the homing.

Another study verifying that showed that β_2 -ADR stimulation lead to an increase sprouting of blood vessels in the skeleton, due to an increase in the vascular endothelial growth factor- α (VEGF- α) expression on osteoblasts and that there was an enhance in Breast cancer bone metastasis [116]. Another study also supported this effect of SNS in extravasation. The β_2 -ADR stimulation of bone marrow stromal cells increase the number of Breast cancer cells that attached to a monolayer of bone marrow-derived endothelial cells in a flow dynamic condition, due to an increase expression of the pro-inflammatory cytokines IL1- β and IL-6 by the osteoblasts, that activate the bone marrow endothelial cells [118].

Therefore, SNS seems to be implicated in the metastatic process of Breast cancer cells to the bone. In fact, targeting the SNS may be highly beneficial as it can reduce bone colonization of Breast cancer cells in two ways (blocking RANK/RANKL migration and decreasing bone vascular formation). However further studies are needed since the impact of α -ADR stimulation as not yet been delineated and a more detailed impact of β -ADR stimulation is still necessary in other cell types of the bone niche such as the osteoclasts. Moreover, the impact of SNS in the growth of the bone metastasis, through the direct effect on breast cancer cells present in bone, needs still to be unravelled.

Models of Breast Cancer Bone Metastasis

In vivo and *in vitro* models are used to study the bone metastatic niche in Breast cancer. Despite their limitations, the higher complexity of the *in vivo* models puts them as a fundamental research tool to understand the overall effect of breast colonization to bone and testing therapeutic approaches. In the case of the *in vitro* platforms, the simplicity achieved allows to thoroughly study the specific impact of a determined pathway, factor or cell-cell interaction. A multitude of *in vitro* models are at disposal where diverse settings can be rearranged to simulate a determined step. Microfluidic platforms are an interesting and recent *in vitro* model due to their unique characteristics such compartmentalization, implementation of dynamic conditions and recreation of close 3-dimensional (3D) environment.

3.1- Animal *in vivo* models

The Breast cancer metastasis to bone and the bone metastatic niche presents unique and peculiar characteristics, so choosing an animal model was to be carefully performed to better mimic the human process of interest as there is inherent genetic and phenotypic interspecies differences (Table 4.1). In this case mouse models are preferentially employed as they have higher genomic similarity and respond faster to pharmacological testing. Different inoculation routes of the cancer cells can also be applied leading to the recapitulation of different stages of the metastatic process. Orthotropic inoculations result in primary tumour growth and the consequent metastatic process. However, the primary tumour volume when metastasis start to appear is not ethically justifiable. This problem can be overcome by the resection of the primary tumour or inoculation of the cancer cells by intracardiac injection. In this case Breast cancer cells are directly injected in the blood circulation mimicking the process of homing, colonization and metastatic tumour growth. The interaction of the tumour cells within the bone environment, or the process of osteolytic lesion can be separately performed with an intraosseous injection in the tibias or femurs. However, during the process of inoculation displacement of bone marrow can occur, which may lead to a reparatory response that may alter the bone microenvironment. Despite that it allows fast tumour growth and the use of a known quantity of cells [120, 121].

Table 2.4- Models of Breast cancer and intrinsic advantages and disadvantages [122].

	Advantage	Disadvantage
Carcinogen-induced	Study of oncogenes or suppressive genes	Different potential to develop distant metastasis No currently available breast cancer bone metastasis
Spontaneous		Quick loss of oestrogen responsiveness No formation of bone metastasis
Genetically induced	Expression of the target gene can be reversibly controlled by external ligands Form distant metastasis	Low metastatic potential
Transplantation/inoculation	Mimic of a specific aspect of the metastatic process	No recapitulation of the complete metastatic process

The biologic relevance of the behaviour and response to a therapy of a tumour is dependent of the primary tumour, allocating further importance on the cell line used. Human Breast cancer cell lines or patient primary cancer cells can be applied improving the biologic similarity to human cancer. Although they need to be inoculated in immunodeficient mice, which consequently removes the immune role in the cancer setting. The presence of an immune system is therefore the major advantage for the use of immunocompetent mice and cancer cells of mouse origin, despite the fewer similar features to human breast cancer [120, 123]. Also human or breast cancer cells are usually bone tropic as the first common site of metastasis in mice are the lungs which could compromise the complete development of bone metastasis (Table 4.2).

Table 2.5- Mouse models for the establishment of Breast cancer bone metastasis. Adapted from [120, 123].

	Mouse strain	Cell type	Inoculation route	Metastatic site	Type of bone lesion	
Immunodeficient mice	BALB/c nude	MDA-MB-231	Intra-cardiac	Mouse long bones	Osteolytic	
		MDA-MB-231-BO2	Intra-cardiac	Mouse long bones spine and jaw	Osteolytic	
			Intra-tibial	Mouse tibiae		
			Intra-arterial	Mouse long bones		
		MDA-MB-231-IV	Intra-cardiac	Mouse long bones, spine and jaw	Osteolytic	
			Intra-tibial	Mouse tibiae		
			Intra-arterial	Mouse long bones		
			Intra-venous			
			Orthotopic	Mouse long bones Human bone X-plants		
		MDA-MB-436				
		MCF7	Intra-cardiac	Mouse long bones	Mixed	
			Intra-tibial	Mouse tibiae		
			Orthotopic	Mouse long bones		
		T47D	Intra-cardiac	Mouse long bones	Mixed	
			Intra-tibial	Mouse tibiae		
			Orthotopic	Mouse long bones		
		ZR-75-1	Intra-cardiac	Mouse long bones and spine	Mixed	
	NOD/SCID	MDA-MB-231	Orthotopic	Mouse tibiae	Osteolytic	
		MDA-MB-231-IV	Orthotopic	Human bone x-plants	Osteolytic	
		SUM 1315	Orthotopic Intra-tibial	Human bone X-plants Mouse tibiae	Osteolytic	
		PDX	Orthotopic	Mouse long bone, lungs, liver, spleen	Mixed	
			Intra-tibial	Mouse tibiae		
		T47D	Orthotopic	Mouse long bones Human bone x-plant	Mixed	
	MF1 nude	MDA-MB-231	Intra-tibial	Mouse tibia	Osteolytic	
		MDA-MB-436	Intraosseous	Mouse tibiae	Osteolytic	
	NSG	MDA-MB-231	Intra-tibial	Mouse tibiae	Osteolytic	
			Orthotopic	Human bone x-plants		
Immunocompet	BALB/c	4T1	Intra-cardiac	Mouse long bones, spine, jaw and lungs	Osteolytic	
			Intra-tibial	Mouse tibiae		
			Orthotopic	Mouse long bones spine, jaw, lungs and spleen		

	KEP	Orthotopic	Mouse long bones and spine	Osteolytic
		Intra-tibial	Mouse tibia	
FVB/N	PyMT MMTV	Intra-cardiac	Mouse long bones, spine, jaw and lungs	Osteolytic
		Intra-tibial	Mouse tibiae	
C57BL/6	E0771	Intra-cardiac	Mouse long bones, spine, jaw and lungs	
		Intra-tibial	Mouse tibia	Mixed
		Orthotopic	Mouse long bones and lungs	
	Py8119	Intra-cardiac	Mouse long bones, spine, jaw and lungs	Mixed
		Intra-tibial		
		Orthotopic	Mouse tibia	
IL2RYC ^{-/-}	KEP	Intra-cardiac	Mouse long bones	Osteolytic
RAG ^{-/-}	KEP	Orthotopic	Spine	Osteolytic

4.2- Microfluidic models

Microfluidic platforms allow high-throughput analyses, specific control over the experimental environment and use of patient derived cells, being also easy to handle and design. To address the bone metastatic niche, first there is a need to correctly simulate the bone environment that is a complex system where a panoply of cells are present (osteoclasts, osteoblasts, immune cells, endothelial cells), through compartmentalization in the microfluidic device the use of different cell types is facilitated [124].

In the case of Breast cancer bone metastasis, microfluidic approaches allow to carefully and meticulously study pathways from different stages of the metastatic process. For the case of the homing the use of separated compartments allows to mimic different tissues and study the preferential metastatic site of the Breast cancer cell line, in dynamic conditions [125]. Representing the vasculature is a major aspect when studying extravasation as it is possible to take into account the interactions of the Breast cancer cells, endothelial cells and bone cells. In the microfluidic platform of Bersini *et al* [126] as three connected and independent channels are design, it is possible to form a vessel like structure and a bone niche and infer about the process/signalling of extravasation. This microfluidic device allowed also to have different structural forms of the endothelial vessel, which increases the resemblance to *in vivo* [127], as 3D conformations has implications in cell behaviour.

The dynamic conditions are also important in cell behaviour, an implementation on the microfluidic is one of the major advantages. Mei *et al*, took advantage of that and designed a microfluidic device with two independent channels, where they seeded on one of the channels the endothelial cells to form a vessel like structure and in the other channel osteocyte like cells. Osteocytes were mechanically stimulated, by physiologically relevant oscillatory fluid flow, which decrease the extravasation of the breast cancer cells onto the osteocyte compartment [128]. Simpler microfluidic settings can be done, as in the case of Hao *et al* [129]

that used a microfluidic chip consisting only in a central reservoir were they allowed osteoblasts to produce bone matrix. After that, introduction of Breast cancer cells resulted in matrix degradation and formation of invadopodia in these cells to reach into deeper layers. This was a simple way to access the osteoblast - Breast cancer cell interaction, since it did not take into account innervation or vasculature.

In relation to the potential of innervation in the Breast cancer bone metastasis it was not yet been explored in the microfluidic platforms. Despite that microfluidic devices are already used to study the impact of osteoblasts in sensory innervation. A microfluidic chip with two independent channels connect with microgrooves was design. These microgrooves allowed the extension of the axons from the neural side to the osteoblasts/bone side. In one compartment a dorsal root ganglia neuron was seeded, where in the other channel MSC were seeded. As osteoblast differentiation takes place the axonal growth was repelled, being show by the retraction of the axons in the microgrooves [130]. In other study sensory innervation had a positive impact in the differentiation of the MSC to the osteoblastic lineage [131].

As new information is gathered, new therapeutic approaches can be developed to target specifically interactions. Microfluidics allow to control chemical gradients, replicate disease stages and allow patient derived cells use, which makes them a valuable tool in drug developing and testing. The microfluidic platforms are a new and simpler tool to research. As the possibility of specifically design the structure, the ease control experimental parameters and increase complexity due to an increase of cell types in 3D culture makes these platforms a key tool to bridge the gap between the *in vitro* 2D models and the *in vivo* conditions.

Chapter 3

Methods

1 - Cell cultures

1.1 - Bone tropic-Breast cancer cell cultures

The mouse breast cancer bone tropic 4T1.2 cell line, is a cell line derived from the parental cell line 4T1 (breast cancer cells) after repeated cardiac inoculation in a mouse, and collection at the bone marrow and in vitro expansion [132], was seeded on 75 cm² flask with complete α -MEM (Gibco) (supplemented with 10% fetal bovine serum (FBS) (Biowest) and 1% Pen/Strep (Labclinics)). Cells were kept at 37°C in a 5% CO₂ atmosphere and medium was changed twice a week.

1.1.1 - Conditioned medium (CM) collection

When cells achieved confluency, they were detached with 0.25% trypsin (Sigma) and resuspend in the respective medium. Cell suspension was seeded at 1×10^4 cells/cm² in T-75 cm² cell culture flasks with complete α -MEM. Cells were kept at 37°C in a 5% CO₂ atmosphere for 24 hours. Cells were treated with 10^{-7} M of JP302 - α_{2C} -ADR antagonist, 40 minutes before treatment with α_{2C} -ADR antagonist NE (0M, 10^{-9} M and 10^{-5} M) and, in serum-free medium (0.5% FBS) for 24 hours at 37°C in a 5% CO₂ atmosphere. After that the conditioned media (CM) was collected, centrifuged at 1200 rpm for 5 min at 4°C (to eliminate cell debris), and kept at -80°C until further use.

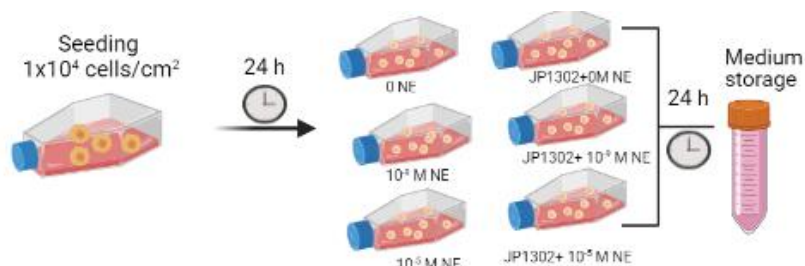


Figure 3.1 - Schematic representation of the NE treatment and condition media collection from 4T1.2 cell line.

1.2 - Osteoblast cultures

Osteoblastic cells were derived from UAMS-P32, a mouse bone marrow stromal cell line stably transfected with a reporter construct derived from a BAC containing the entire murine RANKL gene as well as 120 kb of 5'-flanking region and 14 kb of 3'-flanking region (BAC1). This cell line was kindly provided by Dr. Charles O'Brien (Center for Osteoporosis and Metabolic Bone Diseases, University of Arkansas for Medical Sciences, Little Rock, AR). The UAMS-P32 cell line was seeded on 75 cm² flask with complete α -MEM. Cells were kept at 37°C in a 5% CO₂ atmosphere and medium was changed each two days.

1.2.1 - α_{2C} -ADR agonist - NE and antagonist - JP1302 treatment and CM treatment

When cells achieved confluence, cells were detached with 0.25% trypsin, centrifuge at 1200 rpm for 5 min at RT and suspend in the respective medium. A cell density of 10×10^4 cells/ml was seeded in 24 well-plates. Cells were left to adhere for 24 hours in complete α -MEM at 37°C in a 5% CO₂ atmosphere before treatment. Then media was changed and cells were treated with the α_{2C} -ADR antagonist (10^{-7} M of JP1302) 40 minutes before treatment with the α_{2C} -ADR agonist - NE (0M, 10^{-9} M and 10^{-5} M). After 24 hours, the cells were washed with PBS and incubate for 10 minutes with 100 μ l of 1x reporter Lysis Buffer (RLB) to promote cell lysis. Cell lysate was storage at -80°C for at least 24 hours. After which was applied a vortex of 10-15 seconds and a centrifugation at 12000 rpm for 2 minutes at 4°C. The supernatant was transfer to a new microtubule and stored at -80°C until further use.

For the treatment with the CM the same experimental setup was applied however; cells were left to adhere for 48 hours before treatment with the CM retrieved from the 4T1.2 cell line previously treated with the α_{2C} -ADR agonist and antagonist. The effect of the α_{2C} -ADR pathway in the osteoblast and the effect of the α_{2C} -ADR pathway through the bone tropic Breast cancer cell line were evaluated by the RANKL expression in this cell line.

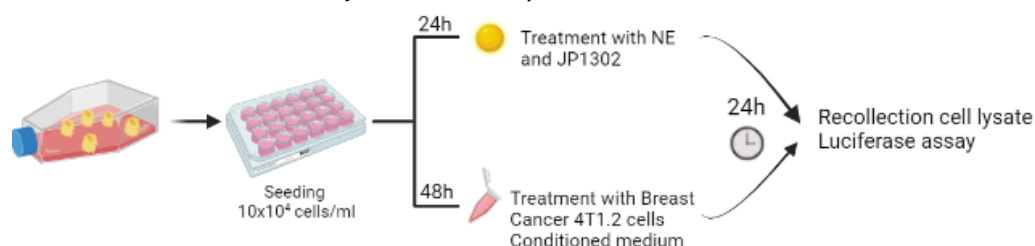


Figure 3.2 - Schematic representation of the NE treatment and CM treatment of UAMS cell line.

1.2.1.1 - Luciferase Assay

In a white/opaque 96-well plate it was added 20 μ l of cell lysate and 100 μ l of reconstitute luciferase assay reagent (Promega). A 2 second measurement delay followed by a 10 second measurement read was performed to quantify luciferase activity. The relative luminescence units (RLU) were normalized to the amount of protein. The protein quantification was performed with BioRad Quantification Kit and the absorbance read at 655nm in a microplate reader.

1.3 - Osteoclast culture

1.3.1 - Isolation

The mouse osteoclast culture was performed as previously described [133]. Femurs and tibias were obtained from Balb/c female mice. Bone marrow was flushed and filtered with a 70 μ m filter to obtain bone marrow cells. Cells were centrifuge at 1600 rpm for 5 minutes and the pellet was resuspended in 3 ml of ACK lysing Buffer (Invitrogen) for 1 minute, after which was added 2 ml of complete α -MEM. Cells were again centrifuge, resuspended and incubated for 24 hours in complete α -MEM supplemented with 10ng/ml of M-CSF (PreproTech). Afterwards, the non-adherent cells were collected to a 50 ml falcon tube and centrifuge with the same parameters, resuspended and incubated for 72 hours in complete α -MEM supplemented with 30ng/ml of M-CSF to generate bone marrow derived macrophages.

After this incubation, cells were washed with fresh complete α -MEM, detached with a cell scraper, centrifuge at 1600 rpm for 5 min and counted in a Neubauer chamber.

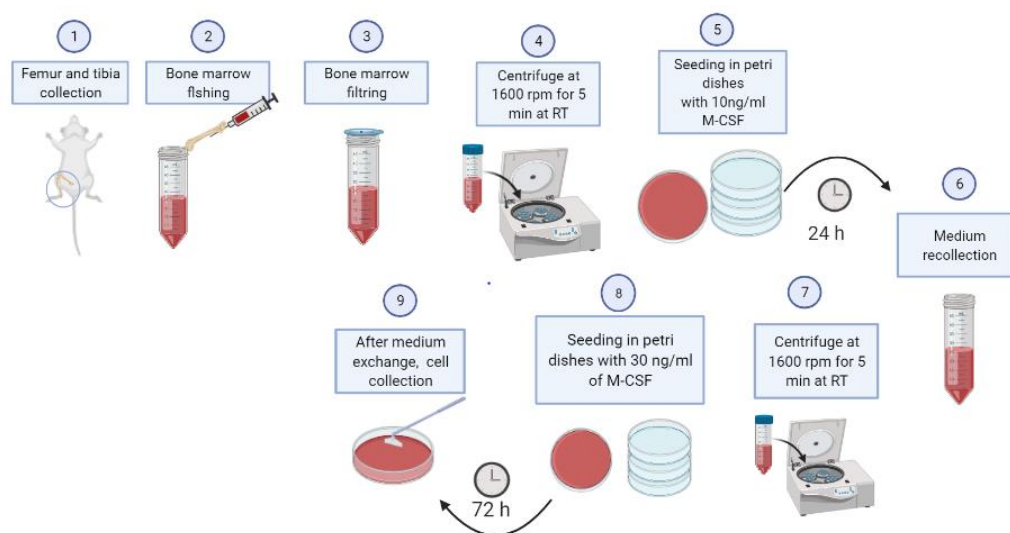


Figure 3.4 - Schematic representation of the osteoclast isolation in the mouse.

1.3.2 - α_{2C} -ADR agonist - NE and antagonist - JP1302 treatment and CM treatment

A cell density of 1.7×10^4 cells/well was seeded in 96-well plates. Cells were seeded with complete α -MEM supplemented with 30ng/ml of M-CSF and 100 ng/ml of RANKL. 4 hours after seeding it was added the α_{2C} -ADR antagonist (10^{-7} M JP302) 40 minutes before treatment with the α_{2C} -ADR agonist NE (0M, 10^{-9} M and 10^{-5} M) to the monoculture. Cells were kept at 37°C and 5% atmosphere. For the differentiation assay, media was exchange at day 3. After 4 days in culture, cells were fixed with 4% Paraformaldehyde (PFA) (Sigma) to immunocytochemistry. In the case of the resorptive activity, cells were seeded on top of bone slices, that were previously incubated in complete α -MEM for 72 hours. Media was change at day 2 and 4. After 7 days of culture, CM was retrieved and bone slices were stained with toluidine blue.

For treatment with the 4T1.2 CM, a cell density of 1.7×10^4 cells/well was seeded in 96-well plates. Cells were seeded with complete α -MEM supplemented with 30ng/ml of M-CSF and 100 ng/ml of RANKL. 4 hours after seeding it was added the CM from the 4T1.2 cells to the

monoculture in 50% of the total volume. The same timeline was applied for the differentiation and resorptive activity evaluation.

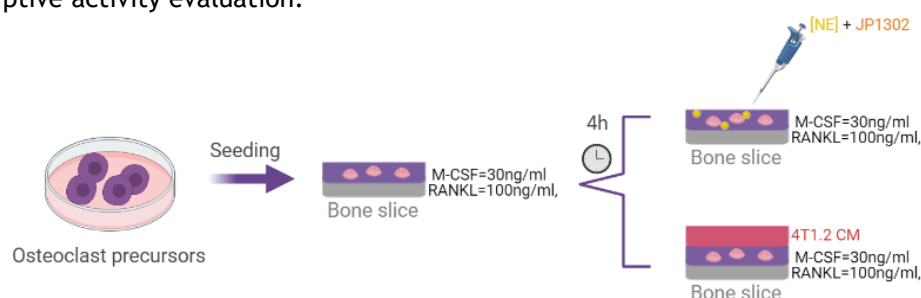


Figure 3.5 - Schematic representation of the NE treatment and CM treatment of monocultures of mouse osteoclast.

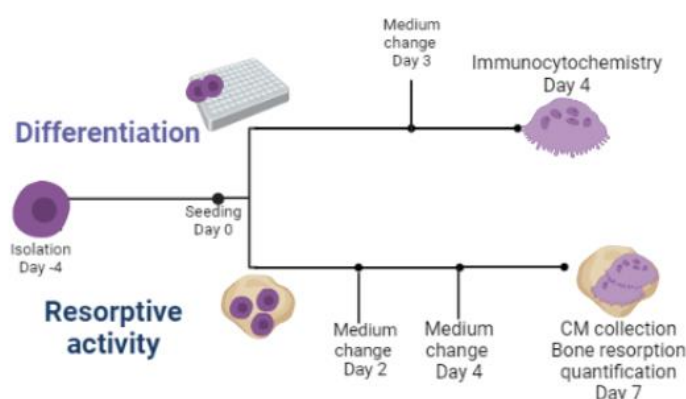


Figure 3.6 - Schematic representation of the timeline of the monoculture of the mouse osteoclasts.

1.3.2.1 - Osteoclast resorptive activity

After 7 days of culture, the medium was collected from the bone slice cultures and stored at -80°C until further use. Three bone slices were washed and left with deionized water for 24 hours. To remove the cells from the bone matrix, deionized water was added to the top of the bone slice and a cotton bud and a clamp were used to detach any cell debris. The bone slices were stained with toluidine blue solution (Sigma-Aldrich) for 15 seconds, and excess solution was removed with a paper towel. The resorption events were quantified by the percentage of total reabsorbed surface, using a 100-point grid (Pyser optics, G50 graticule) placed in the ocular of an optic microscope with a 10x objective. Resorption events were only quantified when they hit the centre of the cross.

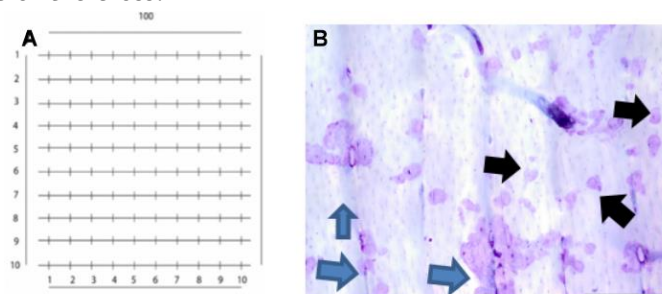


Figure 3.7 - Representative image of bone resorption caused by active osteoclast. A) A 100-point grid is placed in the ocular of the microscope to count the resorption. B) OC seeded on bone slices degrade the bone, forming pits (black arrows) and trenches (blue arrows). (Scale bar 200 μm)

1.4 - Osteoblasts/Osteoclasts co-culture

To mimic the bone niche, it was performed a co-culture of UAMS-32P, a mouse stromal/osteoblastic cell line, and mouse osteoclasts. The monocytes were seeded at a cell density of 1.7×10^4 cells/well, in complete α -MEM supplemented with 10 mM β -Glycerophosphate (β -GP) (Sigma), 50 μ M Ascorbic Acid (AA) (Sigma) and 0.01 μ M Vitamin D3 (Supelco). 4 hours after seeding of the osteoclast lineage, the osteoblastic cell line was seeded at a cell density of 3.4×10^3 cells/well.

1.4.1 - α_{2C} -ADR agonist - NE and antagonist - JP1302 treatment and CM treatment

For understanding the direct effect of the α_{2C} -ADR pathway in the co-culture it was added the α_{2C} -ADR antagonist (0.1 μ M JP1302) 40 minutes before treatment with the α_{2C} -ADR agonist NE (OM, 10^{-9} M and 10^{-5} M) and its' effect through the bone Breast cancer cells it was added the 4T1.2 CM in a percentage 50:50. The same experimental setup of the osteoclast monoculture was applied.

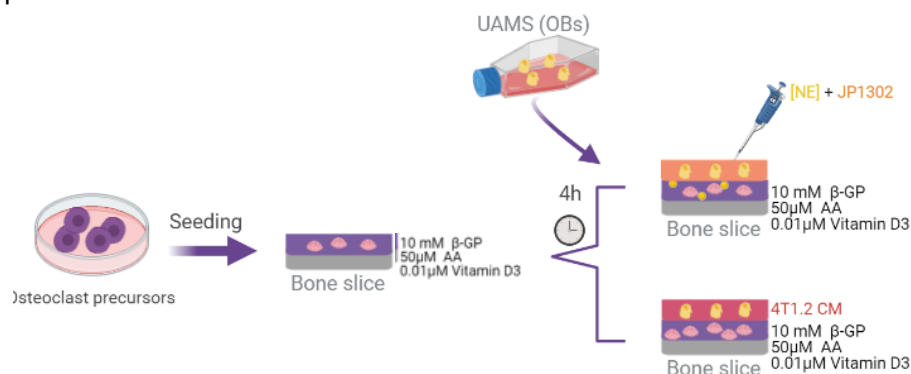


Figure 3.8 - Schematic representation of the NE treatment and CM treatment of co-culture of mouse osteoclast and UAMS.

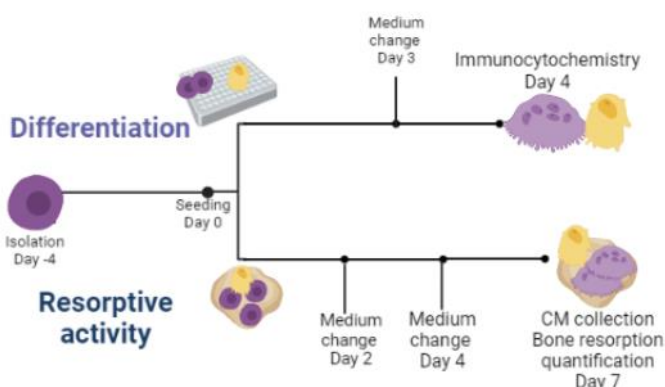


Figure 3.9 - Schematic representation of the timeline of the co-culture of the mouse osteoclasts and osteoblasts.

2 - Proliferation assay - ki67 expression

After 24 h of culture, cells were washed with PBS 1x and fixed for 10 minutes in 4% PFA. Cells were washed and left in PBS until the start of the immunocytochemistry protocol. Once PBS was removed, cells were incubated with 0.1% triton X-100 (VWR) for 10 minutes at RT. Cells were washed twice with PBS, and incubated in 1% Bovine Serum Albumin (BSA) (Merck) in PBS for 1 hour at RT, to block unspecific antibody binding. After removing the BSA, cells were incubated for 24h with primary antibody rabbit anti-ki67 (1:100) at 4°C.

On the next day, cells were washed two times with PBS to remove excess of primary antibody, followed by a 60 min incubation with the secondary antibody anti-rabbit 568 (1:1000) diluted in blocking buffer. Cells were washed again with PBS and were incubated with DAPI (1:10 000) for 5 minutes. Cells were washed and kept in PBS at 4°C, protected from light, until further analysis. The staining's were assessed in a IN Cell Analyser 2000.

3 - Live/Dead assay

After 24 h and 72 hours of culture, cells were rinsed with warmed PBS (37°C). Cells were incubated with Calcein AM (1:1000) for 20 minutes at 37°C in a 5% CO₂ atmosphere. Cells were washed with warmed PBS and incubated for 5 minutes with Propidium Iodide (PI) (1:500) for 5 minutes at 37°C in a 5% CO₂ atmosphere. Another washing step was performed and cells were visualized under fluorescence light in an inverted microscope.

4 - Cell viability- Resazurin assay

After 24 h and 72 hours of culture, it was added 10%(v/v) of resazurin per well and cells were incubated for 4 hours at 37°C in a 5% CO₂ atmosphere. Medium was retrieved and transfer to a black 96 well-plate. Fluorescence was measured (λ_{ex} =530nm, λ_{em} =590nm) in the microplate reader.

5 - Immunocytochemistry analysis

After 4 days in culture, cells were washed with PBS 1x and fixed for 10 minutes in 4% PFA. Cells were washed and left in PBS until the start of the immunocytochemistry protocol. PBS was removed and cells were incubated with 0.1% triton X-100(VWR) for 10 minutes at RT. Cells were washed twice with PBS, and incubated in 1% Bovine Serum Albumin (BSA) (Merck) in PBS for 1 hour at RT, to block unspecific antibody binding. After removing the BSA, cells were incubated for 30 minutes with flash phalloidin green 488 (1:100) at RT. After which two washing steps were performed and cells were incubating with DAPI (1:10000) for 5 minutes. Cells were washed and kept in PBS at 4°C, protected from light, until further analysis. Images were acquire using a IN Cell Analyser 2000.

6 - TRAP activity

TRAP solution containing L- Ascorbic acid (Sigma), DiSodium tartrate (Sigma), 4-nitrophenylphosphate (Sigma), Acetate, Triton X-100, NaCl, EDTA pH=5.5 and MilliQ water, was added to CM of osteoclasts seeded on bone slices, in a 96-well plate. After 40-60 minutes of incubation in the dark at 37°C, NaHO solution was added to stop the reaction. Optical absorbance was measured at 405 nm and TRAP activity was measure against a standard curve of p-nitrophenol solution.

7 - Microfluidic device

7.1 - Preparation of the microfluidic device

Polydimethylsiloxane (PDMS) and the Curing agent (Dow Corning) were weighed in a 10:1 proportion, respectively, and thoroughly mixed. Afterwards, the solution was put in a vacuum chamber and a vacuum pump to allow degassing of entrapped air. Degassed PDMS was poured into the microfluidics mould and polymerized for 1h30 - 2h, at 70°C. The microfluidics were cut out of the mood and punches were made to create the medium reservoirs (Figure 18).

The microfluidic chips were cleaned of dust with tape and placed on top of glass coverslips. The coverslips were washed in Alconox (Sigma) 1% for 2h, at 65°C and 65rpm. Afterward, glass was washed thoroughly in tap water, dH₂O and lastly with EtOH 70%. Then, it was left to dry and sterilized with 15min of UV light. The coverslips were then incubated for 30 min, at 37°C with Poly-D-lysine (PDL) (Sigma) solution and washed with sterile dH₂O. Once dried, the PDMS chips were placed on top and presses gently with forces, to guarantee properly adherence. Once the PDMS chips were prepared, they were transferred to 6 well plates and exposed to UV light for 15 min to sterilize.

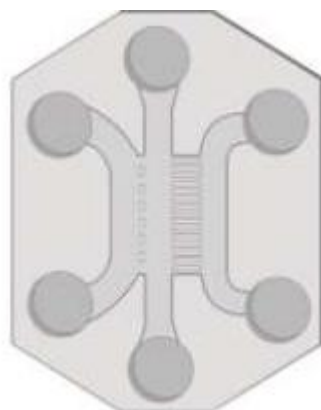


Figure 3.10 -Schematic representation of the microfluidic device.

7.2 - Seeding of 4T1.2 cell line in the microfluidic device - Migration assay

To perform the invasion assay, Matrigel was used to mimic extracellular matrix. Matrigel was added in a percentage of 50:50 to α -MEM complete medium in the microfluidic central channel. To allow polymerization of the Matrigel the microfluidic device was incubated for 5 min at 37°C.

The bone tropic Breast cancer cells (4T1.2 cell line) were seeded in left channel, which is separated from the central channel by structural pillars. Before cell seeding, the channel was washed two times with deionized water, and incubated with PDL for 15 min at 37°C to optimize cell adhesion. Two washing steps with deionized water were performed before medium was added to the channel. Medium was also added to the right channel and to the 6 reservoirs. Then the microfluidic device was incubated for 24 hours at 37°C.

4T1.2 cells were seeded at 7.5×10^4 cells/5 μ l, and left to adhere for 1 hour at 37°C in a 5% CO₂ atmosphere before adding α -MEM complete medium to the reservoirs. 4 hours after seeding α -MEM complete serum free medium was added in the microfluidic channels, and the stimulus was applied to the cells by adding the α_{2C} -ADR antagonist (10^{-7} M JP1302) and α_{2C} -ADR agonist NE (0M, 10^{-9} M and 10^{-5} M). Cells were incubated for 72 h at 37°C in a 5% CO₂ environment. At day 3 of culture, cells were fixed with 4% PFA to immunocytochemistry.

8 - *In vivo* intratibial model of Breast cancer bone metastasis

All animal procedures were approved by the i3S ethics committee and by the Portuguese Agency for Animal Welfare (Direção-Geral de Alimentação e Veterinária) in accordance with the EU Directive (2010/63/EU) and Portuguese law (DL 113/2013). Mice were housed at 22°C with a 12 h light/dark cycle with ad libitum access to water and food.

Immunocompetent Balb/c 4- to 6-week-old female mice were injected in the tibia with 10 μ l of 4T1.2 cells in a density of 1×10^5 cells (bone metastasis (BM) group). As a control, the vehicle PBS was injected in another group of animals (sham-operated (SO) group). BM and SO group of mice were sacrificed at day 3, 5, 7 and 14 and tibias were collected, cleaned off muscles, snap frozen at -20°C in PBS and embedded in gelatin before being cut on a cryostat.

9 - Immunohistochemistry analysis

The immunohistochemistry on frozen sections of the samples obtained from the mice model was performed as previously described. Briefly, 50 μ m-thick frozen sections were left to defrost for 20 min at RT. The cuts were then rehydrated with PBS, for 5 min and incubated with a solution of 0.2% Triton in PBS for 20min, at RT. The sections were incubated with blocking buffer (10% FBS, 1% BSA, 0.2% Triton X-100 in PBS) for 60 min, followed by the incubation with the primary antibodies diluted in the blocking buffer, overnight, at 4°C. The sections were incubated with rabbit anti-TH (1:100) or rabbit anti- α_{2C} -ADR (1:100).

The next day, the slides were washed 3 times with PBS for 5 min, followed by a 75min incubation, at RT, with the secondary antibody anti-rabbit 568 (1:1000) diluted in blocking buffer. Sections were then washed 3 times in PBS for 5 min and incubated with DAPI (1:10 000) for 10 min. The washing step was repeated, and the slides were mounted with Fluoroshield Mounting Medium. Images were taken at a Leica TCS-SP5 inverted confocal microscope (Leica Microsystems).

10- Nerve fiber quantification

The images obtained from the immunohistochemistry analyse were open in ImageJ/Fiji. The red channel image was Z-stacked and transformed in an 8-bit image. The plugin - Neuroanatomy SNT was used to delineated the nerve fibers. After, an analyse was performed choosing the skeletonize and the values for the maxim length were retrieved. The nerve fiber length values were divided by the total area of the picture, and the result was summed with all values that belonged to the same animal.

11- Statistical Analysis

The statistical significance analysis was performed using GraphPad Prism version 8.02 (GraphPad Software, Inc.) through one-way analysis of variance (ANOVA), Kruskal-wallis or Kolmogorov test with comparisons of the means of preselected pairs of columns. The p-values lower than 0.05 were considered statistically significant. Between two statistically significant groups, it was used the following notation: * $p < 0.05$; ** $p < 0.01$.

Chapter 4

Results and Discussion

1 - The role of α_{2C} -ADR signalling pathway in the bone niche

As previously mentioned, the α -signalling pathway can be activated in the different bone resident cells [13]. Despite the great attention being drawn to the effects of the SNS in bone remodelling, not much is known regarding the effects of alpha-ADRs signalling on bone remodelling, as most studies have been predominantly focused on the β_2 -ADR signalling pathway [13]. Nevertheless, the α_{2C} -ADR signalling pathway may offer important knowledge to the complex interactions of SNS and bone, as this ADR is crucial in cases of sympathetic hyperactivation [44, 47, 134]. Furthermore, its' ablation prevented thyrotoxicosis-induced osteopenia, despite the heterogenic phenotype of bone in young mice [82].

In light of that part of the current project focused in the study of the impact of the α_{2C} -ADR signalling pathway in the bone niche, specifically in the osteoclast activity (Figure 4.1). For that, NE was used as the agonist, and two different concentrations were used $10^{-5}M$ and $10^{-9}M$, since it is described that higher doses of NE (like $10^{-5}M$) preferentially bind to β -ADR and lower doses (like $10^{-9}M$) bind to the α -ADR, which allowed to explore both effects of SNS [48]. As NE is not a specific agonist to the α_{2C} -ADR, JP1302 was used as a specific antagonist [135], which by binding to the ADR unable agonist binding and consequent activation of the signalling pathway.

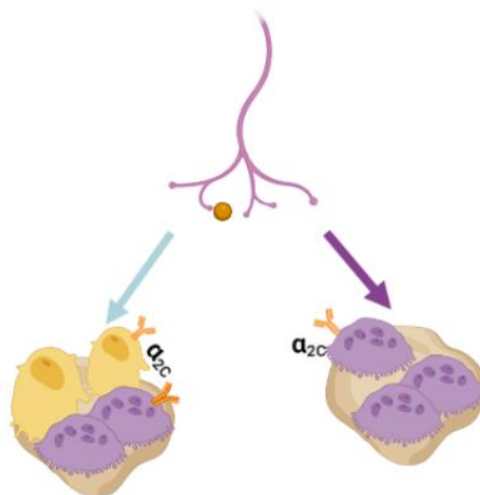


Figure 4.1- Exploring the role of SNS through the of α_{2C} -signalling pathway on osteoclast activity. Targeting the osteoclast directly (purple arrow) or the bone niche (blue arrow). This treatment allows to evaluate how SNS, through the α_{2C} -signalling pathway, impacts osteoclast activity.

1.1 - Effects of α_{2C} - signalling pathway on osteoclast activity

1.1.1 - Establishment of the osteoclast culture and assessment of osteoclast morphology

For this dissertation, murine osteoclasts were derived from primary cells extracted from mouse bone marrow. To generated mature osteoclast, from the obtained bone marrow-derived monocytes (Figure 4.2(A)), cells were culture with 30 ng/ml M-CSF and 100 ng/ml RANKL for 4 days. At day 3 medium was changed and pre-osteoclast are aggregated (Figure 4.2(B)), as the osteoclast is a multinucleated cell formed by cell-cell fusion [29]. In some cases, at day 3 is also possible to note the presence of small osteoclasts. At the end of the process (at day 4-5 of culture) large multinucleated cells can be visualized (Figure 4.2(C)).

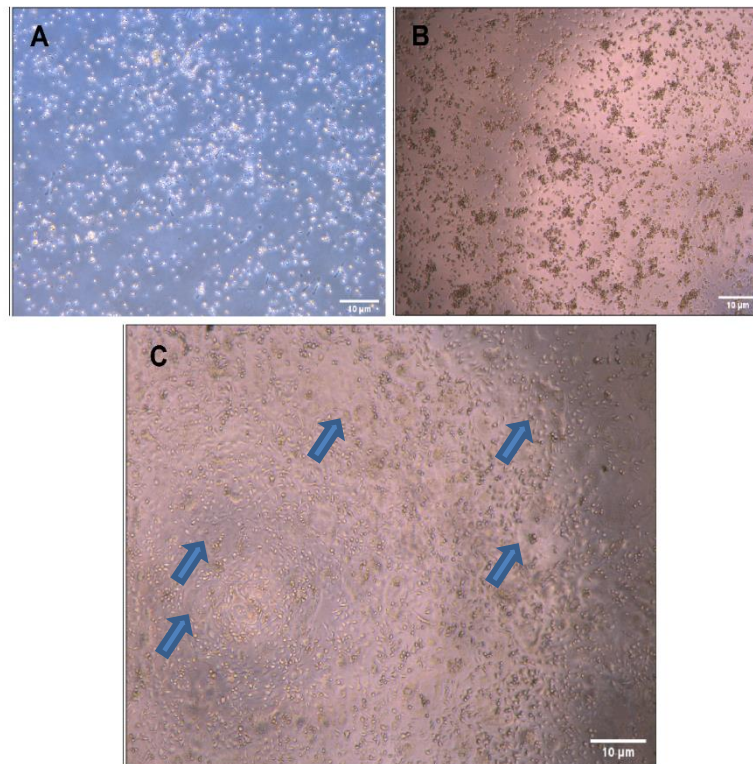


Figure 4.2- Representative images of the osteoclast morphology in the different phases of osteoclastogenesis. (A) Day 0 - bone marrow-derived monocytes that are round or elongated multinucleated cells. (B) Day 3 - presence of pre-osteoclast aggregates (C) Day 4- large and multinucleated osteoclast (blue arrows). (Scale bar =10µm).

Mature osteoclasts are giant multinucleated cells (Figure 4.3), and their resorptive activity is closely linked to the reorganization of their cytoskeleton [29]. In Figure 4.4, different cytoskeletal arrangements of the osteoclast are present as actin rings, podosomes or filipodia can be visualized in mature osteoclasts.

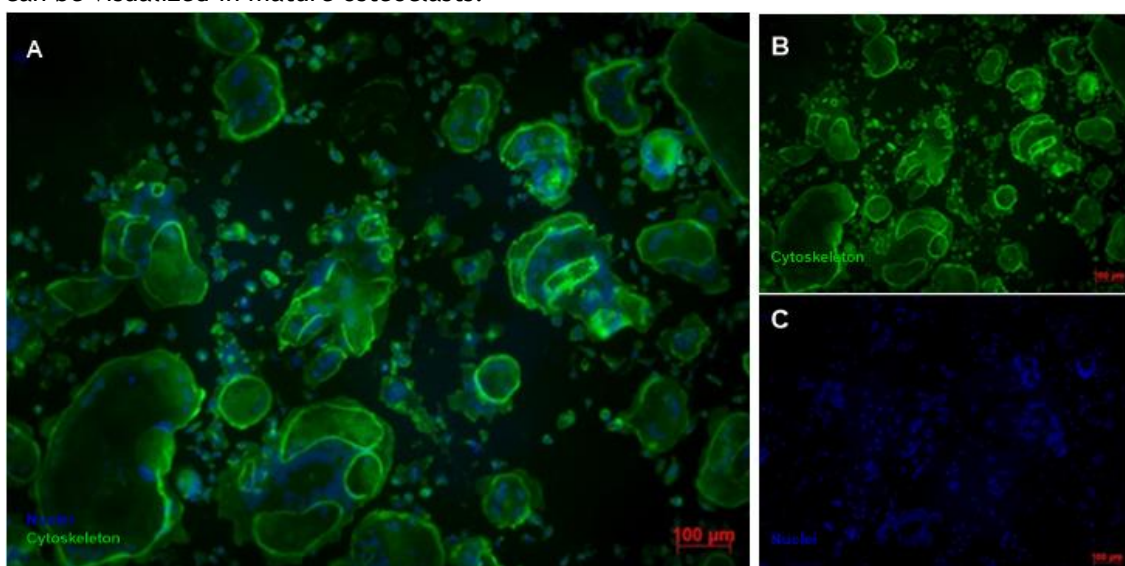


Figure 4.3 - (A)Representative image of day 5 of osteoclast differentiation with F-actin staining. The osteoclast skeleton is represented in green stained with Alexa-Fluor 488(B) and the nuclei in blue with DAPI (C). (Scale bar =100µm)

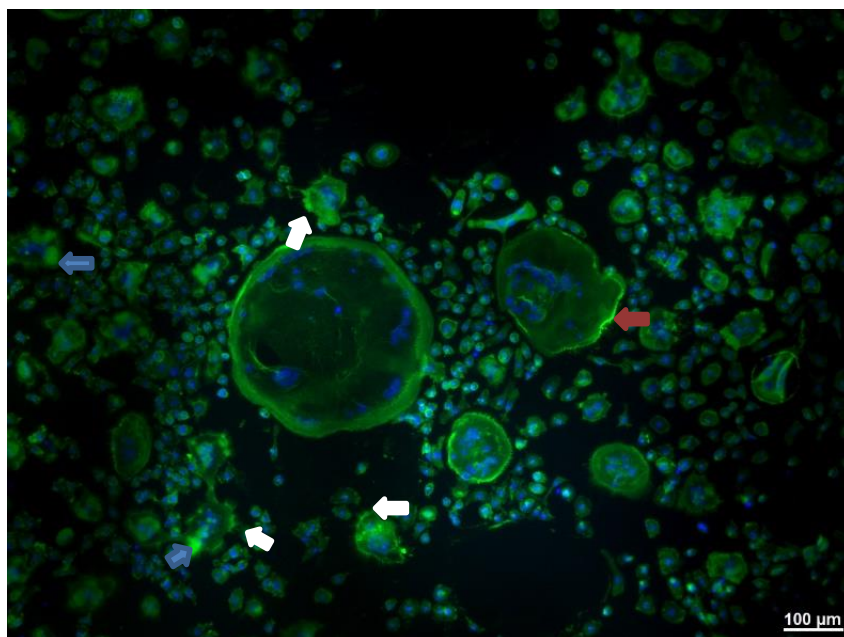


Figure 4.4 - Representative image of the different osteoclast characteristic cytoskeletal structures. Blue arrow: podosomes, white arrows: filopodia, red arrow: actin ring. (Scale bar =100μm)

Another important factor to fully activate the osteoclast is the presence of bone matrix. When osteoclasts are in contact with bone matrix, they polarize their skeleton and are able to reabsorb matrix. There are two types of resorptive events: pits, that are circular excavations, or trenches, that are long and continuous excavations. Osteoclasts when producing trenches have a more aggressive phenotype, since they can reabsorb for longer periods of time, they are able to migrate while reabsorbing and there is a higher rate of collagenolysis in relation to demineralization. This kind of resorptive event increases bone fragility. One interesting aspect is that osteoclasts can switch between these different modes of resorption [136, 137].

In relation to the monoculture, the bone marrow-derived monocytes are seeded on top of bone slices, to mimic the *in vivo* matrix surface, for 7 days (Figure 4.5).

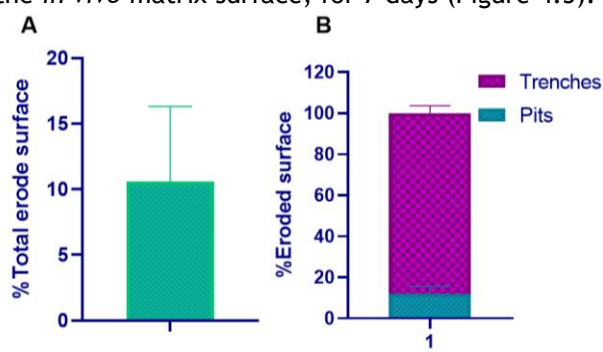


Figure 4.5 - Monoculture establishment resorption. A) Percentage of the total eroded bone surface; B) Pit and trench surface percentage in the total eroded surface; (n=1 with 5 replicates).

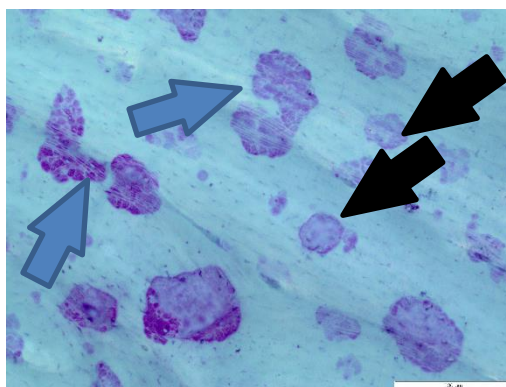


Figure 4.6 -Exemplificative image of the resorptive events: pits (black arrows) and trenches (blue arrows) (Scale bar=200 μ m).

1.1.2 -Osteoclastogenesis/Differentiation

The process of bone remodelling is a balance between the osteoclast function of bone degradation and the process of matrix formation by the osteoblasts [35]. In different bone destructive diseases, there is a disequilibrium once the osteoclast activity is greatly stimulated and osteoblast activity impaired. Mostly, this increase in bone resorption is due to the higher concentration of factors that potentiate osteoclast differentiation and activity, which makes osteoclasts an indirect target [31]. In the case of bone remodelling, when the SNS is brought forward the same behaviour is contemplated in the osteoclast [66-68]. But as more information is gathered, a direct influence of the SNS on the osteoclast is being uncover [67, 73-75].

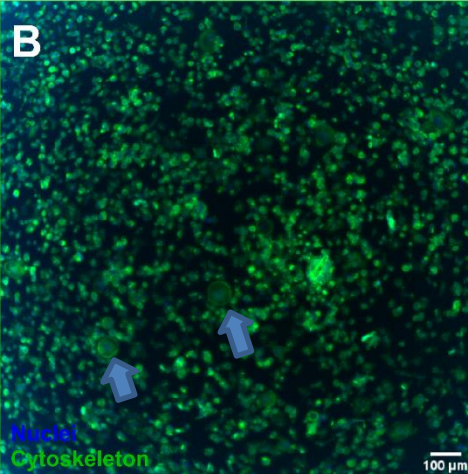
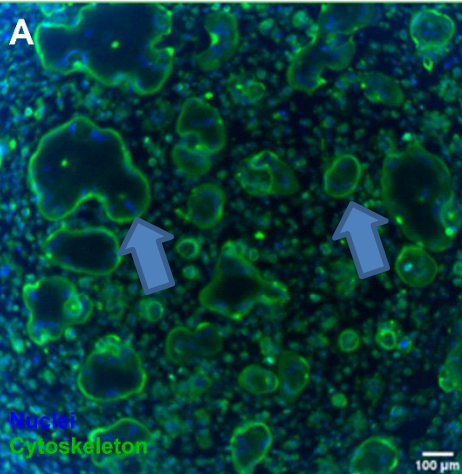
For understanding the direct effect of the α_{2C} -ADR signalling pathway in osteoclastogenesis, the bone marrow derived macrophages were seeded for 4 days with 30 ng/ml M-CSF and 100 ng/ml RANKL in all conditions: (i) 0M NE; (ii) 10^{-9} M NE; (iii) 10^{-5} M NE; (iv) 10^{-7} M JP1302; (v) 10^{-7} M JP1302+ 10^{-9} M NE; and (vii) 10^{-7} M JP1302+ 10^{-5} M NE. The antagonist and agonist were added 4 hours after the seeding, to ensure that it had no impact in osteoclast adhesion to the substrate.

As can be observed in Figure 4.7, when NE is added, the formation of the osteoclasts seems to remain unaltered, as large multinucleated, usually with more than 9 nuclei, osteoclasts are present (Figure 4.7 (A)(C)(E)). However, when the α_{2C} -blocker is added, blocking this signalling pathway, osteoclastogenesis seems to be impaired. In these conditions the osteoclasts are smaller, and seem to present fewer nucleus (Figure 4.7 (B)(D)(F)). Also the cytoskeleton arrangements are also impaired, as formation of the actin ring is only possible to visualize in some cells.

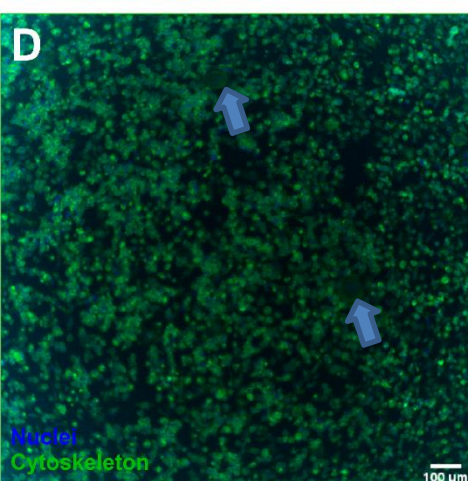
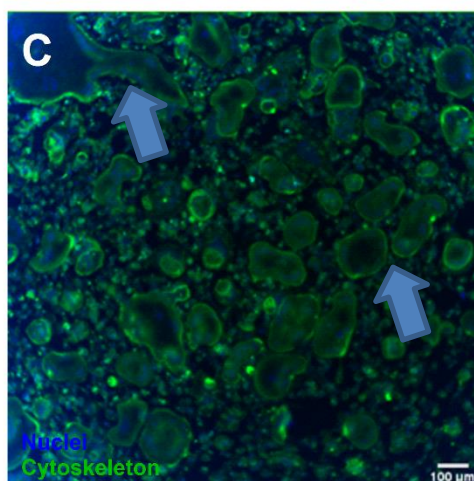
10⁻⁷ M JP1302

+

OM NE



10⁻⁹M NE



10^{-5} M NE

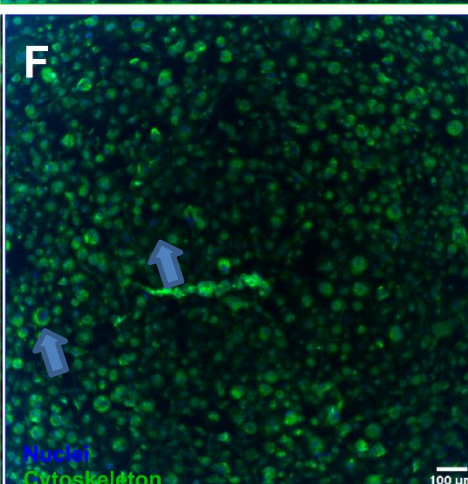
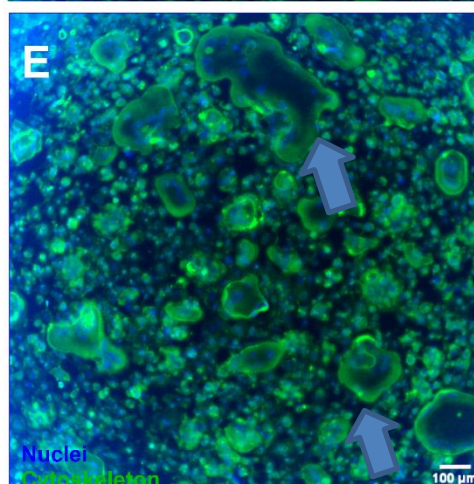


Figure 4.7- Representative images of the osteoclast treated with the α_{2C} -ADR agonist and antagonist. (A) 0M NE-control; (B) 10^{-7} M JP1302; (C) 10^{-9} M NE; (D) 10^{-7} M JP1302+ 10^{-9} M NE; (E) 10^{-5} M NE; (F) 10^{-7} M JP1302+ 10^{-5} M NE (blue arrows=osteoclasts) (Scale bar =100 μ m).

As there seems to be a reduction of osteoclasts formation when the $\alpha_2\text{C}$ -blocker is added, to ensure that it was not due to its' toxicity on the cells, it was performed a live/dead and resazurin assay after 24 and 72 hours of treatment. For that, bone marrow-derived monocytes

were seeded with 30 ng/ml M-CSF and 100 ng/ml RANKL in all conditions: (i) 0 μ M (vehicle H₂O; control); (ii) 0.5 $\times 10^{-7}$ M JP1302 and (iii) 10 $^{-7}$ M JP1302. There appears to be a decrease of the metabolic activity of the osteoclast after de addition of 10 $^{-7}$ M JP1302 in comparison with the control (0 M) (Figure 4.8), however no significant differences are observed between time-points. In fact, when it is analysed the live/dead assay, the number of red-positive cells, which represents the dead cells, is not different from the control (Figure 4.9). Being so, these results suggest that the α_{2C} -ADR signalling pathway do not induce osteoclast apoptosis/death and might have instead a basal influence in the osteoclast formation.

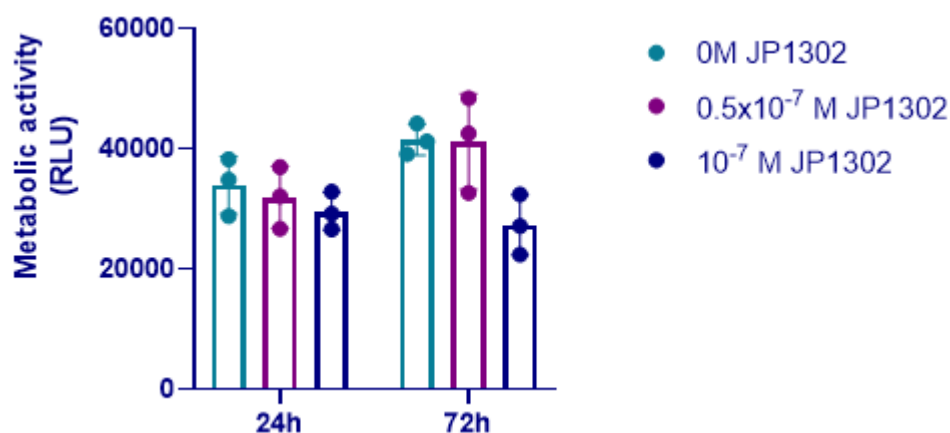


Figure 4.8 - Osteoclast metabolic activity after treatment with JP1302 (n=1 with 3 replicates).

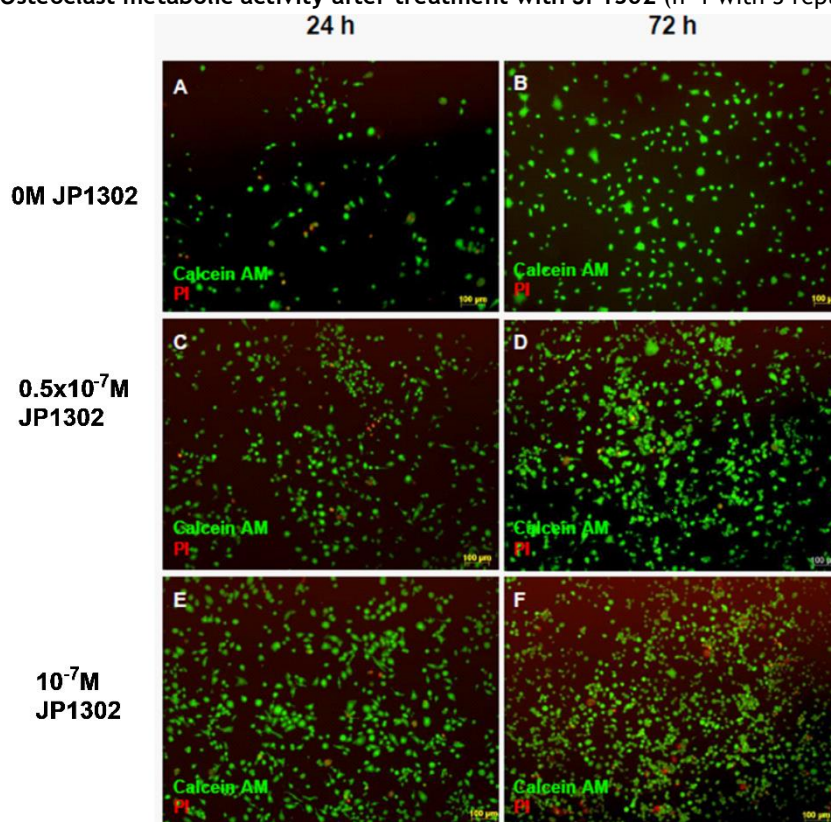


Figure 4.9 - Representative images of the live/dead assay performed after 24 h and 72h of osteoclast treatment with JP1302. (A) treatment with 0M JP1302 after 24 h; (B) treatment with 0M JP1302 after 72 h; (C) treatment with 0.5 $\times 10^{-7}$ M JP1302 after 24 hr; (D) treatment with 0.5 $\times 10^{-7}$ M JP1302 after 72 h; (E) 10 $^{-7}$ M after 24 h; (F) treatment with 10 $^{-7}$ M JP1302 after 72h (Scale bar =100 μ m).

1.1.3 -Osteoclast resorptive activity

The resorptive activity of the osteoclast is fundamental to ensure the renewal of bone and its dysregulation can have a negative impact in health [31]. So to understand the direct effect of the α_{2C} -signalling pathway in osteoclast resorptive activity, the bone marrow derived monocytes were seeded on top of bone slices for 7 days in the same conditions as in the osteoclastogenesis assay.

The total eroded surface was evaluated by the quantification of the number of pits and trenches formed in each bone slice. No significant differences were observed between the different conditions, however when the α_{2C} -signalling pathway is blocked the percentage of total eroded surface seems to be decreased (Figure 4.10 (A)). This effect is more prominent in the condition where the 10^{-9} M NE was also added. As osteoclastogenesis and morphology of osteoclast are affected when the α_{2C} -adrenergic blocker is added, as seen in the differentiation assay, this could explain the decrease resorptive activity. When 10^{-5} M NE was added, it was expected an increase of osteoclast activity due to the activation of the β_2 -ADR, as some studies have pointed at [67, 73-75]. However, that was not the case and on the contrary a slight decrease in the aggressiveness of the osteoclast phenotype was observed, as there were no significant differences between the formation of pits or trenches as in the conditions with 0 M NE- normal activity, and 10^{-9} M NE (Figure 4.10 (B)). In Figure 4.11, it is possible to verify the decline of resorptive activity of the osteoclast when the antagonist is added as the resorptive events seem to be fewer and the presence of larger trenches in the conditions without the JP1302.

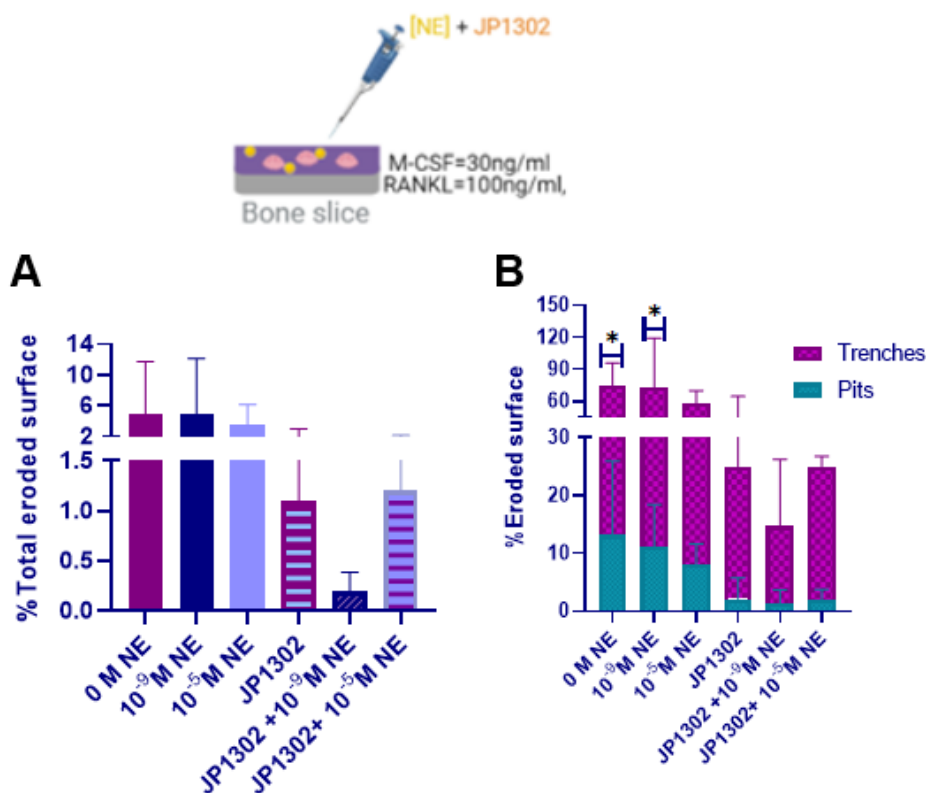


Figure 4.10- Osteoclast resorptive activity after α_{2C} -adrenergic stimulation/blockage. A) Percentage of the total eroded bone surface; B) Pit and trench surface percentage in the total eroded surface; (n=3 with 4 replicates) *p=0.05.

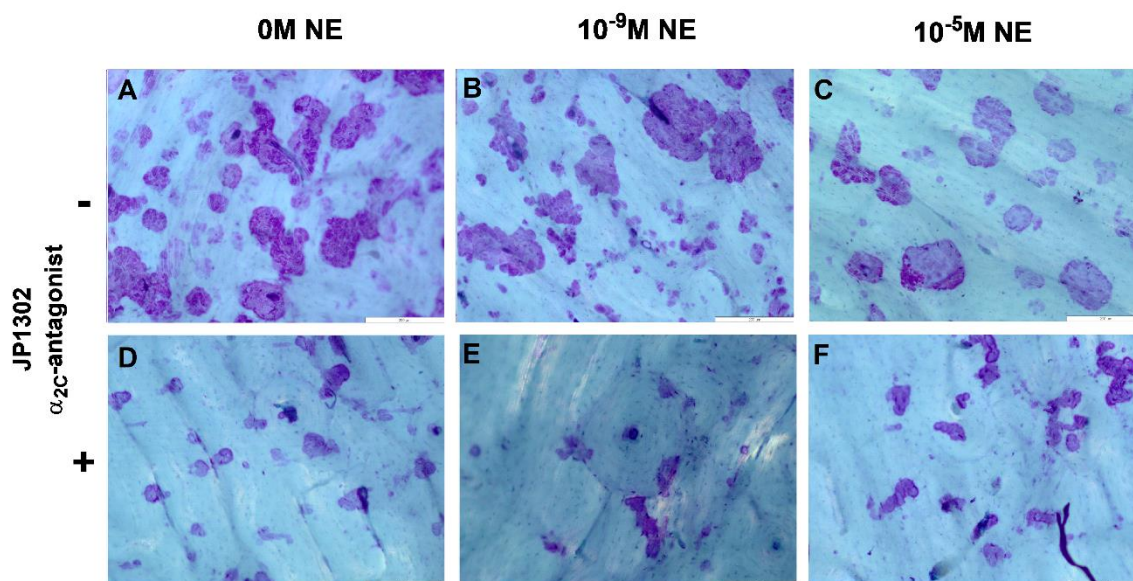


Figure 4.11 - Representative images of osteoclast resorptive events after α_{2C} -adrenergic stimulation/blockage. (A) 0M NE-control; (B) 10^{-9} M NE; (C) 10^{-5} M NE; (D) 10^{-7} M JP1302; (E) 10^{-7} M JP1302+ 10^{-9} M NE; (F) 10^{-7} M JP1302+ 10^{-5} M NE (Scale bar=200 μ m).

During bone resorption osteoclast release different factors to on the Howship lacuna to demineralized bone matrix and degrade the collagen [29]. Therefore, their quantification can be used to further analyse the effect of this pathway in the osteoclast. Thus, media was collect from the bone slices and TRAP quantification was performed (Figure 4.12). Despite the non-significant differences in the conditions, when the α_{2C} -adrenergic blocker was added TRAP activity had a more significant decrease. Other molecules are involved in the process of bone degradation. Thus, it would be interesting to quantify their expression in the secretome, as TRAP activity is also decrease in the conditions were NE was added, but the resorptive activity of the osteoclast does not appeared impaired. These results support a possible beneficial role of α_{2C} -adrenergic blockers in diseases where osteoclast activity is increased.

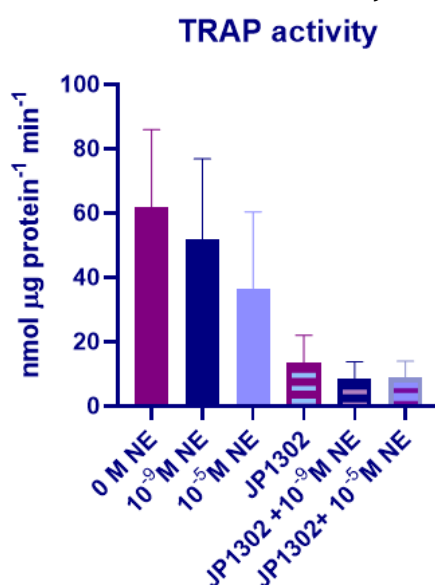


Figure 4.12 - Osteoclast TRAP activity quantification after α_{2C} -adrenergic stimulation/blockage (n=3 with 4 replicates).

1.2 - Effects of $\alpha 2C$ -ADR signalling pathway on osteoclast/osteoblast activity

1.2.1 -Establishment of the osteoclast/osteoblast co-cultures

A co-culture of mouse osteoclast (derived from primary bone marrow-derived monocytes) and osteoblasts (derived from UAMS-32P osteoblastic cell line) was established to increase the complexity and similarity to the *in vivo* bone niche. The co-culture was supplemented with 10 mM B-GP, 50Mm AA to ensure a phenotypic osteoblastic profile of the UAMS-32P cell line and 0.01 μ M Vitamin D3, since it increases RANKL expression on these cell line[138]. In these case UAMS were seeded after 4 hours of the osteoclast lineage seeding to ensure that the bone marrow macrophages were already adhered to the substrate. The co-culture was maintained for 4 days, with medium change at day 3. To evaluate the morphology of osteoclasts in the co-culture, an immunofluorescence staining against actin was performed at day 4. As can be visualized in Figure 4.13, an actin ring delimits the osteoclast, while the osteoblasts are entrapped in matrix produced by them.

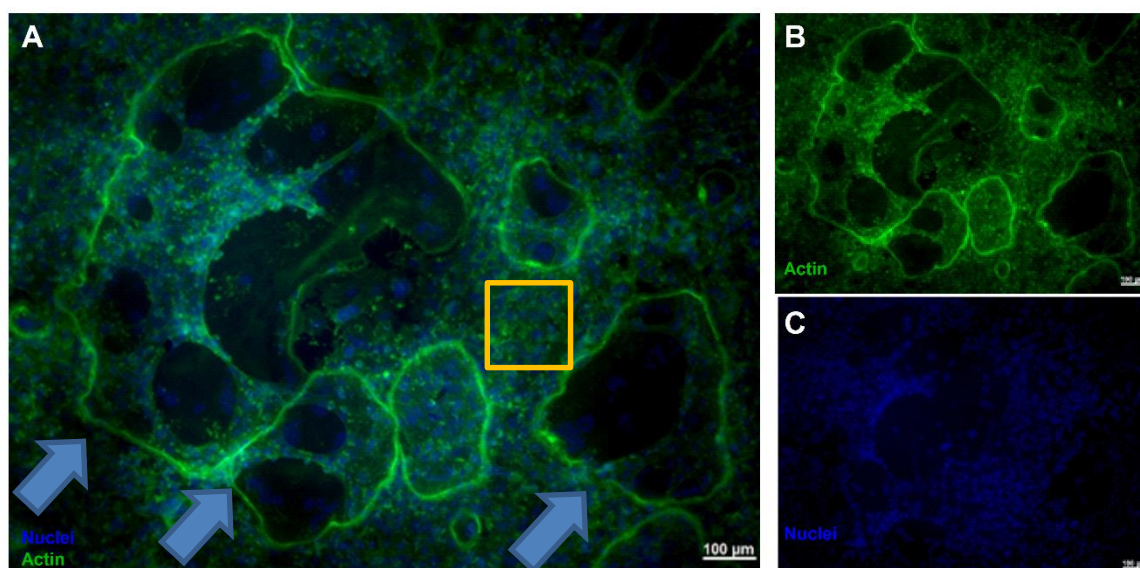


Figure 4.13 - (A) Representative image of day 5 of co-culture of osteoclasts (blue arrows) and UAMS-32P (orange square) with F-actin staining. The osteoclasts and UAMS-32P skeleton is represented in green stained with Alexa-Fluor 488 (B), and the nuclei in blue with DAPI (C). (Scale bar =100 μ m)

The resorptive activity of the osteoclast was also evaluated in the co-culture. As in the monoculture, cells were seeded on top of bone slices and left for 7 days. The presence of osteoclasts and eroded surface (Figure 4.14) indicated the proper function of the established co-culture. Also as there was no necessity to add extra M-SCF or RANKL to ensure osteoclast formation or resorptive activity. Thus, this experimental conditions translated a more accurate *in vivo* bone niche response, as the osteoblasts are the source of these two cytokines.

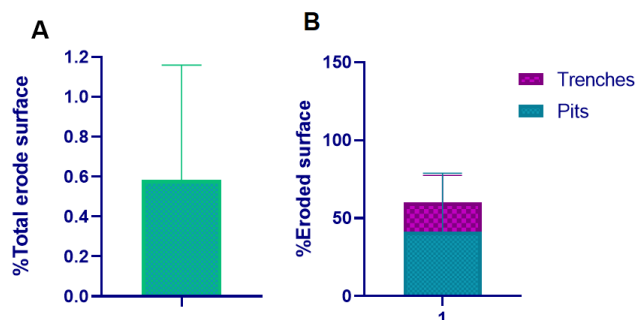


Figure 4.14 - Co-culture establishment resorption. A) Percentage of the total eroded bone surface; B) Pit and trench surface percentage in the total eroded surface (ES); (n=1 with 5 replicates).

1.2.2 -RANKL expression

RANKL/RANK signalling pathway is the most important pathway regulating osteoclast differentiation and activity [31]. Due to that it impacts bone homeostasis, having a relevant role in pathological bone destruction, as in postmenopausal osteoporosis, rheumatoid arthritis (RA) and periodontal diseases, which makes it a valuable target for potential pharmacotherapies [139]. As activation of the β -adrenergic pathways tend to increase RANKL expression on osteoblasts, the use of β -blockers is being investigated [66-68].

As osteoblasts express other ADRs, to better understand the role of SNS, the influence of the α_{2C} - signalling pathway on the expression of RANKL was evaluated using a murine osteoblastic cell line (designated as UAMS-32P). The UAMS-32P mouse bone marrow stromal cell line is a cell line stably transfected with a construct containing the entire murine RANKL gene coupled to luciferase as a reporter gene, which allow detection of RANKL expression in stromal/osteoblastic cell lines.

UAMS-32P cells were stimulated with ADR agonist (NE) and/or the α_{2C} -ADR antagonist (JP1302). Before stimulus addition, the cell line was left to adhere for 24 h. After this period, it was added (i) vehicle (H_2O ; 0M NE); (ii) 10^{-9} M NE; (iii) 10^{-5} M NE; (iv) 10^{-7} M JP1302; (vi) 10^{-7} M JP1302+ 10^{-9} M NE; (vii) 10^{-7} M JP1302+ 10^{-5} M NE and (viii) 0.01 μ M Vitamin D3 (as positive control). As these cells are described to rise the production of RANK when exposed to vitamin D3, since it stimulates the RANKL gene promoter, this condition function as a positive control of the culture.

As presented in the Figure 4.15, there are no significant differences between conditions, even the expected increase in RANKL expression due to the activation of β_2 -ADR (condition with 10^{-5} M NE) was not observed. However, there is a slight increase in RANKL expression when the α_{2C} -adrenergic antagonist is added, which is more pronounced in the condition that was also added 10^{-5} M NE. As in the condition where α_{2C} -ADR was stimulated (10^{-9} M NE) there was a small decrease of the RANKL expression, and the increase RANKL expression in the conditions where it was blocked, the stimulation of α_{2C} - signalling pathway in the osteoblasts might impair RANKL production.

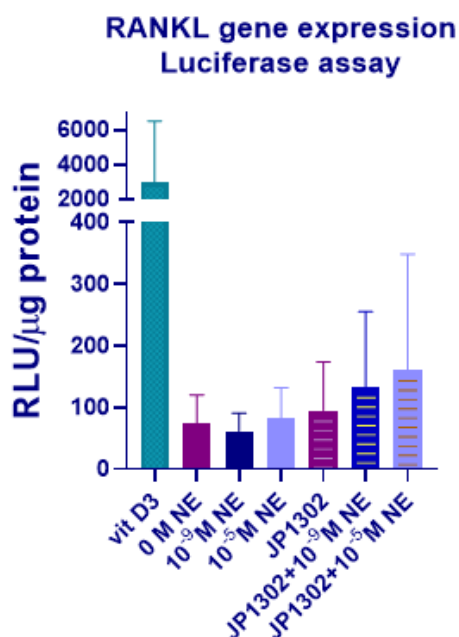


Figure 4.15 - RANKL expression of the UAMS-32P treated with an α_{2C} -ADR agonist and antagonist after 24 hours (n=4 with 4 replicates for each condition).

1.2.3 - Osteoclastogenesis/Differentiation

For understanding the direct effect of the α_{2C} -ADR signalling pathway in the bone niche, the bone marrow derived macrophages were seeded with the referred cytokines in all conditions: (i) Positive control (0M NE); (ii) 10^{-9} M NE; (iii) 10^{-5} M NE; (iv) 10^{-7} M JP1302; (v) 10^{-7} M JP1302+ 10^{-9} M NE; and (vii) 10^{-7} M JP1302+ 10^{-5} M NE. The antagonist and agonist were added 4 hours after the seeding as the osteoblastic cell line as the UAMS-32P, to ensure that it had no impact in osteoclast adhesion to the substrate.

The previous results from the osteoclast and osteoblastic monocultures showed that the α_{2C} -ADR signalling pathway appears to present contradictory effects in relation to its role in the bone degradation phase, since when the α_{2C} -signalling pathway is blocked in the osteoblast, there is a slight increase of the RANKL expression. However, when the blockage is performed in the bone marrow macrophages, osteoclastogenesis seems to be reduced. UAMS-32O cell line present high formation of matrix, and despite the established parameters for the co-culture allowed osteoclast formation, in these experiments that was not observed during the differentiation assay (Figure 4.16).

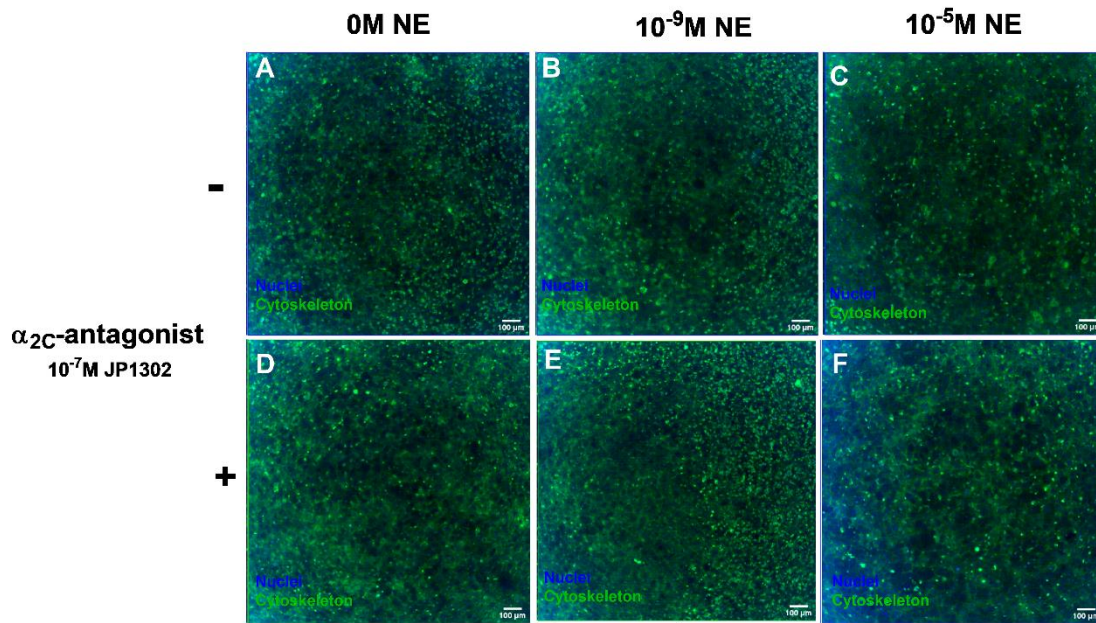


Figure 4.16- Representative images of the co-culture treated with the α_{2C} -ADR agonist and antagonist. (A) 0M NE-control; (B) 10^{-9} M NE; (C) 10^{-5} M NE; (D) 10^{-7} M JP1302; (E) 10^{-7} M JP1302 + 10^{-9} M NE; (F) 10^{-7} M JP1302+ 10^{-5} M NE (Scale bar =100 μ m).

1.2.4 - Osteoclast resorptive activity

The effect of the α_{2C} -ADR signalling pathway in the bone niche was further evaluate by seeding the bone marrow derived monocytes and UAMS-32P on top of bone slices for 7 days in the same previous conditions. Despite no osteoclast formation in the differentiation assay was observed, bone slices present resorptive events. This means that the timeline applied for the resorptive assay can be adapted to the differentiation assay. Thus, the resorptive events were also quantified.

During sympathetic hyperactivation in mouse models there is a decrease in the bone mass, caused by the increase activity of the osteoclast. This effect, as previously referred, is a consequence of the increase expression of RANKL by the osteoblasts, due to activation of the β_2 -ADR. In addition, it is also a consequence of the direct effect of that ADR in the osteoclast [13]. However, that was not observed neither in the monoculture or co-culture (condition with 10^{-5} M NE) (Figure 4.17 (A)). In the case of the α_{2C} -signalling pathway, when it is blocked (conditions with JP1302), the resorptive activity of the osteoclast seems to be decreased in all conditions. This effect is more prominent in the condition with 10^{-9} M NE, and lesser in the condition of with 10^{-5} M NE. However, the formation of trenches appears higher than the formation of pits in these conditions (Figure 4.17(B)). As RANKL expression seems to be increase in the osteoblast cell line, its effect could be on the osteoclast resorptive phenotype by impacting its aggressiveness. This results support the high bone mass phenotype of the mice with ablation of both the α_{2C} -ADR and α_{2A} -ADR, and with the phenotype of the vertebrae of young mice with only ablation of the α_{2C} -ADR [81, 82]. As in the femurs of the young mice the phenotype was contrary, mechanical stimulus might play a role in the effect of this pathway,

as inconsistent results were also found when bone unloading was performed in β_1/β_2 blockage [13]. Examples of the resorptive events for each condition are represented in Figure 4.18.

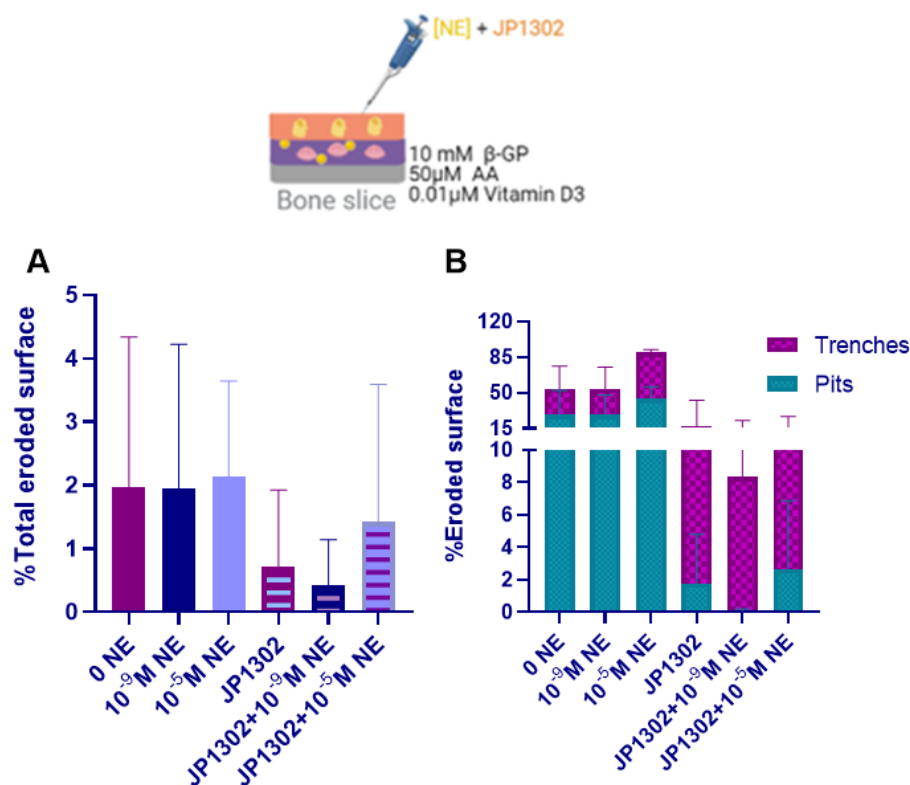


Figure 4.17 - Osteoclast resorption after α_{2c} -adrenergic stimulation/blockage of the co-culture. A) Percentage of the total eroded bone surface; B) Pit and trench surface percentage in the total eroded surface (ES); (n=3 with 4 replicates).

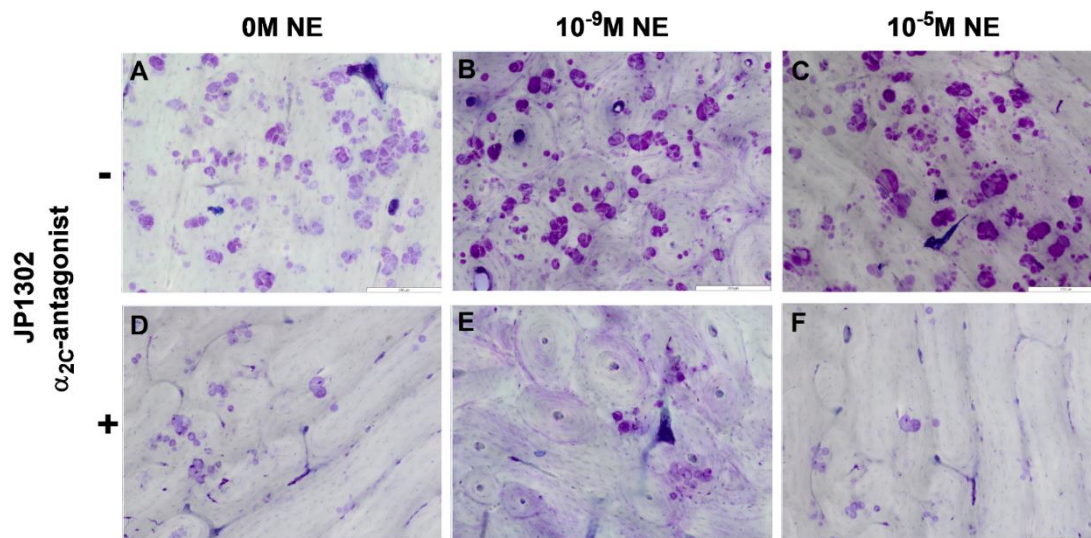


Figure 4.18 - Representative images of osteoclast resorptive events after α_{2c} -adrenergic stimulation/blockage in the co-culture. (A) 0M NE-control; (B) 10^{-9} M NE; (C) 10^{-5} M NE; (D) 10^{-7} M JP1302; (E) 10^{-7} M JP1302 + 10^{-9} M NE; (F) 10^{-7} M JP1302 + 10^{-5} M NE (Scale bar=200 μ m).

To further assess the effect on the osteoclast, the co-culture secretome was retrieved and analysed for the TRAP activity. In all conditions with JP1302 (Figure 4.19), there is a decrease of TRAP activity. The decrease in TRAP activity was expected as it is an important enzyme in

dephosphorylation of bone matrix. As the resorptive profile of the osteoclast appears to be more aggressive, the quantification of molecules involved in the collagenolysis [137], as MMPs, should be assessed.

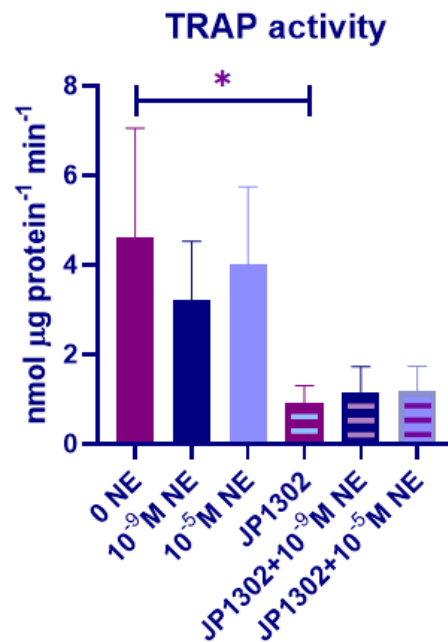


Figure 4.19 - Osteoclast TRAP activity after α_{2C} -adrenergic stimulation/blockage of the co-culture. (n=3 with 4 replicates) *p=0.01.

2 - The role of α_{2C} -ADR signalling pathway in the breast cancer bone metastatic niche

As previously referred, breast cancer patients have worse prognosis when they present sympathetic hyperactivation. This is due to an increase tumour burden and metastasis [6-8]. The stimulation of α -ADR is linked with increase aggressiveness of the tumour directly by increasing its' proliferation and invasiveness, and indirectly by promoting a pro-tumorigenic environment through the stromal or immune cells [140]. In the case of the breast cancer bone metastasis, few is known since only the effect of SNS through β -ADR signalling in Breast cancer homing to bone was the focus of studying [117, 118]. As the same in bone remodelling process, the effect of α -ADR is still poorly understood in Breast cancer bone metastasis. So, the second part of this work consisted in exploring the effect of α_{2C} -ADR pathway in Breast cancer bone metastasis (proliferation, migration) and how its influence in the breast cancer bone tropic cells is translated to the bone niche, specifically how it impacts the osteoclast activity and consequently bone destruction (Figure 4.20).

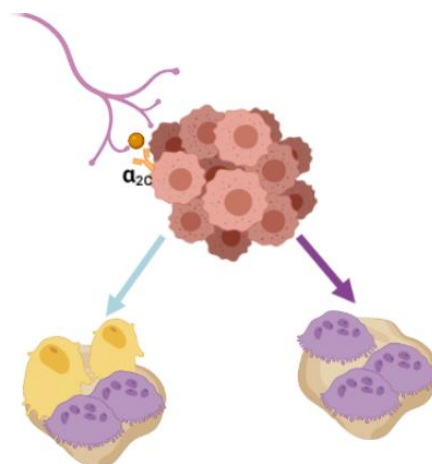


Figure 4.20 - Exploring the role of SNS on Breast cancer-induced bone metastasis - the osteoclast activity. The effects provoked by SNS on Breast cancer-induced bone metastasis were assessed by first treating Breast cancer cells with NE and an α_{2C} -ADR blocker, using this CM to afterwards to treat osteoclasts (purple arrow) and the bone niche (blue arrow). This treatment allows to evaluate how SNS, after influencing Breast cancer, can impair osteoclast function.

2.1 - Effect on the bone tropic breast cancer cell

2.1.1 - Proliferation

To study the direct effect of the α_{2C} -signalling pathway in the Breast cancer bone tropic cells, 4T1.2 cell line was treated α_{2C} -ADR agonist (i) 0M NE (ii) 10^{-9} M NE; (iii) 10^{-5} M NE; and α_{2C} -ADR antagonist (iv) 10^{-7} M JP1302; (vi) 10^{-7} M JP1302+ 10^{-9} M NE; (vii) 10^{-7} M JP1302+ 10^{-5} M NE for 24 hours in α -MEM free serum (0.5% FBS) complete medium. The lack of serum was to ensure that the cells response was due only to the stimulation/blockage of the α_{2C} -ADR.

Blocking the α_{2C} -signalling pathway in the bone Breast cancer cell, decrease the number of nucleus/cells present (Figure 4.21) after 24 hours. In particular, in the condition where only the α_{2C} -ADR blocker is added. The α -ADR stimulation was expected to increase the proliferation of the cells, since increased [^{3} H]-thymidine incorporation was observed in some Breast cancer cells, however it was not observed in this case. In the conditions where the α_{2C} -ADR blocker was added, when NE was also present the effect of the blockage seems to be mitigate, due to the affinity of NE- major neurotransmitter of SNS, for other ADRs.

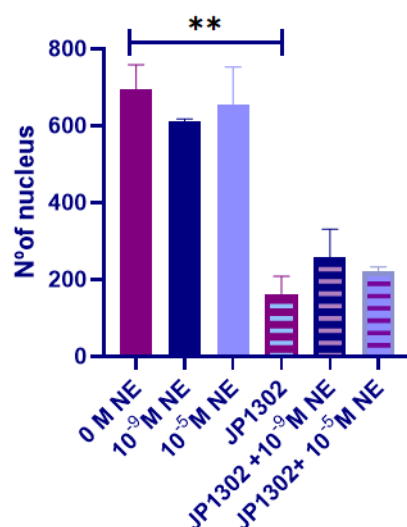


Figure 4.21- Quantification of nucleus of bone tropic Breast cancer cells treated with the α_{2c} -ADR agonist and antagonist; **p=0.01.

The decrease of cells might be due to an impact on proliferation or survival of Breast cancer cells. Ki67 is a proliferative gene, that is being indicated to be used as a predictive marker in Breast cancer [16, 18-21]. So a high presence of this gene indicates a high proliferation rate. As can be seen in Figure 4.22, despite the decrease in the number of cells, the Ki67 is present in the same percentage in all the conditions where the α_{2c} -blocker is added. So the effect of its blockage might be in a short period of time, and when the Ki67 staining was performed Breast cancer cells could already have overcome its effect, due to their high metabolic capacity. Thus an earlier time point should be performed. Nevertheless, to understand the effect of the α_{2c} -signalling pathway, a live/dead assay (toxicity/apoptosis) and resazurin assay (metabolic activity) should be performed.

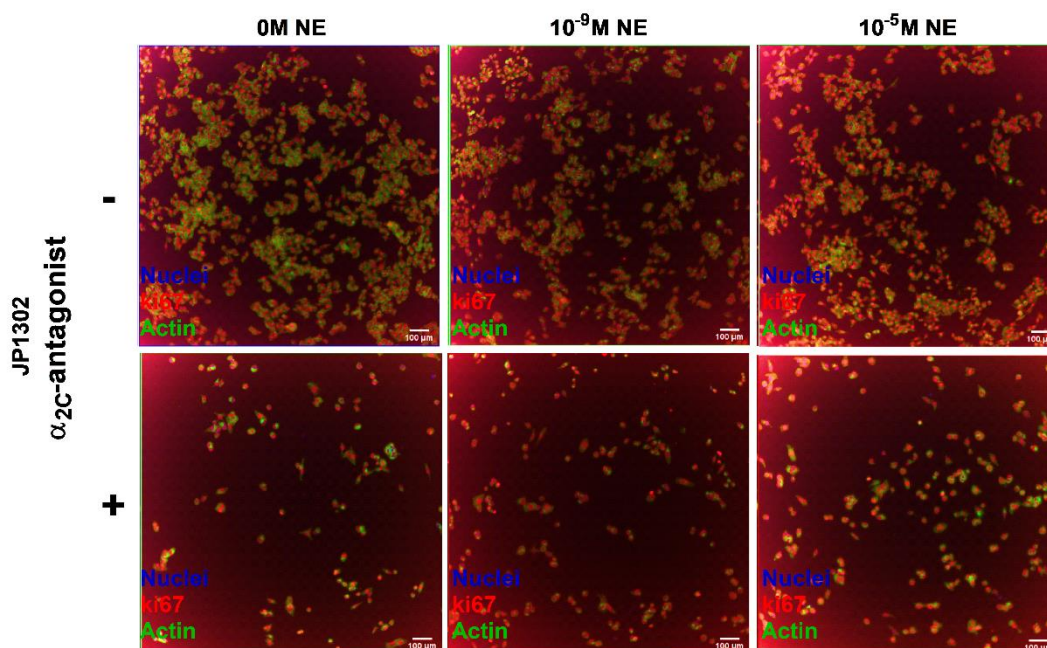


Figure 4.22 - Representative images of the ki67 staining. (A) 0M NE-control; (B) 10⁻⁹M NE; (C) 10⁻⁵M NE; (D) 10⁻⁷M JP1302; (E) 10⁻⁷M JP1302+10⁻⁹M NE; (F) 10⁻⁷M JP1302+10⁻⁵M NE (Scale bar =100μm).

2.1.2 - Migration

The capacity of the cells to migrate is associated with a more aggressive profile, as they are capable of invade to other tissues [24]. To understand the effect of the α_{2C} -signalling pathway the bone tropic Breast cancer cells were seeded in a microfluidic device and after 4 hours treated with α_{2C} -ADR agonist (i) 0M NE (ii) 10^{-9} M NE; (iii) 10^{-5} M NE; and α_{2C} -ADR antagonist (iv) 10^{-7} M JP1302; (vi) 10^{-7} M JP1302+ 10^{-9} M NE;(vii) 10^{-7} M JP1302+ 10^{-5} M NE for 72 hours in serum depleted medium.

B-stimulation in Breast cancer is linked to increase invasive potential of Breast cancer cell. In Figure 4.23, it is shown some migration observed in the different conditions. When only the α_{2C} -adrenergic pathway is blocked, cells appear to not be capable of migrate, as no cytoskeletal projections were observed in the Matrigel compartment. So this pathway could impair the production of MMPs, that are important for matrix degradation, which consequently impacts the migration capability of the cancer cells. However, in these experiments no conclusions can be drawn as in some cases the seeding of cells or polymerization of the Matrigel was not good.

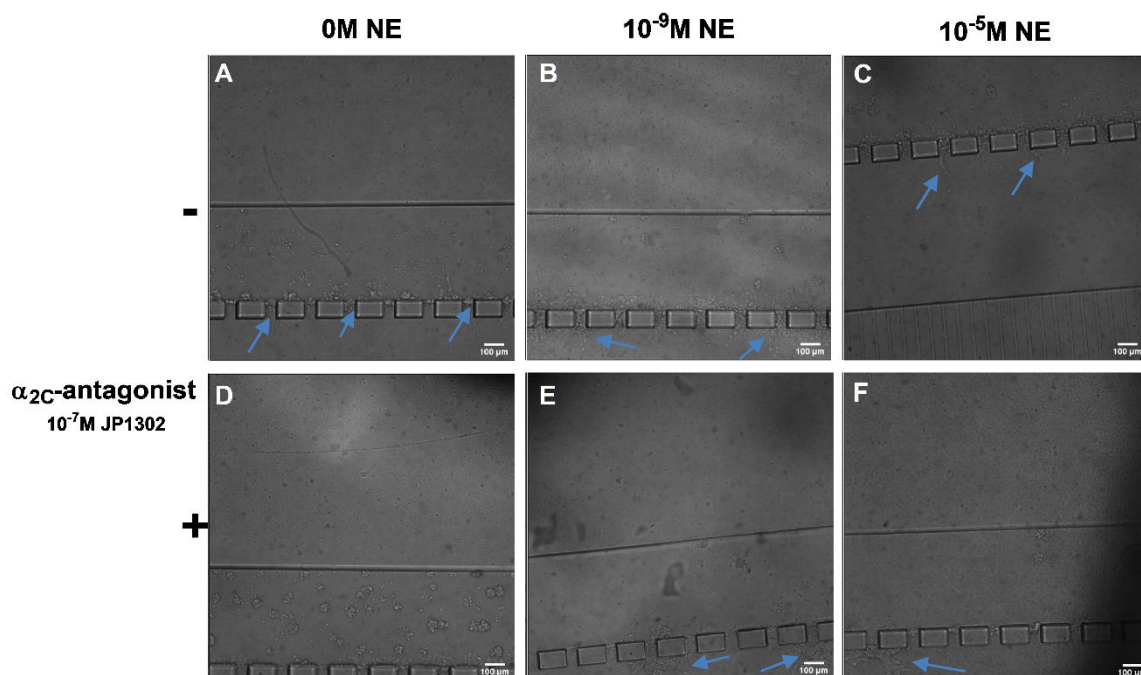


Figure 4.23- Representative images of the migration assay of bone tropic Breast cancer after α_{2C} -adrenergic stimulation/blockage. (A) 0M NE-control; (B) 10^{-9} M NE; (C) 10^{-5} M NE; (D) 10^{-7} M JP1302; (E) 10^{-7} M JP1302+ 10^{-9} M NE;(F) 10^{-7} M JP1302+ 10^{-5} M NE. Blue arrows point to the cells that were capable of migrate in the Matrigel (Scale bar =100 μ m)

2.2 - Effect of breast cancer secretome under the influence of α_{2C} -ADR on the osteoclast activity

The osteoclast is the major target of Breast cancer bone osteolytic metastasis, and the current treatments are only palliative since they try to mitigate the increase resorptive activity of the osteoclast [38, 39]. Due to this, new alternatives are necessary, and as previously mentioned the use of B-blockers is being studied as a new therapeutic approach. So to

understand how the α_{2C} -ADR signalling pathway influences the osteoclast through the bone Breast cancer cells, mouse osteoclasts were seeded with 30 ng/ml M-CSF and 100 ng/ml RANKL in all conditions: (i) α -MEM complete- CTRL+; (ii) α -MEM complete (5% FBS) - CTRL Media (iii) 4T1.2 CM; (iv) 4T1.2 treated with 10^{-9} M NE CM; (v) 4T1.2 treated with 10^{-5} M NE CM; (vi) 4T1.2 treated with 10^{-7} M JP1302 CM; (vii) 4T1.2 treated with 10^{-7} M JP1302 + 10^{-9} M NE CM; and (viii) 4T1.2 treated with 10^{-7} M JP1302+ 10^{-5} M NE CM.

2.2.1 - Osteoclastogenesis/Differentiation

As shown in Figure 4.24 (B), the depletion of serum seems to have an effect on the morphology of the osteoclast, as cytoskeletal structures (actin ring) are not present, but they are still large. When the CM is added in the conditions where Breast cancer cells were treated with the antagonist, the osteoclasts react similarly as when they were directly treated with the α_{2C} -blocker as they appear to be smaller, and present less nuclei. Regarding the treatment with NE in the breast cancer cells, it seems to not present great differences between the conditions, although the morphology of the osteoclasts seems to be similar to the positive control, especially in the condition with 10^{-5} M NE was added to the Breast cancer cells (Figure 4.24).

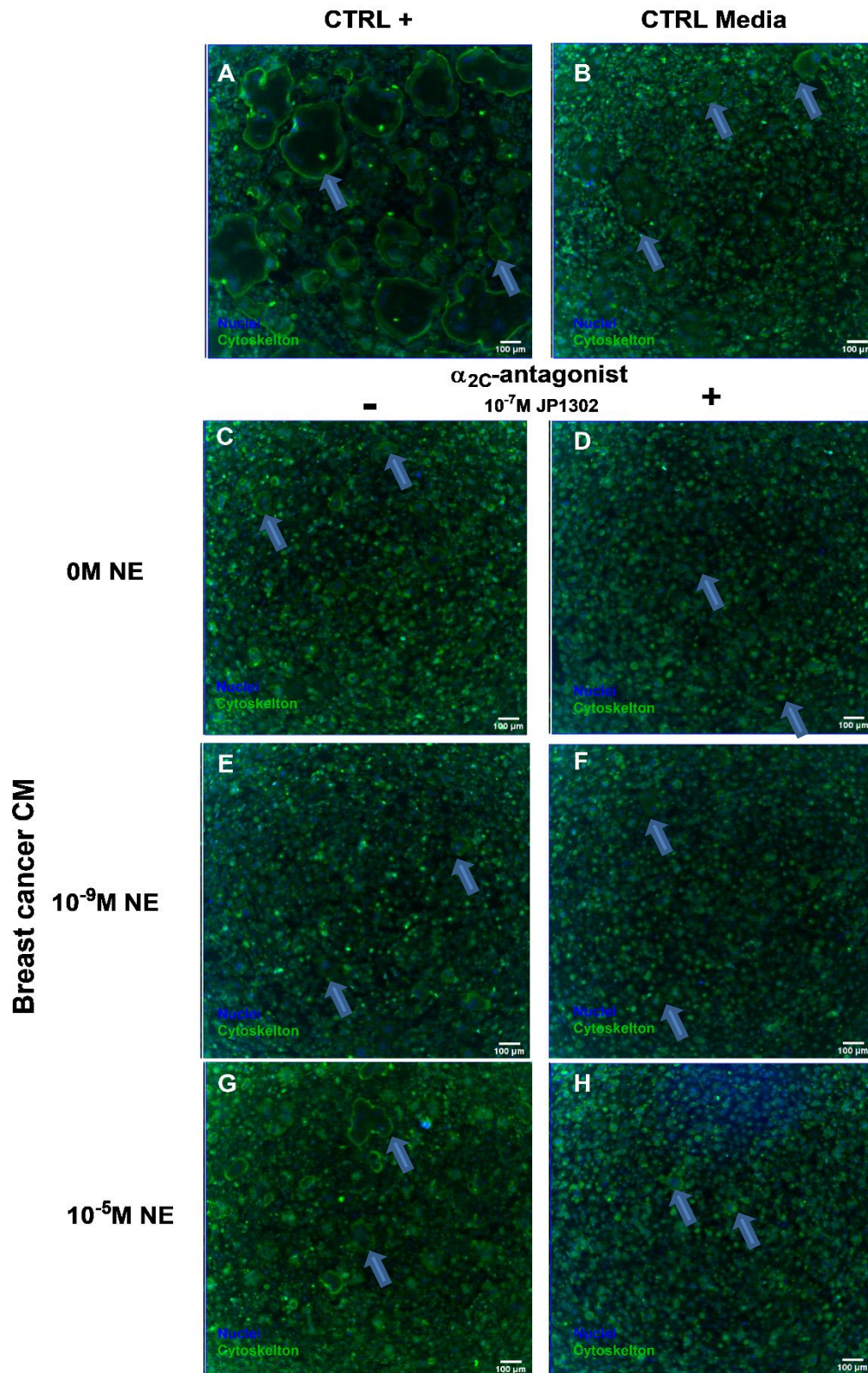


Figure 4.24 - Representative images of the osteoclasts treated with Breast cancer CM. (A) Positive control (B) α-MEM complete (5% FBS) - CTRL (C) Breast cancer CM; (D) Breast cancer CM treated with 10⁻⁷M JP1302 CM; (E) Breast cancer CM treated with 10⁻⁹M NE; (F) Breast cancer CM treated with 10⁻⁷M JP1302 + 10⁻⁹M NE; (G) Breast cancer CM treated with 10⁻⁵M NE; (H) Breast cancer treated with 10⁻⁷M JP1302+10⁻⁵M NE CM; (Blue arrows=osteoclasts). (Scale bar =100μm).

2.2.2 - Resorptive activity

During the Breast cancer bone metastasis, when the Breast cancer cells enter the bone niche it is established a “vicious cycle”. The increase of the osteoclast resorptive activity is not only a consequence of the indirect effect of the Breast cancer cells through the osteoblasts, but also due to the direct effect of the cytokines release by the cancer cells [38, 40]. In Figure 4.25, it is possible to verified that there seems a decrease of the osteoclast activity due to reduction of the serum. Despite that there is an increase of the resorptive activity when the not treated Breast cancer CM is applied. However, it has unexpected that the Breast cancer CM treated with the 10^{-5} M NE seem to decrease the osteoclast activity, since in the differentiation osteoclast present a normal morphology. In addition, sympathetic hyperactivation tends to favour metastasis to bone and risk of cancer burden [141]. This effect seems to be slightly counterbalanced when the α_{2C} -ADR signalling pathway is blocked in the Breast cancer cell line. However, when the α_{2C} -ADR blocker is added the tendency is to a decrease in the osteoclast resorptive activity, but no significant differences were observed. Nevertheless, osteoclast present the same resorptive profile in all conditions (Figure 4.25 (B)), as the formation of trenches was the most frequent resorptive event. In Figure 4.26, it is represented the resorptive events of each condition, as is possible to verified that trenches appear to become smaller when culture media has serum depleted and a tendency to a decrease in the resorptive events is also seen when α_{2C} -blokage is performed in the Breast cancer cells.

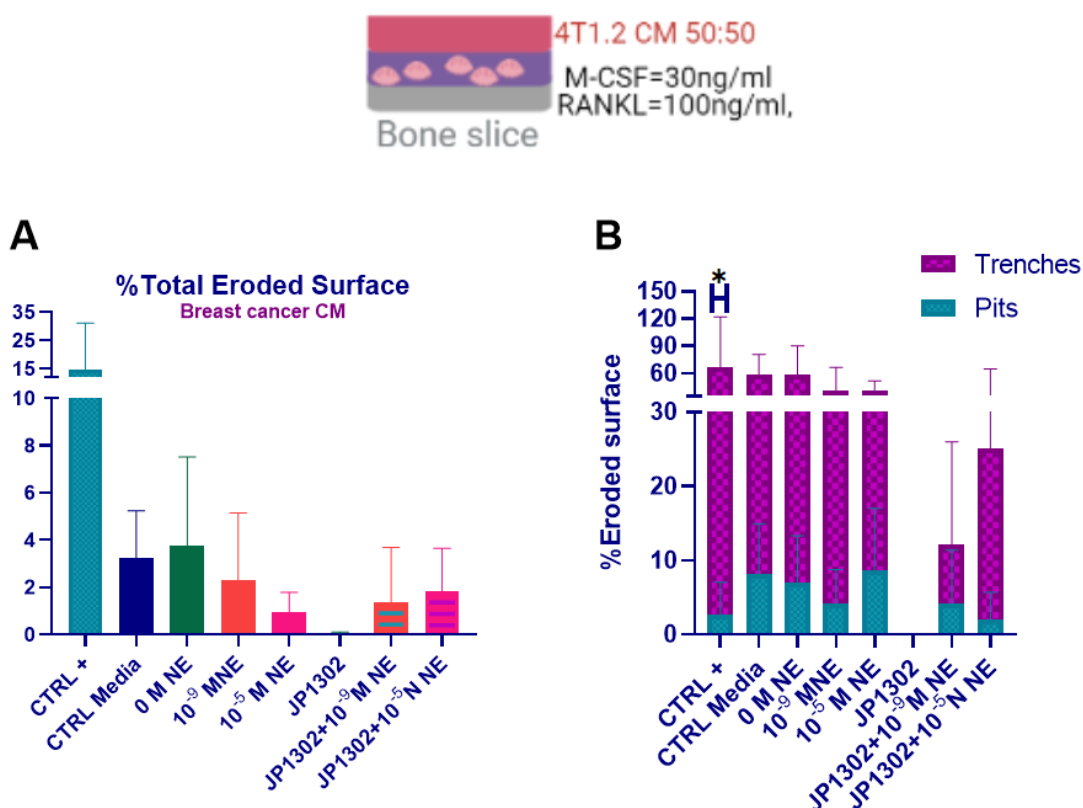


Figure 4.25 - Monoculture establishment resorption after treatment with Breast cancer CM. A) Percentage of the total eroded bone surface; B) Pit and trench surface percentage in the total eroded surface (ES); (n=3 with 4 replicates).

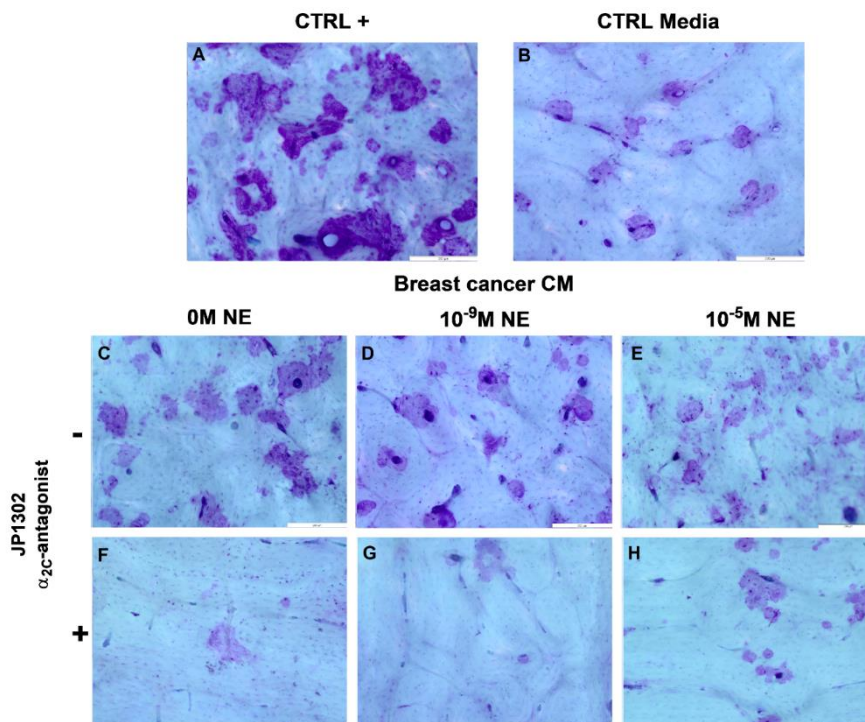


Figure 4.26 - Representative images of osteoclast resorptive events after treatment with Breast cancer CM. (A) Positive control (B) α -MEM complete (5% FBS) - CTRL (C) Breast cancer CM; (D) Breast cancer CM treated with 10^{-9} M NE; (E) Breast cancer CM treated with 10^{-5} M NE; (F) Breast cancer CM treated with 10^{-7} M JP1302 CM; (G) Breast cancer CM treated with 10^{-7} M JP1302 + 10^{-9} M NE; (H) Breast cancer CM treated with 10^{-7} M JP1302 + 10^{-5} M NE CM. (Scale bar=200 μ m)

As same as in the direct effect of the α_{2C} -ADR pathway, the secretome was also evaluated (Figure 4.27). Additionally, TRAP activity is being studied to evaluate the effects of radiotherapy on the osseous progression of bone metastasis in patients [142]. In Figure 4.27, there is an apparent decrease of its activity in the conditions where the α_{2C} -ADR blocker was added, except in the condition where 10^{-5} M NE was also added to the bone Breast cancer cells. These results support a possible beneficial role of the use of α_{2C} -blockers for the treatment of bone metastasis as the osteoclast activity is reduced.

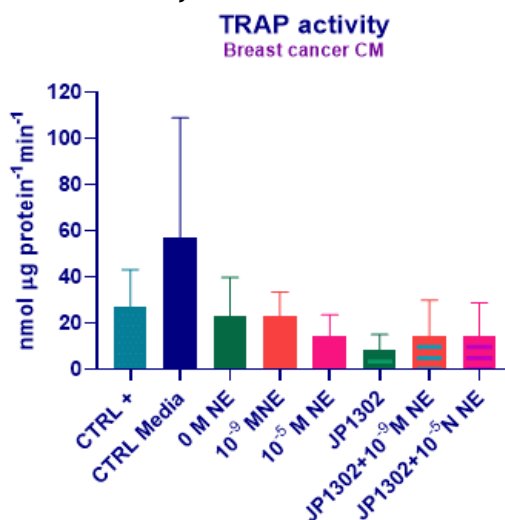


Figure 4.27 - Monoculture TRAP activity after treatment with Breast cancer CM (n=3 with 4 replicates).

2.3 - Effect of breast cancer secretome under the influence of α_{2C} -ADR on osteoblast/osteoclast co-cultures

2.3.1 - RANKL expression

During the vicious cycle the increase of RANKL expression by the osteoblasts due to the Breast cancer cells is the most predominant effect, which consequently increases osteoclast activity. Due to that desonumab is used to counteract the increase of RANKL production, aiming to alleviate Breast cancer bone metastasis patient's symptoms [38, 40]. Also, RANKL/RANK pathway seems to be implicated in the homing of the Breast cancer cells in bone, with or without SNS implication [117, 119]. So its expression has negative effects to patient health.

To study how the α_{2C} -ADR pathway affects RANKL expression by the osteoblasts through the bone-tropic breast cancer (4T1.2) cells, UAMS-32P were treated with the secretome (CM - conditioned media) of 4T1.2 breast cancer cells. First, UAMS-32P cell line was seeded for 48h before the stimulus application, since the Breast cancer CM (secretome) was depleted of serum. After this period, it was added (i) α -MEM complete (5% FBS) - CTRL (ii) 4T1.2 CM; (iii) 4T1.2 treated with 10^{-9} M NE CM; (iv) 4T1.2 treated with 10^{-5} M NE CM; (v) 4T1.2 treated with 10^{-7} M JP1302 CM; (vi) 4T1.2 treated with 10^{-7} M JP1302 + 10^{-9} M NE CM; (vii) 4T1.2 treated with 10^{-7} M JP1302+ 10^{-5} M NE CM and (viii) 0.01 μ M vitamin D3-positive control.

As showed in Figure 4.28 and contrary to the direct effect of the α_{2C} -ADR pathway there seems to be a slight decrease on RANKL expression, except in the condition with 10^{-5} M NE, when the α_{2C} -ADR is blocked in the Breast cancer cells, especially in the condition where no NE is added.

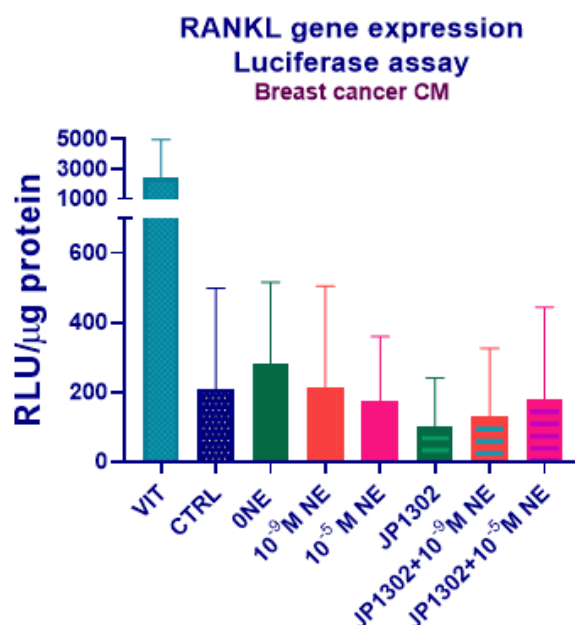


Figure 4.28 - RANKL expression of the UAMS-32P 24 hours after treatment with Breast cancer CM. (n=3 with 4 replicates for each condition.)

2.3.2 - Osteoclastogenesis/Differentiation

The “vicious cycle” is a complex set of interactions between the Breast cancer cells and the bone resident cells, as previously referred. So to increase the similarity and better understand the *in vivo* bone metastatic niche a co-culture was performed with 10 mM B-GP, 50 μ M Ascorbic Acid (AA) and 0.01 μ M Vitamin D3 in all conditions previously described. As in the co-culture of the α_{2C} -ADR direct effect, the Breast cancer CM and osteoblast seeding was done after 4 hours of the osteoclast lineage seeding.

As it happened during the direct effect of the α_{2C} -ADR in the co-culture, it was not possible to observe osteoclasts formation (Figure 4.29). As the osteoblast cell line produces a great amount of matrix, and the activity of the bone marrow cells is already decrease due to the reduced concentration of the serum these are the possible explanations for the absence of the large multinucleated osteoclasts. Despite that, positive cells for CTSK, an important enzyme in bone degradation, were present (Figure 4.30).

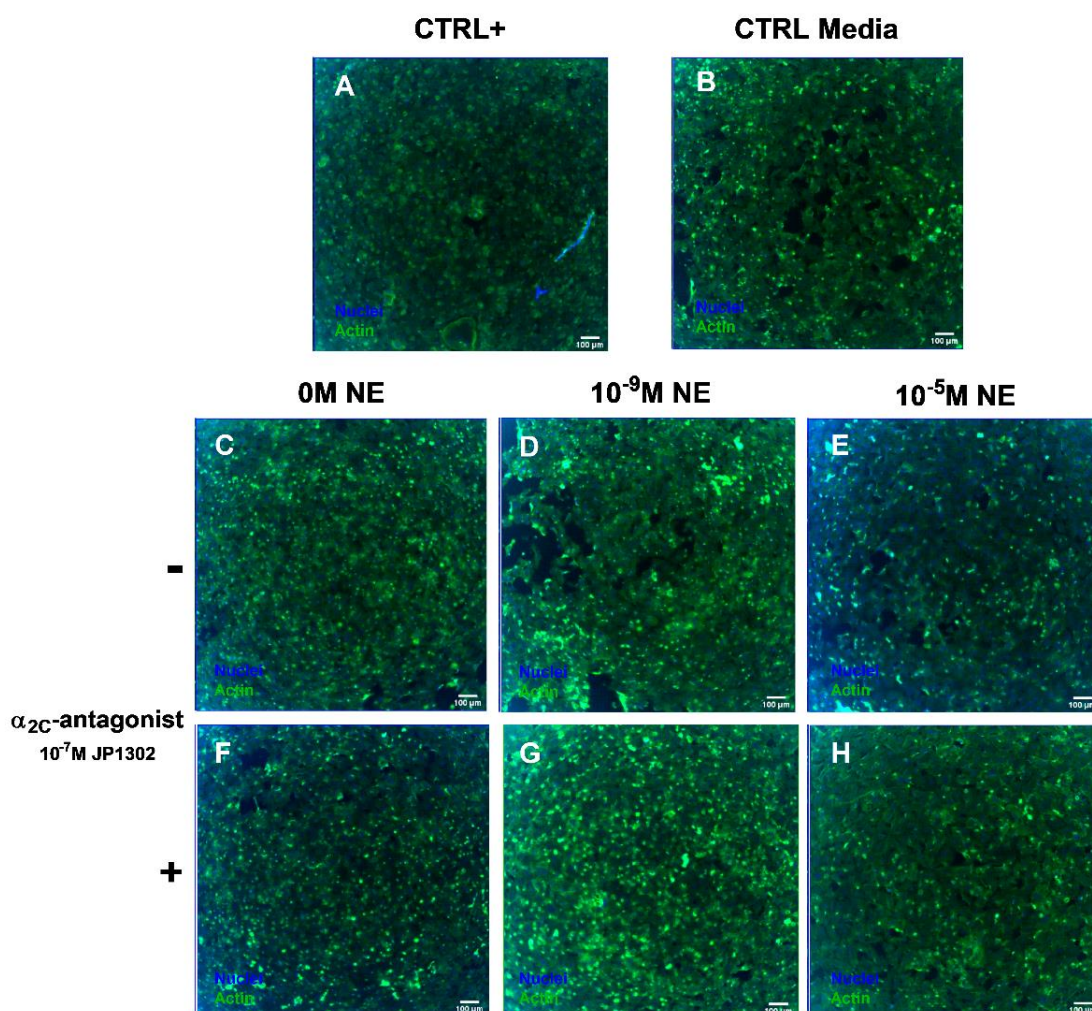


Figure 4.29 - Representative images of the co-culture treated with Breast cancer CM. (A) Positive control (B) α -MEM complete (5% FBS) - CTRL (C) Breast cancer CM; (D) Breast cancer CM treated with 10^{-9} M NE; (E) Breast cancer CM treated with 10^{-5} M NE; (F) Breast cancer CM treated with 10^{-7} M JP1302 CM; (G) Breast cancer CM treated with 10^{-7} M JP1302 + 10^{-9} M NE; (H) Breast cancer treated with 10^{-7} M JP1302 + 10^{-5} M NE CM (Scale bar=100 μ m)

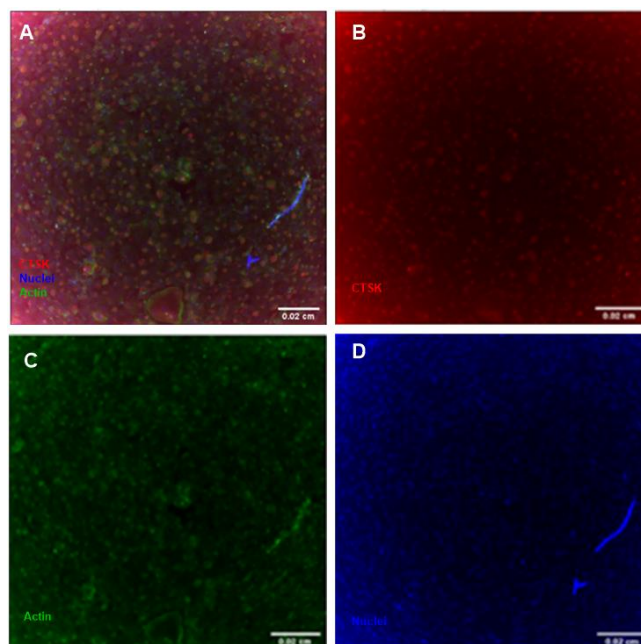


Figure 4.30-Representative image of the positive control group of the co-culture(A). (B) represents the staining for CTSK; (B) represents the staining for the actin; (C) nuclei (Scale bar=100µm)

2.3.3 - Osteoclast resorptive activity

As there was no formation of osteoclasts during the 5 day of the differentiation assay, the probability of having resorptive events in the bone slice was reduced. Despite the fact that in the direct effect of the α_{2C} -ADR, resorption was present, which implied osteoclast formation and maturation, in this case there was none. So, in regard of the indirect of the α_{2C} -ADR signalling pathway through the Breast cancer in the co-culture on the osteoclast it was not possible to evaluated. As the osteoblasts seem to have normal proliferation and activity (matrix production) a possible solution was to change the cell density of these lineage, as in the case of the direct effect of the α_{2C} -ADR pathway in the bone niche the osteoblast proliferate in a high rate which might not allow preosteoclast fusion. Furthermore, it could be added cytokines as M-CSF and RANKL, since the reduction of FBS might impair the activity and fusion of osteoclasts precursors.

3 - *In vivo* expression profile of sympathetic nerve fibers and α_{2C} -ADR

In vivo models are still the ultimate step before pharmacological trials in human. This is due to their increase complexity, as the effects will be observed in all of the organism systems. Not only they offer insight on the effects of the drug tested, but can also be used to mimic pathological conditions and to study specific aspects related to them [120, 123]. In the case of Breast cancer bone metastasis, different steps of the process can be imitated and be carefully analysed. SNS is being increasingly studied in Breast cancer, and higher tumour burden was associated with sympathetic nerve fibers in relapsed patients [84, 141].

Therefore, part of this work was also focused on studying the presence of sympathetic innervation in breast cancer-bone metastasis and the expression of α_{2C} -ADR in the vicinity of

the bone metastatic niche/microenvironment, using 50 μ m-thick bone cryosections obtained from an intratibial breast cancer bone metastasis model (a female mouse *in vivo* model of the late stage of Breast cancer bone metastasis). Briefly, bone-tropic breast cancer cells (4T1.2 cells) were inoculated into the tibia of immunocompetent 4- to 6-week old female mice (and sham-operated mice were injected with PBS - vehicle). As the immune system presents a preponderant effect in cancer, the *in vivo* model utilized was immunocompetent.

3.1 - TH- positive nerve fibers presence and quantification

The presence of nerve fibers in bone is confirmed by several studies [13, 48]. Sympathetic innervation in bone is usually associated with blood vessels, controlling the vascular hemodynamics in the bone marrow [13]. TH is an enzyme involved in NE synthesis, the major neurotransmitter of the sympathetic nerve fibers. In consequence, TH-staining allows to analyse the presence of sympathetic nerve fibers in bone metastasis.

To standardize the analysis from all samples, the tibia has been divided into 4 different zones. As show in Figure 4.31, the Z1 and Z2 at the epiphysis site englobe both the growth plate and the initial part of the tibia where 4T1.2 cells were inoculated; and at the diaphysis Z3 englobes the area of tumour growth, and finally Z4 the most distal part of the tibia.

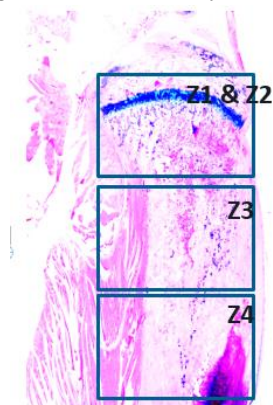


Figure 4.31- Schematic representation of the division of the mouse tibia.

Both in the BM group and SO group, sympathetic nerve fibers were only found in Z3 and Z4 at day 3 and 5 (Figure 4.32). As previously mentioned, sympathetic nerve fibers are associated with blood vessels, and as can be observed in Figure 4.31, fibers present the characteristic spiral-like structure and are located in the centre of bone marrow and in the border with the cortical bone. Although no fibers were found in the tumour, their presence in the bone environment can still have an indirect impact in its progression and growth, as sympathetic activation promotes bone metastasis by modulating the bone environment, and it is also linked to favour tumour by inducing a pro-tumorigenic immune environment[141]. Furthermore, sympathetic activation is associated with an increase resorptive activity of the osteoclasts, consequently increasing bone destruction[13].

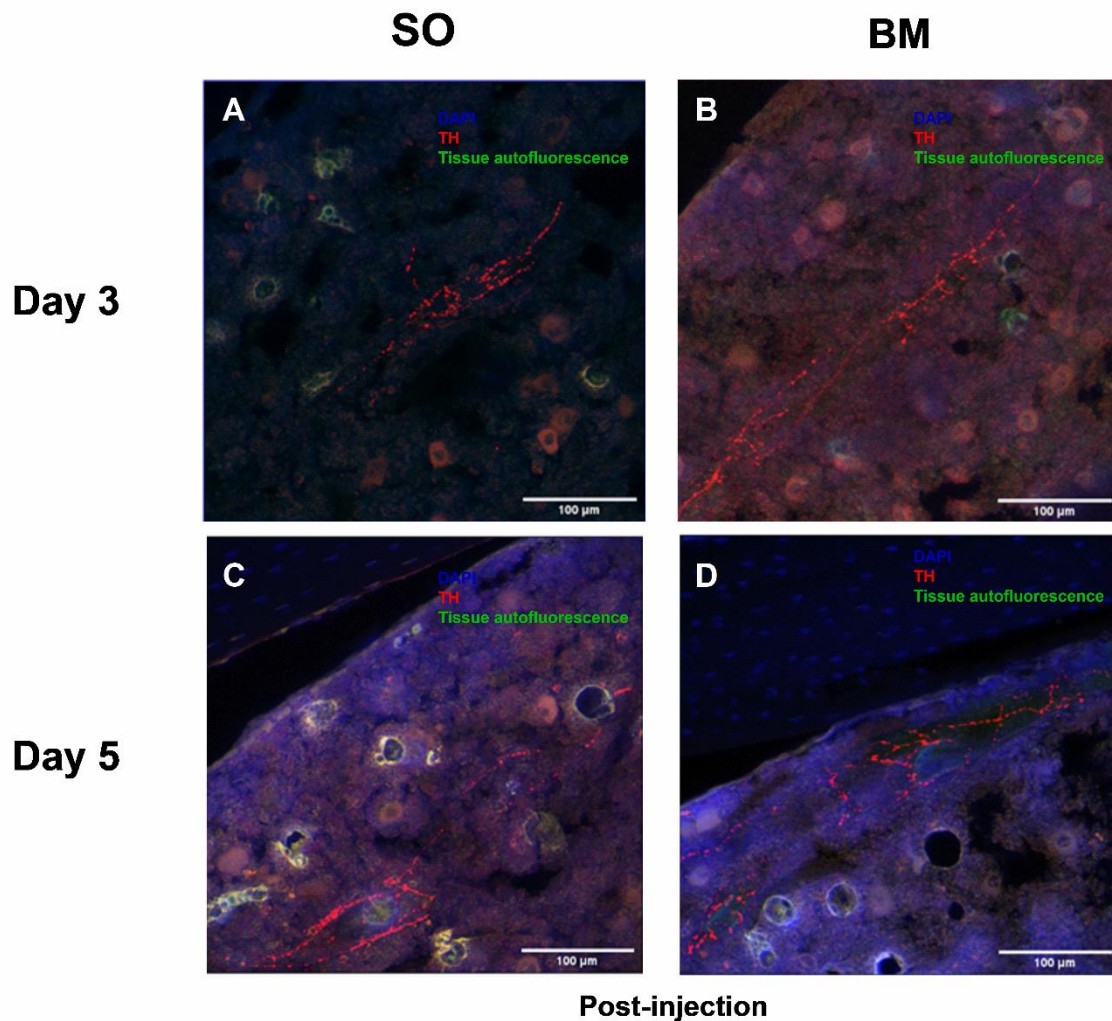


Figure 4.32- Representative images of the TH-staining at day 3 and 5 of the SO group and BM group after injection. (A) SO group at day 3 post-injection; (B) BM group at day 3 post-injection; (C) SO group at day 5 post-injection; (D) BM group at day 5 post-injection (Scale bar =100μm).

Small TH-positive nerve fibers were only observed by day 7 near the tumour (Figure 4.32). As tumour grows, there is an increase need for nutrients and oxygen. Thus, the tumour cells modulate the behaviour of the cells in its environment to allow formation of an aberrant network of vasculature. As sympathetic nerve fibers are closely related with the blood vessels, and sympathetic activation has been shown to be a player in neoangiogenesis, the presence of TH-positive fibers in the tumour might be related to the new blood vessels formed [140]. At day 14 (Figure 4.33), sympathetic nerve fibers were also observed in the tumour. So as sympathetic activation is also linked with the increase invasion of the cancer cells, the presence of the nerve fibers in the tumour can have implications in its intravasation and homing to other tissues[140]. Furthermore, in these time-point in both conditions it was observed sympathetic nerve fibers in the periosteum.

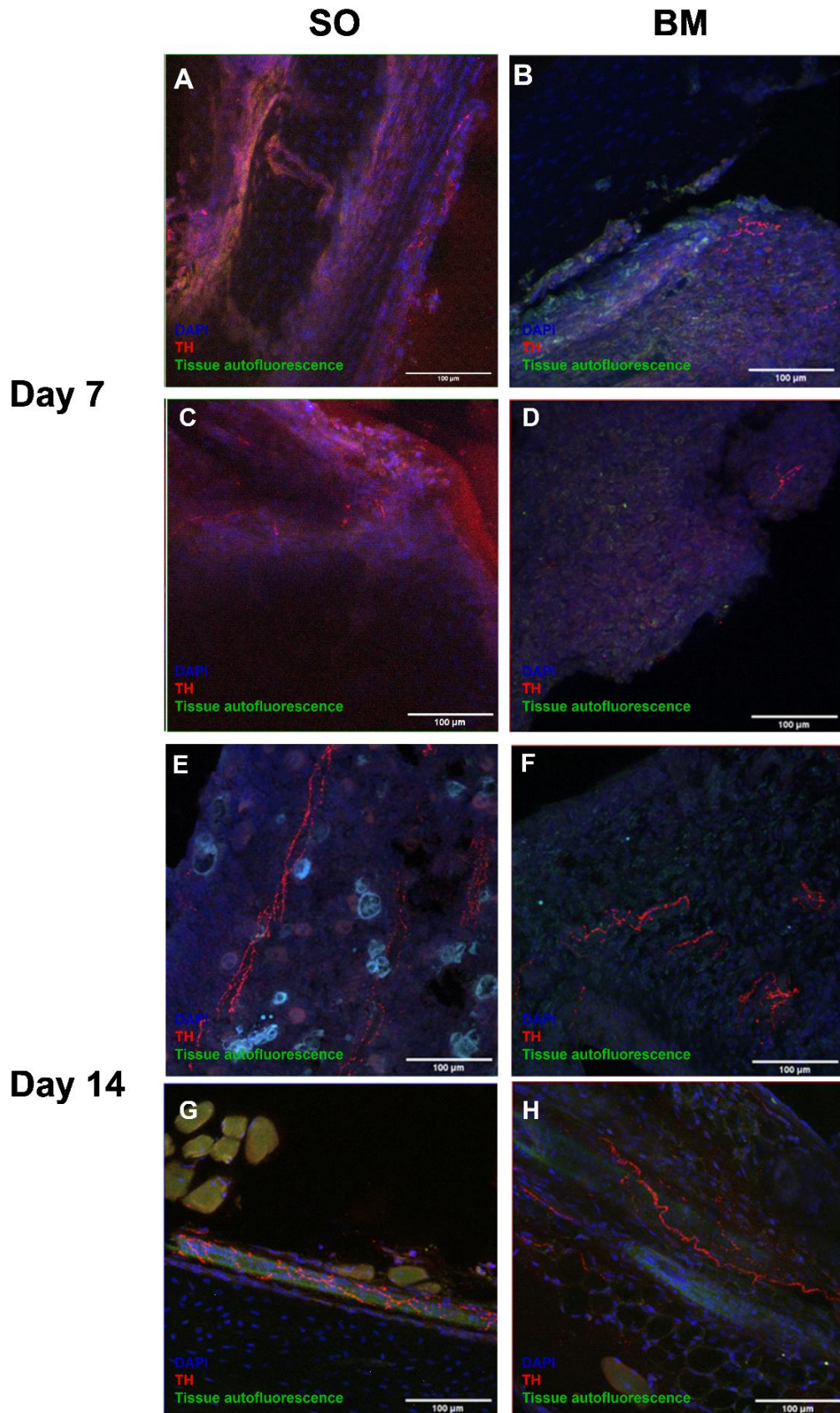


Figure 4.33 - Representative images of the TH-staining at day 7 and 14 of the SO group and BM group after injection. (A)(C) SO group at day 7 post-injection; (B)(D) BM group at day 7 post-injection; (E)(G) SO group at day 14 post-injection; (F)(H) BM group at day 14 post-injection (Scale bar =100µm)

To better understand these differences, the amount of TH-positive nerve fibers were quantified. During 14 days, tumour increase size as well as bone destruction, however no significant differences were observed in terms of the quantity TH-positive nerve fibers in the different days and between conditions (Figure 4.34). It is possible to verify that in the same condition values are disperse, making it hard to draw any conclusions. The optimal comparison of the quantification of the TH-nerve fibers, would imply that all samples were retrieved from the same position of the bone, however due to the bone structure and the presence of the bone metastasis, that increases bone fragility, during the process of cutting the bone samples this cannot be complete fulfilled. Also, sympathetic nerve fibers can be entangled in the vessels, and one nerve fiber can be cut in two small fibers as they do not necessarily follow a linear path, which can disguise the true results. Also, it would be necessary to increase the number of animals per condition and time-points.

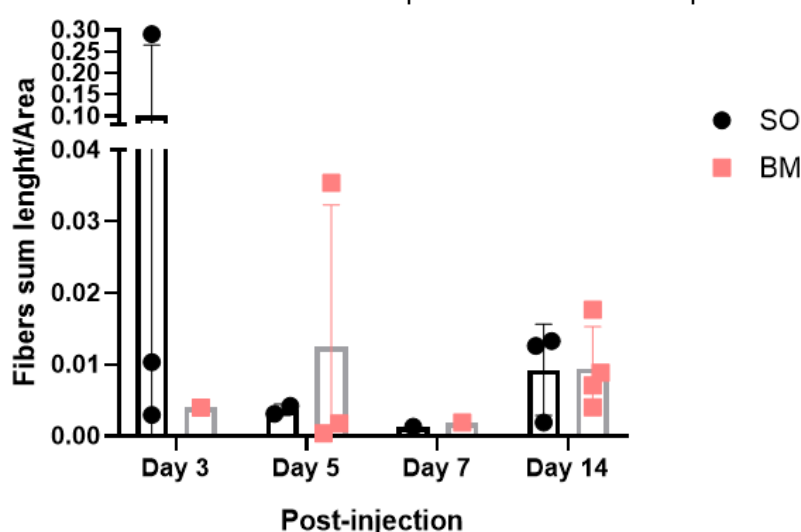


Figure 4.34- TH-positive nerve fiber quantification from the different days.

3.2 - α_{2C} -ADR expression profile

The presence of sympathetic nerve fibers in bone [13, 48] and the existence of ADR in the bone resident cells gives support to a role of the SNS in bone remodelling [13, 58, 63-66]. Not only a direct effect of the SNS was observed in the bone niche, but also in the leptin regulation of the bone remodelling [58, 63]. Furthermore, the α_{2C} -ADR was show not only to be involved in the direct effect of SNS in bone remodelling, but also to play a role in thyrotoxicosis-induced osteopenia [82]. Both of these evidences uncover a more complex role for the SNS in the regulation of bone metabolism. In addition, in Breast cancer the sympathetic signals may alter tumour growth and proliferation. Therefore, as SNS might be implicate in both bone remodelling and Breast cancer metastasis to bone, understanding the expression of the α_{2C} -ADR in the bone metastatic niche could increase knowledge of the possible interaction between the SNS in Breast cancer bone metastasis.

The α_{2C} -ADR expression was evaluated at day 14. Despite the fact that sympathetic innervation was not significantly different between the two groups, the different expression of the α_{2C} -ADR would also have implications on the SNS role on the bone metastatic niche. Its

expression and activation was linked to an increase malignance of colon cancer cells [143]. In both groups α_{2C} -ADR was express in the cortical bone and in the periosteum (Figure 4.35). In the SO group the staining had a dot like resemblance and the way it was distributed might point to the presence of a sympathetic nerve fiber, as they are autoreceptors. The expression of the α_{2C} -ADR in the bone niche appears to be more prominent in the bone marrow near the border with the cortical bone. Regarding the BM group, α_{2C} -ADR expression was observed in the tumour, being equally distributed in its centre and peripheries. So the tumour might be influence by the SNS as both TH-positive fibers and the α_{2C} -ADR are present within it.

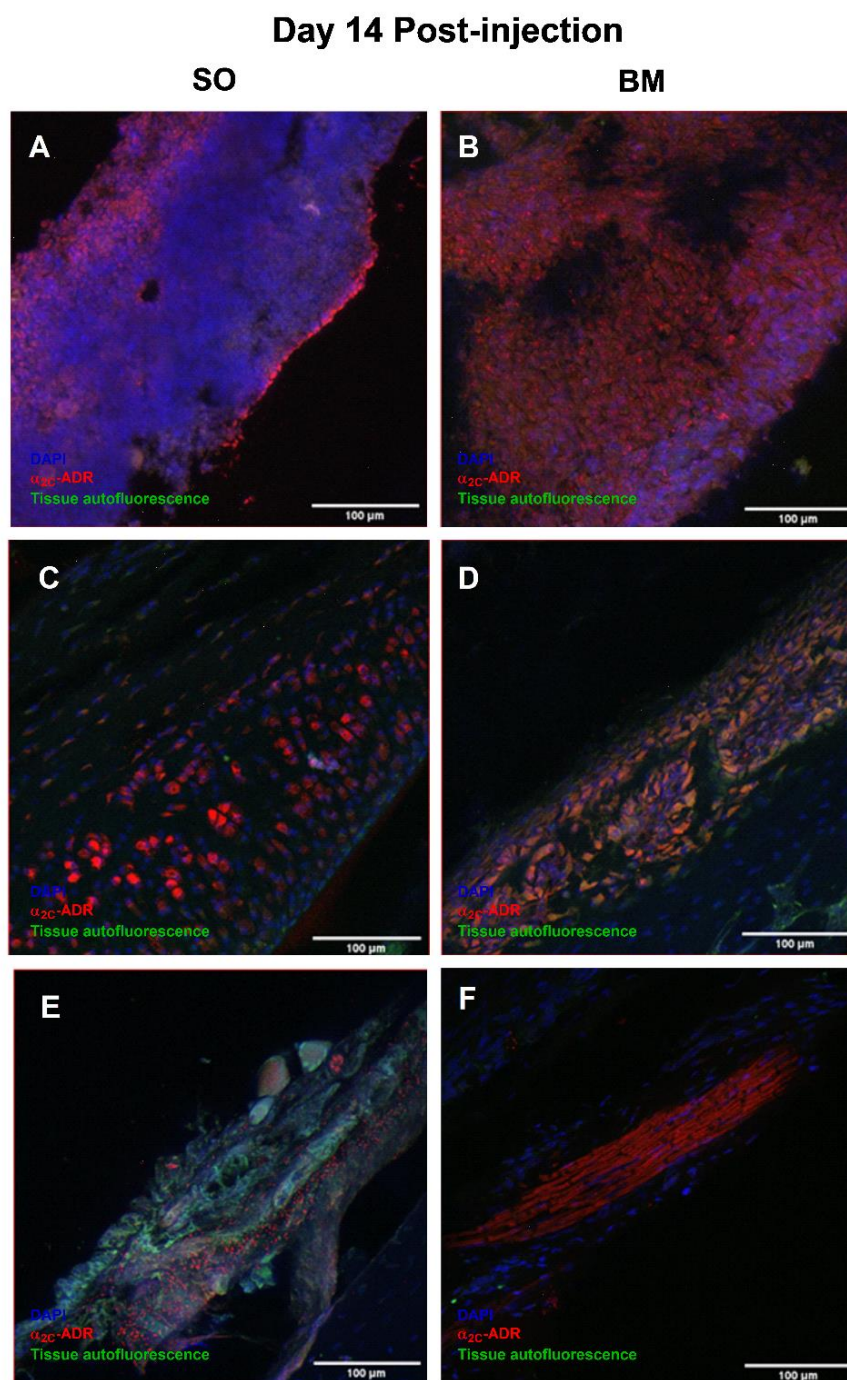


Figure 4.35- Representative images of the α_{2C} -ADR staining on the SO group and BM group after 14 days' post-injection. (A) bone marrow (C) cortical bone (E) periosteum SO group at day 14 post-injection; (B) tumour (D) cortical bone (F) periosteum BM group at day 14 post-injection. (Scale bar =100 μ m).

To better understand if the expression of the α_{2C} -ADR is increased in the bone metastatic niche, a comparison between the intensity of α_{2C} -ADR staining in both groups should be performed. However, due to the thickness of the samples in some cases nuclei were not well defined and thus hindered the analyses. Also the relationship of the α_{2C} -ADR expression and the sympathetic nerve fibers should be evaluated during the different days of the bone metastasis growth.

Chapter 5

Conclusion and Future Perspectives

The SNS is a complex system that regulates the majority of human organ systems, by the release of catecholamine neurotransmitters, such NE. However long periods of acute SNS activation have deleterious consequences in health. In light of that a potential role for SNS in bone is being explored, as sympathetic nerve fibers and catecholamine ADR are present in bone niche. Additionally, SNS is also being implicated in cancer incidence and proliferation through the activation of α -ADR and β -ADR signalling pathways.

Thus, in this work it has been proposed to evaluate the mechanisms underneath α_{2C} -ADR activation in bone remodelling and its impact on osteoclast activity in the context of Breast cancer bone metastatic disease. For that, it has been considered two approaches: i) a direct effect of the α_{2C} -ADR signalling pathway on bone niche and ii) an indirect effect of the α_{2C} -ADR signalling pathway through Breast cancer cells on bone niche.

To evaluate the effects on osteoclast function, a monoculture was performed and direct activation/blockade of the α_{2C} -ADR was done by using NE, as an α_{2C} -ADR agonist and JP1302, as an α_{2C} -ADR blocker. The blockage of this receptor seem to impact negatively osteoclast activity. Firstly, by the apparent decrease on the osteoclastogenesis and impairment of the osteoclast morphology, which could have consequences in its resorptive activity. As toxicity of the α_{2C} -ADR blocker was not observed, this pathway might have some basal influence in the osteoclast, as further support was found when the tendency to a decrease of resorptive activity was higher when the concentration of 10^{-9} M NE was also added.

As in the bone niche, different resident cells express α_{2C} -ADR, a co-culture was also performed to increase similarity to the *in vivo* scenario. In this case the effect of the α_{2C} -ADR blockage on the osteoblast seems to increase the RANKL expression, which was more pronounced at the higher concentration of NE (10^{-5} M). As the RANKL expression is increased, this can explain the slight increase of eroded surface on the co-culture in relation to the monoculture in that condition of α_{2C} -blockade and 10^{-5} M NE. However, the same was not observed in the other conditions where the α_{2C} -ADR antagonist was added. As at the concentration of 10^{-5} M, NE binds preferentially to β -ADR, this suggests that its activation

mitigates the effect of the α_{2C} -signalling pathway on the osteoclast, both directly and indirectly. These results further support the role of SNS in bone remodelling [13]. Nevertheless, increasing the sample size is necessary as values present variability. Furthermore, a different approach to the osteoclastogenesis in the co-culture need to be performed as it was not possible to infer it due to the high activity of the osteoblasts. A possible solution is to exploit the same timeline of the resorptive activity assay, but reduce the time of culture.

These results further support the role of SNS in bone remodelling. Additionally, SNS is also being implicate in cancer incidence and proliferation [140, 141]. Therefore, a relationship between the bone metastatic niche and SNS is an opportunity to uncover new information in the Breast cancer bone metastasis and develop new therapeutic strategies.

Thus, the effect of the α_{2C} -signalling pathway in Breast cancer bone metastasis was assessed. Firstly, it was evaluated its impact in the breast cancer cell line. Bone tropic Breast cancer cells (4T1.2 cell line) were stimulated with NE, an α_{2C} -ADR agonist, and JP1302, an α_{2C} -ADR blocker and it was assessed its' effect on proliferation and migration. The blockage of the α_{2C} -signalling pathway decrease cell density, however the expression of the ki67 marker was the same. Due to that the effect of the blocker might have impact in the first hours after addition, so this assay should be repeated at an earlier time-point. Regarding the migration, the 72-hour period was not enough to observe migration through the Matrigel compartment. As no further stimulus was applied, medium change could be done after 24 hours to allow more exposure to stimulus.

Secondly, as there is a gap of knowledge regarding the mechanisms of osteoclast in bone of the SNS involvement in the Breast cancer bone metastasis, it was analysed the effects of the α_{2C} -ADR activation in the bone Breast cancer cells by treating a monoculture of osteoclasts or a co-culture of osteoclasts and osteoblasts with the Breast cancer CM. The serum free media had impact on the osteoclast, but it was possible also to observe the impact of the Breast cancer secretome, since there was an apparent increase in osteoclast activity was found when the non-stimulated Breast cancer CM was added to the culture. As happened in the case of the direct effect of the α_{2C} -adrenergic pathway, osteoclastogenesis, osteoclast morphology and resorptive activity seem to be affect when this pathway was blocked in the Breast cancer cells.

Regarding the co-culture, not only it was not possible to evaluate osteoclastogenesis, but also quantification of the resorptive activity of the osteoclast was not assessed as no resorption was observed in the bone slices. So possible solutions, might implicate addition of extra M-CSF and RANKL to the culture, or modification of the time-points of medium change to increase stimulus. For the case of bone resorption, treatment with the CM could be only performed at day 3 since only at that time-point osteoclast start to appear in culture. Although a slight decrease was observed in RANKL expression in the osteoblasts, when α_{2C} -blockage was performed in the Breast cancer cells.

In vivo models represent a fundamental tool in research. As B-ADR stimulation of the bone environment increases Breast cancer engraftment to bone, and the high sympathetic nerve density is associated with worse clinical outcomes[140, 141], the expression profile of TH-positive fibers was evaluated in a Breast cancer bone metastatic mouse model. This model

represents the late stage of the Breast cancer bone metastasis as bone tropic Breast cancer cells were directly inoculated in the tibia and had the advantage of being immunocompetent, since the immune system also plays a role in SNS mediation of cancer. The presence of TH-positive nerve fibers was assessed at different time-points. No significant differences were observed between the SO group and the BM group and, in both, sympathetic nerve fibers were predominantly found in Z3 or Z4 in the spiral-like shape, that is characteristic of this nerve fibers, as they are usually associated with vessel structures. In the BM, small TH-positive fibers were only found at day 7 and 14 near and in the tumour. As at that time metastasis is well established the presence of the nerve fibers might indicate vasculature or the potential intravasation to other sites. Despite the non-significant difference of sympathetic fibers, the expression of α_{2C} -ADR could be distinct, which would have implications in the SNS interaction in the bone metastatic niche. Thus, it was assessed the expression of α_{2C} -ADR at day 14, when tumour had already potentiated large bone destruction. The expression of this receptor was equally found in the cortical bone and periosteum of both groups. In the SO group the ADR was also found in the periphery of the bone marrow with cortical bone. Whereas in the BM group it was present in the tumour in an equitable spatial distribution. Due to the thickness of the samples, comparison of intensity of α_{2C} -ADR staining/nucleus was hindered.

Globally, this study suggests that the SNS stimulation through the α_{2C} -ADR in bone remodelling could possibly affect: (i) osteoclast differentiation, function and its respective resorptive capacity, (iii) RANKL expression in osteoblasts. In regard of SNS stimulation in bone Breast cancer metastasis it may affect (i) Breast cancer survival; and consequently bone metastasis; (ii) osteoclast differentiation, function and its respective resorptive capacity, (iii) RANKL expression in osteoblast; and (iv) SNS can interact with the tumour through the α_{2C} -adrenergic pathway.

As future perspectives, regarding both the effect of SNS in bone remodelling and SNS in bone Breast cancer metastasis increasing sample size is necessary. Further, manually analyse of the number of osteoclast with the different nucleus range and area should be performed. Also, it would be interesting to analyse the proteins present in the osteoclast medium of the differentiation assay. Additionally, analyses of secretome components during the bone resorption, such MMP9 and CTSK should be performed in order to obtain more information on the mechanisms behind the of α_{2C} -signalling pathway on the osteoclast resorptive activity.

Microfluidic devices are an important tool in research that is being used as a bridge between the *in vitro* and *in vivo* data. Thus, they can be used to better mimic the bone metastatic niche, by removing the need to use CM. For that two different compartments can be design, where one simulated the bone niche, by culturing the bone cells and in the other breast cancer cells can be added and stimulated.

Regarding the effect of the SNS in Breast cancer bone metastasis, analyses of the RNA expression of molecules involved in the “vicious cycle” after stimulation/blockage of the α_{2C} -signalling pathway in the bone tropic Breast cancer cells. Increasing sample size in proliferation and migration assay are also necessary. A resazurin assay should be performed to understand the effect of α_{2C} -ADR pathway in the metabolic activity of the cancer cells and a live/dead to understand the toxicity. An invasion assay could also be performed by using the same

microfluidic device, taking advantage of the third channel to culture osteoclasts and osteoblasts, mimicking that way the bone niche.

Finally, for the TH-positive fibers profile and α_{2C} -ADR expression increase sample size is necessary and animals per condition. For the sympathetic nerve fiber profile it would be valuable to add another time point between the day 7 and 14, to further understand the sympathetic innervation profile of the tumour. In relation to the α_{2C} -ADR expression, it should also be evaluated in the other time-points. In the first days to understand the possible effect of SNS in the bone niche, as fibers were only found in the zones where tumour was not present, and after to infer if the presence of sympathetic nerve fibers influence this ADR expression in the tumour cells. Lastly, understanding how sympathetic hyperactivation influences TH-positive nerve fibers profile and α_{2C} -ADR expression during Breast cancer bone metastasis would be an important and valuable future perspective.

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