

MESTRADO EM ONCOLOGIA
ESPECIALIZAÇÃO EM ONCOLOGIA MOLECULAR

Exploring the role of sympathetic nervous system in breast cancer-induced bone metastasis – the osteoclast side

Catarina Martins Lourenço

M
2018



Catarina Martins Lourenço
Exploring the role of SNS in breast cancer-induced bone metastasis – the osteoclast side



M.ICBAS 2018

Exploring the role of sympathetic nervous system in breast cancer-induced bone metastasis – the osteoclast side

Catarina Martins Lourenço



Catarina Martins Lourenço

Exploring the role of sympathetic nervous system in breast cancer-induced bone metastasis – the osteoclast side

Dissertação de Candidatura ao grau de Mestre em Oncologia submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientador – Doutora Daniela Sousa

Categoria – Post-doc researcher

Afiliação – Instituto de investigação e inovação em saúde (I3S) da Universidade do Porto/Instituto de engenharia biomédica (INEB).

Co-orientador – Doutora Cármen Jerónimo

Categoria – Professora Associada convidada com Agregação

Afiliação – Departamento de Patologia e Imunologia Molecular, Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto (ICBAS); Investigadora Auxiliar; Coordenadora do Grupo de Epigenética e Biologia do Cancro IPOP.

Agradecimentos

Durante a realização da minha tese, muitas foram as pessoas que, de uma maneira ou de outra me ajudaram a concluir esta etapa da minha vida. Como tal, não posso deixar de agradecer:

À minha orientadora, a Doutora Daniela Sousa! Por todas as coisas que aprendi contigo, tanto a nível científico como pessoal. Obrigada por seres a pessoa que és, por estares sempre pronta a ajudar nas mais pequenas coisas. Por toda a paciência, boa disposição e positividade que me transmitiste quando mais precisava. Não podia ter imaginado ninguém melhor para me guiar nesta etapa tão importante, obrigada!

A todos restantes membros do Nesk group, por estarem sempre prontos a ajudar e por me terem feito sentir em casa! À Professora Meriem Lamghari, por me ter concedido a oportunidade de pertencer ao seu incrível grupo. À Ju por toda a calma, bondade e atenção, foste a melhor companheira de secretária! Ao Luís, obrigada por todas as brincadeiras e *Maltesers*, vou pensar sempre em ti quando marcar *ependorfs*! À Estrela, pelo teu incrível sorriso e boa disposição! Ao Francisco, por toda a paciência que tiveste comigo, por estares sempre disponível para tirares todas as minhas dúvidas e por tudo que aprendi contigo! Ao Miguel, pelo companheirismo, por estar sempre pronto para me ajudar e por partilhar comigo tudo o que sabia.

Às meninas do Neuro Meals! Sofia, Marília, Natália, Eva, Spencer e Ana obrigada por me inserirem no vosso grupo! Por todas os almoços, jantares e lanches, pelas conversas, risos e conselhos que partilharam comigo. Por toda a ajuda dentro e fora do laboratório, vou ter mesmo muitas saudades vossas!

A todas as restantes pessoas do 214.S1, sempre repleto de boa-disposição e espírito de entreajuda, não podia ter pedido melhor local de trabalho, partilhado com as melhores pessoas. A todos os outros membros do I3S que sempre se mostraram disponíveis para ajudar, entre eles, a Dalila, Joana, Maria e André Maia. Todos vocês facilitaram o meu percurso nesta grande casa!

À minha co-orientadora, a Professora Cármen, por me ter aconselhado este incrível projeto e por toda a disponibilidade que sempre mostrou em ajudar no que fosse preciso. E claro, à Helena, por ser uma pessoa incrível e super amorosa, por ouvir todas as minhas lamúrias e incertezas e por até ao fim se mostrar pronta para me ajudar. Está mesmo quase!

Por fim, não posso de deixar de agradecer a todas as pessoas, que sem conseguirem entender muito bem, sempre me apoiaram e me deram força para poder continuar, por isso aqui vai:

Mãe, Pai e Filipe, nunca conseguirei exprimir o quão importantes foram para mim durante este percurso, e durante toda a minha vida na verdade! Por isso obrigada por todos os momentos que partilhamos, por entenderem a minha má disposição (ocasional), o meu cansaço e por todo o apoio e força que me deram, sem vocês era impossível! Não posso deixar de agradecer às minhas Avós e aos meus Avôs, sou a pessoa que sou hoje por me terem ajudado a criar e educar.

Aos restantes membros da Academia do Aloquete! Todos vocês, Padrinhos, Tios, Primos, Leonor, Jorge, a minha Edu, Diana e João, Pequenitos! De uma maneira ou outra, nos pequenos olhares e abraços apertados, por toda a boa disposição, pela força e apoio, por todas as gargalhadas até chorar, e todos os momentos que muitas vezes fizeram desaparecer qualquer vestígio de stress, o meu sincero obrigada!

Resumo

O cancro da mama (BrCa) permanece como o cancro mais diagnosticado nas mulheres em todo o mundo. E apesar de quando precocemente detetado apresentar bom prognóstico, um número substancial de pacientes ainda é detetado com cancro da mama metastático ou o cancro acaba por reaparecer após remoção do primário. Apesar de existirem diferentes subtipos de BrCa que estão associados a padrões de metastização únicos, o esqueleto permanece como o principal local de metastização. As doenças metastáticas do osso causadas pelo ciclo vicioso do BrCa são uma grande causa de morbilidade nestes pacientes, que até estes dias permanece sem estratégias de tratamento que limitem a progressão da doença óssea.

Curiosamente, a ativação de vias α - e β -adrenérgicas, pela libertação de catecolaminas em resposta à ativação do sistema nervoso simpático (SNS) tem sido associado com aumento de incidência de metástases do BrCa no osso. Já que a maioria dos estudos se foca no papel dos osteoblastos, o papel dos osteoclastos (OCs) permanece pouco reconhecido. Deste modo, o melhor conhecimento das vias moleculares e celulares envolvidas neste processo é imperativo para poder explorar terapias novas e mais eficientes para tratar eventos ósseos, melhorar a sobrevivência e diminuir o risco de recorrência em pacientes com BrCa.

Neste sentido, este estudo investigou o papel de cada subtipo de BrCa na diferenciação e atividade dos OCs através da ativação do SNS (tratamento com norepinefrina (NE)). Assim sendo, cinco linhas celulares do BrCa (MCF-7, BT-474, SK-BR-3, MDA-MB-468 e MDA-MB-231), cada uma representando um subtipo, e uma linha metastática de osso (Bo-1833) foram usadas para realizar estes estudos. A caracterização do seu perfil simpático foi analisada por qRT-PCR e imunofluorescência, revelando que de facto, diferentes subtipos de BrCa expressam recetores adrenérgicos (ARs) e enzimas simpáticas distintas.

Além disso, NE (agonista global dos ARs) foi adicionada a cada linha celular de cancro da mama. Esse meio foi recolhido e usado para tratar OCs, de modo a avaliar os efeitos causados pela ativação do SNS, na diferenciação e atividade das células que reabsorvem o osso. OCs humanos foram diferenciados através de monócitos isolados de *buffy-coats* de doadores saudáveis, que dependendo do estudo, foram postos em cultura em superfícies TCPS ou em cima de um pedaço de osso bovino. De modo a avaliar estes parâmetros, imunofluorescência, quantificação da fosfatase ácida târtaro-resistente (TRAP), expressão das metaloproteínas (MMPs) e ensaios da atividade de reabsorção foram realizados.

Antes de avaliar os efeitos do SNS em interações BrCa-OC, os efeitos diretos que a ativação do SNS tem na diferenciação e atividade dos OCs foi avaliada, revelando que os números de OCs aumentam gradualmente quando as células são tratadas com doses mais baixas NE e diminuem com a adição de doses altas. A área dos OCs é semelhante em todos os tratamentos e a atividade de reabsorção diminui com o aumento das concentrações de NE.

Em relação aos efeitos do meio condicionado (CM) das células do BrCa nos OCs, quando o CM foi adicionado, a reabsorção aumentou e este resultado foi comprovado pelos dados da atividade da TRAP. Apesar dos tratamentos das células de BrCa com NE também afetarem a morfologia e função dos OCs, os efeitos dependem do subtipo de BrCa presente. Enquanto que os subtipos luminais e HER2⁺ quase não causaram alterações na percentagem de superfície erodida quando NE foi adicionada, os CMs do subtipo triplo negativo provocaram um ligeiro aumento com a adição de NE com doses mais elevadas.

Por estas razões, este estudo preliminar sugere que o SNS tem um papel nas metástases ósseas do BrCa, através da ativação dos OCs. No entanto, mais estudos precisam de ser realizados para aumentar o tamanho da amostra e assim confirmar os resultados obtidos neste projeto.

Abstract

Breast cancer (BrCa) remains as the most commonly diagnosed cancer in women worldwide. And although when early detected, most BrCa cases have good prognosis, a substantial number of BrCa patients end-up relapsing or are still detected with metastatic BrCa at the time of diagnosis. While there are different BrCa subtypes which are associated with unique metastatic patterns, the skeleton remains as one of the major sites for metastasis. Metastatic bone diseases caused by the BrCa vicious cycle are a major cause of morbidity in these patients, that until this day lacks proper treatment strategies to surpass the progression of bone disease.

Interestingly, the activation of α - and β -adrenergic pathways by release of catecholamines in response to sympathetic nervous system (SNS) activation have been linked with the incidence rate of BrCa metastases in bone. Since most studies mainly focus on the role of osteoblasts (OBs), the role played by osteoclasts (OCs), the only bone-resorbing cells, remains poorly understood. Therefore, there is a need to better understand the molecular and cellular pathways involved in this process to explore new and more efficient potential therapies to treat bone events, improve survival and decrease recurrence in BrCa patients.

With this in mind, this exploratory study investigated the role of each BrCa subtype on OC differentiation and activity through SNS activation (e.g. norepinephrine (NE) treatment). Therefore, five BrCa cell lines (MCF-7, BT-474, SK-BR-3, MDA-MB-468 and MDA-MB-231), each representing a BrCa subtype, and a BrCa bone-tropic cell line (Bo-1833), were used to perform these studies. Their SNS profile characterization was analysed through qRT-PCR and immunostaining analysis, revealing that indeed different BrCa subtypes express distinct adrenoreceptors (ARs) and SNS enzymes.

Moreover, NE (a global AR agonist) was added to each BrCa cell line. This medium was collected and used to treat OCs, in order to evaluate the effects caused by SNS activation, on differentiation and activity of the bone resorbing cells. Human OCs were differentiated from monocytes isolated from buffy coats of healthy donors, and depending on the study, these cells were cultured on TCPS surfaces or seeded on top of bone slices. To evaluate these parameters, F-actin immunostaining, tartrate-resistant acid phosphatase (TRAP) quantifications, metalloproteinase (MMP) expression and resorption activity assays were performed.

Before assessing the effects of SNS on BrCa-OC interactions, the direct effects that SNS activation has on OC differentiation and resorptive activity were evaluated, revealing that OC numbers gradually increase when cells are treated with lower doses of NE and decrease with the addition of highest doses. OC area is similar in all NE treatments and OC activity decreases with increasing NE concentrations.

Regarding the effects of BrCa condition media (CM) on OCs, when CM of BrCa cells was added to OCs, the resorption was increased, and this result was complemented with TRAP activity data. Although treatments of BrCa cells with NE also affected OC morphology and function, the effects differed in each BrCa subtype. While luminal and human epidermal growth factor receptor 2 (HER2)-enriched subtypes almost did not alter the percentage of eroded surface when NE was added, triple-negative cells provoked a small decrease with the addition of increasing doses of NE.

Therefore, this preliminary study suggests that SNS have a role to play in BrCa bone metastasis, through OC activation. However, more studies with different donors need to be performed to increase the sample size and confirm the results obtained in this project.

Index

Agradecimientos	i
Resumo	iii
Abstract.....	v
List of Figures	ix
List of Tables	xii
Abbreviations	xiii
I. Introduction	1
1. Breast cancer (BrCa).....	3
1.1. Epidemiology and risk factors	3
1.2. BrCa subtypes	4
1.3. Patterns of metastatic BrCa.....	5
2. Bone microenvironment and bone metastasis.....	8
2.1. Bone structure and remodelling	8
2.2. Bone metastasis.....	13
2.3. BrCa bone metastasis	14
2.4. Therapeutic approaches to bone metastasis.....	15
3. SNS.....	16
3.1. Stress response	16
3.2. SNS in BrCa-bone metastasis	19
II. Aims	21
III. Materials and methods	25
1. Cell cultures	27
1.1. BrCa cultures	27
1.2. OCs cultures	28
2. Gene expression analysis	30
3. Immunocytochemistry analysis.....	31
4. Cell morphology analysis.....	32

5. Bone resorption quantification	32
6. TRAP quantification.....	33
6.1. TRAP staining.....	33
6.2. TRAP activity	33
7. Zymography analysis	33
8. Scanning electron microscopy (SEM) analysis.....	34
9. Statistical Analysis	34
IV.Results and discussion	35
1. Characterization of SNS expression profile	37
1.1. BrCa	37
1.2. OCs	43
2. Role of SNS in OCs (direct effects)	46
2.1. Effects of NE treatment on OC differentiation	46
2.2. Effects of NE treatment on OC resorption activity	54
3. Role of SNS in BrCa-OC interactions (indirect effects)	58
3.1. RANKL/RANK expression in BrCa.....	58
3.2. Effects of BrCa NE treated CM on OC differentiation	60
3.3. Effects of BrCa NE treated CM on OC resorption activity	74
Concluding remarks and Future perspectives	83
References	87
Annexes	95

List of Figures

Figure I. 1 - Anatomy and histology of the breast.....	3
Figure I. 2 –Metastatic events of BrCa cells from the primary tumour to bone.	6
Figure I. 3 – The distribution of most common metastatic sites by each BrCa intrinsic subtype.	8
Figure I. 4 – Steps of bone remodelling.	9
Figure I. 5 – Osteoclastogenesis	11
Figure I. 6 – Different OC morphologies during non-resorptive and resorptive phases.....	12
Figure I. 7 – BrCa bone metastatic vicious cycle.	14
Figure I. 8 - Pathways involved in stress response.	17
Figure I. 9 – Signalling pathways, localization and effects of each AR subtype.	18
Figure II. 1 - Exploring the role of SNS on BrCa-induced bone metastasis – the OC activity....	23
Figure III. 1 – Workflow of isolation and differentiation of CD14+ monocytes from buffy coats into fully matured OCs	29
Figure III. 2 – Scheme of OC treatments.....	30
Figure III. 3 – Quantitative real time Polymerase Chain Reaction protocol.....	31
Figure III. 4 – Representative image of resorptive events caused by active OCs.	32
Figure IV. 1 – Representative images of 2D and 3D morphologies of each BrCa cell line used.	38
Figure IV. 2 – Expression of $\alpha 1$-, $\alpha 2A$-, $\beta 2$-ARs and TH in each BrCa cell line by immunostaining.	40
Figure IV. 3 – ARs and catecholamine synthesizing enzymes gene expression profile of the 6 BrCa cell lines.....	42
Figure IV. 4– Representative images of OC morphology during the different stages of osteoclastogenesis.	43
Figure IV. 5 – Representative images of mature OC' cytoskeletal arrangements.	44
Figure IV. 6 – SEM images of OCs seeded for 72h on top of bovine bone slices.	45
Figure IV. 7 – Gene expression by qRT-PCR of ARs and enzymes expressed by OCs.	46
Figure IV. 8 - TRAP staining of pre-OCs treated (since day 5) with different NE concentrations until day 9 of differentiation.	47
Figure IV. 10 – Overview protocol for OC quantification.	49
Figure IV. 11 – Representative images of F-actin staining with OC treated (since day 5) with increasing doses of NE.	50

Figure IV. 12 - Morphological analysis of OCs treated, since day 5, with different NE concentrations.	51
Figure IV. 13 – Representative images of F-actin staining of OCs treated (since day 9) with increasing NE concentrations.....	52
Figure IV. 14 - Morphological analysis of OCs treated, since day 9, with different NE concentrations.	53
Figure IV. 15 – Direct effect of NE treatment on OC activity.	55
Figure IV. 16 – Quantification of TRAP enzyme activity from media of OCs treated with NE. ..	56
Figure IV. 17 - Zymography analysis of MMP-2 and MMP-9 activity from OC media treated with NE.....	57
Figure IV. 18 – RANKL and RANK gene expression profile of each BrCa cell line.	59
Figure IV. 19 – Quantification of OC treated, since day 5, with CM of MCF-10A cells treated with 0, 10^{-8} and 10^{-6} M of NE.....	60
Figure IV. 20 - Quantification of OC treated, since day 9, with CM of MCF-10A cells treated with 0, 10^{-8} and 10^{-6} M of NE.	61
Figure IV. 21 – Quantification of OC treated, since day 5, with CM of MCF-7 cells treated with 0, 10^{-8} and 10^{-6} M of NE.	62
Figure IV. 22 - Quantification of OC treated, since day 9, with CM of MCF-7 cells treated with 0, 10^{-8} and 10^{-6} M of NE.	63
Figure IV. 23 – Quantification of OC treated, since day 5, with CM of BT-474 cells treated with 0, 10^{-8} and 10^{-6} M of NE.	64
Figure IV. 24 - Quantification of OC treated, since day 9, with CM of BT-474 cells treated with 0, 10^{-8} and 10^{-6} M of NE.	65
Figure IV. 25 – Quantification of OC treated, since day 5, with CM of SK-BR-3 cells treated with 0, 10^{-8} and 10^{-6} M of NE.	66
Figure IV. 26 – Quantification of OC treated, since day 9, with CM of SK-BR-3 cells treated with 0, 10^{-8} and 10^{-6} M of NE.	67
Figure IV. 27 – Quantification of OC treated, since day 5, with CM of MDA-MB-468 cells treated with 0, 10^{-8} and 10^{-6} M of NE.....	68
Figure IV. 28 - Quantification of OC treated, since day 9, with CM of MDA-MB-468 cells treated with 0, 10^{-8} and 10^{-6} M of NE.....	69
Figure IV. 29 – Quantification of OC treated, since day 5, with CM of MDA-MB-231 cells treated with 0, 10^{-8} and 10^{-6} M of NE.....	70
Figure IV. 30 - Quantification of OC treated, since day 9, with CM of MDA-MB-231 cells treated with 0, 10^{-8} and 10^{-6} M of NE.....	71
Figure IV. 31 – Quantification of OC treated, since day 5, with CM of Bo-1833 cells treated with 0, 10^{-8} and 10^{-6} M of NE.	72
Figure IV. 32 - Quantification of OC treated, since day 9, with CM of Bo-1833 cells treated with 0, 10^{-8} and 10^{-6} M of NE.....	73

Figure IV. 33 – Effect of MCF-10A CM treated with NE on OC activity.	74
Figure IV. 34 – Effect of CM of luminal and HER2-enriched subtypes treated with NE on OC activity.....	75
Figure IV. 35 – Effect of triple negative subtypes treated with NE on OC activity.	76
Figure IV. 36 - Zymography analysis of MMP-2 and MMP-9 activity in MCF-10A CM.....	77
Figure IV. 37 - Zymography analysis of MMP-2 and MMP-9 activity in MCF-7 CM before and after OC contact.	78
Figure IV. 38 - Zymography analysis of MMP-2 and MMP-9 activity in BT-474 CM before OC contact.	79
Figure IV. 40 - Zymography analysis of MMP-2 and MMP-9 activity in MDA-MB-468 CM before OC contact.....	79
Figure IV. 41 - Zymography analysis of MMP-2 and MMP-9 activity in MDA-MB-231 CM before and after OC contact.	80
Figure IV. 42 - Zymography analysis of MMP-2 and MMP-9 activity in Bo-1833 before and after OC contact.....	81
Figure A. 1 – Effects of direct NE treatment on OCs at early (A-B) and late (C-D) differentiation stages.	98
Figure A. 2 - Direct effect of NE treatment on OC activity of all 6 donors.....	99
Figure A. 3 – Effects of BrCa cells treated with NE CM on OCs number at early differentiation stage.....	100
Figure A. 4 – Effects of BrCa cells treated with NE CM on OCs area at early differentiation stage.....	101
Figure A. 5 – Effects of BrCa cells treated with NE CM on OCs number at late differentiation stage.....	102
Figure A. 6 – Effects of BrCa cells treated with NE CM on OCs area at late differentiation stage.	103

List of Tables

Table I.1 – Characteristics of BrCa intrinsic subtypes	5
Table III. 1 – List of BrCa cell lines and respective culture media	27
Table A.1 - Primers used in qRT-PCR analysis.	97

Abbreviations

A

ACTH – Adrenocorticotrophic hormone

ALP – Alkaline phosphatase protein

ANS – Autonomous nervous system

AR – Adrenoreceptor

B

B2M – Beta-2-microglobulin

BMPs – Bone morphogenetic proteins

BMU – Basic multicellular unit

BRC – Bone remodelling compartment

BrCa – Breast cancer

BSA – Bovine Serum Albumin

C

Ca²⁺ – Calcium

cDNA – complementary DNA

CK – cytokeratins

CM – Conditioned media

CNS – Central nervous system

CRF – Corticotrophin-releasing factor

D

DBH – Dopamine beta-hydroxylase

DMEM – Dulbecco's Modified Eagle Medium

DNA – Deoxyribonucleic acid

DTC – Disseminated tumour cells

E

E – Epinephrine

ECM – Extracellular matrix

EGF – Epidermal growth factor

EMT – Epithelial to mesenchymal transition

ER – Estrogen

F

FBS – Fetal bovine serum

FHS – Fetal horse serum

G

GPCR – G-protein coupled receptor

H

HCS – High-content screening

HER2 – Human epidermal growth factor receptor 2

HPA – Hypothalamic pituitary adrenal

HR – Hormonal receptor

HRT – Hormone replacement therapy

HSC – Hematopoietic stem cells

I

IGF – Insulin Growth Factor

IL – Interleukin

M

M-CSF – Monocyte/macrophage colony–stimulating factor

MMP – Metalloproteinase

MSC – Mesenchymal stem cells

N

N – Norepinephrine

O

OB – Osteoblast

OC - Osteoclast

OPG – Osteoprotegerin

P

PBMC – Peripheral blood mononuclear cells

PBS – Phosphate-buffered saline

PFA – Paraformaldehyde

PGE2 – Prostaglandin E2

PNMT – Phenylethanolamine N-methyltransferase

PR – Progesterone

PTHrP – Parathyroid Hormone-related

Q

qRT-PCR - quantitative real time polymerase chain reaction

R

RANK – Receptor activator of nuclear factor- κ B

RANKL – Receptor activator of nuclear factor- κ B ligand

rBM – reconstituted Basal Membrane

RIPA – Radioimmunoprecipitation assay buffer

RNA – Ribonucleic acid

S

SNS – Sympathetic nervous system

T

TGF β – Transforming growth factor- β

TH – Tyrosine-hydroxylase

TNBC – Triple negative BrCa

TNF – Tumour necrosis factor

TRAP – Tartrate-resistant acid phosphatase

V

VEGF – Vascular endothelial growth factor

α-MEM – α-Modified Eagle Medium

I. Introduction

1. Breast cancer (BrCa)

1.1. Epidemiology and risk factors

BrCa is the most diagnosed cancer in women worldwide and is expected to represent around 30% of all cancer cases diagnosed in females [1, 2]. Despite the large number of new cases, mortality rate has been slowly decreasing in developed countries, with the implementation of earlier diagnosis and improvement of adjuvant therapies [3-5].

Although the breast is composed by a variety of tissues, the majority of breast tumours occur in the mammary gland, with tumours beginning either in the lobules, which are the glands that produce milk, or in the ducts, the canals in which milk reaches the nipple (Figure I.1). The stromal tissues of the breast, such as the adipose or fibrous connective tissues are less commonly affected [6].

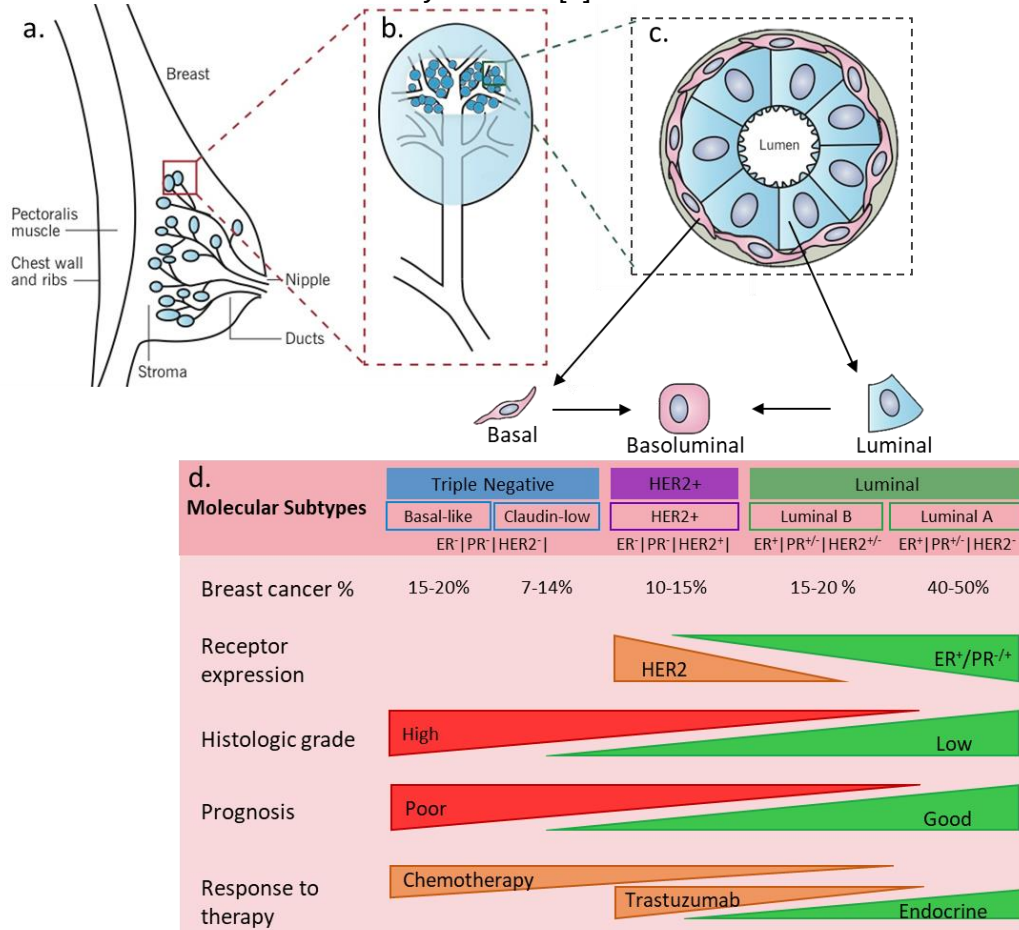


Figure I. 1 - Anatomy and histology of the breast. The breast is composed by the mammary gland, fat and connective tissues (a.). BrCa usually develops in the terminal duct lobular units of the mammary gland, which are constituted by two epithelial cell types (b.). In the inner layer, the luminal cells are responsible for the production of milk, while the outer layer is filled by myoepithelial/basal cells, which have contractile properties and help in milk ejection during lactation (c.). Depending on the cell type that suffers genetic mutations and originates the malignant tumour, BrCa can be classified into 3 major intrinsic subtypes: luminal, HER2⁺ and triple-negative tumours. Each subtype comprises specific characteristics, including prognosis and response to therapy (d.). Adapted from [7].

BrCa can be triggered by a genetic abnormality, but only 5-10% of BrCa patients inherit these mutations. The majority of BrCas, however, are a multifactorial disease caused by an intricate correlation of factors such as gender, age, reproductive and hormonal history, and environmental and lifestyle factors, among others [3, 8-10].

1.2. BrCa subtypes

Although breast carcinomas are originated from the epithelial cells that line the milk ducts, the luminal and the myoepithelial cells (Figure I.1), BrCa is a highly complex and heterogenous disease [11]. This heterogeneity has been noted for a long time by accessing histologic samples and patients' outcomes. With the development of molecular profiling techniques, this heterogeneity was ensured, and it is now possible to classify BrCa within, at least, 3 subtypes: luminal, HER2 enriched and basal-like, as detailed in Table I.1 [11, 12]. Each subtype has different biological characteristics, including risk factors, prognosis, response to therapies and a preferential metastasis' site. The patients' clinical outcome has been successfully improved with the implementation of BrCa stratification, since it allowed for new treatment approaches depending on the intrinsic subtypes, including endocrine therapy, chemotherapy, radiotherapy and HER2 antibody therapy [13].

The tumours that express receptors of estrogen (ER) and progesterone (PR) are called luminal tumours since their hormonal profile is similar to the luminal epithelial cells of the mammary gland. These tumours are divided into 2 subtypes: the luminal A and luminal B tumours, which have different proliferation status, with the luminal B tumours being more proliferative than the luminal A [14].

The other subtypes represent ER negative tumours and usually have worse prognosis than luminal tumours. The HER2-enriched subtype exemplifies the tumours which have a high expression of HER2 gene and other genes related to its pathway. Since these subtypes are classified based on their molecular profiling, and not all gene mutations show transcriptional changes, some HER2-enriched tumours present a negative immunohistochemical profile [13]. A Basal-like tumour mimics the expression profile of the myoepithelial cells and usually lack the expression of both hormonal receptors (HR) and HER2, being often referred as triple-negative BrCa (TNBC). Importantly, not all basal-like tumours are triple-negative [15]. Therefore, recently, a new subtype was described to represent the tumours that, besides not expressing the HRs, also lack cell-cell adhesion and tight junction's markers, such as claudins, denominated claudin-low subtype (Table I.1) [16, 17].

Table 1.1 – Characteristics of BrCa intrinsic subtypes. Adapted from [4, 13, 16-21].

		Expression profile	Other characteristics
Luminal	Luminal A	ER ⁺ PR ^{+/−} HER2 [−] and Ki67 ^{low} High expression of luminal markers: cytokeratins (CK) 8 and 18	Most common subtype (40-50%) Good prognosis: • Low grade, mitotic activity, relapse rate • Low expression of proliferative genes • Treatment: Hormonotherapy Commonly metastasizes to bone
	Luminal B	ER ⁺ PR ^{+/−} HER2 [−] and Ki67 ^{high} or ER ⁺ PR ^{+/−} HER2 ⁺ Lower expression of luminal markers	Accounts for 15-20% of all BrCas 20-30% are HER2 ⁺ Intermediate prognosis: • Higher grade, relapse rate • Higher proliferative index Treatment: • HER2[−]: Hormonotherapy combined with chemotherapy • HER2⁺: use of anti HER2 therapy
HER2+	HER2+	ER [−] PR [−] HER2 ⁺ and Ki67 ^{high} Amplification and over-expression of the <i>ERBB2</i> proto-oncogene	10-15% of BrCa subtypes Poor prognosis: • High grade, nuclear grade, relapse rate • High proliferative index Treatment: • Trastuzumab therapy (anti-HER2 monoclonal antibody) • Sensitive to neoadjuvant therapy (cytotoxic agents) Frequently metastasizes to brain and visceral organs
Triple-negative	Basal-like	ER [−] PR [−] HER2 [−] and Ki67 ^{high} High expression of myoepithelial markers: CK 5, 6, 14 and 17, laminin, P-cadherin	15-20% of BrCa cases Poor prognosis: • High grade, mitotic index, relapse rate • Highly proliferative rate Treatment: • Chemotherapy (currently lack a targeted therapy) High metastatic rate to brain and lungs
	Claudin-low	ER [−] PR [−] HER2 [−] and Ki67 ^{low} Low expression of claudins 3, 4 and 7, occludin and E cadherin. High expression of stem cell signatures and epithelial to mesenchymal transition (ECM) genes.	Least common subtype (7-14% incidence rate) Poor prognosis: • High histological grade • High mitotic index • High relapse rate • Lower expression of proliferative genes Treatment: • Intermediate response to chemotherapy

Ki67 (proliferating cell nuclear antigen)

The new information provided by the new molecular profiling techniques has allowed to better understand each breast tumour. In the future, the aim is to achieve individualized therapies which will be translated in improvement of patients' outcome.

1.3. Patterns of metastatic BrCa

As stated above, BrCa mortality rate has been declining since the 90s, however around 14% of cancer deaths in women are due to breast tumours [1]. As with the majority of cancer types, metastasis is the main cause of BrCa deaths and not the primary tumour. Nevertheless, despite the advances made in prevention, detection and improvement of therapies, some women are still diagnosed with advanced metastasis, and a significant number of patients end-up relapsing and eventually die from metastatic

BrCa. In fact, only 26% of American women survive in the first 5 years after being diagnosed with metastatic BrCa [1].

Therefore, recurrence is still a major problem in these patients. This occurs when BrCa cells gain the ability to leave the primary tumour, by invading the basement membrane, intravasate into blood or lymphatic vessels, where they circulate until arriving in a new distant tissue. Upon arrival, cells need to extravasate and colonize the new organ, in order to transform into a macrometastasis (Figure I.2).

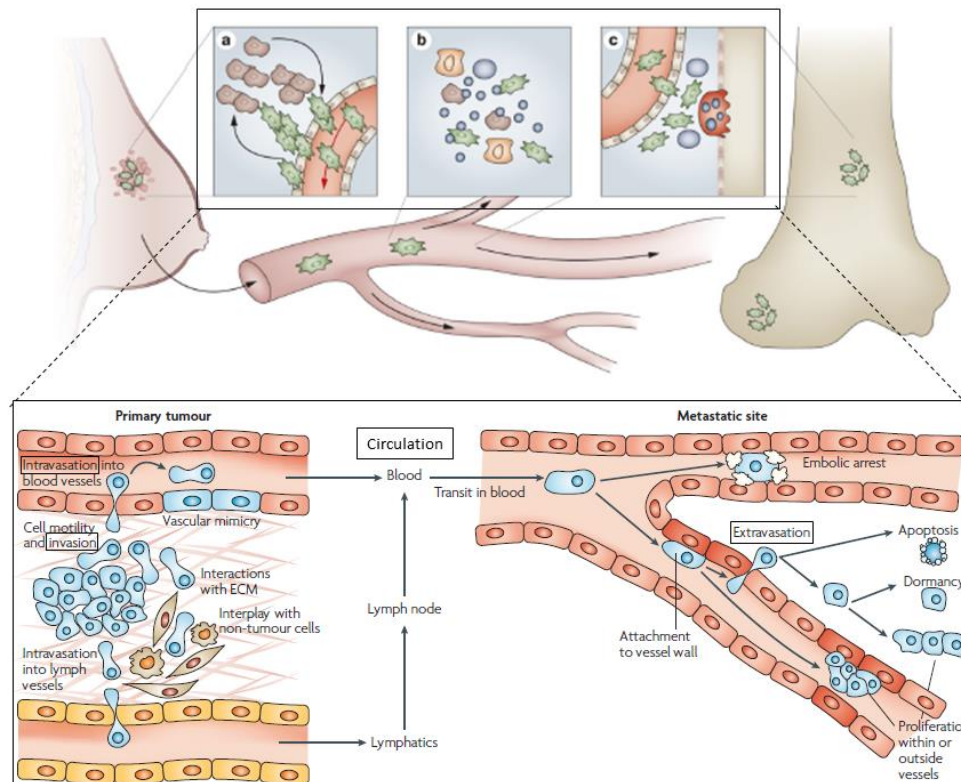


Figure I. 2 –Metastatic events of BrCa cells from the primary tumour to bone. The metastatic process is comprised by a series of steps that must be completed in order for cancer cells to leave the primary tumour and go develop a new tumour in a distant site. This cascade initiates with the invasion and migration of primary tumour cancer cells to adjacent healthy tissues. After migration into surrounding tissues, cancer cells intravasate into the circulatory or lymphatic system (a.). Migration is facilitated by degradation of extracellular matrix (ECM) and because of EMT. Thenceforth, circulation step initiates and cancer cells need to survive in the numerous inhospitable conditions of vascular environment, like high toxicity or lymphocytes' presence (b.). This critical step ends up creating a selection where the surviving cells are particularly aggressive and resistant. Sometimes, and if cancer cells manage to survive the circulation conditions and reach the new organ, cells can penetrate the endothelium through degradation by proteolysis, in a similar process as intravasation, denominated extravasation (c.). In an alternative process, cells start to proliferate in the lumen of the vessel and due to its growth, the walls are destroyed and cancer cells enter in the new organ. Lastly, for metastasis to be well succeeded, the cancer cells that arrived at the new organ need to colonize it, initiate tumour growth and be able to adapt to the new organ's conditions in order to transform into a clinically detectable macrometastasis [22-24]. Adapted from [25, 26].

In the end, metastasis is an inefficient multifactorial process where the ability for a primary tumour to metastasize to a specific organ depends on a variety of factors, including the cancer cell type, the primary organ and the microenvironment of the secondary site. The intrinsic characteristics of cancer cells and the cellular and cytokine

profile of the tissue of origin dictate how these cells will migrate, survive and proliferate. The tissue's microenvironment to which metastatic cells eventually home also plays a significant role in this process. Most importantly, the relation between the primary organ and the secondary site dictate the success of metastasis [27].

The distribution of metastatic sites does not seem to be random. Organ-specific metastases were firstly described by Paget in 1889 in the “seed and soil” hypothesis, where he, after evaluating BrCa patients' autopsies, stated that “in cancer of the breast, the bones suffer in a special way”. He proposed that these patterns were due to the seed dependency (cancer cell) for the soil (environment factors in the new organ) [28]. In general, BrCa cells have been described to commonly metastasize to bone, brain, lungs and liver [29], despite this organotropism, bone seems to be a preferential organ for metastasis, accounting for approximately 70-80% of patients with terminal BrCa [30].

The prediction for development of metastasis can be dictated by some risk factors, such as grade, nodal involvement and size of the tumour. However, these factors do not predict the specific sites or patterns of metastasis. It has been hypothesized that the primary tumour can give insight about the organ that they eventually home to, having the possibility to influence the therapeutic and survey strategies for each patient since the time of primary diagnosis. Tumours with positive ER and PR show a strong incidence of bone metastasis, take longer to relapse and have a longer survival in the presence of metastasis. Whereas tumours with negative HR show mostly metastasis in visceral organs and are least present in bone, these tumours rapidly recur [31-33].

In fact, the majority of studies showed that the luminal subtypes have a preference to metastasize to bone, contrary to the TNBC subtypes, which rarely have bone metastasis and are usually present in brain and lungs. Regarding the HER2⁺ subtype, most metastasis occur in visceral organs (Figure 1.3) [34-38]. Thus, besides influencing the primary tumour characteristics, such as aggressiveness and response to treatments, BrCa subtypes also show different metastatic behaviour and could help in the development of follow-up and surveillance strategies for newly diagnosed patients, allowing for different options of adjuvant therapies.

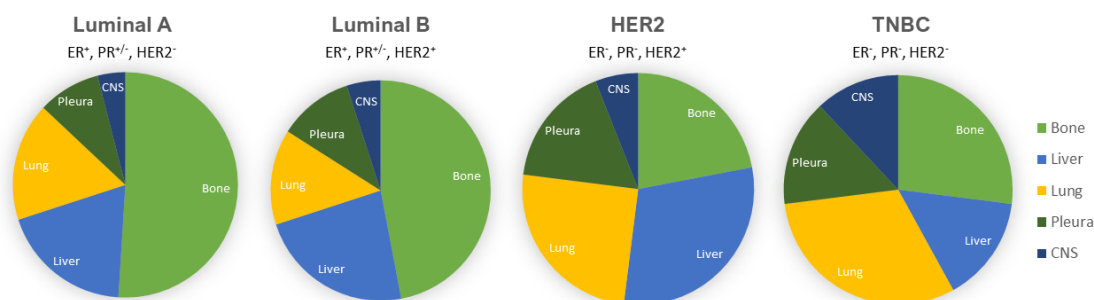


Figure I. 3 – The distribution of most common metastatic sites by each BrCa intrinsic subtype. CNS, central nervous system; HER2, human epidermal growth factor 2; TNBC, triple-negative BrCa. Adapted from [34].

2. Bone microenvironment and bone metastasis

2.1. Bone structure and remodelling

Bone is a rigid but dynamic tissue which is under constant remodelling and is predominantly composed by mineral crystals and organic matrix, with cells representing only 10% of bone. There are two types of bone: the cortical and the trabecular bone. The cortical bone gives mechanical function and protects the organism, whereas the trabecular bone is where bone remodelling occurs. Therefore bone diseases, either bone loss or bone deposition are mostly localized in trabecular bone [39].

Bone is also a reservoir of calcium, phosphate and growth factors, which are released during bone remodelling. Bone remodelling is essential to maintain all the functions of a healthy skeleton [40]. This process occurs when bone that is damaged or old is replaced by new bone. It is described as a cycle that begins with degradation of bone by the bone-resorbing cells (OCs) and ends with bone deposition by bone-forming cells (OBs) (Figure I.4). Remodelling of bone is a physiological process in healthy mature bones where a tight balance is kept between bone destruction and bone formation, to guarantee no differences in bone mass or strength after each cycle [39]. Bone mass reaches a peak in young adults and then decreases with age. Each year, in adulthood, 10% of bone is renewed. Normally, women in menopause suffer a high loss of bone mass, due to a reduction of ER levels. However, in a pathologic situation such as bone metastasis, this process is accelerated and the tight balance between OCs and OBs' activities is weakened [41].

Bone remodelling takes place in the bone remodelling compartment (BRC), and is carried out by 4 main cell types: OCs, OBs, osteocytes and bone-lining cells [40].

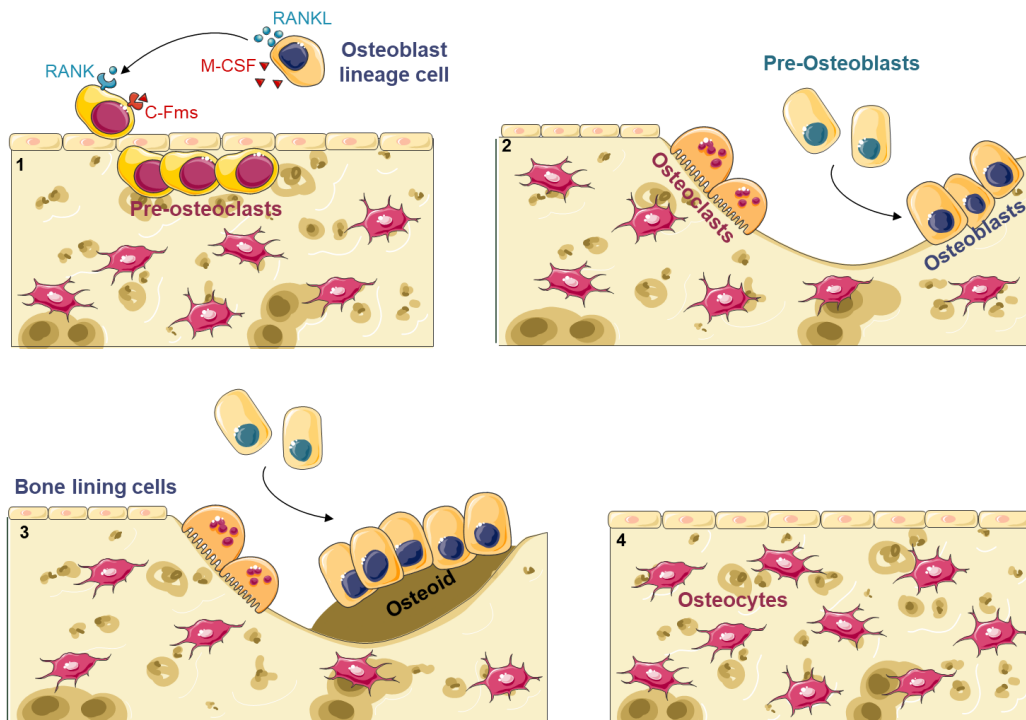


Figure I. 4 – Steps of bone remodelling. Under normal conditions, bone resorption starts with the coupling process, where OB lineage cells differentiate pre-OCs into activated OCs through the production of RANKL and M-CSF that bind into their receptors, RANK and c-fms, respectively. After OC precursors arrive at the BRC, either by crossing the bone lining cells canopy or by capillary penetration, they differentiate into highly specialized multinucleated large OCs that attach to the BRC (1). Under the bone lining cells, OCs create a lacuna and expose the organic matrix, resulting in degradation of bone matrix and dissolution of the bone minerals, due to release of proteolytic enzymes, including cathepsin k, acid and collagenases (2). After the beginning of bone resorption, MSCs and osteoprogenitors start to be recruited into the BRC, also either by the bone marrow or from the capillaries. While the differentiation of MSCs and osteoprogenitors into pre-OBs and OBs start, OC formation and bone resorption continue. After activation, OBs start to follow the OCs and reform the bone matrix by synthesising osteoid, which is composed by collagen, osteonectin and other non-mineral molecules, that start to mature and will mineralize after some time (3). This process continues after bone resorption stops to maintain the balance between the amount of bone resorbed and bone formed. When the osteoid is mineralized, the bone remodelling cycle comes to an end (4). Adapted from [41].

Cells of the osteoblastic lineage derive from mesenchymal stem cells (MSC) and can differentiate into OBs, osteocytes and bone-lining cells. The growth factors that direct this differentiation are stored in the bone matrix and include, transforming growth factor- β (TGF β), WNT proteins and bone morphogenetic proteins (BMPs). Depending on their differentiation state, these cells transition into different phenotypic and genetic patterns, and also differ in their location in bone. OBs are cuboidal cells located on the bone surface, which contain a well-developed Golgi complex and endoplasmic reticulum, showing its important functions in the synthesis and secretion of collagenous and non-collagenous proteins, such as collagen type 1 and osteocalcin, respectively. Once matured, OBs start expressing alkaline phosphatase protein (ALP), which is a

phosphotransferase that hydrolyses a calcification inhibitor, the pyrophosphate. Therefore, ALP increases phosphates required for calcification, regulating bone matrix mineralization [42].

After bone formation, OBs can undergo apoptosis, differentiate into osteocytes and remain in the matrix, or revert the differentiation into bone-lining cells. The bone-lining cells function is not very clear, they cover the bone surface and their retraction seems vital for the beginning of bone resorption. On the other hand, osteocytes, the most abundant cells in the bone, are terminally differentiated OBs that at the end of the bone remodelling cycle become buried within the bone matrix. A lot of studies point to a pivotal role of osteocytes in the initiation of remodelling, since it has been proposed that osteocytes function as mechano-sensing cells that send signals to OC precursors when they detect bone deformations or micro-damages in old bones, initiating bone remodelling [43].

OCs are giant multinucleated cells which originate from the fusion of hematopoietic stem cells (HSCs). These cells have numerous mitochondria, vesicles and lysosomes and can reach 100 μm of diameter. HSCs differentiate into cells of monocyte/macrophage lineage which further develop into mononuclear pre-OCs. To become bone-resorbing cells, OCs precursors need to fuse to become functional multinucleated OCs. This differentiation process, denominated as osteoclastogenesis is detailed in Figure I.5, and is directed by two factors: the monocyte/macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor- κB ligand (RANKL), that bind to pre-OCs after being released by cells from OB lineage [39]. These cytokines induce the expression of genes, such as TRAP and cathepsin K, that leads to OC activation [44].

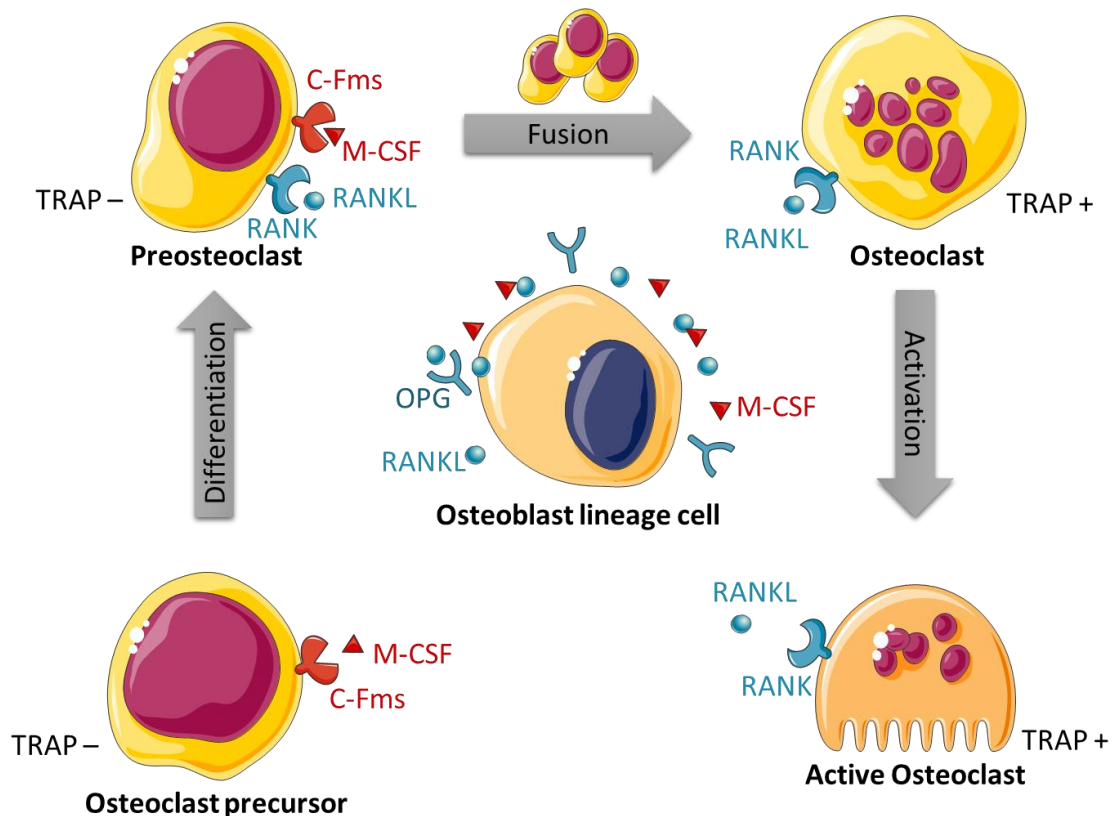


Figure I. 5 – Osteoclastogenesis. Growth factors M-CSF and RANKL, which are released from cells of OB lineage, play a pivotal role in OC maturation. M-CSF binds to C-fms present on early OC precursor cells and prone them to proliferate and differentiate. Besides expressing the receptor for M-CSF, pre-OCs also express RANK on their surfaces, which bind to RANKL and further promotes osteoclastogenesis. Osteoprotegerin (OPG) also intervenes in osteoclastogenesis by neutralizing RANKL, slowing down the maturation process. After the fusion into multinucleated cells, although still inactivated, OCs start expressing typical bone resorbing enzymes, such as TRAP. After further stimulation by OB lineage cells, OCs are activated and start to resorb the bone matrix. Adapted from [44].

After differentiating, OCs need to adhere to the bone ECM and migrate to bone active surfaces. Once activated, OCs become polarized cells, presenting internal changes, such as rearrangement of the cytoskeleton and formation of a sealed compartment, preparing the cell to resorb the bone (Figure I.6). In order to migrate, OCs develop cell projections, named filopodia and before resorption, F-actin reorganizes in podosomes which are dot-like structures that cover the periphery of the cell. During the resorption phase, the OCs presents 4 domains in their membrane: the clear/sealing zone, the ruffled border, the basolateral and the secretory membrane. The clear/sealing zone is responsible for the attachment of bone resorbing cells to the bone matrix and presents numerous actin filaments, which form a characteristic actin ring seen in phalloidin stainings. The ruffled border is responsible for bone resorption. This region is composed by microvilli, which contain H^+ -ATPases that acidify the resorption lacuna, allowing for degradation of mineral components. The released protons reduce the pH, leading to the activation of bone-degrading enzymes, such as MMP-9, TRAP and cathepsin K, that also digest the organic components, such as the collagen of the matrix. These elements are all comprised in the Howship's lacuna and contribute to bone matrix

degradation. Although the other two regions are not in direct contact with the bone matrix, they contribute in osteoclastogenesis by receiving cytokines from the microenvironment, including the ones released by OBs and the products released in the Howship's lacuna, during bone resorption [42, 45].

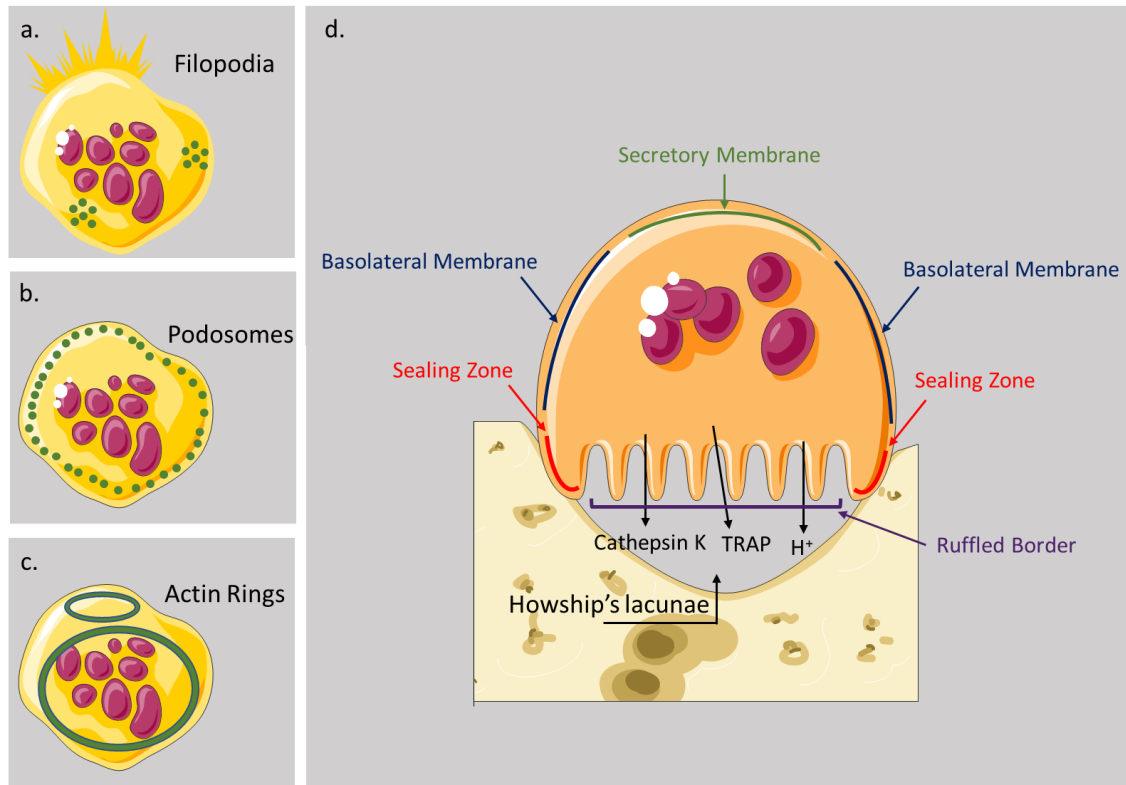


Figure I. 6 – Different OC morphologies during non-resorptive and resorptive phases. Once OCs are activated, they become polarized cells that suffer cytoskeletal arrangements. (a.) In order to attach and migrate to bone matrix, OCs develop cell projections named filopodia and the actin filaments are arranged in clusters. (b.) Actin filaments also reorganize in belt-like structures to form podosomes. (c.) The actin rings prone to attachment to the bone matrix, forming the sealing zone. (d.) in the resorptive phase, the OC membrane can be divided in 4 regions: the sealing zone, the ruffled border and the basolateral and secretory membranes. In the ruffled border of the OC protons and bone resorptive enzymes, such as TRAP and cathepsin K, are released into the Howship's lacunae, resulting in resorption of bone. Adapted from [45].

RANKL plays a major role in bone remodelling, belongs to the tumour necrosis factor (TNF) family and is released by OB lineage cells. Osteoprotegerin (OPG), which is also produced by OBs, is a decoy receptor for RANKL that also belongs to the TNF receptor family. OPG blocks the binding between RANKL with RANK, preventing OC activation. These factors' ratio regulates the formation and activation of OCs. It is known that RANK/RANKL/OPG cytokine triad, influences physiological osteoclastogenesis of bone remodelling, thermoregulation, development of the mammary gland, regulation of adaptive immunity and vascular calcification, and also plays a role in pathological processes like tumorigenesis and metastasis [46].

The discovery that the factors, M-CSF and RANKL, secreted by osteoblastic lineage cells stimulate OC differentiation, created the idea that the bone cells may have

the capacity to communicate with each other. When it was described that factors released by OCs during bone resorption also have the ability to control bone formation, this idea was further intensified. This communication process is known as 'coupling', and is kept in an appropriate balance with the involvement of many coordinated signalling pathways [40]. An imbalance occurs in a pathological condition, resulting in an aberrant bone remodelling, leading to the development of bone disorders.

2.2. Bone metastasis

Bone metastasis are common in a lot of different cancer types. However, breast, thyroid, kidney and prostate cancers have a predilection for the skeleton. As discussed above, the initial steps of the metastatic process require an intricate interplay of the intrinsic characteristics of tumour cells, the stromal cells of the primary tissue and the surrounding ECM. The success of the colonization of bone, on the other hand, relies on the intrinsic characteristics of the disseminated tumour cells (DTCs) that came from the primary tumour, and their interaction with the bone microenvironment properties, in a way that promotes their survival, colonization and growth. The inherited characteristics provided by the bone biology are unique and influence the tumour development in a way that is unlikely available in other metastatic organs [40].

Bone has multiple characteristics, including a long list of cells and factors, that make it a preferential organ for metastasis. Firstly, bone is richly vascularised and favours the arrest and establishment of circulating cancer cells in the skeleton. Secondly, bone microenvironment has a multicellular nature with high mobility of both resident cells and transient cells. The resident bone cells also secrete cytokines and other ECM proteins that promote the homing and retention of metastatic cancer cells [47].

After the establishment of DTCs in the bone surface, the equilibrium of bone remodelling can be tipped toward different directions, depending on the type of interaction between the tumour cells and the bone microenvironment. This interaction originates a vicious cycle which can result in very different bone metastatic effects, that range from an increased lysis of bone (osteolytic lesions) or towards an excessive formation of bone (osteoblastic lesions) [48]. Most of BrCa patients with bone metastasis have dominant osteolytic and destructive lesions, only 25% of bone metastatic BrCa cases are diagnosed with osteoblastic lesions, identical to those seen in the majority of prostate cancer patients [48].

Tumour cells, OCs, OBs and the bone matrix are the main players in the propagation of the vicious cycle. Tumour cells start by releasing a variety of factors which

will interact with the bone stromal cells, inducing recruitment and activation of these cells. The bone matrix resorbed by OCs will release different growth factors, previously synthesized by OBs, which enable the attraction of tumour cells to bone and further promote tumour metastatic growth [43].

2.3. BrCa bone metastasis

The breast carcinoma bone metastasis is one of the best described vicious cycles, where the interaction between tumour cells and the bone niche is vital for the later formation of osteolytic lesions. These effects occur when breast tumour cells arrive in the bone microenvironment and, in order to activate osteoclastogenesis, release OC-activating factors. These factors can act directly on OCs or act indirectly on cells from OB lineage to induce the production of RANKL, which will activate the bone resorbing cells (details in Figure I.6) [49].

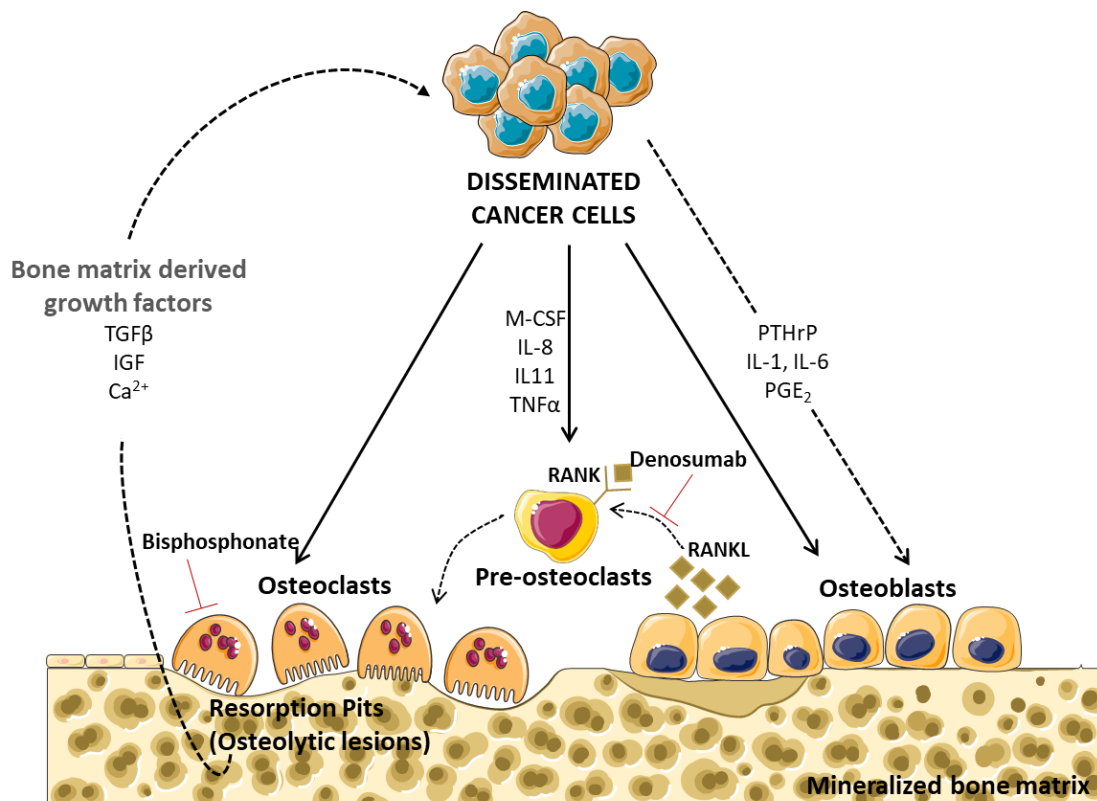


Figure I. 7 – BrCa bone metastatic vicious cycle. To activate osteoclastogenesis, breast tumour cells arrive in the bone microenvironment and release OC-activating factors. Tumour necrosis factor alpha (TNF-α), Interleukin (IL)-11, IL-8 and M-CSF act directly on OCs. On the other hand, Parathyroid Hormone-related Protein (PTHrP), interleukins (IL-1, and IL-6) and prostaglandin E2 (PGE₂), act on osteoblastic stromal cells and induce the production of OC-activating factors, such as RANKL, that will bind with its receptor RANK on the OC surface, initiating bone resorption. With the activation of OCs, bone matrix starts to be resorbed, and it is the turn of the mineralized bone matrix to release growth factors, such as transforming growth factor β (TGF-β), Insulin Growth Factors (IGFs), and ionized calcium (Ca²⁺) that will increase the survival and proliferation of tumour cells. This further stimulates the production of osteolytic factors by cancer cells. This feed-back increases osteolysis once again, perpetuating this cycle. Bisphosphonates and denosumab are the most commonly administered drugs on bone metastasis. Adapted from [49].

Cancer cells can also inhibit the differentiation and adhesion of OBs, increase their apoptosis and delay collagen synthesis. Therefore, predominantly lytic lesions are

the result of extreme bone degradation and deficient bone replacement since the osteoblastic response is always impaired and is not capable of forming the amount of resorbed bone. With the activation of OCs, bone matrix starts to be resorbed, and it is the turn of the mineralized bone matrix to release growth factors and ionized calcium, which will further stimulate the expression of bone resorption factors by cancer cells. This self-perpetuating cycle leads to bone loss and tumour growth and is designated by the “vicious cycle” of BrCa bone metastasis [50].

2.4. Therapeutic approaches to bone metastasis

This disease is incurable, with most of patients surviving only 2 years after emergence of metastasis. When metastasis remains confined to the bone, the cause of death is due entirely to bone complications, which include pain, hypercalcemia, fractures and spinal nerve compression. All these complications hugely affect quality of life, increasing both morbidity and mortality in BrCa patients [51]. Therefore, drugs usually administered on bone loss diseases, such as osteoporosis, are used to treat the symptoms of bone metastasis. Bisphosphonates and Denosumab are the most commonly administered drugs and reduce the rate of bone loss, decrease the incidence of skeletal complications and can even alleviate bone pain. Although clinical administration is still palliative [52], it has been suggested that these drugs might be potential adjuvant therapies.

Bisphosphonates, which are OC inhibitors, are the standard treatment for bone metastasis [53]. They bind to hydroxyapatite of the bone matrix that is resorbed by OCs (Figure 1.7). When OCs ingest these drugs, it affects their survival, cytoskeletal dynamics and osteoclastogenesis, causing a reduction in osteolytic lesions [30]. However, the use of bisphosphonates as adjuvant therapy on early BrCa is not supported by clinical findings, as only postmenopausal BrCa patients seem to benefit from this therapy, showing a reduction in bone recurrence and increased survival [54-56].

Denosumab, is a human neutralizing monoclonal antibody that binds to RANKL and blocks RANKL/RANK binding, inhibiting OC differentiation. This drug shows superior decreases in both bone pain and skeletal complications, in comparison to bisphosphonates [57]. Furthermore, denosumab seems to be very effective in targeting the subtypes of breast and prostate cancer that express RANKL. However, recent reports state that denosumab did not improve bone metastasis free survival among patients with early BrCa (Coleman, data not published).

Therefore, it is necessary to discover new therapies that prevent the development of bone metastasis. Recently, several preclinical and epidemiologic studies [58-61] have

shown that β -blocker drugs, commonly used to treat hypertension, have been linked with reduced BrCa metastasis and improvement of patients' survival. β -blockers, are drugs that inhibit the activation of β -ARs, which are activated in a stress situation by stimulation of SNS. These receptors are present in bone cells and their activation was shown to cause alterations in bone microenvironment [62, 63]. β 2-AR activation leads to production of RANKL which activates OCs [64], decreasing bone mass, OC number and activity. Therefore, β -blockers may have similar effects to RANKL blockade and seem promising in metastatic prevention.

3. SNS

3.1. Stress response

Patients diagnosed with BrCa are at a higher risk of feeling emotional stress [65, 66]. These symptoms may lead up to a psychiatric disorder, such as anxiety or depression, that can develop several years after the diagnosis of the disease [67]. The link between cancer and emotional disorders has been suggested since the ancient times, but the nature of this association has only started to be revealed in the last century [68]. A few studies show an association between stress and the incidence of cancer, however, there seems to be a stronger and more consistent relationship between psychological factors and the progression of already-existing tumours [69-72].

Stress is a complex process where both psychosocial and environmental factors trigger a cascade of information-processing pathways in central nervous system (CNS) and in peripheral nervous system. These systems will generate the fight-or-flight stress response in the autonomous nervous system (ANS), which is mediated by the SNS, and the defeat/withdrawal responses in the hypothalamic pituitary adrenal (HPA) axis (Figure 1.8) [73]. Although both systems have been implicated in cancer, there is an increasing body of studies linking SNS mediators with cancer progression [71, 72, 74].

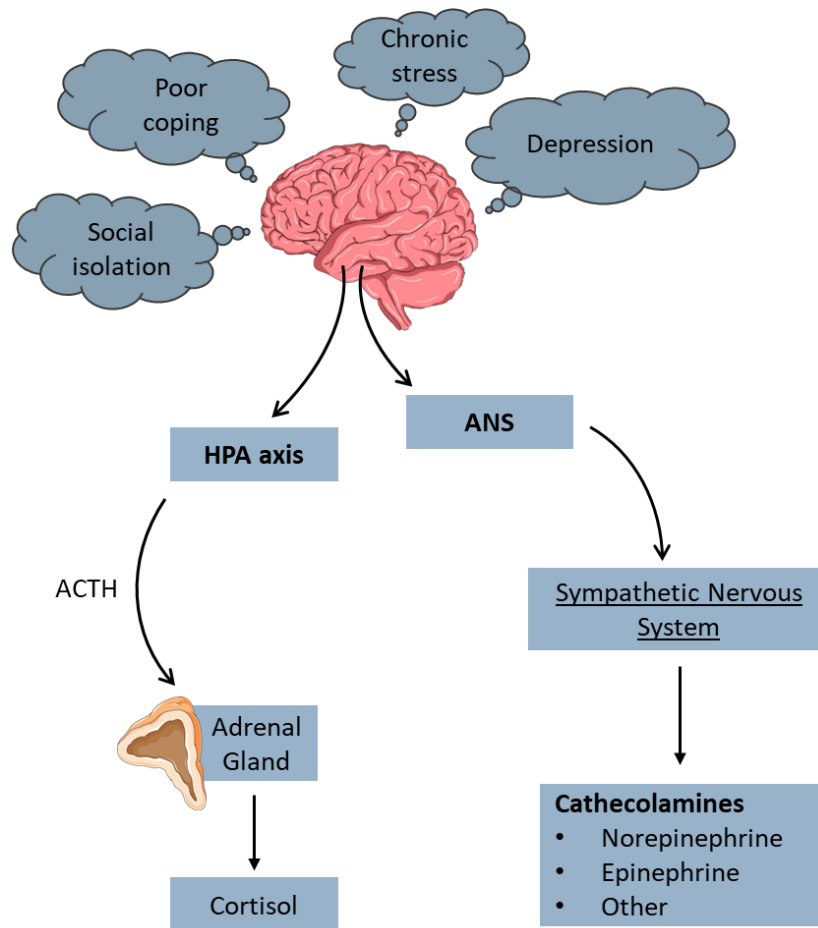


Figure I. 8 - Pathways involved in stress response. Responses to stressful experiences activate the pathways from CNS, ANS and the HPA axis. The defeat/withdrawal responses from the HPA are mediated by the hypothalamic productions of arginine vasopressin and corticotrophin-releasing factor (CRF), which will then activate the secretion of pituitary hormones such as adrenocorticotrophic hormone (ACTH). In its turn, this hormone induces the release of glucocorticoids like cortisol from the adrenal cortex, which can influence the metabolism, growth, immune function and regulation of basal function and stress reactivity. ANS responses, on the other hand, are mainly regulated by the SNS which will lead to the secretion of catecholamines, such as NE and epinephrine (E), from sympathetic neurons and adrenal medulla. Adapted from [72].

The SNS controls involuntary body functions, and therefore virtually regulates all human organs. The activation of SNS leads to the secretion of catecholamines, such as NE and epinephrine (E), that regulate these functions and have two possible signalling pathways. On one hand, there is the localized release of NE from the sympathetic nervous terminals, that directly innervate the target organs; whereas the other pathway mainly involves the hormonal regulation of E through the capillaries [75]. These catecholamines will bind to different AR subtypes [74], which are G protein-coupled receptors (GPCR). The ARs show distinct patterns of tissue distribution, have diverse functions and can even originate opposite actions, but generally regulate physiological homeostasis (Figure I.9). The two main classes of AR are the alpha (α -AR) and the beta (β -AR), but these can be further subdivided in 9 subtypes: α 1AR (α 1A, α 1B, and α 1D),

α 2AR (α 2A, α 2B, and α 2C) and β -AR (β 1, β 2, β 3) [74, 76]. The α 1- and β -ARs are excitatory receptors, while the α 2-ARs have inhibitory pathways.

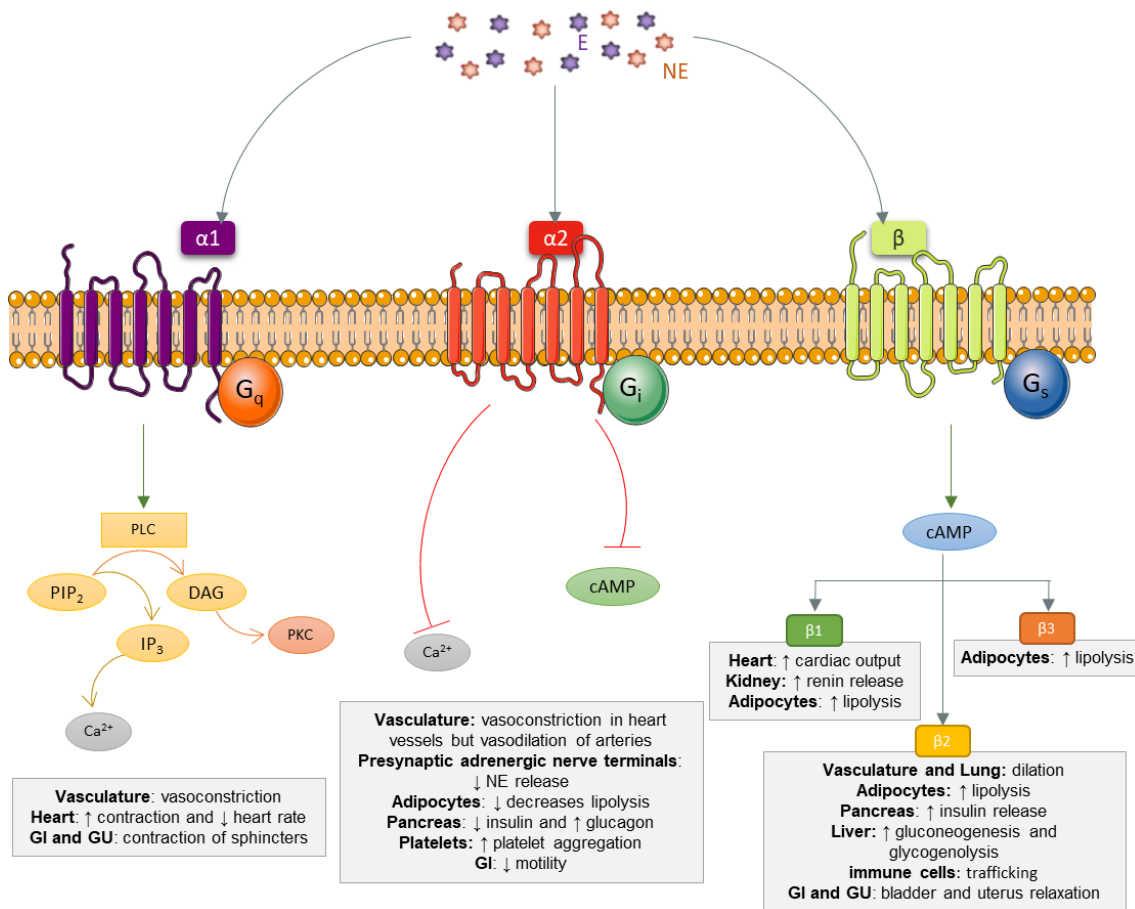


Figure I. 9 – Signalling pathways, localization and effects of each AR subtype. Both NE and E bind to the ARs subtypes, with different affinities, resulting in activation of their signalling pathways. AR are GPCRs, which are multifactorial proteins with multiple effectors: α 1-AR usually links with members of the G_q family, α 2-AR links to G_i and β -ARs link to members of the G_s family. Adapted from [76]. PIP_2 , phosphatidylinositol biphosphate; IP_3 , inositol phosphate; Ca^{2+} , calcium; DAG, diacylglycerol; cAMP, 3',5'-cyclic adenosine monophosphate; ATP, Adenosine triphosphate.

During the SNS acute fight-or-flight responses, the E and NE levels can increase by 10 times. This causes rapid physiological changes in respiratory, cardiovascular, muscular, immune and neural systems, increasing the blood flow to muscles and lungs, and preparing the body for alert situations. The catecholamines levels return to baseline in a very short amount of time (20-60 minutes), therefore, the activation of the acute stress responses is considered adaptive [75]. On the other hand, in chronic stress conditions, the physiological systems are exposed to glucocorticoids and catecholamines for too long, negatively affecting the organism. This is showed by a decrease in health conditions, such as increased risk of infections and cardiac diseases, decreased wound healing and eventually death [77].

Lately, behavioural stress has also been pointed as an accelerator of cancer progression, encouraging the investigation of the effects of SNS on cancer biology. This resulted in the identification of various cellular and molecular processes that may mediate SNS effects on tumour progression [72, 75].

3.2. SNS in BrCa-bone metastasis

As previously stated, severe depression and chronic stress conditions have been linked to poor prognosis, increased recurrence and reduced survival in BrCa patients [65-67]. Although the association between cancer and emotional disorders has been suggested since the ancient times, the link between psychological factors and the progression of already-existing tumours [69-72] has only started to be revealed in the last century [68].

Several studies have confirmed that in general cancer cells express α - or β -ARs, as well as enzymes involved in catecholamine biosynthesis, such as tyrosine-hydroxylase (TH), phenylethanolamine N-methyltransferase (PNMT) or dopamine beta-hydroxylase (DBH) [78]. Besides cancer cells, various bone stromal cell types, such as OCs [79], OBs [80] and MSCs [81], also express ARs, supporting the premise that the peripheral sympathetic neurons play an important role in bone remodelling and other bone physiologic processes [82].

Additionally, stress response is partly mediated by the activation of the ARs. The most described α -AR in BrCa is the α_2 -AR subtype, which caused an increased proliferation of BrCa cell lines [67, 83, 84], conferred chemoresistance [85], and lead to tumour growth [84, 86, 87] and metastasis [88, 89] of different BrCa cells. Interestingly, one study showed that SNS activation can influence tumour growth and metastasis through the α_2 -AR present on stromal cells of the tumour microenvironment [89]. The β -ARs were the firsts to be described in BrCa tissues [90] and cell lines [91], with the β_2 -AR subtype being the most expressed of the β -AR subtypes. According with its stimulatory G-protein nature, β -AR showed promotion of tumour growth in various BrCa studies [92-94], however inhibited proliferation in many others [84, 95]. Besides proliferation, β -AR has mainly been associated with BrCa cells migration [96, 97] and metastasis [98]. Studies also provided evidences for a direct β -adrenergic signalling pathway in promotion of tumour angiogenesis [99].

The link between stress and cancer progression was strengthened when pharmaco-epidemiological studies showed that the use of β -blockers, at the time of diagnosis, was associated with improved survival, decreased tumour invasion,

metastasis, recurrence and mortality [58-61]. However, not every populational study has seen this association [100].

Therefore, in the last few years, a few studies that focused on BrCa showed that SNS activation can have an effect in the process of BrCa bone metastasis. However, little is known about the mechanism by which chronic stress leads to dissemination of metastatic cancer cells from breast primary tumour to distant organs.

Sloan et al. demonstrated that the neuroendocrine signalling directly affected breast tumour cells, resulting in an augmented tumour invasiveness and metastasis [98]. However, some studies have addressed the fact that by influencing bone homeostasis, SNS can also act on bone marrow stromal cells and indirectly affect the behaviour of metastatic BrCa cells [101]. The activation of β 2-AR expressed in OBs seems to play a crucial role in mediating the SNS signals in bone, throughout the production of cytokines such as RANKL and vascular endothelial growth factor (VEGF) [101, 102]. The role that these cytokines play in bone metastasis suggest that when chronic stress or depression activate the SNS, the bone marrow microenvironment is transformed into a more favourable tissue for establishment of metastasis [68]. These results suggest that BrCa cells affinity to the skeleton can increase when there is an increase of sympathetic signals in bone.

Therefore, BrCa patients who suffer from emotional stress, often show a high sympathetic tone with a reduced survival, and a poorer prognosis. And there seems to be an association between these patients and high expression of RANKL in bone with an augmented incidence of bone metastasis.

In summary, several reports support the hypothesis that chronic SNS activation plays a critical role in the establishment of metastatic BrCa cells into the skeleton [51, 68]. Studies point towards an indirect effect of SNS on the bone stromal cells, via NE and β 2-AR expressed by OBs. However, there may be also a significant direct effect on metastatic cells [103]. Thus, it is still not clear how SNS is involved in BrCa bone metastasis, specifically through the osteoclastic pathway.

II. Aims

The main aim of this project is to uncover the effects of SNS activation in the development of bone metastasis by each BrCa subtype.

In order to do achieve the main objective, the project was divided into two parts:

1. Characterization of the adrenergic profile of the different BrCa subtypes

Since each BrCa subtype presents its own characteristics, different cell lines representing each intrinsic subtype (Luminal A and B, HER2-enriched, Basal-like and Claudin-low) and a bone-tropic cell line were used to assess the expression of the ARs and adrenergic metabolic enzymes present in each BrCa subtype.

2. Evaluation of the effects of SNS on BrCa-OC interactions

To study the effects of SNS in OC activity through BrCa, OCs were treated with the conditioned medium collected from each BrCa cell line treated with increasing doses of NE, a global AR agonist (Figure II.1a).

Since OCs also express ARs, the direct effect of NE in OC differentiation and resorptive activity was also evaluated (Figure II.1b).

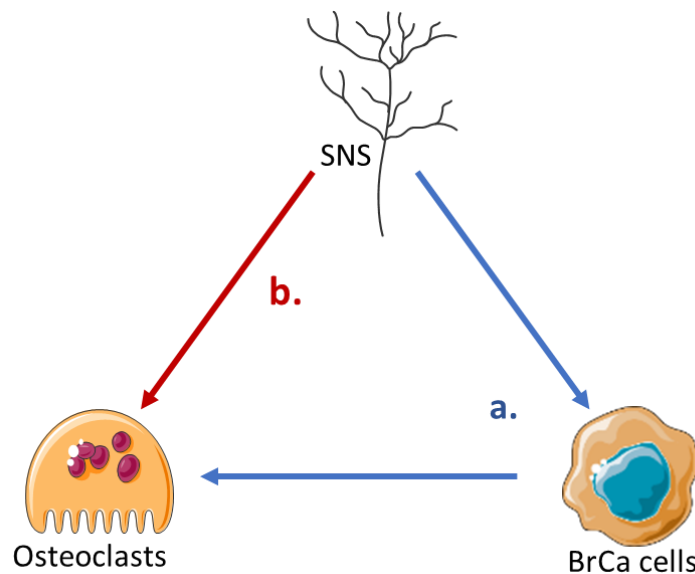


Figure II. 1 - Exploring the role of SNS on BrCa-induced bone metastasis – the OC activity. This image represents the interplay of the three systems studied on this project. (a.) The effects provoked by SNS on BrCa-induced bone metastasis were assessed by first treating BrCa cells with NE, using this CM to afterwards treat OCs (blue arrows). This treatment allows to evaluate how SNS, after influencing BrCa, can impair OC function. (b.) In order to evaluate the direct effects that SNS has on OCs, the bone resorptive cells were treated with NE (red arrow).

III. Materials and methods

1. Cell cultures

1.1. BrCa cultures

1.1.1. 2D and 3D cultures

MCF-10A, BrCa cell lines (MCF-7, MDA,468, MDA-MB-231, BT-474, SK-BR-3) and Bo-1833 cell line were seeded on a T75 cm² flask (details on Table III.1). The media were supplemented with 10% fetal bovine serum (FBS) (Biowest) and 10 µg/mL Pen/Strep. In the case of MCF-10A the medium was supplemented with 5% Fetal Horse Serum (FHS) (ThermoFisher Scientific), 100 µg/mL epidermal growth factor (EGF) (Sigma), 1 mg/mL hydrocortisone (Sigma), 1 mg/mL cholera toxin (Sigma), 10 mg/mL insulin (Sigma). All cells were kept at 37°C in a 5% CO₂ atmosphere and medium was changed twice a week. Morphologies were assessed with optic microscope Olympus CKX41 at 10x objective.

Table III. 1 – List of BrCa cell lines and respective culture media.

<i>Cell Lines</i>	<i>Tumorigenic property</i>	<i>Primary Subtype</i>	<i>Immunoprofile</i>	<i>Culture medium</i>	<i>ATCC Reference</i>
MCF-10A	Non-Tumorigenic	–	–	DMEM/F12 +Glutamax	CRL-10317™
MCF-7	Tumorigenic	Luminal A	ER ⁺ , PR ^{+/-} , HER2 ⁻	DMEM/F12	HTB22™
BT-474	Tumorigenic	Luminal B	ER ⁺ , PR ^{+/-} , HER2 ⁺	DMEM High Glucose	HTB20™
SK-BR-3	Tumorigenic	HER2 enriched	ER ⁻ , PR ⁻ , HER2 ⁺	DMEM High Glucose	HTB30™
MDA-MB-468	Tumorigenic	Basal-like	ER ⁻ , PR ⁻ , HER2 ⁻	DMEM High Glucose	HTB132™
MDA-MB-231	Tumorigenic	Claudin-low	ER ⁻ , PR ⁻ , HER2 ⁻	DMEM High Glucose	HTB26™
Bo-1833	Bone Metastatic	–	–	DMEM High Glucose	MSKCC

For 3D cultures, 2D monolayered cells were detached with 0.25% trypsin and resuspended in the respective medium. Cell suspension was seeded at 5x10⁴ cells/mL (1x10⁴ cells per well) in ultra-low attachment round-bottom 96-well plates (Corning Costar). For optimal spheroid formation, 2.5% of reconstituted basal membrane (rBM) (Matrigel, Corning) was thawed overnight at 4°C and added to the cell suspension with

ice-cold pipette tips. Immediately after cell seeding, the plates were centrifuged at 1200 rpm for 10 minutes, in order to force spheroid formation, and spheroids were allowed to grow for 4 days until analysis.

1.1.2. NE treatment

When cells achieved confluency, 2D cultures of BrCa cells were treated with increasing doses of NE ($0,10^{-8}$ and 10^{-6} M) in serum-free medium (0.5% FBS) for 24 hours at 37°C in a 5% CO₂ atmosphere. After 24 hours the conditioned media (CM) was collected and kept at -80°C until use.

1.2. OCs cultures

1.2.1. Isolation

Human peripheral blood mononuclear cells (hPBMC) were isolated from buffy coats of female healthy blood donors, provided by Hospital São João, under a protocol approved by i3S ethical committee. Cells were isolated by Ficoll gradient centrifugation, where the buffy coats were diluted in Phosphate-buffered saline (PBS) and layered over Ficoll-Paque PLUS (GE Healthcare) solution. The sample was then centrifuged at 900 g for 20 minutes at RT, without accelerator or brake, in order to create a density gradient. The PBMCs layer, which corresponds to the interphase layer between the plasma and the Ficoll-Paque PLUS solution, was collected, washed twice with PBS and centrifuged at 900 g for 12 minutes at RT, with both accelerator and brake. The pellet was then resuspended in incubation buffer (0.5% Biotin-free bovine serum albumin (BSA) (Sigma) in PBS with 2 mM EDTA (Invitrogen)). To enrich the PBMCs with the monocyte fraction, the cell suspension was incubated with BD IMag™ Magnetic Particles conjugated with anti-Human CD14 antibody (BD Biosciences), magnetically separating the CD14-positive cells. This solution was incubated with the cell suspension for 30 minutes at RT, in order to incorporate the beads. After this, the tubes were transferred to an EasySep extremely strong magnet (StemCell) for 10 minutes. While still inside the magnet, the supernatant was discarded, and the cells were washed twice with the incubation buffer. The CD-14 positive cells were resuspended in α -Modified Eagle Medium (α -MEM) (Gibco) with 10% FBS and 10 μ g/mL Pen/Strep, supplemented with 25 ng/ml M-CSF (R&D Systems). The cells were cultured in a T75 flask and incubated at 37°C with 5% CO₂. For the following 9 days of OC differentiation, the medium was changed and

supplemented with 25 ng/ml M-CSF and 25 ng/ml RANKL (R&D Systems) as described in Figure III.1.

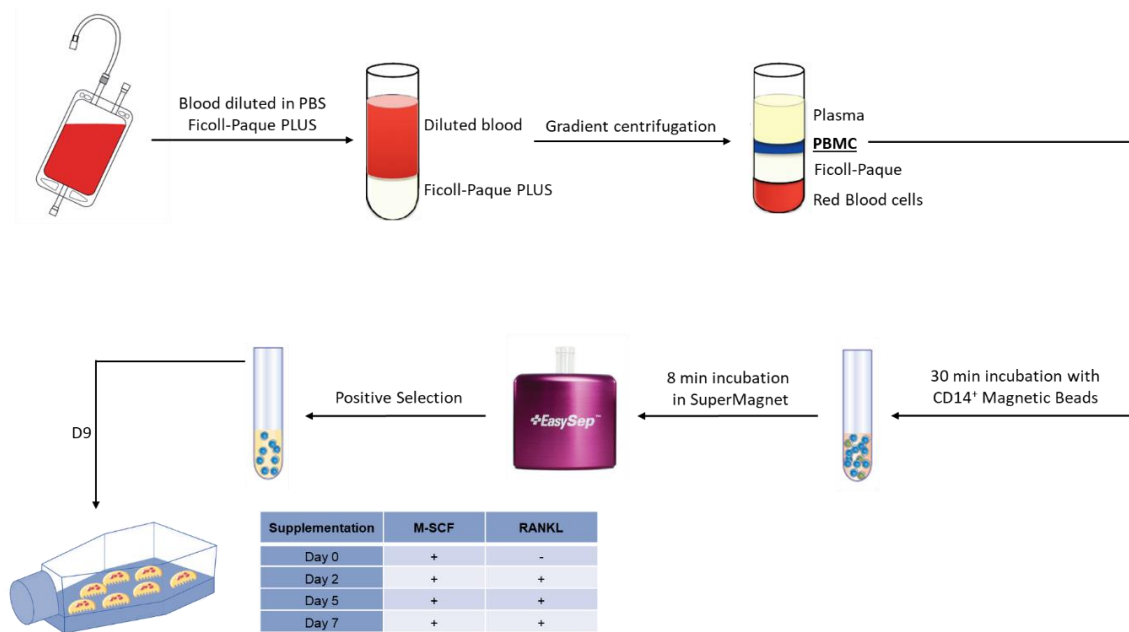


Figure III. 1 – Workflow of isolation and differentiation of CD14⁺ monocytes from buffy coats into fully matured OCs. The monocytes were isolated with a Ficoll-Paque PLUS density centrifugation and were incubated at 37°C with 5% CO₂ for 9 days. On day 2 the culture medium was collected, centrifuged and the cell pellet was resuspended and placed again in the culture flask. Fresh osteoclastic medium was added every 2-3 days thereafter.

1.2.2. NE and CM treatment

When needed, OCs were detached from the flasks after 10 minutes of incubation with accutase solution (Gibco). A density of 5×10^5 cells/mL were seeded in a TCPS 96-well plate in order to evaluate OC morphology or on top of bovine bone slices (IDS Nordic, Herlev, Denmark and Boneslices.com, Jelling, Denmark) to assess OC resorption activity. In the case of NE treatment, OCs were treated with increasing concentrations of NE (0, 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} M). As for CM treatment, the CM collected from BrCa cell lines were added in a 1:1 proportion to OCs cultures (50% CM + 50% α -MEM).

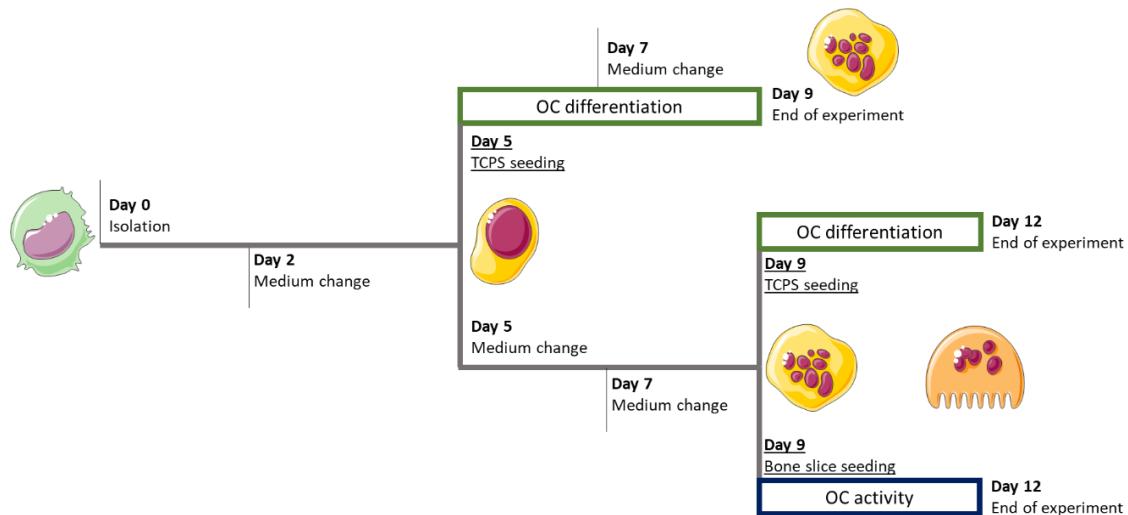


Figure III. 2 – Scheme of OC treatments. After monocyte isolation, cells were differentiated by adding osteogenic cytokines as previously described. In order to assess the effects of SNS activation on OCs, both OC morphology and OC activity were evaluated.

2. Gene expression analysis

RNA extraction and quantification: RNA was extracted immediately after culture. To lyse the cells, 500 μ L of TRIzol reagent (Life Technology) was added. The samples were then stored at -80°C until further usage. Direct-zol RNA Miniprep kit (Epigenetics) was used for RNA purification and the protocol was followed accordingly to manufacturer's instructions. Starting with the addition of 1 volume of 96% sterile ethanol, the sample homogenate was mixed and loaded on top of a Zymo-Spin IIC Column in a Collection Tube and successive washing steps preceded centrifugations at 12000 g for 1 minute. The RNA samples were then eluted in DNase/RNase-Free water and Nanodrop ND 1000 determined the samples' concentration and purity. Until cDNA conversion, the samples were stored at -80°C .

cDNA synthesis: NZY First Strand Synthesis Kit (NZYtech) was used to synthesize complementary DNA (cDNA) through an incubation period of 10 minutes at 25°C followed by a 30 minutes incubation at 50°C in a Thermocycler (Biometra). Afterwards, the reaction was inactivated by heating at 85°C for 5 minutes and posterior chilling on ice. In order to eliminate remnant RNA, 1 μ L of RNase H was added to each tube and incubated at 37°C for 20 minutes. cDNA was stored at -20°C until further use.

Quantitative real time-PCR (qRT-PCR): Primers were designed in NCBI/Primer-BLAST to amplify and detect the adrenergic profile of different BrCa subtypes and OCs. The corresponding primer sequences are summarized in Table A.1, in annexes. 1 μ L of cDNA and 9 μ L of iTaq Universal SYBR Green Supermix (BioRad) containing the specific

primer pairs were used to perform the qRT-PCR. The PCR protocol used is described in Figure III.2. PCR products were analysed using the CFX Maestro software (Bio-Rad) and the expression of the markers was normalized using the Δ CT method to a Beta-2-microglobulin (B2M) reference gene.

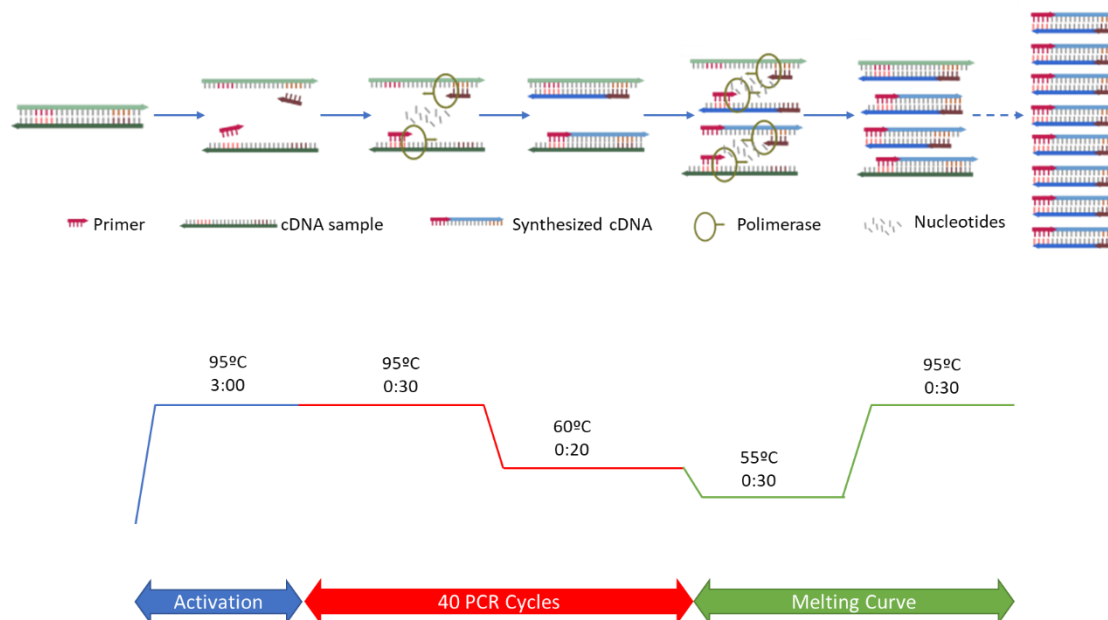


Figure III. 3 – Quantitative real time Polymerase Chain Reaction protocol – PCR runs started with an activation step of 95°C for 3 minutes. The 40 amplification cycles had a denaturation step of 95°C for 30 seconds, followed by an annealing step of 60°C for 20 seconds. The melting curve acquisition started at 55°C with a 0.5°C temperature increment until it reached 95°C.

3. Immunocytochemistry analysis

After culture of 2D BrCa cell lines in an 8 multi-well μ Slide (LabTek), cells were washed twice with PBS, and fixated with paraformaldehyde (PFA) (Sigma-Aldrich) 4% (w/v) for 10 minutes. After another washing step, the cells were permeabilized with ice-cold Triton X-100 1% (v/v) for 5 minutes. After washing twice with PBS, cells were incubated for 1 hour at 37°C with 1% (w/v) BSA in PBS, in order to block unspecific antibody binding. After 1 hour incubation with 1% BSA in PBS solution, cells were incubated with rabbit polyclonal α 1-AR (Abcam), α 2A-AR (Abcam), β 2-AR (ProteinTech) and TH (Millipore) antibodies at a dilution of 1:100 in BSA/PBS solution for 30 minutes. After washing again with PBS, the cells were stained with a solution of 1 μ g/ml DAPI (Sigma) in PBS. The samples were kept at 4°C in the dark until further analysis. The stainings were assessed in a Leica TCS SP2 Confocal Microscope (Leica Microsystems, Inc.). Two representative images were acquired for each cell line, with a resolution of 2048x2048 pixels and a z-

step of 5 μm for the spheroids. The image projection in z-axis was obtained in Fiji (ImageJ Software).

4. Cell morphology analysis

OCs were washed, fixed and permeabilized as previously described for 2D BrCa cells. After BSA blocking, OCs were incubated with Flash Phalloidin Green 488 (BioLegend) 1:100 in BSA/PBS solution for 1 hour at RT in the dark. After washing again with PBS, the cells were stained with a solution of 1 $\mu\text{g/ml}$ DAPI and 1 $\mu\text{g/ml}$ high-content screening (HCS) CellMask™ (Invitrogen) in PBS. The samples were kept at 4°C in the dark until further analysis. Cell morphology was assessed using a high-throughput scan (InCell Analyzer 2000, GE Healthcare). OC quantification was performed on the IN Cell Developer Toolbox 3.1 (GE Healthcare) software.

5. Bone resorption quantification

After 72 hours of culture, the medium was collected and stored at -80°C until further analysis. Water was added on top of bone slices for 24 hours in order to remove the cells from the bone matrix. With the help of a cotton bud and a clamp, the bone slice surfaces were cleaned. For 15 seconds the bone slices were stained with toluidine blue (Sigma-Aldrich). The resorption events (Figure III.4) were quantified by the percentage of total resorbed surface, using a 100-point grid (Pyser optics, G50 graticule) placed in the ocular of an optic microscope with a 10x. objective.

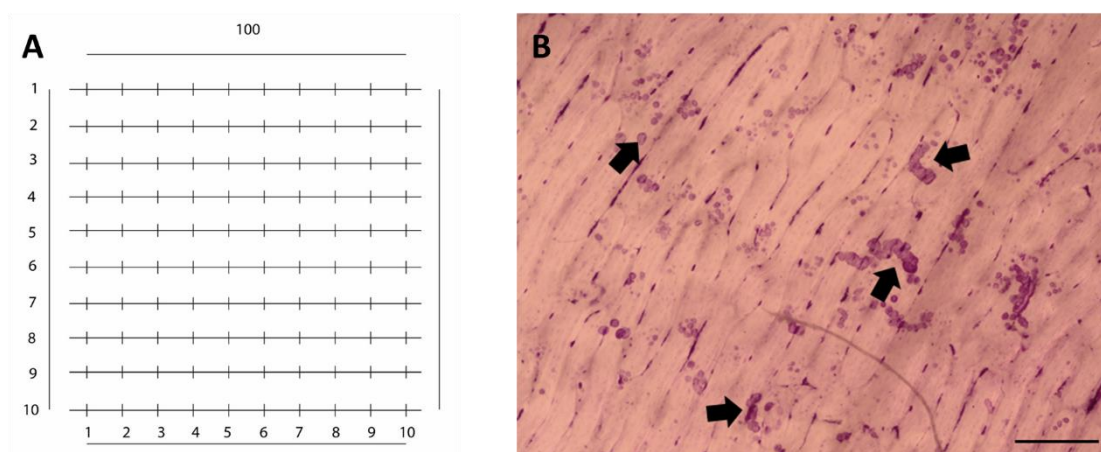


Figure III. 4 – Representative image of resorptive events caused by active OCs. A 100-point grid placed in the microscope ocular is used to count resorptive events (A). OCs are seeded on top of bovine bone slices for 72 hours, where they are activated and start to excavate the bone matrix, forming pits or trenches (arrows) (B). Scale bar 200 μm .

6. TRAP quantification

6.1. TRAP staining

Leukocyte Acid Phosphatase Kit (Sigma) was used to perform TRAP staining, on OCs seeded on a TCPS surface, according to manufacturer's instructions. Cells were fixed with a Citrate/Acetone Solution for 30 seconds at RT. After being rinsed with deionized water, the cells were left to air dry and then were incubated with TRAP Solution, consisting of water, acetate, naphthol, tartrate and 1 capsule of fast garnet salt GBC. The plate was incubated for 1 hour at 37°C in the dark. Cells were washed with water and were incubated with Acid Haematoxylin solution for 5 minutes at RT. Cell morphology was assessed using a high-throughput scan (InCell Analyzer 2000, GE Healthcare).

6.2. TRAP activity

TRAcP 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 OCs seeded on bone slices, in a 96-well plate. After 1 hour incubation in the dark at 37°C, NaHO solution was added to stop the reaction. Optical absorbance was measured 405 nm and TRAcP activity was measure against a standard curve of p-nitrophenol solution.

7. Zymography analysis

10% acrylamide separating gels which contained 0.1% of gelatine (Sigma-Aldrich) and 5% acrylamide (Bio-Rad) stacking gels, were used to perform the zymography. Protein content was quantified with the Bio-Rad DC Protein Assay kit (Bio-Rad). After sample loading buffer (SDS 0.1% plus 0.04% sucrose in Tris buffer 0.25 M, pH 6.8) was added to 10 µg of sample, the solution was loaded to the gelatine-SDS polyacrylamide gels. Precision Plus Protein™ Dual Color Standards (Bio-Rad) were used as protein standards and the gels ran at 80 V until the marker for 25 kDa reached the bottom of the resolving gel. Gels were washed twice with a 2% Triton X-100 solution for 30 minutes in agitation. After washing with distilled water (dH₂O) for 5 minutes, the gels were left on overnight incubation in MMP substrate buffer (50 mM Tris-HCl, pH7.5; 10 mM CaCl₂), at 37°C under gently agitation. Afterwards, the gels were once again washed with dH₂O, stained with a Coomassie Blue solution for 30 minutes under

agitation and washed with dH₂O until the bands were clear. Gels were scanned in a GS800 calibrated densitometer (Bio-Rad) and analysed with Quantity One version 4.6.9 software (Bio-Rad).

8. Scanning electron microscopy (SEM) analysis

After washing with PBS, 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate solution was added to OCs cultured on top of bone slices. The samples were fixated at RT for 30 minutes under gentle shaking (50 rpm), in an orbital shaker. After three washing steps with cacodylate buffer, samples were dehydrated in 10 minutes incubation in a dilution series of ethanol solutions 50, 60, 70, 80, 90 and 99% (v/v). Samples were then transferred to hexamethyldisilazane solution, air-dried at room temperature overnight and mounted onto appropriate supports with araldite glue. Samples were then sputtered coated with Au/Pd thin film for 80 s, using the SPI Module Sputter Coater equipment and examined using a High-Resolution Scanning Electron Microscope with X-Ray Microanalysis (JEOL JSM 6301F/ Oxford INCA Energy 350 at CEMUP facilities).

9. Statistical Analysis

Through software GraphPad Prism version 6.01 (GraphPad Software, Inc.), statistical significance was calculated using one-way analysis of variance (ANOVA) applying multiple comparisons test between all experimental groups. Data was expressed as mean values $\pm$ standard deviation. p-Values less than 0.05 were defined as statistically significant. A significant difference between two experiment groups is denoted as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

IV. Results and discussion

1. Characterization of SNS expression profile

1.1. BrCa

BrCa heterogeneity plays a pivotal role in patients' outcome. Therefore, the awareness that diagnosing the intrinsic BrCa subtype allows for a more appropriate treatment decision, has been increasing. These subtypes present different prognosis, response to therapy, patterns of metastasis, among other characteristics [13, 19, 104]. With that in mind, 5 different BrCa cell lines (MCF-7, BT-474, SK-BR-3, MDA-MB-231 and MDA-MB-468), each representing a distinct subtype, and a bone-tropic cell line (Bo-1833) were used to study the effects of SNS activation on bone metastasis driven from the different BrCa subtypes. When needed, MCF-10A (a non-tumorigenic cell line) was used as a control. Bo-1833 cell line, which represents the metastatic cancer cells present in bone, was derived from a parental MDA-MB-231 cell line, and was obtained from repeated intracardiac inoculations with subsequent isolation of tumour cells from bone lesions, until their metastatic phenotype was confirmed [105].

1.1.1. BrCa cells and morphology

As depicted in Figure VI.1, MCF-7 cells (luminal A) grew rapidly and homogeneously in the TCPS surface. Both BT-474 (luminal B) and SK-BR-3 (HER2 enriched) had a lower proliferative rate and grew in aggregates. Bo-1833, which is the bone tropic cell line, and its parental cell line, MDA-MB-231 (claudin-low) presented very similar proliferative and morphological characteristics. These cells rapidly achieved confluency and showed a more elongated form. Similarly to MDA-MB-231, MDA-MB-468 (triple-negative) had high proliferative rates, which goes in accordance with their aggressive phenotype.

It has been confirmed that cell spheroids better mimic the properties and environments found in tumours [106-108]. Therefore, 3D morphology, with and without rBM was analysed. rBM is a preparation extracted from a mouse sarcoma rich in ECM proteins. Its presence facilitates the natural aggregation of cells, by difficulting cell movement with its viscosity and stabilizing the structure formed in the centrifugation by interacting with cell receptors' [109]. This way, the ability that each cell line has to aggregate can be compared between the presence and absence of rBM.

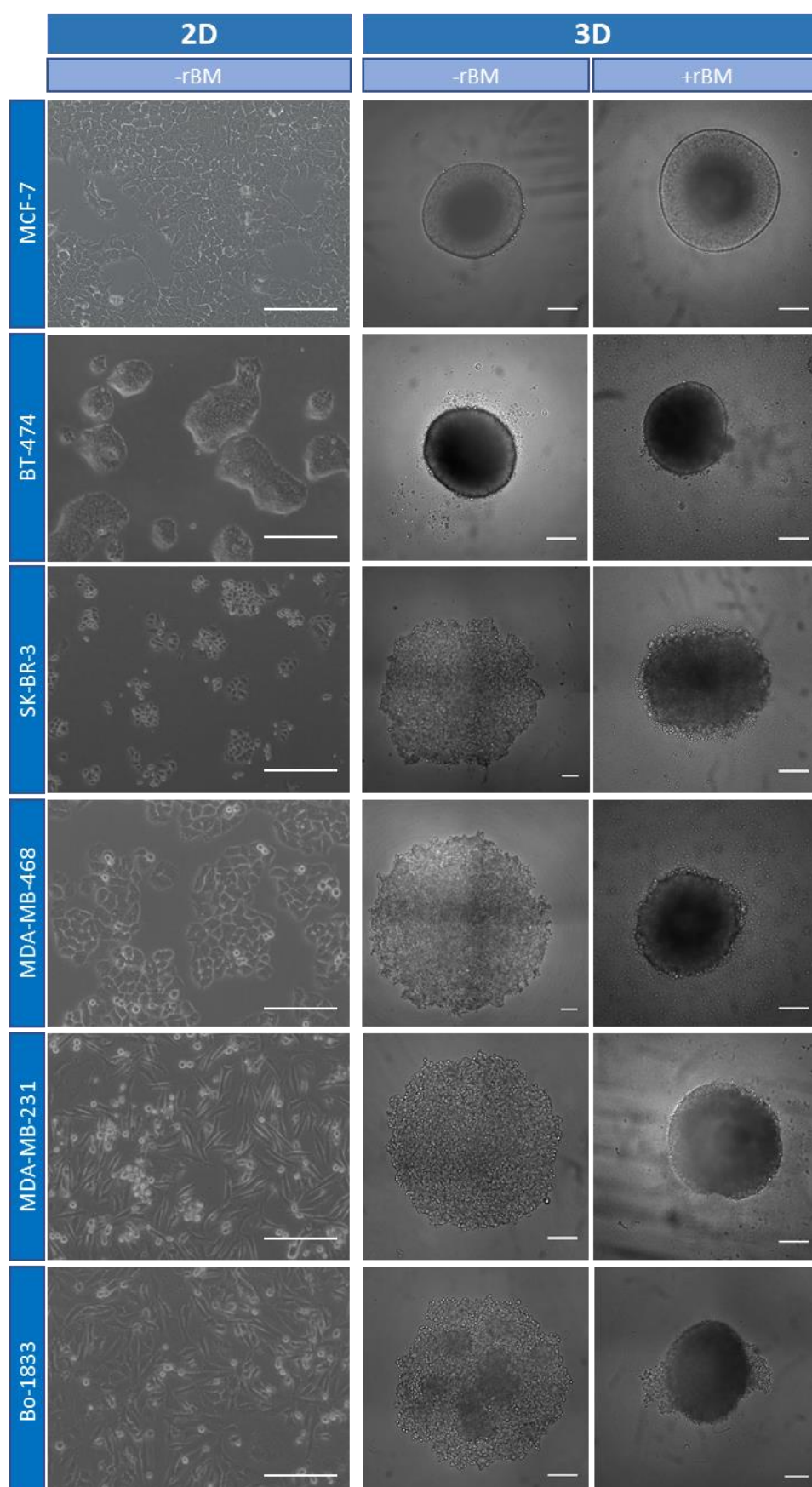


Figure IV. 1 – Representative images of 2D and 3D morphologies of each BrCa cell line used. 2D cells were cultured on T75 flasks. 3D cultures were cultured in absence and presence of rBM. Scale bar 200 μ m.

Only the luminal subtypes, MCF-7 and BT-474 were able to form a compact spheroid in the absence of rBM. The other cell lines were only able to form a 3D structure when rBM was added. This can be explained by luminal cell lines' ability to express proteins such as E-cadherins, which facilitate their aggregation [110]. On the other hand, triple-negative cell lines lack the expression of these tight junction and cell-cell adhesion proteins [17]. Even in the presence of rBM, the luminal cells are the ones that form smaller, more compact spheroids. The other 3D structures are less spherical and present cell projections and individual cells separated from the main aggregates.

1.1.2. Adrenergic expression profile

The ARs are activated by different concentrations of NE and E released by SNS, in response to stress situations. These catecholamines are synthesized by enzymes, such as TH, DBH and PNMT. TH is involved in the beginning of dopamine synthesis, which will be converted into NE by DBH. And PNMT is responsible to convert NE into E [111]. Catecholamines have the ability to activate 9 different subtypes of ARs, which are present in multiple tissues, including bone [80, 112, 113], and have also been described in different cancer types [114].

Since the link between SNS activation and cancer progression has been made, studies have described that BrCa cells also express ARs [67, 83, 115, 116], however most studies usually assess the effects of SNS activation by studying the effects of AR-agonists or antagonists, not specifying the subtype that is being stimulated [84, 88, 89, 97, 98, 117]. Therefore, the pathways that are being activated by SNS activation in cancer are still not fully understood. In order to confirm the presence of different ARs on the membrane of BrCa cells from different subtypes, immunostaining and gene expression analysis by qRT-PCR were performed.

For the immunostaining, one antibody per each class of AR ($\alpha 1$, $\alpha 2A$ and $\beta 2$) and TH were analysed (Figure IV.2).

Since ARs are distributed across the membrane, besides analysing the presence or absence of ARs, the immunostaining also allowed to once again verify the different morphologies of each cell line. For instance, the ability that BT-474 cells have for natural aggregation is verified in Figure IV.2E-H.

The immunostaining images show that MCF-7 and BT-474 express $\alpha 1$ and $\alpha 2A$ -AR, however there is almost no staining for $\beta 2$ -AR (Figure IV.2A-C, E-G). And while BT-474 show a strong staining for TH, MCF-7 does not (Figure IV.2D, H, L). $\alpha 2A$ -AR has a weak

staining in SK-BR-3 cells, but Bo-1833 on the other hand, mainly express this receptor (Figure IV.2J,V). However, MDA-MB-468 and MDA-MB-231 seem to express the four proteins (Figure IV.2M-T).

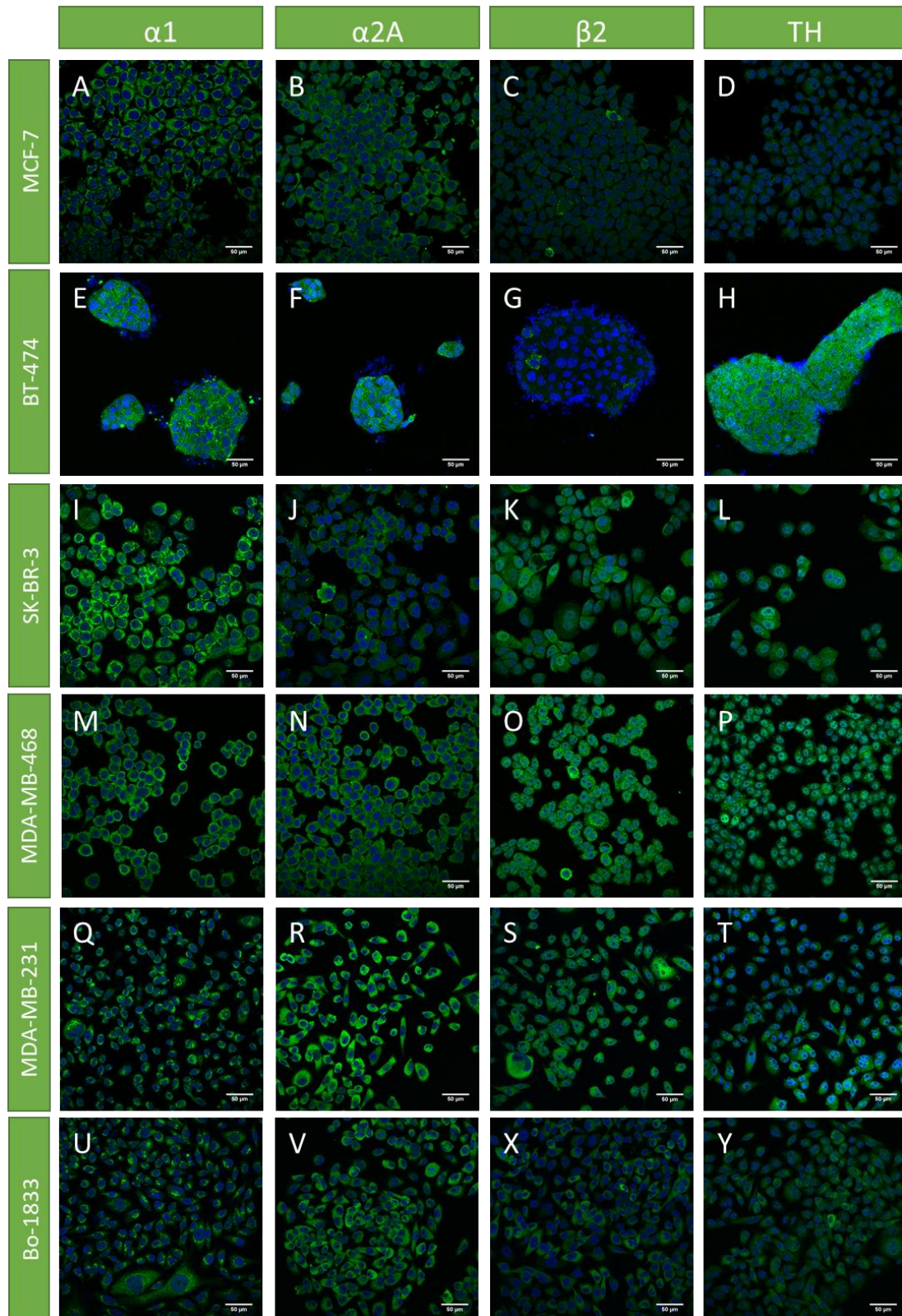


Figure IV. 2 – Expression of $\alpha 1$ -, $\alpha 2A$ -, $\beta 2$ -ARs and TH in each BrCa cell line by immunostaining. Representative images of AR expression profile of MCF-7 (A-D), BT-474 (E-H), SK-BR-3 (I-L), MDA-MB-468 (M-P), MDA-MB-231 (Q-T) and Bo-1833 (U-Y) cell lines.

To complement the results obtained by immunofluorescence, the 6 BrCa cell lines were analysed by qRT-PCR analysis, but before performing this technique, RT-PCR revealed in agarose electrophoresis gel was performed to assess the functionality of the primers. Since $\alpha 2B$ and $\alpha 2C$ primers were not working, new primers were designed to perform qRT-PCR analysis.

Therefore, primers for the 9 AR subtypes, TH, PNMT and DBH were designed in NCBI/Primer-BLAST to perform qRT-PCR and analyse the adrenergic profile of each BrCa cell line. B2M was the housekeeping gene used.

qRT-PCR results show that both luminal cells express a $\alpha 2$ -AR subtype and adrenergic metabolic enzymes. MCF-7 cells express $\alpha 2C$ -AR and TH (Figure IV.3A). And BT-474 cells express $\alpha 2A$ -AR and the enzymes PNMT and TH (Figure IV.3B). In fact, this was the only cell line that expressed PNMT. Besides expressing $\beta 1$ -AR, SK-BR-3 cells also expressed TH (Figure IV.3C). $\beta 1$ - and $\beta 2$ -AR were expressed by all triple-negative cells (Figure IV.3D-F). And Bo-1833 and its parental cell line (MDA-MB-231) also expressed $\alpha 2A$ -AR and TH. Bo-1833 was the only cell line that expressed $\alpha 1B$ -AR.

A few studies have already assessed the expression of some ARs in BrCa cell lines. MCF-7 cell line also showed expression of $\alpha 2C$ -AR in a study by Vazquez et al. [67]. However, this cell line was also described to express $\alpha 2B$ -, $\beta 1$ - and $\beta 2$ -ARs [67, 94]. BT-474 and SK-BR-3 were described to also express $\beta 2$ -AR [115] and triple-negative cell lines, MDA-MB-231 and MDA-MB-468 besides expressing $\beta 2$ -AR [84, 94], showed expression of $\alpha 2B$ -AR [67] and $\beta 1$ -AR [94], respectively. Furthermore, one study evaluated the expression of ARs in tissues of primary BrCa, linking luminal tumours with $\beta 2$ -AR expression and basal-like tumours with $\alpha 2C$ -AR [116]. In Figure VI.3, the agarose PCR runs, which is the method mostly performed in these studies, also show $\beta 1$ - and $\beta 2$ -AR expression in every cell line. However, regular RT-PCR is not as specific as the quantitative method.

Most studies only describe the presence of ARs by the effects caused by non selective AR-agonists or antagonists on cell proliferation [84] or tumour progression [88, 89, 97, 98].

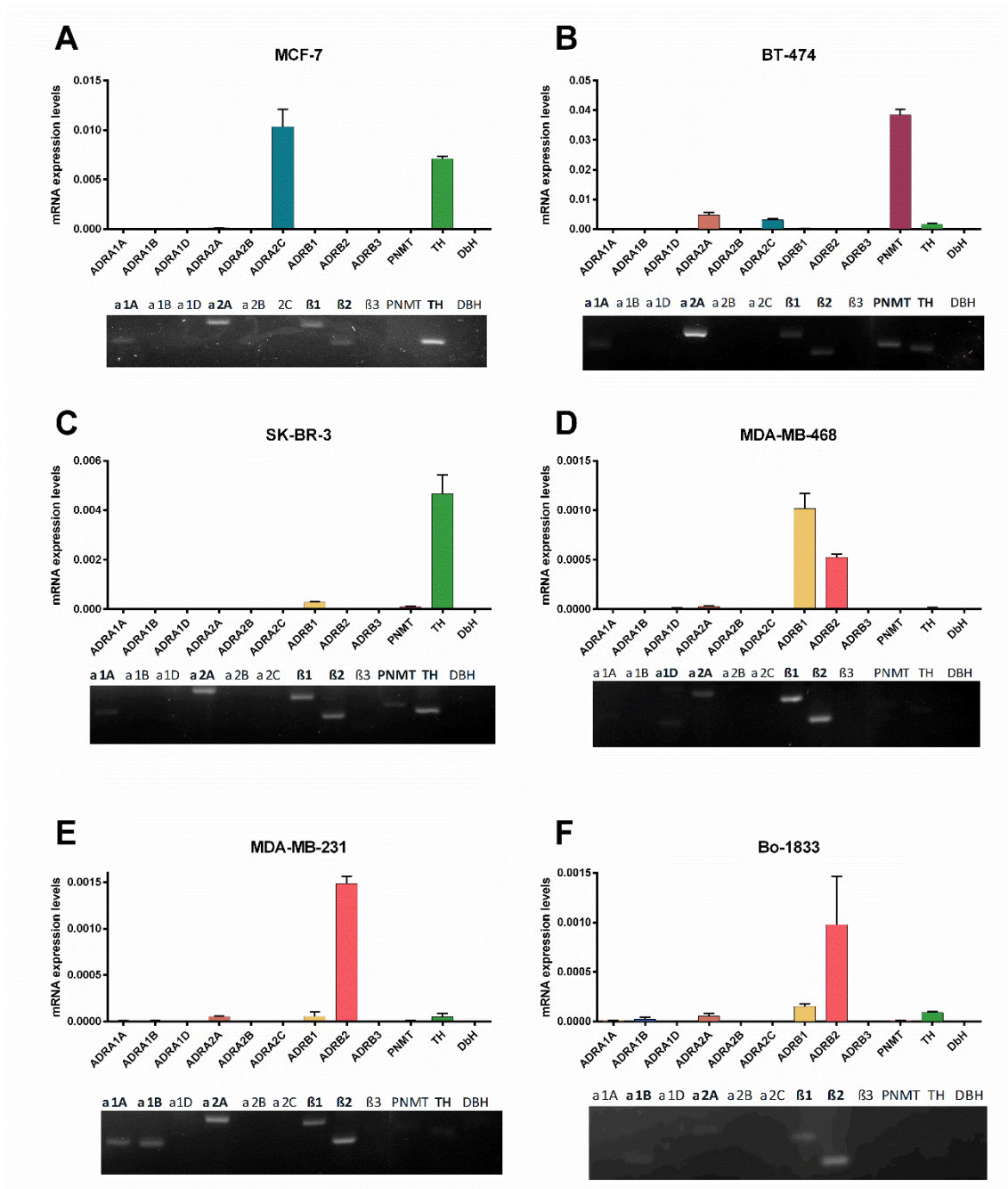


Figure IV. 3 – ARs and catecholamine synthesizing enzymes gene expression profile of the 6 BrCa cell lines. Gene expression of ARs and enzymes involved in catecholamine synthesis were analysed by qRT-PCR analysis (upper image) and PCR revealed in agarose gel electrophoresis (lower image) for each BrCa cell line (A-F). The expression values of each gene on qRT-PCR were normalized with expression of B2M gene. Data is expressed as mean $\pm$ SD (n=3).

There are some differences when comparing the results from the immunostaining with the gene expression analysis. However, it was possible to verify that each BrCa cell line presents different expression profiles of ARs, which can lead to different effects once SNS is activated.

In the future, other methods, such as immunostaining with more specific antibodies and protein quantification by western blotting, will be tested to better understand the AR expression profile of each BrCa subtype.

1.2. OCs

1.2.1. OCs differentiation and morphology

After monocyte isolation these cells undergo a multi-step differentiation process, leading to the formation of mature OCs capable of resorbing bone matrix (Figure IV.4). By addition of the osteogenic cytokines M-CSF and RANKL, monocyte lineage cells differentiate into pre-OCs that fuse to form multinucleated cells, that once activated gain the ability to resorb bone matrix.

M-CSF mainly induces the proliferation of monocyte lineage cells (Figure IV.4A). After addition of RANKL, cells start to differentiate and on day 5 it is already possible to see the presence of OC-like cells (Figure IV.4B). Mononuclear cells start to fuse into multinuclear OCs, and on day 9 of differentiation the culture surface is almost completely covered by these cells (Figure IV.6D). At this point, OCs are ready to resorb bone matrix, and by addition of a bone slice, resorption studies can be performed.

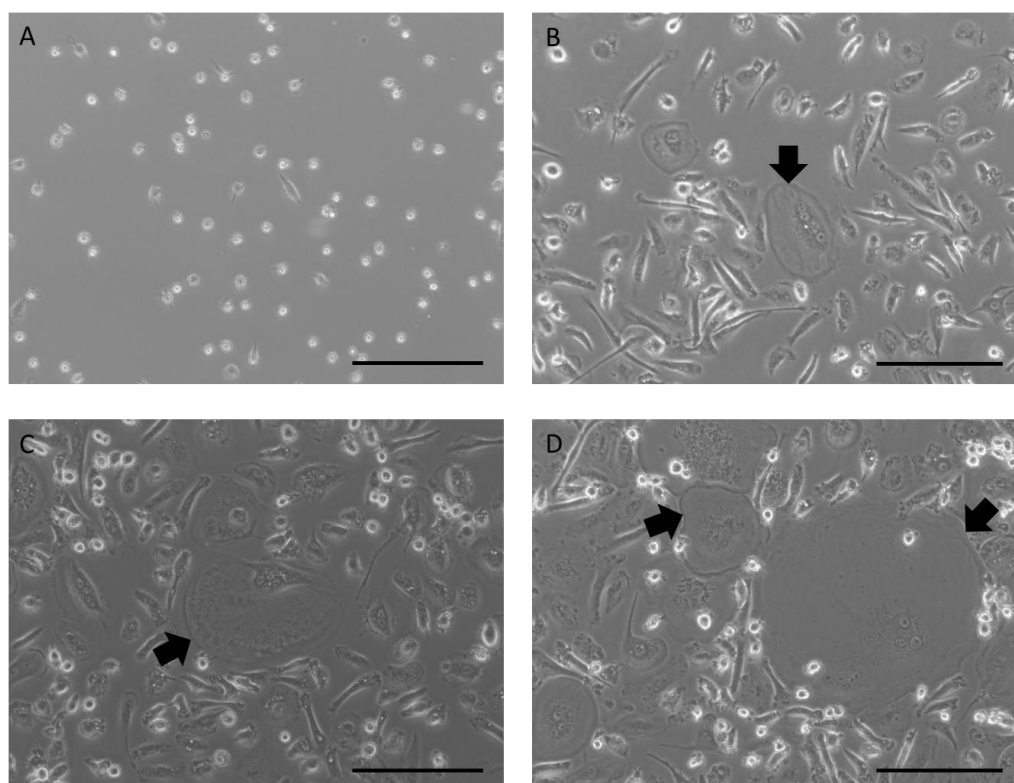


Figure IV. 4– Representative images of OC morphology during the different stages of osteoclastogenesis. A. Day 2: mononuclear cells are small and round, resembling monocyte cell features. **B.** Day 5: different cell populations can be distinguished, most cells are elongated but some cells already present OC-like appearance. **C.** Day 7: larger multinucleated cells can be seen on this maturation stage. **D.** Day 9: at this stage, the flask surface is covered by giant multinucleated OCs. Arrows: OC-like cells. Scale bar 200 μ m.

In order to become fully activated, OCs modulate the actin filaments present in their cytoskeleton [45]. Therefore, Figure IV.5 shows the different cytoskeletal arrangements formed by mature OCs on day 12 of differentiation, marked by F-actin immunostaining. It was possible to recognize 3 different actin modulations: the filopodia cell projections (Figure IV.5A), the podosomes, dot like structures arranged as a belt (Figure IV.5B) and the formation of an actin ring in the periphery of the cell (Figure IV.5C). Since there is no bone matrix, the actin structures found during the resorption phase, such as the sealing zone, are absent on TCPS surfaces.

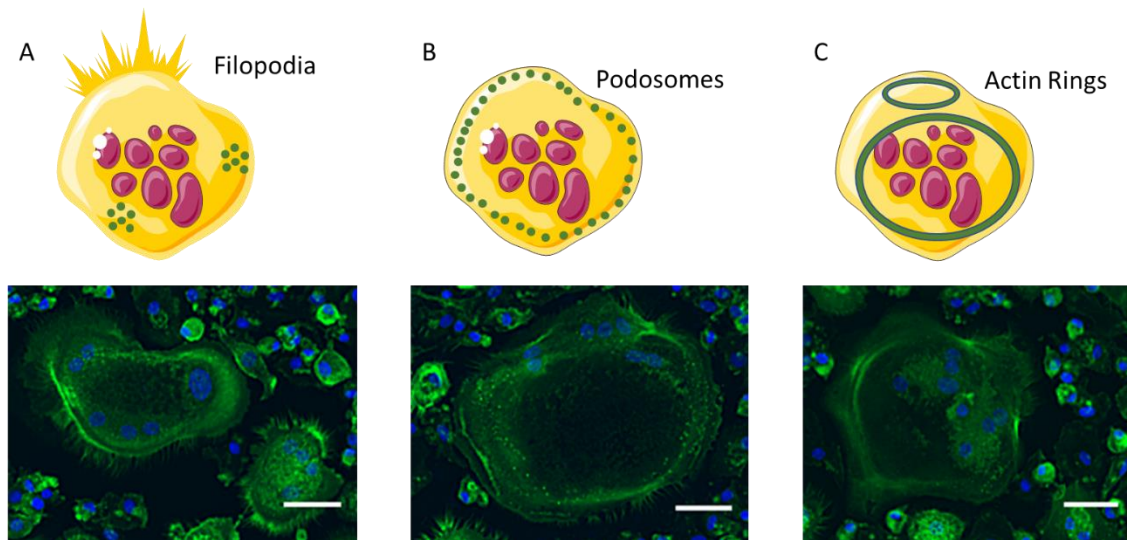


Figure IV. 5 – Representative images of mature OC' cytoskeletal arrangements. Upper images show a scheme of cytoskeletal modulation. Mature OCs cultured for 72h in a TCPS surface presented filopodia (A), podosomes (B) and actin ring (C) structures (Bottom images). Scale bar: 50 μ m.

Since OCs need bone matrix to become fully activated, these cells were seeded on top of bovine bone slices that provide an *in vivo* bone substrate for OC resorption of bone. In Figure IV.6, SEM images demonstrate the cells covering the bone surface (Figure IV.6A), the erosion caused by their activation (Figure IV.6B,C,E) and OC morphology is showed in Figure IV.6D and F, extending their membrane projections in order to resorb the bone.

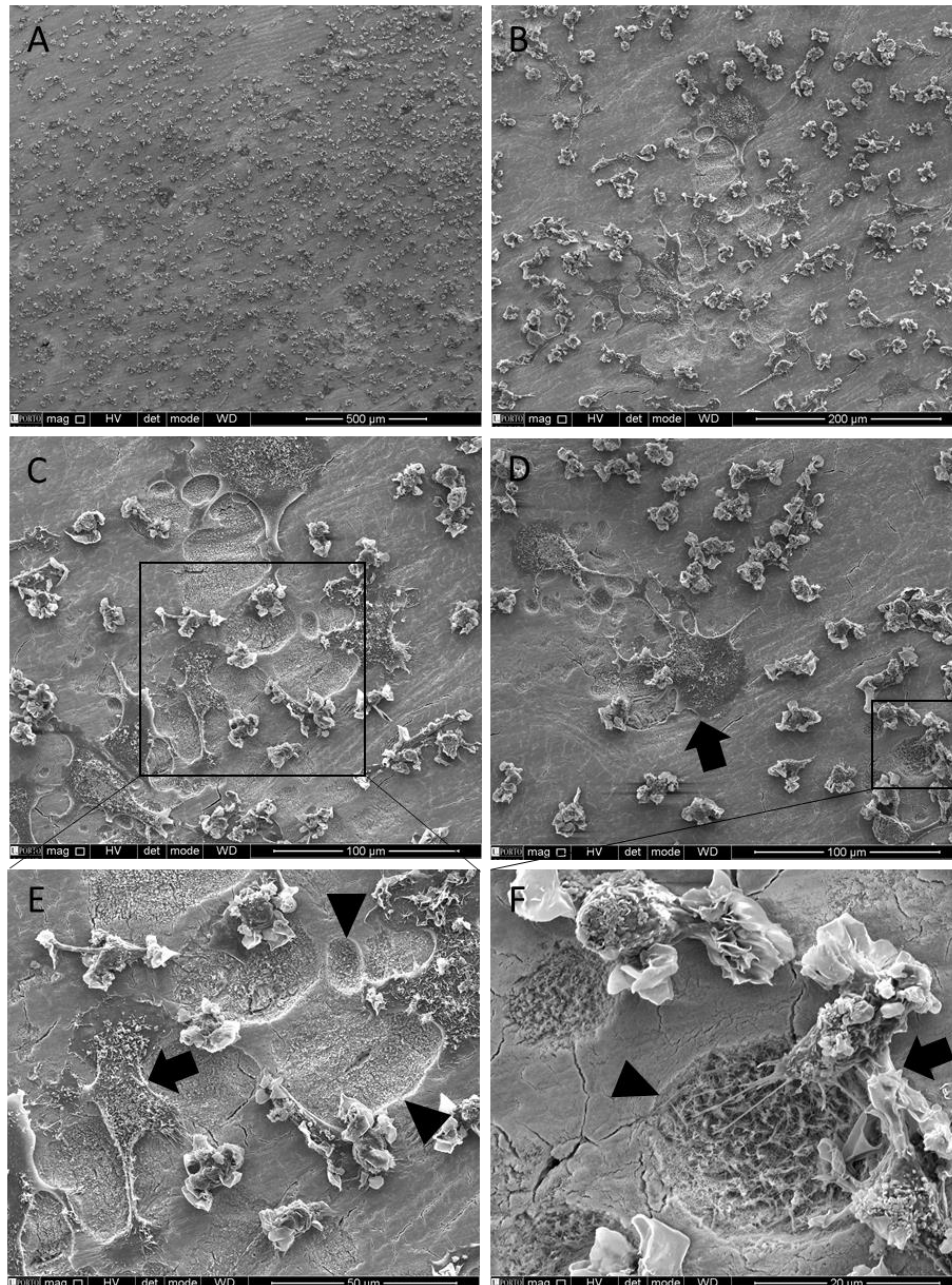


Figure IV. 6 – SEM images of OCs seeded for 72h on top of bovine bone slices. A. Close-up of the bone slice surface covered by OCs. **B.** Effects of bone resorption caused by OCs activation. **C, E.** Details of resorption events caused by OCs. **D.** OC on top of the bone matrix. **F.** Detail of OC spread on top of a resorption event. Arrows: OCs. Arrow heads: resorption events.

1.2.2. Adrenergic expression profile

OCs also express ARs [79, 113, 118], however most studies focus only on β -ARs. The AR gene expression profile of OCs presented on Figure IV.7, shows that OC express $\alpha 1A$ -AR, all three $\alpha 2$ -AR subtypes and β -ARs. TH and DBH were also expressed by these cells. In a recent study, mouse OC-like cell line RAW264.7, also expressed all $\alpha 2$ -AR subtypes [119]. Although there is one study with human OC-like cells that

described expression of α 1B-, α 2B- and β 2-ARs [79], the protocol of OC isolation differed from the one used on this project.

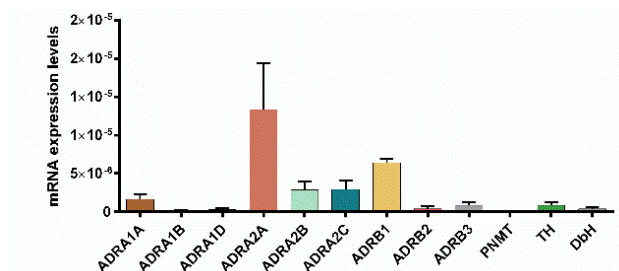


Figure IV. 7 – Gene expression by qRT-PCR of ARs and enzymes expressed by OCs. The expression values of each gene were normalized with expression of B2M gene. Data is expressed as mean $\pm$ SD (n=3).

2. Role of SNS in OCs (direct effects)

After SNS activation, NE and E reach the cells, mainly through sympathetic nerve fibers and circulation, respectively. Although SNS has the ability to affect the tumour progression by its two pathways, either by local or circulating catecholamines, growing evidence suggests that the local release of NE from SNS nerve terminals plays a dominant role [120, 121].

BrCa vicious cycle usually results in osteolytic lesions and loss of bone mass. And one of the main intervenients on this vicious cycle is the OC, since it is the only cell capable of bone resorption.

However, the direct effects of SNS on OC activity cannot be excluded. Therefore, firstly, OCs were directly treated with increasing doses of NE, in order to mimic the activation of SNS in bone, and the differentiation and resorption capacity of OC was evaluated.

2.1. Effects of NE treatment on OC differentiation

To evaluate how the differentiation process was affected by SNS activation, treatments of OCs cultured with 0, 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} and 10^{-5} M of NE started at early (day 5) and late (day 9) stages of differentiation.

In order to evaluate how SNS affected early differentiation, day 5 after monocyte isolation was chosen to represent pre-OC phase, since presents a large number of mononuclear cells (Figure IV.4B). And OCs were considered mature on day 9, when most of the cell monolayer was constituted by multinucleated cells, as represented in Figure IV. 4D. Therefore, this day was chosen to evaluate the effects caused by SNS activation on late OC differentiation.

A method that easily evaluates OC morphology is qualitative analysis of TRAP expression. This phosphatase is only expressed by multinucleated cells (both inactive and active OCs) therefore, the fact that in Figure VI.8B all wells show TRAP+ cells, means that cells treated in all conditions were able to differentiate. Moreover, the wells treated with smaller or no dose of NE present more TRAP+ cells than wells treated with higher NE concentrations.

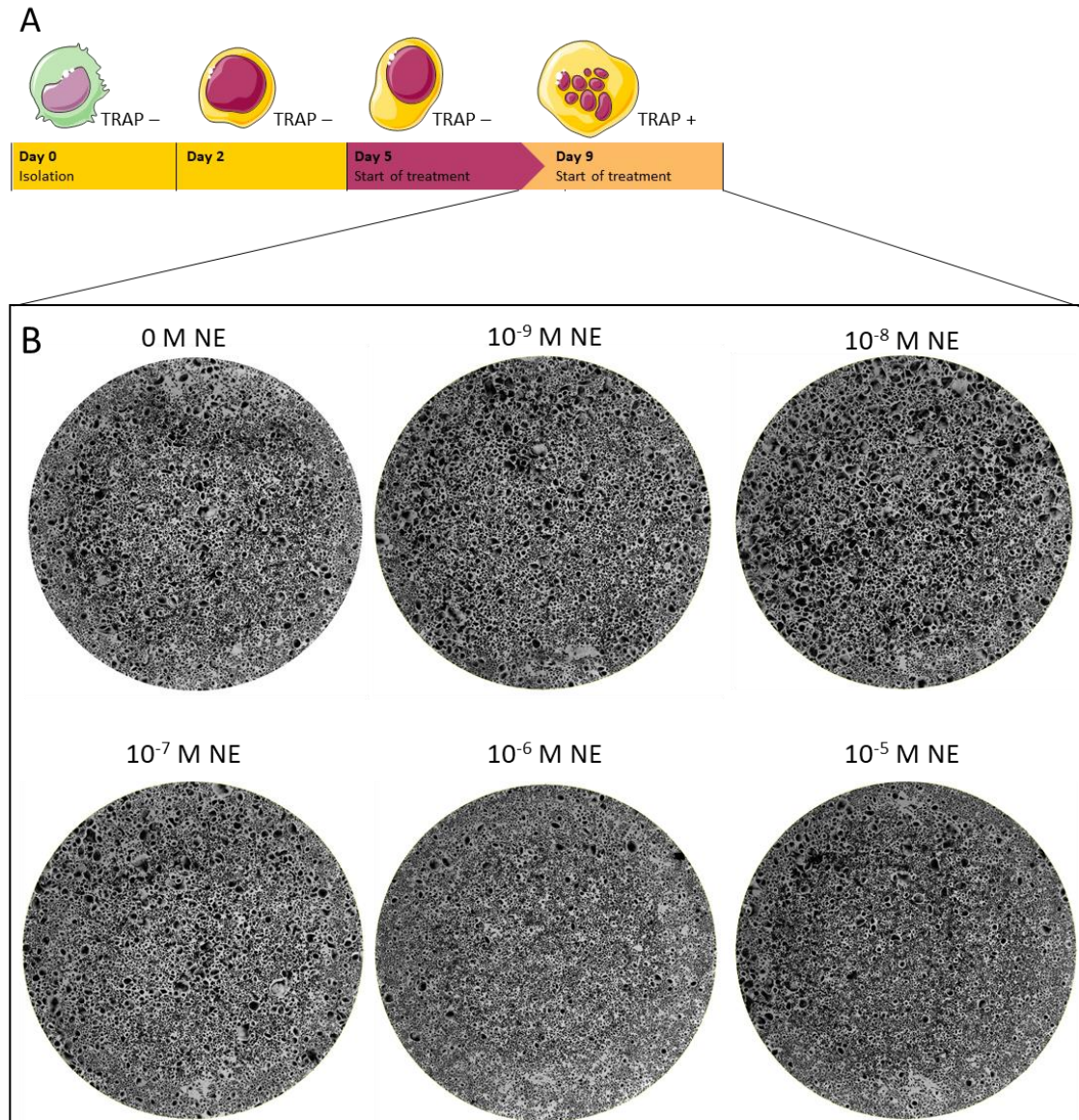


Figure IV. 8 - TRAP staining of pre-OCs treated (since day 5) with different NE concentrations until day 9 of differentiation. **A.** Scheme of OC treatment for TRAP staining. Treatment was initiated on day 5 when most cells are TRAP-negative OCs. **B.** Representative images of TRAP staining of each condition.

In Figure IV.9 treatment started later in the differentiation process (day 9) and shows, like the previous time point, that in all conditions there are mature OC. However, it seems that OC treated with 10^{-8} and 10^{-7} M of NE present more TRAP+ cells than the other conditions.

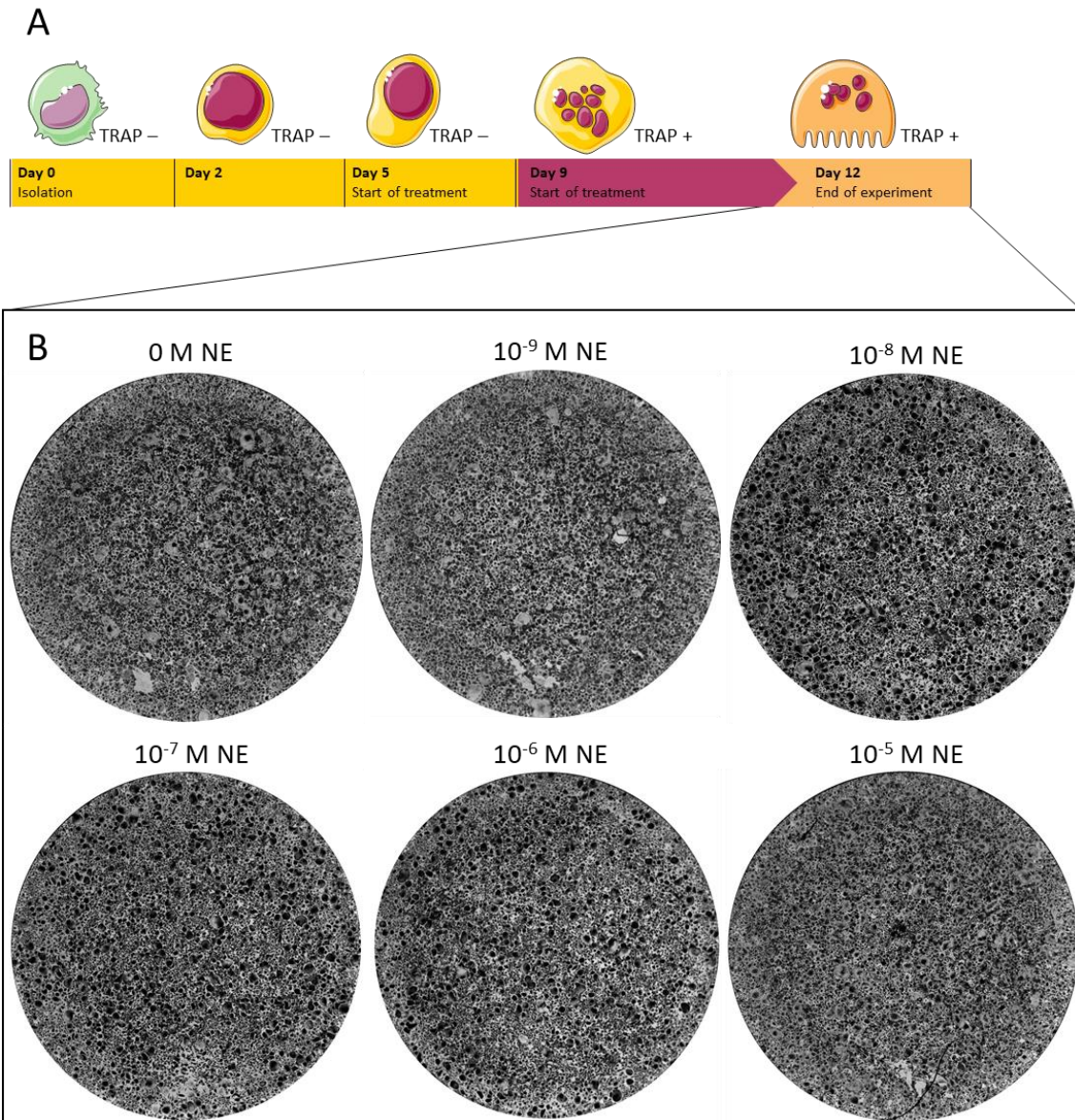


Figure IV. 9 – TRAP staining of OCs treated (since day 9) with different NE concentrations for 72h on TCPS surfaces. A. Scheme of OC treatment for TRAP staining. Treatment was initiated on day 9 when there are already TRAP-positive multinucleated OCs. **B.** Representative images of TRAP staining in each condition.

Furthermore, as expected, there are more TRAP+ cells on these wells, compared to the assay performed on pre-OCs, since they were still differentiating (Figure IV.8B). In this assay, treatment started on a later differentiation phase and by the time TRAP staining is performed OCs already completed the maturation process on non-resorbing surfaces.

To evaluate the effects caused by the increasing doses of NE, OC quantification was performed, through F-actin and HSC cell mask immunostained images, by practical high-throughput image softwares, *ilastik* and *Cell profiler* (Figure IV.10). *ilastik* allows for image segmentation, labelling background and cells of a few representative images (Figure IV.10B) [122]. After the output generated by *ilastik*, *Cell profiler* is able to measure cell area, texture and intensity (Figure IV.10C) [123], generating the output required for OC quantification (Figure IV.10D).

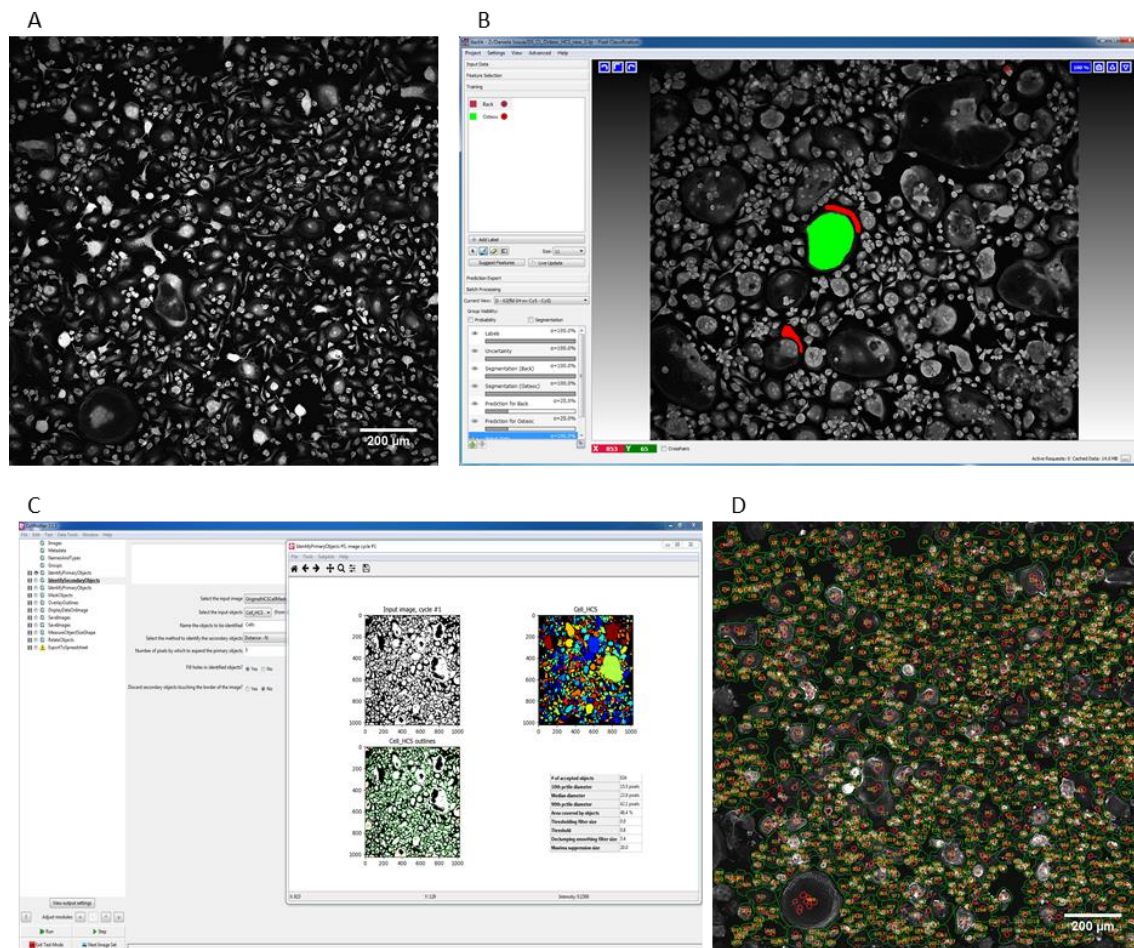


Figure IV. 10 – Overview protocol for OC quantification. (A). Representative HSC cell mask image used as model for OC quantification. *ilastik* (B) and *Cell profiler* (C) processed images, generating the outputs required for OC quantification. (D) Example of merged F-actin and *Cell profiler* output images.

Figure VI.11 shows representative images of OC morphology treated with every NE dose since day 5 of differentiation, and no major differences can be seen in these images. However, when these cells were quantified, not every treatment presented the same cell number or area (Figure VI.12).

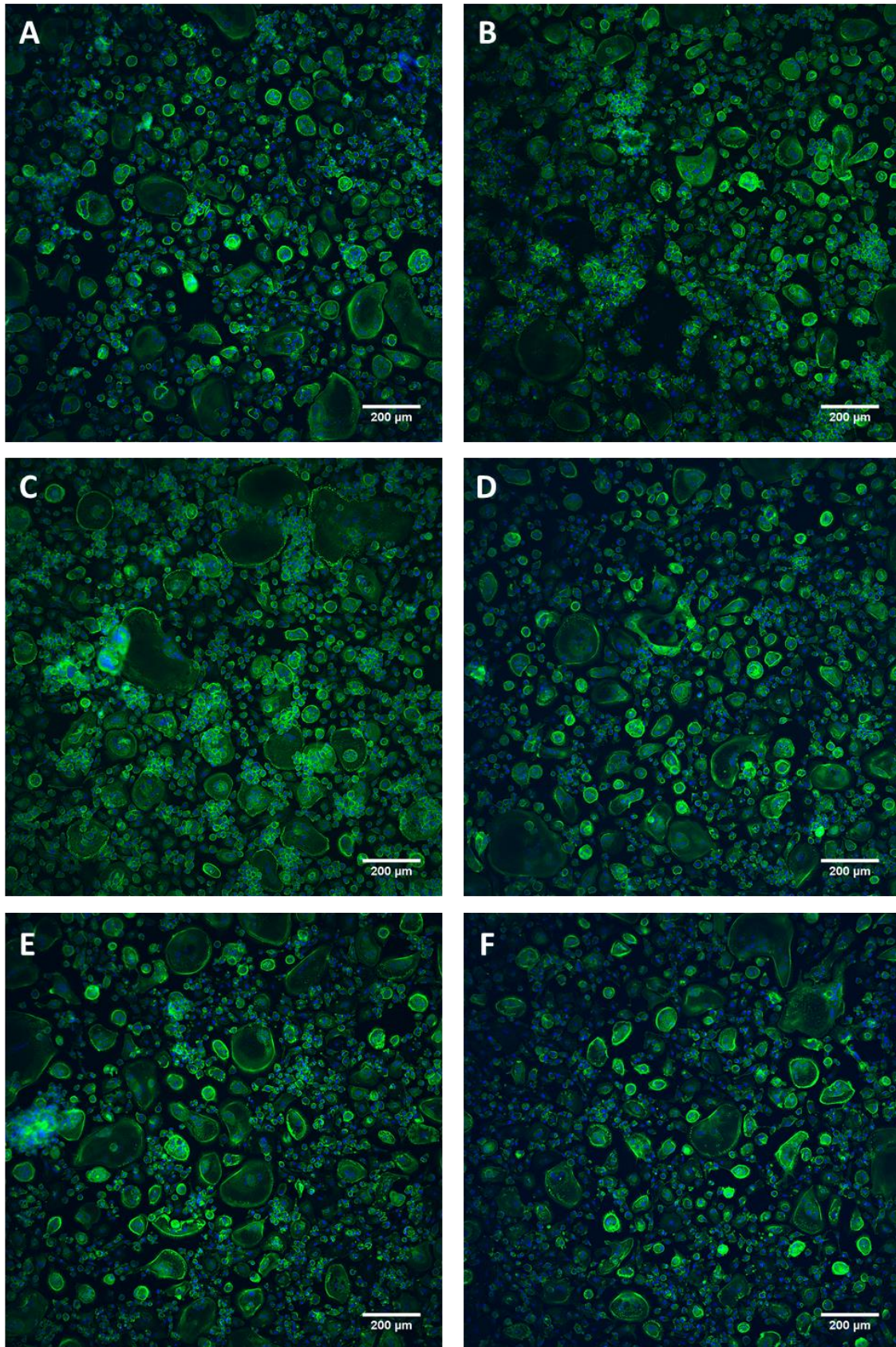


Figure IV. 11 – Representative images of F-actin staining with OC treated (since day 5) with increasing doses of NE. On day 5 after monocyte isolation, pre-OCs were seeded on 96-well plates and phalloidin immunostaining was performed on day 9. Images show OC morphology of OCs treated with 0 (A), 10^{-9} (B), 10^{-8} (C), 10^{-7} (D), 10^{-6} (E) and 10^{-5} (F) M of NE. Scale bar=200 µm.

Since OCs are characterized for being multinucleated cells, only cells with more than 2 nuclei were analysed as OCs, and cells were divided into two nuclei groups of $3 \leq n \leq 7$ and $n \geq 8$, in order to better characterize the differences between each condition. Global OC ($n \geq 3$) quantifications of early and late differentiation are in Figure VI. A1 in annexes.

The number of OCs with $3 \leq n \leq 7$ nuclei is similar with treatments of every NE concentration. The bigger differences on OC number were found on cells with $n \geq 8$, where cell number gradually increased in the lowest NE doses (10^{-8} to 10^{-9} M). The highest doses of NE however, caused a decrease in OC number (Figure IV.12A). Cell area was similar in every condition, except in wells with 10^{-5} M of NE treatment. Although this condition presents fewer cells, the area of these cells is bigger than OCs found in other treatments (Figure IV.12B).

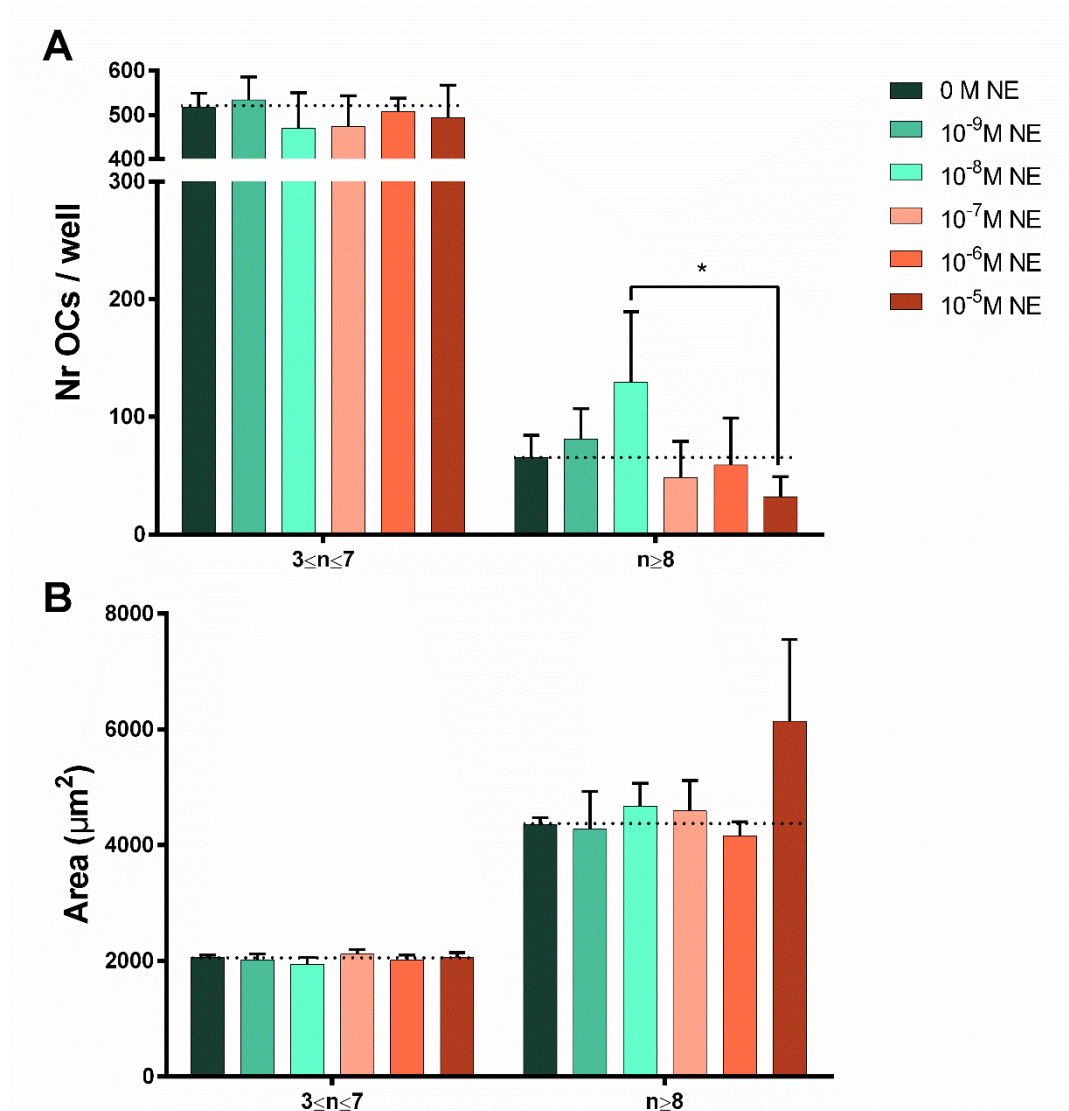


Figure IV. 12 - Morphological analysis of OCs treated, since day 5, with different NE concentrations. Number (A) and area (B) of OCs per well with 3 to 7 nuclei ($3 \leq n \leq 7$) and with 8 or more nuclei ($n \geq 8$); ($n=5$ wells per condition).

Contrarily to Figure VI.11, the representative images of mature OCs stained with phalloidin, suggest that OCs treated with NE are bigger than OCs subjected to no NE treatment (Figure IV.13).

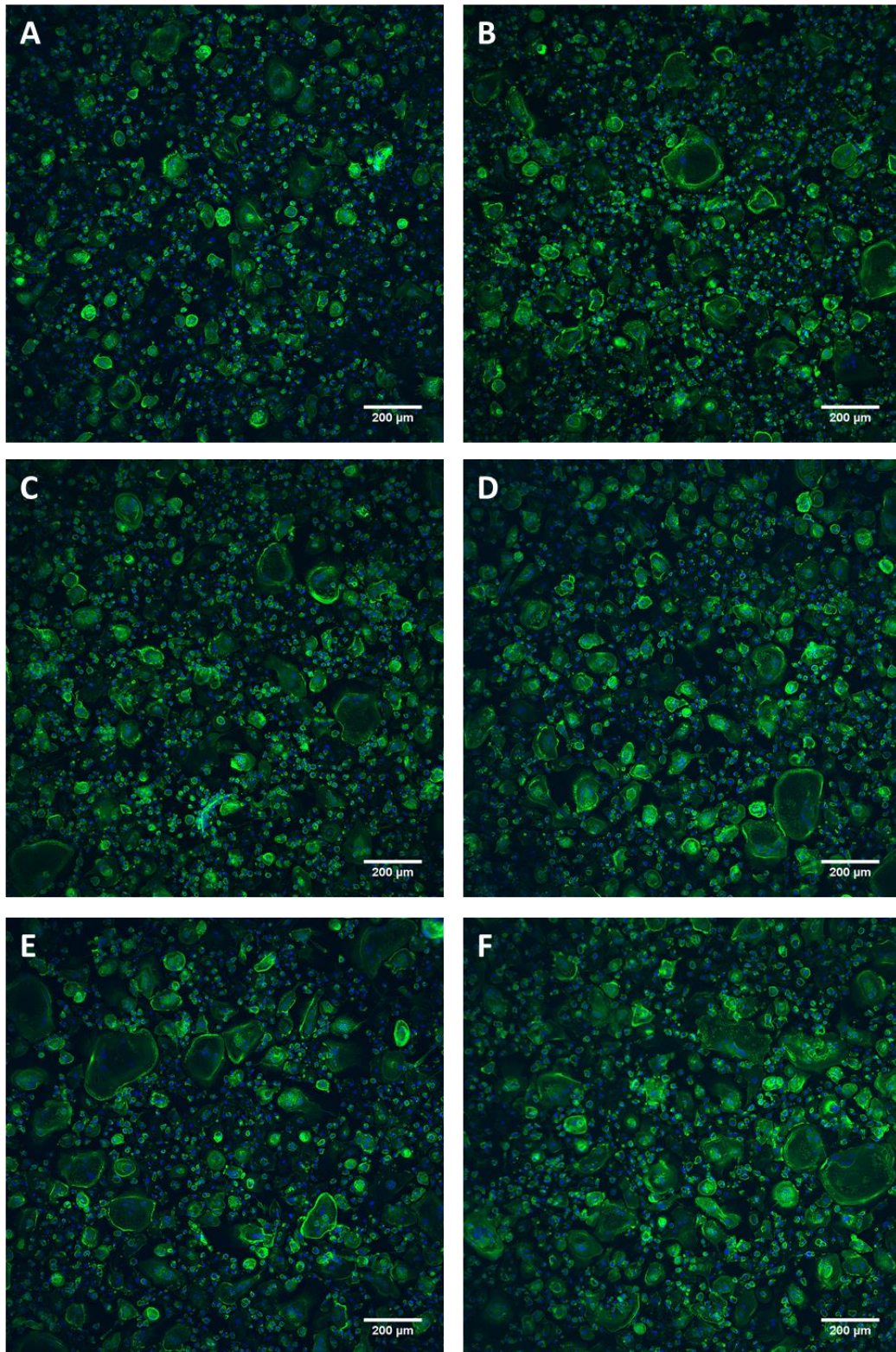


Figure IV. 13 – Representative images of F-actin staining of OCs treated (since day 9) with increasing NE concentrations. On day 9 after monocyte isolation, mature OCs were seeded on 96-well plates and phalloidin immunostaining was performed on day 12. Images show OC morphology of OCs treated with 0 (A), 10^{-9} (B), 10^{-8} (C), 10^{-7} (D), 10^{-6} (E) and 10^{-5} (F) M of NE. Scale bar=200 μm.

Both number and area of cells show bigger differences in the group with more nuclei ($n \geq 8$). Although there are no significant differences, the number of cells with $3 \leq n \leq 7$ gradually increases, from OCs without NE treatment to OCs treated with 10^{-8} M of NE. From this NE concentration until the highest dose (10^{-5} M NE), the number of OCs with $3 \leq n \leq 7$ decreases (Figure IV.14A). There are almost no differences between the area of cells treated with different NE treatments, however OCs present on wells treated with no NE are smaller than cells treated with all NE doses, as observed in F-actin stained images (Figure IV.14B).

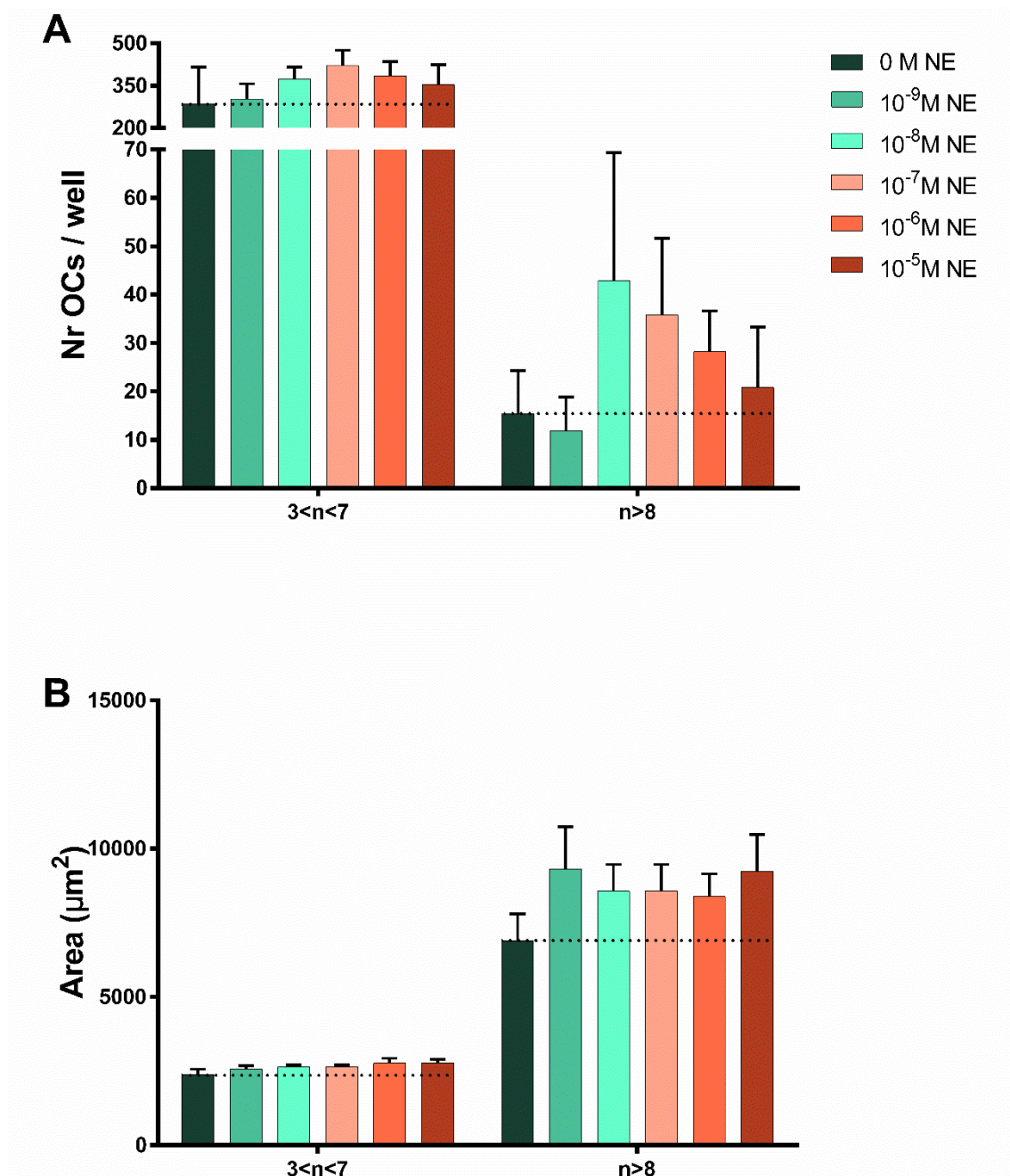


Figure IV. 14 - Morphological analysis of OCs treated, since day 9, with different NE concentrations. Number (A) and area (B) of OCs per well with 3 to 7 nuclei ($3 \leq n \leq 7$) and with 8 or more nuclei ($n \geq 8$). ($n=5$ wells per condition).

Therefore, in both treatment time points, multinucleated OCs can be found in every condition, surrounded by a large number of pre-OCs (Figure VI.11 and 13). Treatment of OCs with lower doses of NE (10^{-8} M) cause an increase of OC number, both when treatment starts at early or late differentiation phases. And this number starts to decrease when high doses of NE (10^{-6} and 10^{-5} M) are administered (Figure VI.12A and Figure VI.14A). A similar modulation was already described in OCs isolated from Dark Agouti rats, where NE induced a dose-dependent effect: 10^{-6} inhibited OC formation and 10^{-8} M of NE showed stimulatory effects [124].

Besides the increase in the area of OCs treated with 10^{-5} M of NE during early differentiation, the area of both experiments remains unchanged through the different NE concentrations (Figure VI.12B and Figure VI.14B). However, cell size is bigger in every NE treatment when compared to the control condition, during late differentiation. These results suggest that NE administration affects both early and late OC differentiation, suggesting a direct role of SNS activation on OC function.

Furthermore, OCs from late differentiation treatments with no NE suffer a decrease in cell number and an increase on cell area when compared to early differentiation (Figure A.1, annexes). This is expected, since early differentiated mononuclear cells have fused to become giant multinucleated OCs.

2.2. Effects of NE treatment on OC resorption activity

Once OCs are matured, they can be activated and start to resorb bone. This process is of most importance to renew bone that is damaged or old. However, once this process is deregulated a lot of bone events, such as fractures and bone pain, start to appear [125]. It is described that SNS is capable of modulating bone microenvironment by activating ARs on OBs, which by activating OCs will lead to a decrease of bone mass [126]. However, little is known about the direct effects that SNS activation has on OC function.

Therefore, mature OCs were seeded on top of bone slices for 72h, in order to evaluate the direct effects caused by administration of NE on OC resorptive activity. Two NE doses were selected (10^{-8} and 10^{-6} M) to perform the following experiments, since has been described that NE at 10^{-8} M mainly activates α -ARs and that NE at 10^{-6} M mostly activates β -ARs [127].

OCs were obtained through monocyte isolation from buffy coats of 6 different donors and the individual resorption profile from each donor is presented in Figure A.2,

in annexes. Despite the high variability between samples and although there are no statistical differences, there is a slight decrease of bone resorption when OCs are treated with increasing NE concentrations (Figure IV.15).

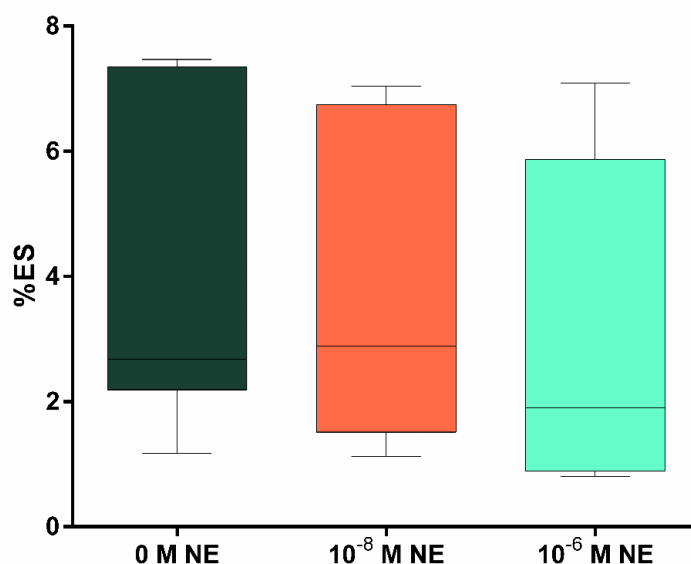


Figure IV. 15 – Direct effect of NE treatment on OC activity. Resorptive activity was quantified by the percentage of eroded surface (ES) per total bone surface. OCs were treated with 0, 10^{-8} and 10^{-6} M of NE. Data is expressed as min to max (n=6 donors, 5 bone slices per condition).

Since the sample size was small, it is not possible to fully understand the role of SNS stimulation on bone resorption by OC activation. This sample was composed by 6 different donors and each of these donors presented different resorption profiles (Figure A.2, in annexes). This variability is a major obstacle to obtain true statistical values for bone scientific research [128]. Therefore, there is need to increase the sample size to understand if the trend set by these 6 donors is verified. If that is the case, when NE is released, OCs decrease their activity and resorb less bone.

TRAP is released during resorption phase to degrade organic compounds present on bone matrix. Therefore, medium of OCs seeded on top of bone slices was collected to analyse the activity of TRAP enzyme released in the medium of each condition, complementing the results obtained in the eroded surface assay.

Although there is no significative difference, the activity of TRAP enzyme seems to decrease when NE is added to OCs (Figure VI.16). This profile is similar to the results obtained in resorption assay (Figure VI.15), which means that untreated OCs resorbed more matrix and this is associated with more degradation of bone organic components, such as osteopontin, by TRAP activation during resorption [129].

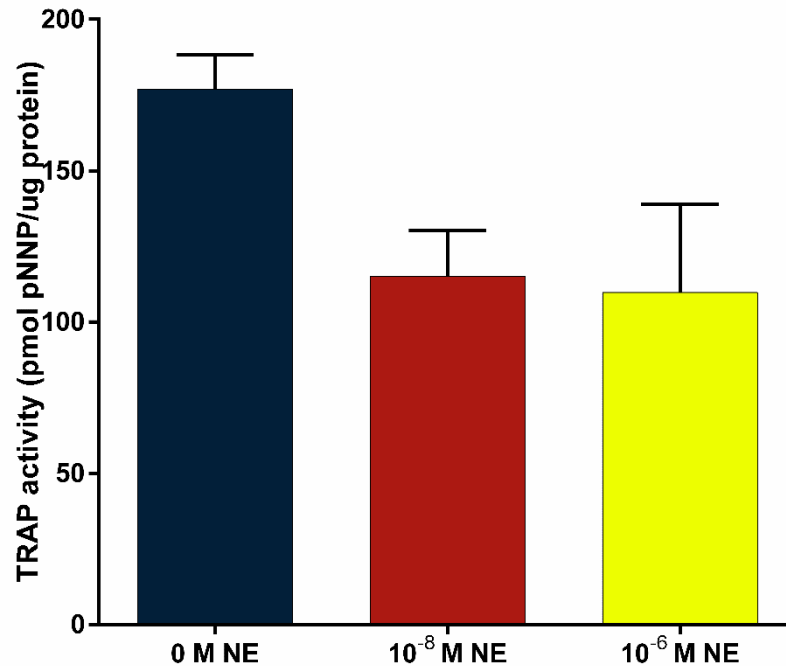


Figure IV. 16 – Quantification of TRAP enzyme activity from media of OCs treated with NE. Media of OCs cultured with 0, 10⁻⁸ and 10⁻⁶ M of NE for 72h were quantified. Data is expressed as mean ± SD, n=3 donors (5 bone slices per condition).

Another aspect that can be analysed to evaluate OC activity is the quantification of MMPs. MMP-2 and MMP-9 are two gelatinases found in bone. They participate in various physiological and pathological processes, mainly regarding the cleavage of ECM [130]. MMP-9 is also very important in OC maturation process, since it is also involved in resorption of organic components in bone matrix. Therefore, zymograms of the CM from NE treated OCs were analysed to quantify the MMPs released during resorption.

As expected, there was a strong activity of MMP-9, which precludes the distinction between pro and active bands (Figure IV.17A). In Figure VI.17B is showed that this activity is not changed by NE treatments. Both forms of MMP-2 are present in all samples and OCs treated with 10⁻⁸ M of NE present more MMP-2 activity than OCs treated with other conditions. In fact, the lack of MMP-2 was related to a decrease of OC numbers in mice [131], which is in accordance with results obtained in both early and late differentiation, where 10⁻⁸ M of NE presented elevated OC numbers and also caused an increase of MMP-2 activity. 10⁻⁶ M of NE, on the other hand, presented decreased OC numbers, which is also reflected by a decrease of pro-MMP-2 activity (Figure VI.12, Figure VI.14 and Figure VI.16).

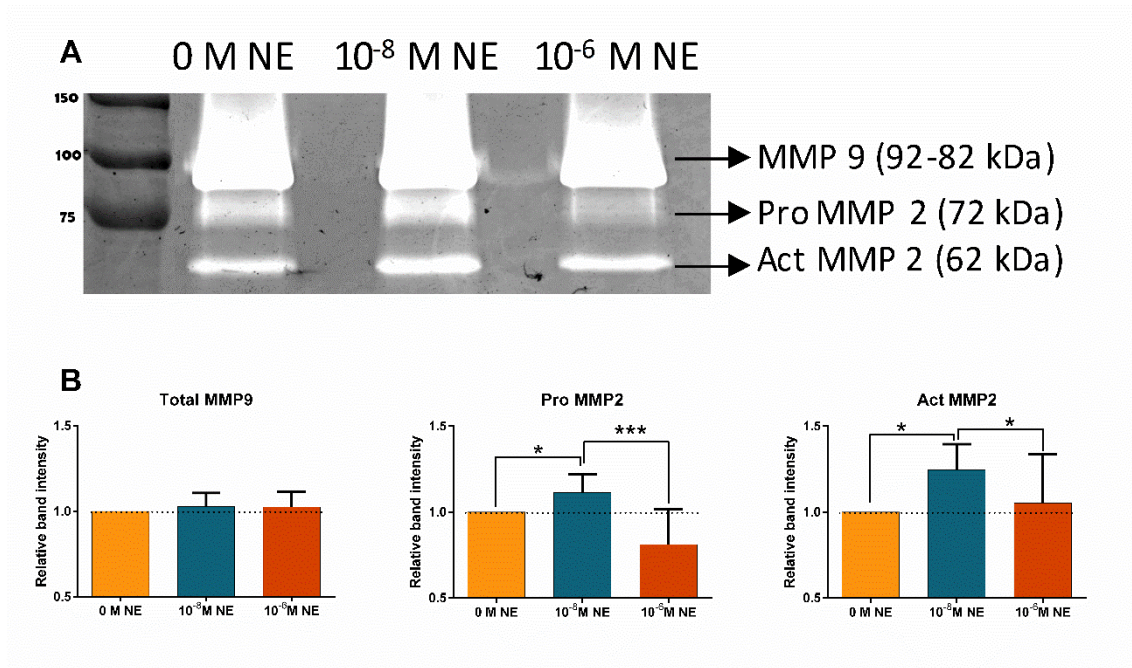


Figure IV. 17 - Zymography analysis of MMP-2 and MMP-9 activity from OC media treated with NE. A. Zymogram gelatine gel of OCs treated with 0, 10^{-8} and 10^{-6} M of NE. The bands were identified by their molecular weight [130]. **B.** Relative quantity of MMP-9 and MMP-2 activity was obtained through band volume using Image Lab. The values were normalized with the intensity of 0 M of NE band. Data is expressed as mean $\pm$ SD, * $p < 0.05$, *** $p < 0.0001$, $n=5$ donors (5 replicates per condition).

Although both MMP-9 and TRAP are enzymes that degrade organic elements of bone matrix during bone resorption, these results suggest that the decrease of bone resorption is, at least in part due to a decrease of TRAP activity, while MMP-9 remains unchanged. Although MMP-2 function remains relatively unknown, this MMP also seems to be involved in OC formation.

3. Role of SNS in BrCa-OC interactions (indirect effects)

Once BrCa cells home to bone they release OC-activating factors, promoting osteoclastogenesis and bone degradation. This excessive bone degradation leads to bone lesions and eventually to fractures which are a major problem for BrCa patients [132, 133].

Therefore, different subtypes of BrCa cells CM, previously treated with NE, were added to OCs, as described in material and methods.

3.1. RANKL/RANK expression in BrCa

RANKL is a cytokine typically released by OBs that binds to RANK expressed on the membrane of OCs, stimulating their activation. This interaction plays a critical role in bone remodelling, prompting the start of bone matrix resorption. However, these factors' expression is not restricted to bone cells. Some epithelial cells, including the ones found in breast show expression of both RANKL and RANK, since they intervene in the normal development of lactation [134]. It is also described that some BrCa cell lines are able to express these factors, normally found in bone, promoting the homing and facilitating survival in this organ since they easily adapt to its microenvironment [135-137].

To further confirm these observations, qRT-PCR analysis was performed to quantify the gene expression pattern of both RANKL and RANK by each BrCa cell line used in this study (Figure IV.18). MCF-7 and BT-474 cells, which represent luminal subtypes, expressed the higher levels of RANKL among BrCa cell lines, with MCF-7 expression being significantly higher than MDA-MB-468 expression. The triple-negative cell lines, MDA-MB-468 and MDA-MB-231, had the lowest RANKL expression (Figure IV.18A). This expression pattern can help explain the metastatic site distribution of BrCa subtypes, since luminal subtypes, which have bone as a major metastatic site [37], showed higher RANKL expression.

In addition, triple negative cells showed the lowest expression of RANK, with this gene being mostly expressed by BT-474 and SK-BR-3 cell lines (Figure IV.18B). Once again, triple-negative cells showed a profile less favourable for bone metastasis, which is in accordance with the literature [34, 35, 138]

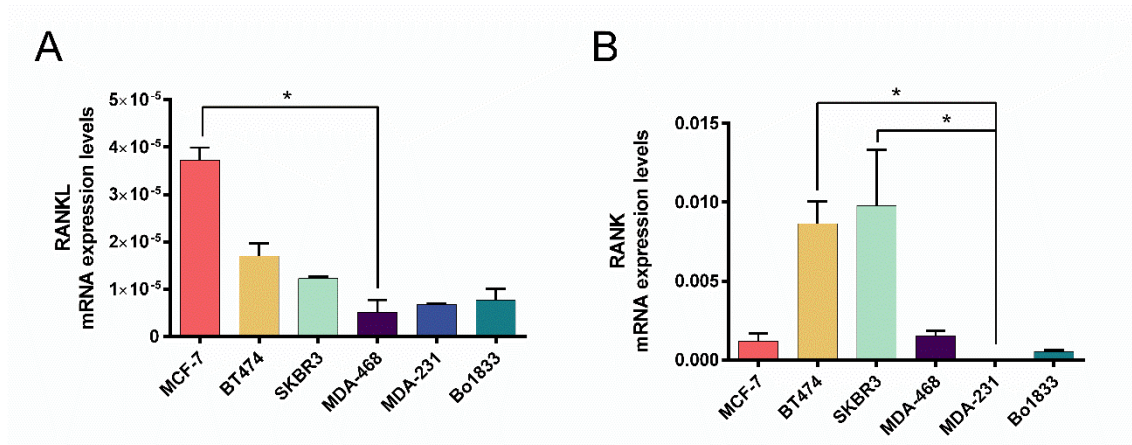


Figure IV. 18 – RANKL and RANK gene expression profile of each BrCa cell line. The mRNA expression levels of RANKL (**A**) and RANK (**B**) by each BrCa cell line were analysed by qRT-PCR. The expression values of each gene were normalized with expression of B2M gene. Data is expressed as mean $\pm$ SD, * $p < 0.05$ ($n=3$).

3.2. Effects of BrCa NE treated CM on OC differentiation

In order to evaluate if the treatment of OCs with CM of BrCa cells with or without NE treatment affected OC differentiation, F-actin immunostaining was performed, similarly as for the direct effects of NE on OCs. The treatment conditions were compared with OCs treated with the normal medium used to culture the respective BrCa cell line.

Since all BrCa cells were cultured with OCs differentiated from the same donor, the effects of different cell lines can be compared. The global OC ($n \geq 3$) quantifications of early and late differentiation are in Figure VI. A3-6, in annexes.

• MCF-10A CM

When treatment started on day 5, the addition of MCF-10A CM treated with or without NE and DMEM/F12 P only, completely altered OC differentiation. No giant multinucleated OCs are found in any of the wells treated with these conditions (Figure VI.19A-D). Actually, only one condition presented a few OCs with $n \geq 8$, in wells treated with DMEM/F12 P (Figure VI.19E). Between the CM conditions, although not significative, MCF-10A without NE treatment presents the smallest OC number and area (Figure VI.19F).

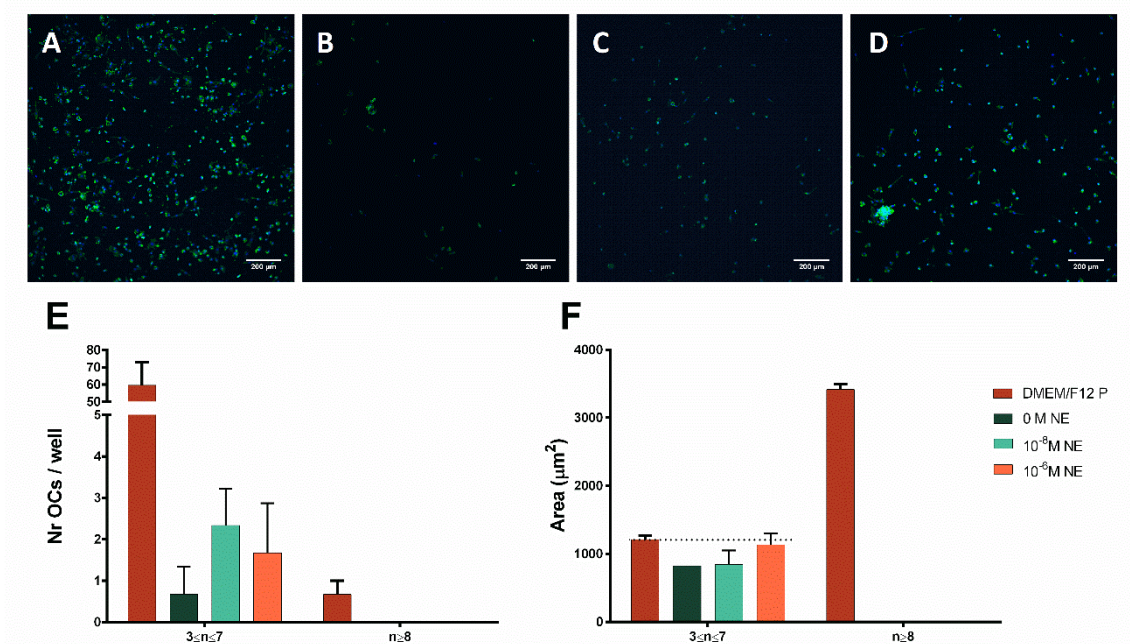


Figure IV. 19 – Quantification of OC treated, since day 5, with CM of MCF-10A cells treated with 0, 10^{-8} and 10^{-6} M of NE. Representative images of F-actin stained OCs treated with DMEM/F12 P (A), 0 (B), 10^{-8} (C) and 10^{-6} (D) M of NE. The number (E) and area (F) of OCs per well in each condition were quantified using ilastik and Cell profiler softwares. Data is expressed as mean $\pm$ SD ($n=3$ wells per condition).

As seen in early OC differentiation, this cell line completely altered OC morphology (Figure VI.20A-D). Both DMEM/F12 P and MCF-10A treated with or without NE CM caused a huge decrease in OC number. The highest number of OCs was 30 cells per well, in DMEM/F12 P condition. With the addition of MCF-10A the number was further decreased. Furthermore, OCs with $n \geq 8$ were not found in some conditions (Figure VI.20E). Area of OCs with $3 \leq n \leq 7$ treated with NE suffer a slight increase from OCs treated with 0 M of NE (Figure VI.20F).

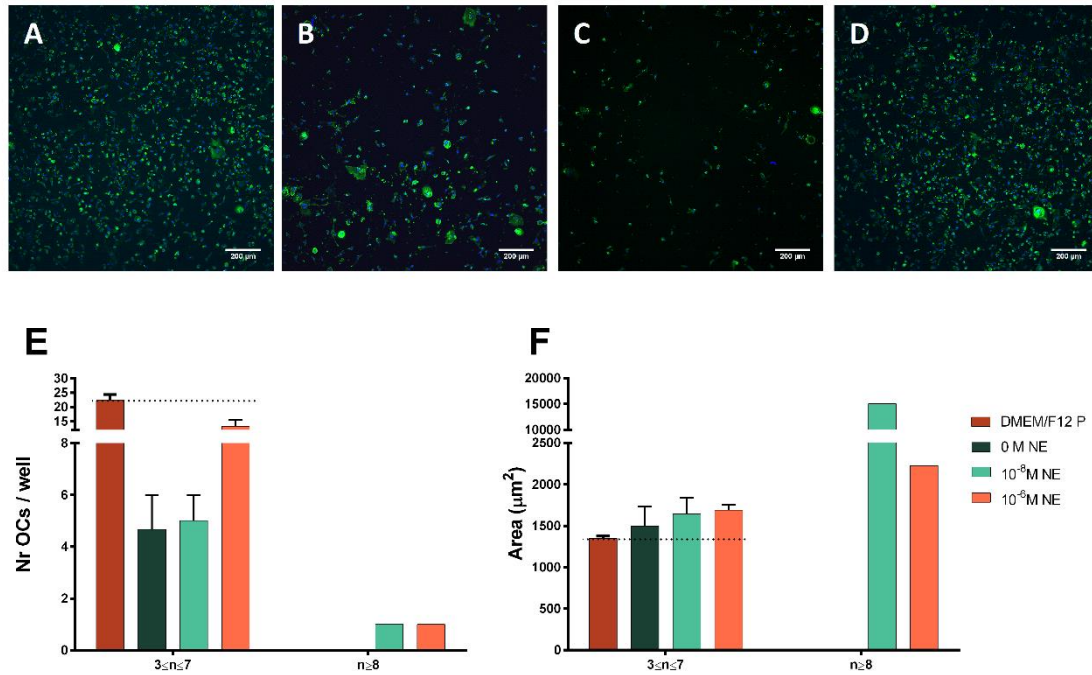


Figure IV. 20 - Quantification of OC treated, since day 9, with CM of MCF-10A cells treated with 0, 10^{-8} and 10^{-6} M of NE. Representative images of F-actin stained OCs treated with DMEM/F12 P (A), 0 (B), 10^{-8} (C) and 10^{-6} (D) M of NE. The number (E) and area (F) of OCs per well in each condition were quantified using ilastik and Cell profiler softwares. Data is expressed as mean $\pm$ SD (3 wells per condition).

• MCF-7 CM

No major differences can be seen in OC morphology by addition of MCF-7 CM (Figure VI.21A-B). However, in Figure VI.21D, when the highest dose of NE is added, OC morphology is altered.

To confirm these results, OC number and area were quantified (Figure VI.21E-F). In fact, and although not significative, the biggest difference was found in the wells with 10^{-6} M of NE treatment. These wells presented a decrease in OC number within both nuclei groups (Figure VI.21E). However, the area of these cells was bigger than the area of OCs found in the other treatments (Figure VI.21F).

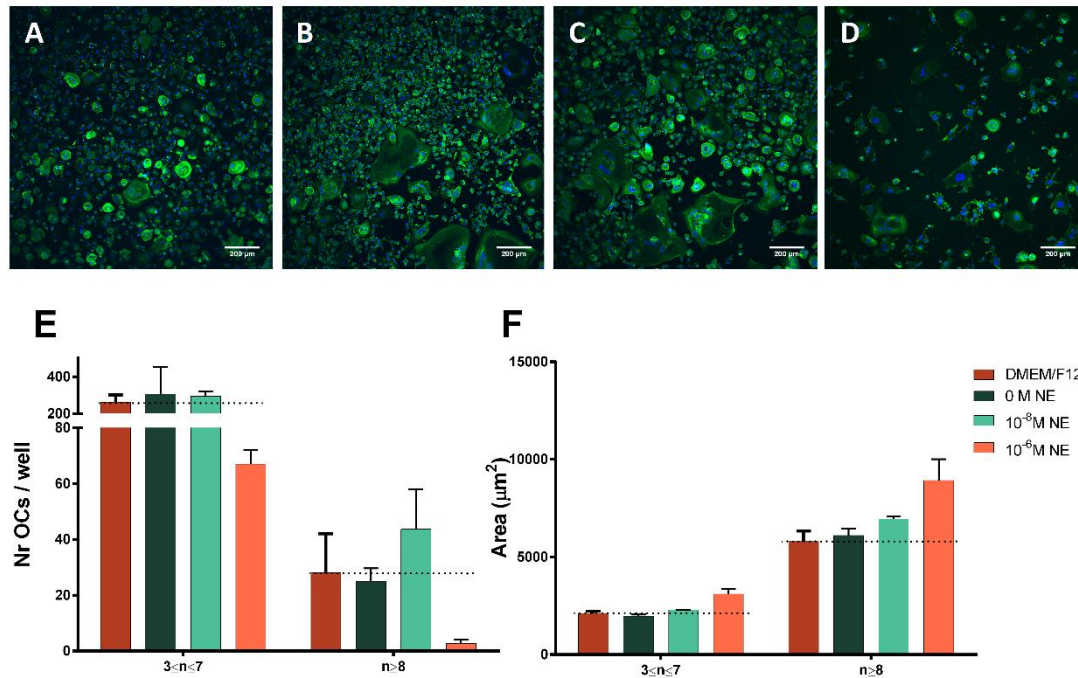


Figure IV. 21 – Quantification of OC treated, since day 5, with CM of MCF-7 cells treated with 0, 10^{-8} and 10^{-6} M of NE. Representative images of F-actin stained OCs treated with DMEM/F12 (A), 0 (B), 10^{-8} (C) and 10^{-6} (D) M of NE. The number (E) and area (F) of OCs per well in each condition were quantified using ilastik and Cell profiler softwares. Data is expressed as mean $\pm$ SD (n=3 wells per condition).

When treatment starts at day 9, F-actin images show bigger cells in the wells treated with MCF-7 CM treated with 0 and 10^{-6} M of NE (Figure IV.22A-D). Furthermore, there are more OCs with $3 \leq n \leq 7$ in the wells treated with DMEM/F12 than in wells treated with all conditions of MCF-7 CM. In OCs with $n \geq 8$, wells treated with MCF-7 CM without NE, have more OCs than any other condition, and this number decreases with increasing doses of NE (Figure IV.22E). And although the area of OCs treated with DMEM/F12 and MCF-7 treated with 10^{-8} M of NE CM is similar, OCs treated with MCF-7 without NE and 10^{-6} M of NE CM are bigger (Figure IV.22F).

Once again, the treatment of MCF-7 with 10^{-6} M of NE CM caused a reduction of OCs with $n \geq 8$ but caused an increase in cell area (Figure IV.21 and 22E-F).

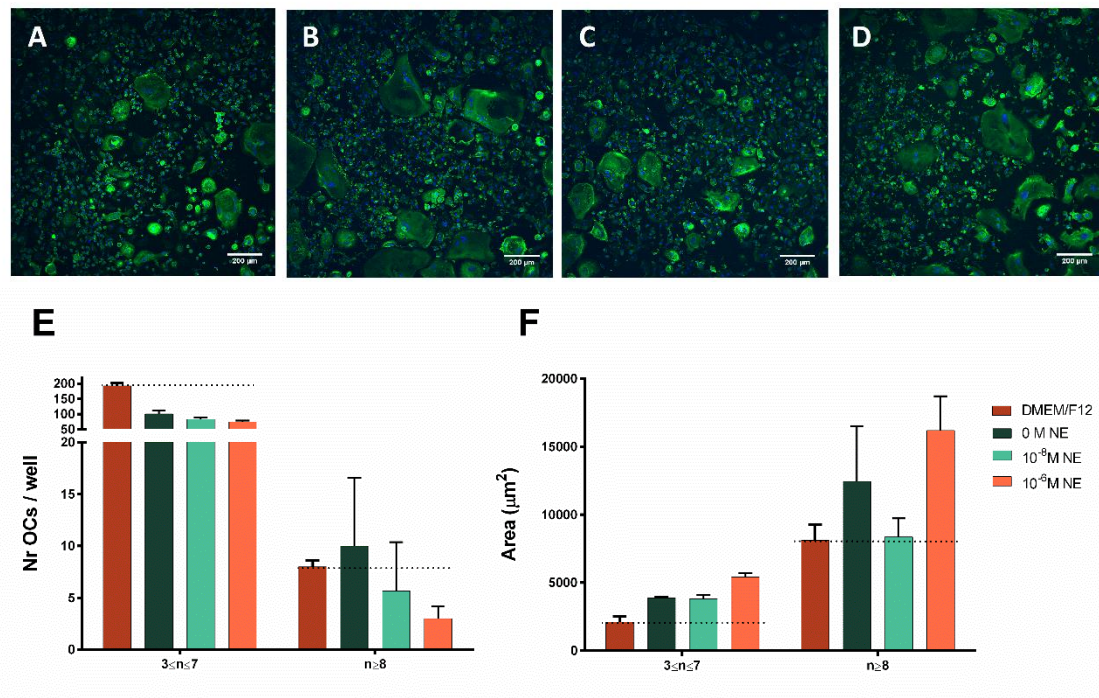


Figure IV. 22 - Quantification of OC treated, since day 9, with CM of MCF-7 cells treated with 0, 10^{-8} and 10^{-6} M of NE. Representative images of F-actin stained OCs treated with DMEM/F12 (A), 0 (B), 10^{-8} (C) and 10^{-6} (D) M of NE. The number (E) and area (F) of OCs per well in each condition were quantified using ilastik and Cell profiler softwares. Data is expressed as mean $\pm$ SD (n=3 wells per condition).

- **BT-474 CM**

OCs treated with BT-474 CM with or without NE, since day 5, seem bigger than the ones treated with DMEM only (Figure VI.23A-D). This is confirmed by area measurements, where DMEM wells present smaller OCs than the other 3 conditions, and this is verified in all groups of OCs (Figure VI.23F). Inversely, DMEM wells have more OCs and the number of OCs decreases when the CM is added with or without NE, although there is a bigger difference when NE is absent (Figure VI.23E).

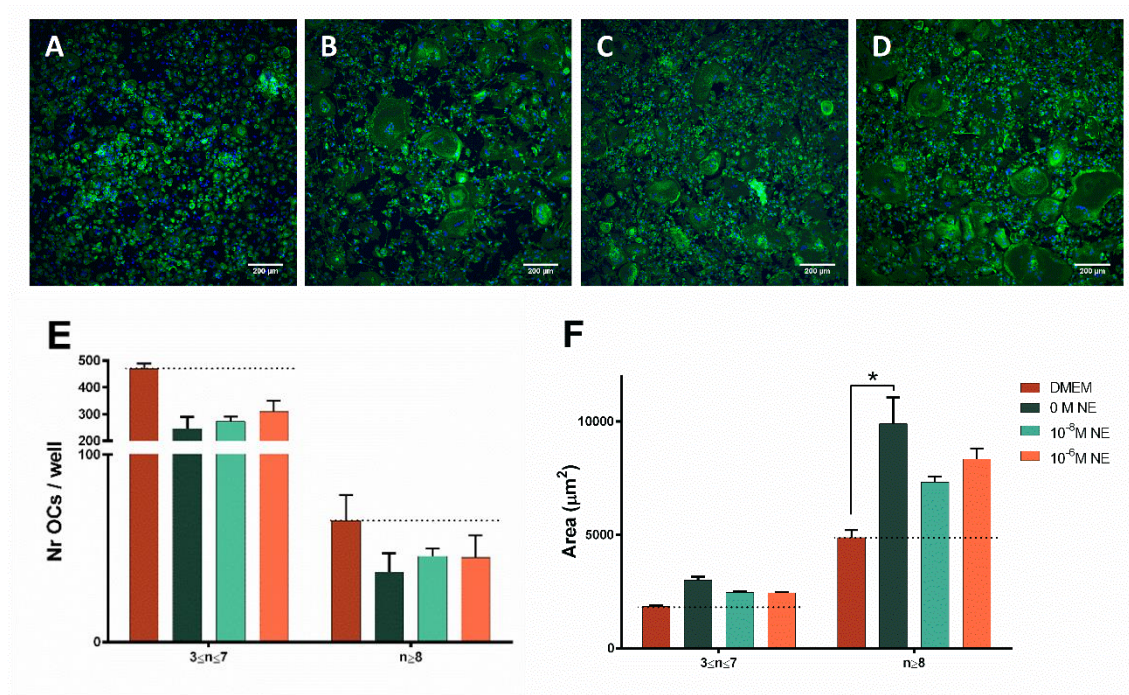


Figure IV. 23 – Quantification of OC treated, since day 5, with CM of BT-474 cells treated with 0, 10^{-8} and 10^{-6} M of NE. Representative images of F-actin stained OCs treated with DMEM (A), 0 (B), 10^{-8} (C) and 10^{-6} (D) M of NE. The number (E) and area (F) of OCs per well in each condition were quantified using ilastik and Cell profiler softwares. Data is expressed as mean $\pm$ SD (n=3 wells per condition).

During late differentiation, in F-actin images, more OCs seem to be present in wells treated with BT-474 with or without NE CM, than the ones found in DMEM condition (Figure VI.24A-D). Actually, OC quantification confirmed that, contrarily to MCF-7 CM, the presence of BT-474 without NE CM generated more OCs than DMEM wells. NE slightly decreased OC number to DMEM levels. OCs with $n \geq 8$ showed a more pronounced decrease in BT-474 treated with NE CM (Figure VI.24E). No major differences are seen in area of OCs (Figure VI.24F).

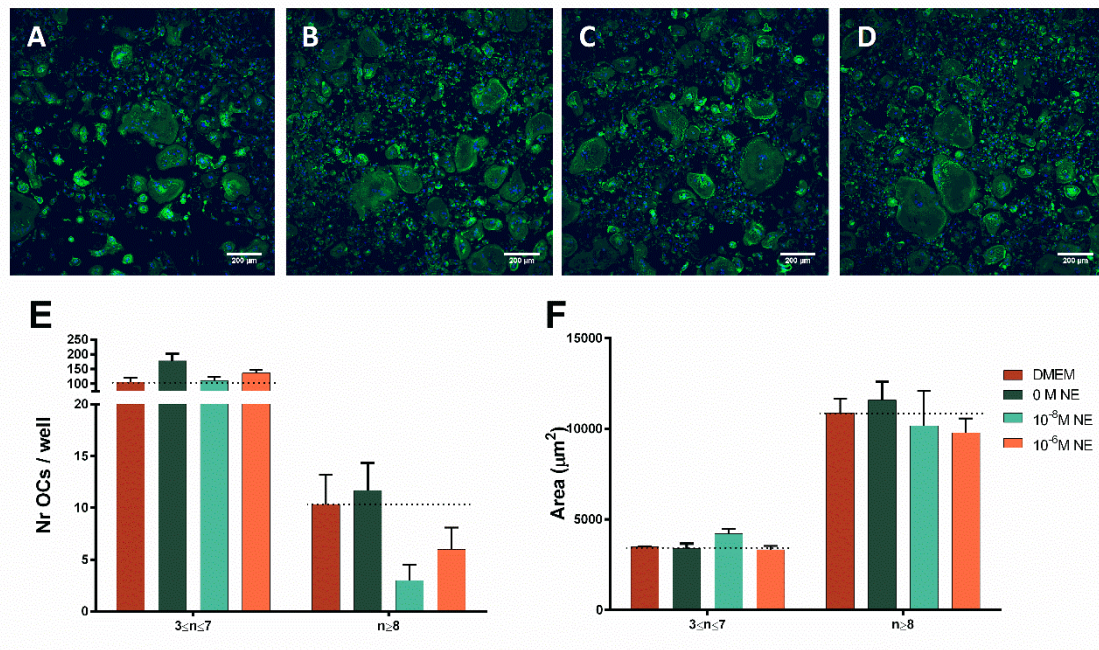


Figure IV. 24 - Quantification of OC treated, since day 9, with CM of BT-474 cells treated with 0, 10^{-8} and 10^{-6} M of NE. Representative images of F-actin stained OCs treated with DMEM (A), 0 (B), 10^{-8} (C) and 10^{-6} (D) M of NE. The number (E) and area (F) of OCs per well in each condition were quantified using ilastik and Cell profiler softwares. Data is expressed as mean $\pm$ SD (n=3 wells per condition).

• SK-BR-3 CM

In the F-actin images, it seems that OCs treated since day 5 with CM of SK-BR-3 cells treated 24h with 10^{-8} M of NE are bigger than in other conditions (Figure VI.25A-D). When the cell area was quantified, OCs in this condition showed indeed a size increase when compared to DMEM treated OCs (Figure VI.25E). In cells with more nuclei however, area was mostly increased in OCs treated with SK-BR-3 without NE CM (Figure VI.25F). The number of OCs with less nuclei decreased in every CM treatment, either with or without NE (Figure VI.25E). In cells with more nuclei, 10^{-8} M of NE treated CM inhibited the effect caused when only SK-BR-3 cells CM was added to OCs, presenting the same OC number as DMEM wells. However, when the highest dose of NE was added, the CM caused a decreased in OCs number similar to CM without NE (Figure VI.25F).

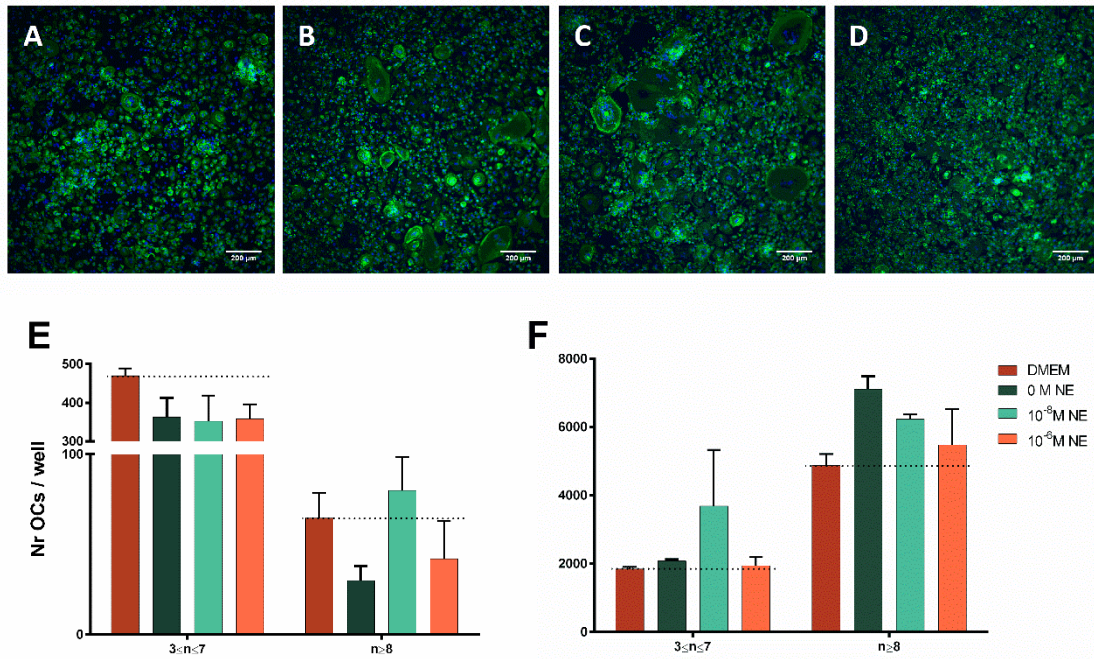


Figure IV. 25 – Quantification of OC treated, since day 5, with CM of SK-BR-3 cells treated with 0, 10^{-8} and 10^{-6} M of NE. Representative images of F-actin stained OCs treated with DMEM (A), 0 (B), 10^{-8} (C) and 10^{-6} (D) M of NE. The number (E) and area (F) of OCs per well in each condition were quantified using ilastik and Cell profiler softwares. Data is expressed as mean $\pm$ SD (n=3 wells per condition).

Figure VI.26B shows a decrease of multinucleated cells when SK-BR-3 CM is added to OCs, compared to wells of the other conditions (Figure VI.26A,C-D). In fact, in OC number quantification, there is a low number of OCs with a lot of nuclei in SK-BR-3 treated with no NE CM. Furthermore, these cells are smaller when compared to the other treatments (Figure VI.26F). The presence of both NE doses increased OC number (Figure VI.26E).

When treatment starts on day 5, OC number decreases with SK-BR-3 with or without NE CM when compared to DMEM wells (Figure VI.25E). On the other hand, these conditions have more OCs, compared with DMEM wells when treatment starts at day 9 (Figure VI.26E).

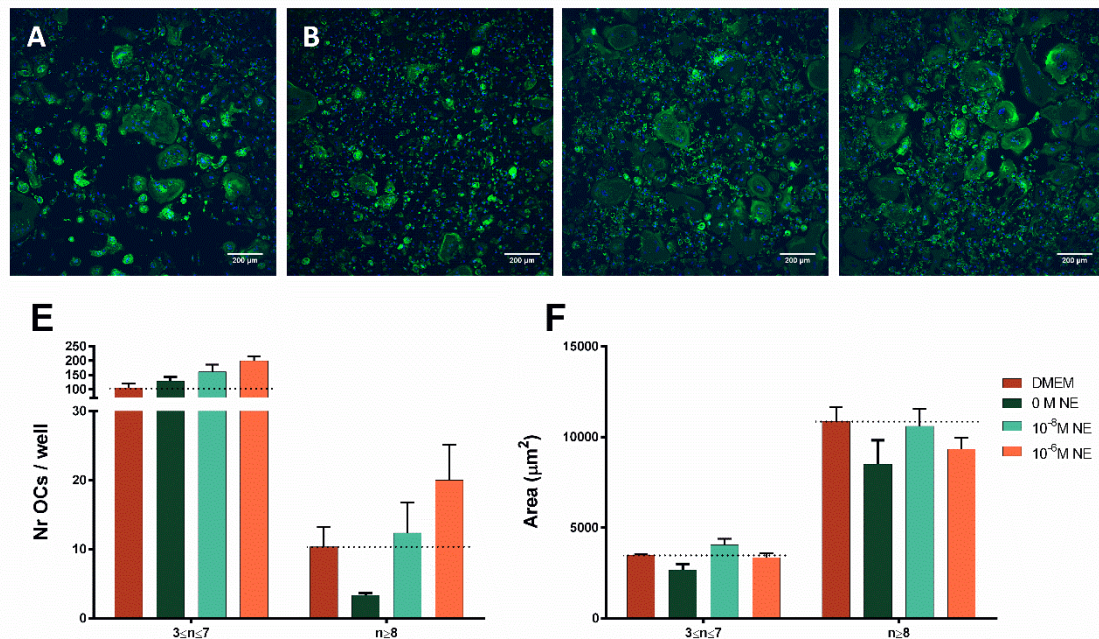


Figure IV. 26 – Quantification of OC treated, since day 9, with CM of SK-BR-3 cells treated with 0, 10⁻⁸ and 10⁻⁶ M of NE. Representative images of F-actin stained OCs treated with DMEM (A), 0 (B), 10⁻⁸ (C) and 10⁻⁶ (D) M of NE. The number (E) and area (F) of OCs per well in each condition were quantified using ilastik and Cell profiler softwares. Data is expressed as mean ± SD (n=3 wells per condition).

- **MDA-MB-468 CM**

In Figure VI.27B, is evident that MDA-MB-468 CM causes an alteration of OC morphology. Furthermore, the number of cells in every group of OCs decreases when the CM is added. This decrease occurs with or without NE treatment, however the effect is more prominent without the presence of NE on MDA-MB-468 cells (Figure VI.27E). The opposite trend occurs in cell area, where MDA-MB-468 treated with 0 M of NE CM has a significant increase in area when compared with OCs treated with DMEM only. When NE is present, OCs are bigger than DMEM treated OCs but smaller than the ones found in the wells treated with MDA-MB-468 CM (Figure VI.27F).

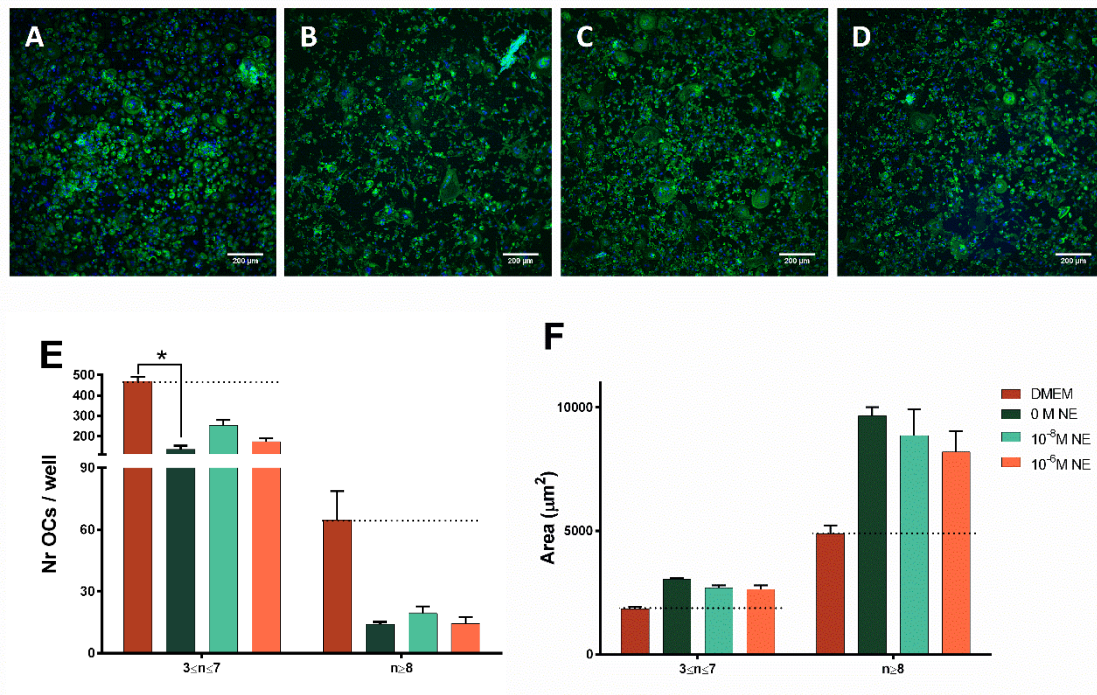


Figure IV. 27 – Quantification of OC treated, since day 5, with CM of MDA-MB-468 cells treated with 0, 10^{-8} and 10^{-6} M of NE. Representative images of F-actin stained OCs treated with DMEM (A), 0 (B), 10^{-8} (C) and 10^{-6} (D) M of NE. The number (E) and area (F) of OCs per well in each condition were quantified using ilastik and Cell profiler softwares. Data is expressed as mean $\pm$ SD. * $p < 0.05$ ($n=3$ wells per condition).

When treatment starts at day 9, number of OCs with $n \geq 8$ decreases with the addition of MDA-MB-468 CM treated with NE (Figure VI.28E). The number of OCs with $3 \leq n \leq 7$ treated with or without NE CM was higher than cell number found in DMEM wells (Figure VI.28E). The presence of MDA-MB-468 CM with or without NE caused a small decrease of OC area, independently of the number of nuclei (Figure VI.28F). Once again, the effects caused on OC differentiation depended on the time point (Figure VI.27 and 28E-F).

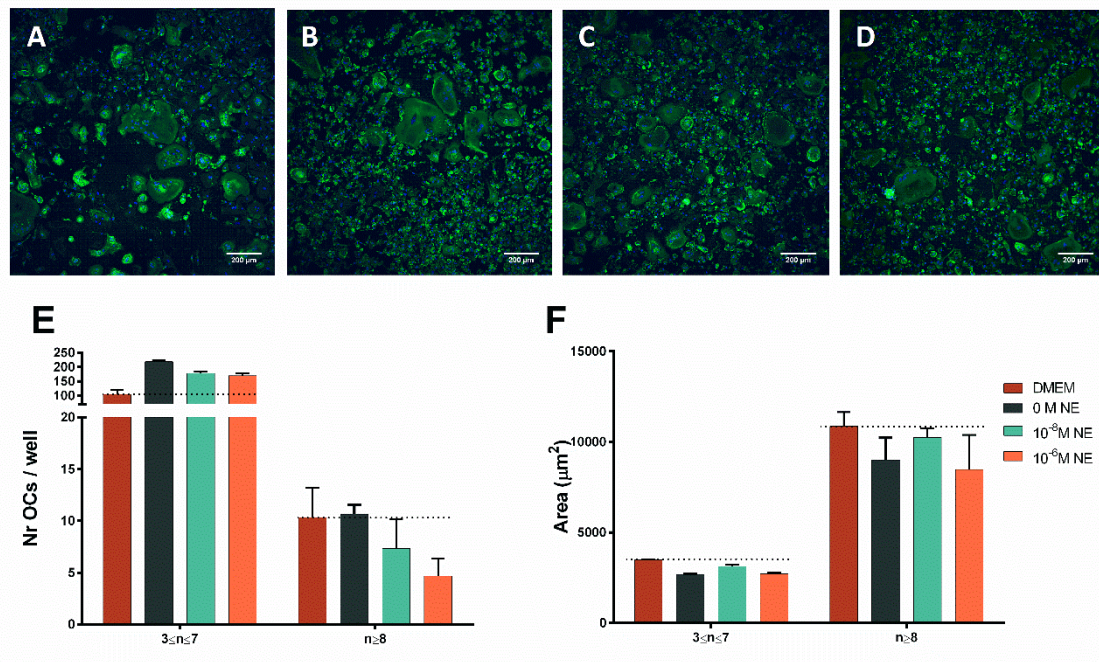


Figure IV. 28 - Quantification of OC treated, since day 9, with CM of MDA-MB-468 cells treated with 0, 10^{-8} and 10^{-6} M of NE. Representative images of F-actin stained OCs treated with DMEM (A), 0 (B), 10^{-8} (C) and 10^{-6} (D) M of NE. The number (E) and area (F) of OCs per well in each condition were quantified using ilastik and Cell profiler softwares. Data is expressed as mean $\pm$ SD (n=3 wells per condition).

- **MDA-MB-231 CM**

Wells treated with CM of MDA-MB-231 treated with or without NE seem to present bigger cells than OCs treated with DMEM. And cell morphology also seems to be altered, with cells presenting a more elongated form (Figure VI.29A-D). The results from OC quantification indicate that the number of OCs decreases when the CM of MDA-MB-231 cells is added. Once again, this decrease is less accentuated on wells with NE treated CM (Figure VI.29E). Area measurements confirm what is observed in F-actin stained images, cells treated with CM with or without NE are bigger than DMEM treated cells (Figure VI.29F).

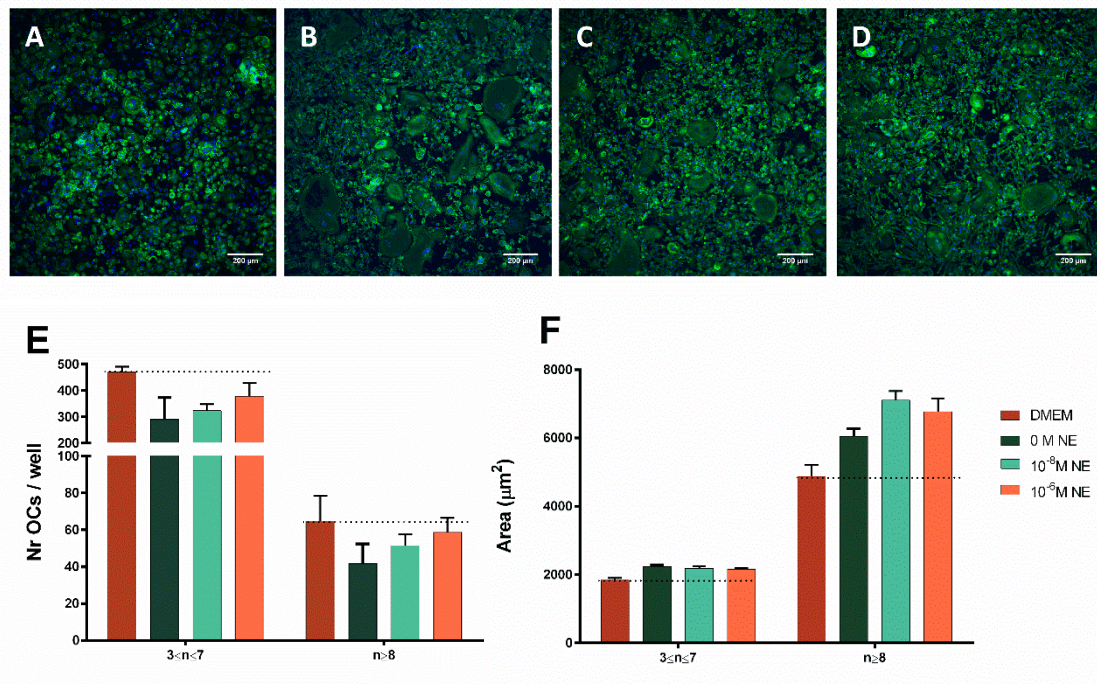


Figure IV. 29 – Quantification of OC treated, since day 5, with CM of MDA-MB-231 cells treated with 0, 10^{-8} and 10^{-6} M of NE. Representative images of F-actin stained OCs treated with DMEM (A), 0 (B), 10^{-8} (C) and 10^{-6} (D) M of NE. The number (E) and area (F) of OCs per well in each condition were quantified using ilastik and Cell profiler softwares. Data is expressed as mean $\pm$ SD (n=3 wells per condition).

In late differentiation, OC number increases with the addition of MDA-MB-231 CM and this effect is maintained when MDA-MB-231 are treated with 10^{-8} M of NE. When MDA-MB-231 are treated with 10^{-6} M of NE however, the number of OCs decreases to a similar number found on DMEM wells (Figure VI.30E). OCs treated with MDA-MB-231 cultured with 10^{-8} M of NE CM, are bigger than OCs in every other condition ($n \geq 8$) (Figure VI.30F). Furthermore, in the representative images, the morphology of OCs remains abnormal.

While treatment starting at day 5 caused a decrease of cell number with the addition of CM with or without NE (Figure VI.29E), the addition of the same conditions on day 9 leads to an increase of cell number, when compared to DMEM wells (Figure VI.30E), similarly to what happens in OCs treated with MDA-MB-468 CM (Figure VI.27E and 28E).

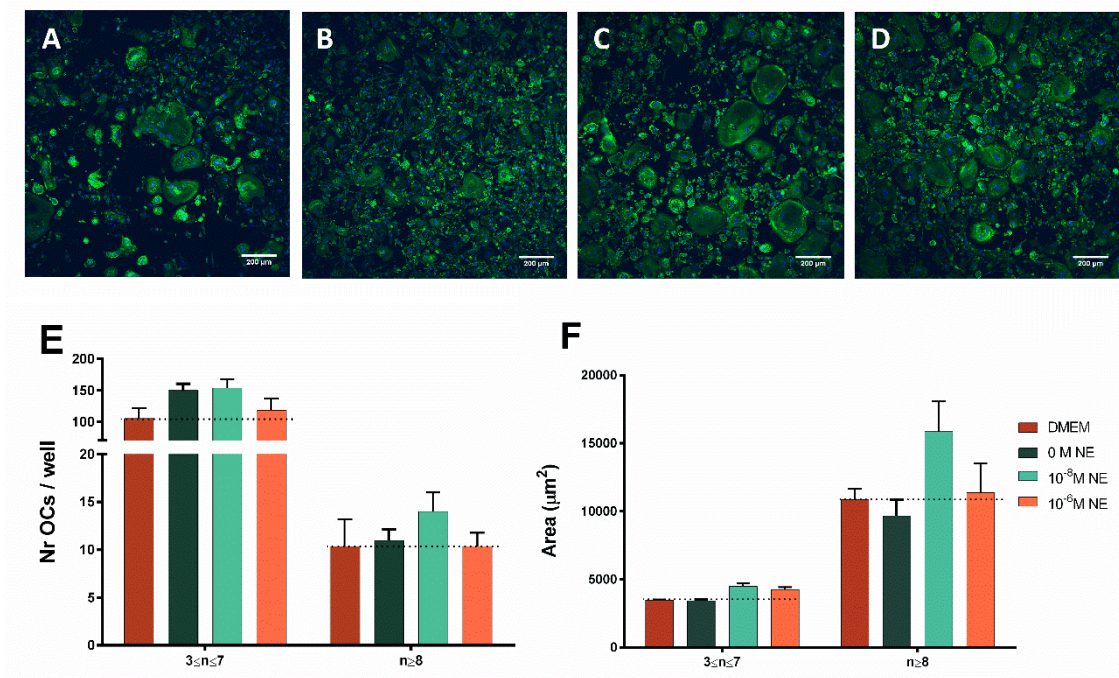


Figure IV. 30 - Quantification of OC treated, since day 9, with CM of MDA-MB-231 cells treated with 0, 10⁻⁸ and 10⁻⁶ M of NE. Representative images of F-actin stained OCs treated with DMEM (A), 0 (B), 10⁻⁸ (C) and 10⁻⁶ (D) M of NE. The number (E) and area (F) of OCs per well in each condition were quantified using ilastik and Cell profiler softwares. Data is expressed as mean ± SD (n=3 wells per condition).

- **Bo-1833 CM**

Once again in F-actin images, a more elongated, fibroblastic morphology can be seen in cells treated with Bo-1833 CM, with or without NE presence (Figure VI.31A-D). Besides morphology, the 3 conditions with CM present a decrease in OC number, more pronounced when no NE was added to Bo-1833 (Figure VI.31E). This decrease in cell number is accompanied by a similar increase in cell area. All conditions present bigger cells than OCs treated with DMEM (Figure VI.31F).

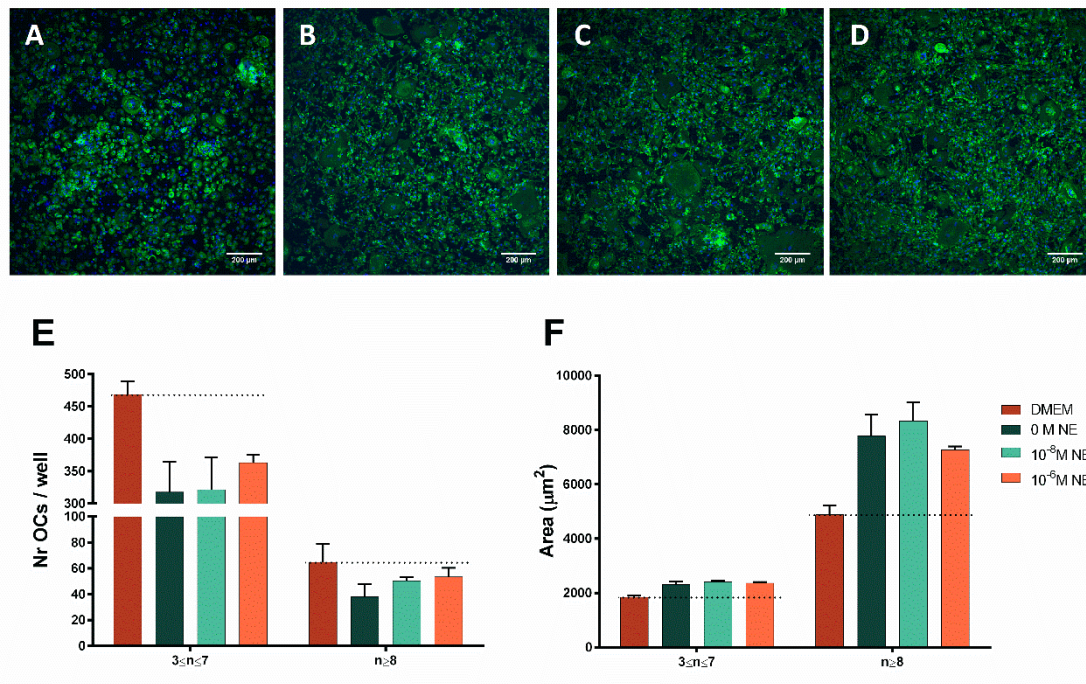


Figure IV. 31 – Quantification of OC treated, since day 5, with CM of Bo-1833 cells treated with 0, 10^{-8} and 10^{-6} M of NE. Representative images of F-actin stained OCs treated with DMEM (A), 0 (B), 10^{-8} (C) and 10^{-6} M (D) of NE. The number (E) and area (F) of OCs per well in each condition were quantified using ilastik and Cell profiler softwares. Data is expressed as mean $\pm$ SD (n=3 wells per condition).

In F-actin images, is possible to observe that the wells treated with Bo-1833 CM treated with NE, present bigger cells than Bo-1833 CM without NE (Figure VI.32A-D), although they no longer, except for Bo-1833 CM, alter OC morphology. OC number is not greatly affected by the addition of Bo-1833 CM, except in cells with $n \geq 8$, which is slightly decreased. When CM treated with NE is added however, there is a slight increase of OC numbers, mainly with 10^{-8} M of NE condition (Figure VI.32E). The area of cells with $n \geq 8$ treated with Bo-1833 with 0 M of NE CM show a small increase, that is decreased with the addition of NE (Figure VI.32F).

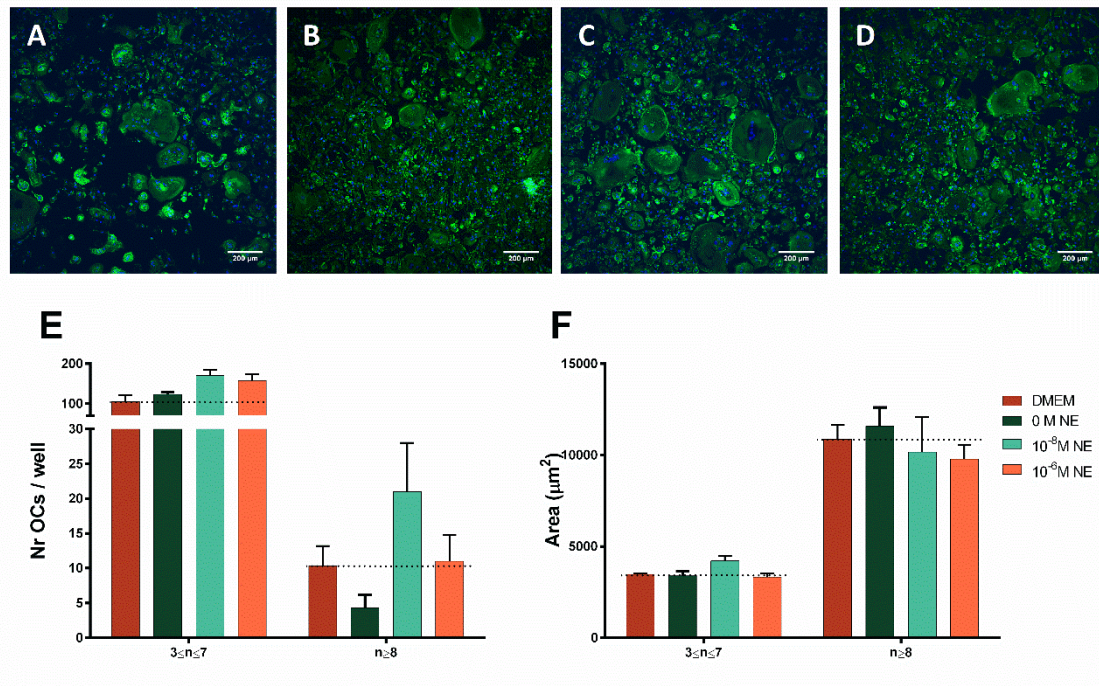


Figure IV. 32 - Quantification of OC treated, since day 9, with CM of Bo-1833 cells treated with 0, 10^{-8} and 10^{-6} M of NE. Representative images of F-actin stained OCs treated with DMEM (A), 0 M (B), 10^{-8} M (C) and 10^{-6} M (D) of NE. The number (E) and area (F) of OCs per well in each condition were quantified using ilastik and Cell profiler softwares. Data is expressed as mean $\pm$ SD (n=3 wells per condition).

With F-actin staining, it was possible to verify that the addition of BrCa CM affected OC differentiation. Furthermore, OC quantification allowed to understand that, except for MCF-7, CM of every other cell line caused a decrease in OC ($3 \leq n \leq 7$) number at day 5 of treatment (Figure VI.21 and Figure VI.23,25,27,29,31). The cell area usually presents the opposite result of OC number (Figure VI.20, 23, 25, 27 29 and 31E-F) mainly in early differentiation. Interestingly, cell morphology is noticeably changed in OCs treated with triple-negative cell lines (Figure VI.27-32A-D), which is a translation of their aggressive phenotype.

Although these results can only represent the profile of one donor, the CM of BrCa cells treated with NE seems to affect OC morphology, even when treatment starts on mature OCs. Furthermore, the effects vary depending on the BrCa subtype. However, to verify these results, this study has to be repeated with more OC donors.

3.3. Effects of BrCa NE treated CM on OC resorption activity

In order to investigate the effects provoked by SNS on BrCa-bone metastasis, the evaluation of bone degradation caused by the different BrCa subtypes, with the absence and presence of NE was performed.

Firstly, to evaluate the effects of SNS activation on OCs treated with the CM of BrCa cells, MCF-10A which is a non-tumorigenic cell line was used as control (Figure VI.33). Interestingly, there was an extreme reduction of eroded surface, comparing with the activity of OCs directly treated with NE (Figure VI.15). Although there is an increase of resorption on OCs treated with MCF-10A NE CM, maximum resorption was below 1%, with some bone slices not presenting any resorptive events (Figure VI.33A-B).

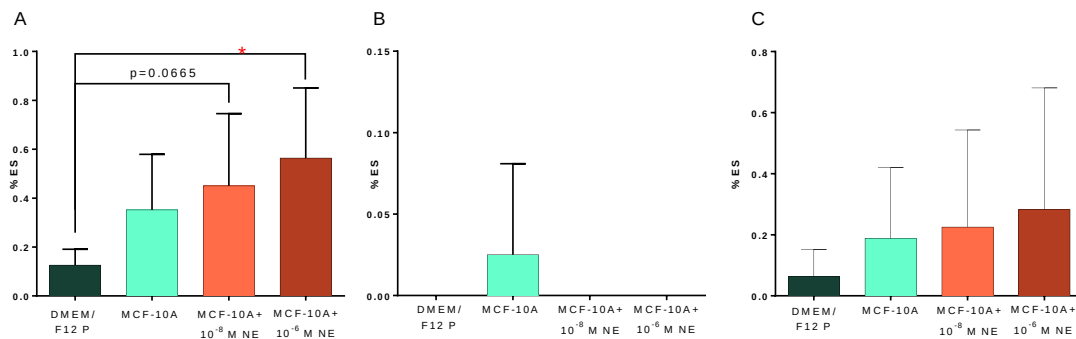


Figure IV. 33 – Effect of MCF-10A CM treated with NE on OC activity. Resorptive activity was quantified by the percentage of eroded surface (ES) per total bone surface. OCs were treated with the DMEM/F12 P, MCF-10A CM with 0, 10⁻⁸ and 10⁻⁶ M of NE. Graphs on the right and middle represent individual donors (**A** and **B**), graph on the left (**C**) represents media of both donors. Data is expressed as mean ± SD. *p < 0.05, n=2 donors (5 bone slices per condition).

MCF-7 cell line belongs to luminal A subtype, which represents the subtype mostly found in bone [139]. The resorption results for this cell line were very contradictory, while there were no significative differences in the first donor (Figure IV.34A), the second donor shows an increased resorption of bone when OCs were treated with CM of BrCa cells treated with the highest concentration of NE (Figure IV.34B). Therefore, when combining the results from both donors, there seems to be no differences between treatments (Figure IV.34C). The variability can be seen by comparing the results of resorption of DMEM/F12 of both donors. In the first donor, OCs treated with DMEM/F12, where there is no cancer cell influence, has approximately 8% of eroded surface, while the second donor has basal resorption of 1%.

The BT-474 cell line, which accounts for the other luminal subtype, also showed no differences with or without NE treatment (Figure IV.34F). However, the CM increased the resorption when compared with the DMEM condition (Figure IV.34D).

The HER2+ subtype cell line showed an increased bone resorption when OCs were treated with the lowest NE concentration, compared with the absence of cancer cells, by the two donors (Figure IV.34G-H). suggesting that SK-BR-3 treated with 10^{-8} M of NE CM causes a slight increase of OC resorption.

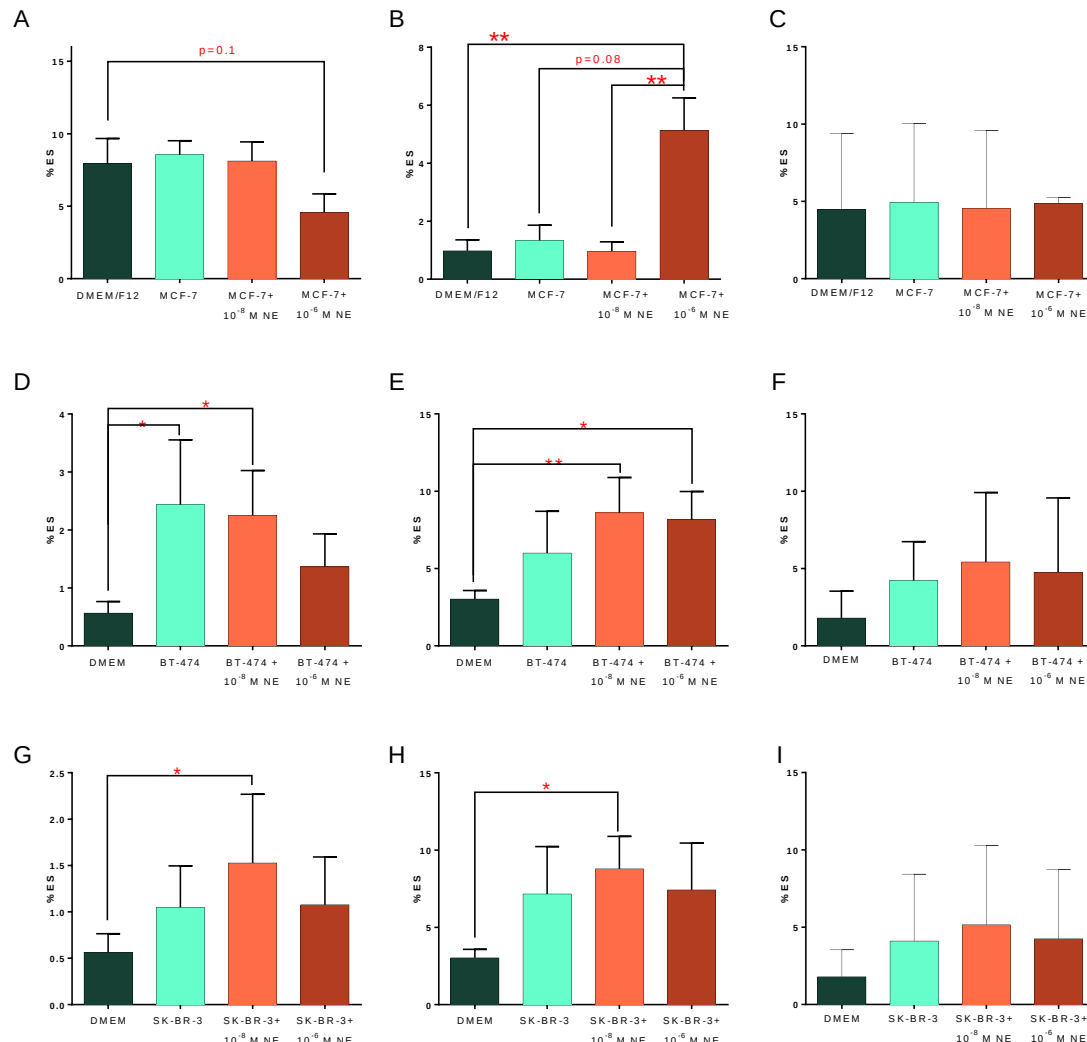


Figure IV. 34 – Effect of CM of luminal and HER2-enriched subtypes treated with NE on OC activity. Resorptive activity was quantified by the percentage of eroded surface (ES) per total bone surface. OCs were treated with the DMEM/F12, MCF-7 CM with 0, 10^{-8} and 10^{-6} M of NE (A-C), DMEM, BT-474 CM with 0, 10^{-8} and 10^{-6} M of NE (D-F) and DMEM, SK-BR-3 CM with 0, 10^{-8} and 10^{-6} M of NE (G-I). Graphs on the right and middle represent individual donors, graph on the left represents media of both donors. Data is expressed as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, $n = 2$ donors (5 bone slices per condition).

Although bone is not the major metastatic site for TNBC, the presence of MDA-MB-468 CM highly increased OC activity (Figure IV.35A-B). On the second donor, this effect decreased when the highest NE dose was added to MDA-MB-468. Together, there is a slight decrease of bone resorption with the increasing doses of NE on MDA-MB-468 (Figure IV.35C).

The OC activity of OCs treated with MDA-MB-231 CM seems similar to MDA-MB-468 cells. These are both basal like cells, which are characterized by their

aggressiveness. Although the MDA-MB-231 CM increased bone resorption, this activity was decreased when increasing doses of NE were added to the cells (Figure IV.35D-E).

The Bo-1833 cell line represents the metastatic BrCa cells found in bone. The first donor showed a high increase of resorption when the CM of Bo-1833 was added and this increase, similarly to one of MDA-MB-468 donors, was inhibited by the highest concentration of NE (Figure IV.35G). However, the second donor showed no differences between the conditions tested (Figure IV.35H). Overall, by combining the data of the two donors, there seems to be an increase of resorption when Bo-1833 CM is added, and this increase is slightly inhibited by the addition of increasing doses of NE (Figure IV.35I).

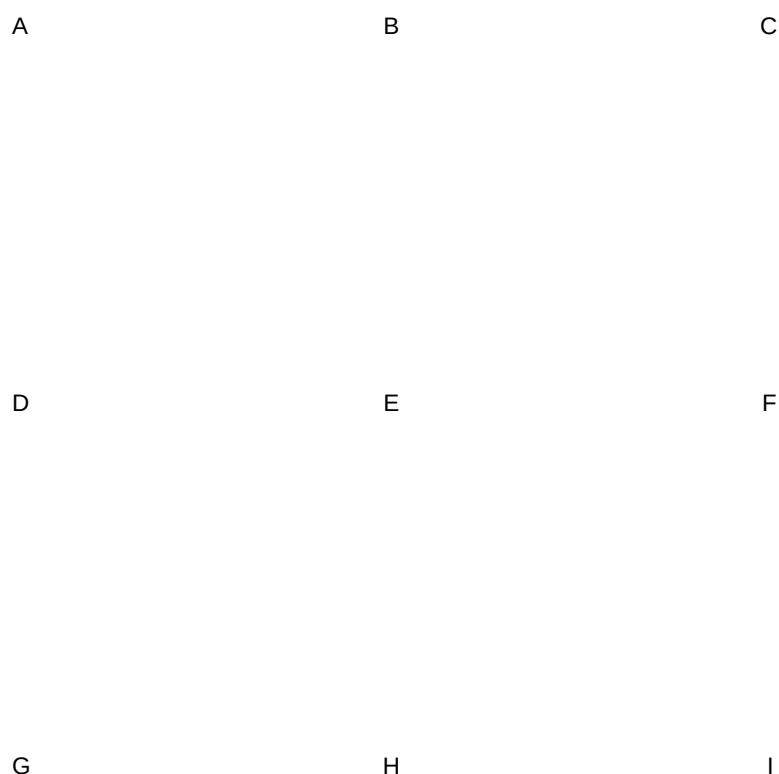


Figure IV. 35 – Effect of triple negative subtypes treated with NE on OC activity. Resorptive activity was quantified by the percentage of eroded surface (ES) per total bone surface. OCs were treated with the DMEM/F12, MCF-7 CM with 0, 10^{-8} and 10^{-6} M of NE (**A-C**), DMEM, BT-474 CM with 0, 10^{-8} and 10^{-6} M of NE (**D-F**) and DMEM, SK-BR-3 CM with 0, 10^{-8} and 10^{-6} M of NE (**G-I**). Graphs on the right and middle represent individual donors, graph on the left represents media of both donors. Data is expressed as mean $\pm$ SD. **p < 0.01, ***p < 0.001, n=2 donors (5 bone slices per condition).

When OCs were treated with BrCa CM, OC activity was always increased, except for MCF-7 CM (Figure IV.34 and 35). These results are in accordance to what is described in the literature. For example, an increase of OC resorption has been described when BrCa cells are cultured with OCs obtained from murine bone marrow [140]. Bo-1833 CM added to OCs originated from mice bone marrow increased OC resorption [141]. And in human OCs originated from bone marrow, MDA-MB-231 CM also caused an increase of OC resorption[142], as depicted in Figure VI.35D-I.

Since there are only 2 donors per cell line, no major conclusions can be made. However, the trends set by each BrCa cell line cultured with NE CM differ. While the triple negative cell lines show a gradual decrease of resorption from 0 to 10^{-8} and to 10^{-6} M of NE (Figure VI.35), luminal A and B and SK-BR-3 cell lines show almost the same percentage of eroded surface in the 3 conditions. BT-474 and SK-BR-3, however, show a slight increase in 10^{-8} M of NE (Figure VI.34).

It is well known that the variation between OCs originated from different blood donors is an obstacle in bone research, since it difficulties the ability to obtain accordance between different experiments. Therefore, the number of donors need to be increased to see if these results are confirmed.

Besides its role in bone, MMPs have also been related to BrCa. BrCa expresses a large number of MMP subtypes and some of them have been indicated as possible markers for cancer progression [143]. Namely, MMP-2 and MMP-9 can be found in the serum of BrCa patients [144].

MCF-10A cells were used as control and only presented one band, correspondent to the pro-MMP-2 molecular weight (Figure IV.36A). This band showed slightly more intensity with 10^{-8} M of NE treatment (Figure IV.36B).

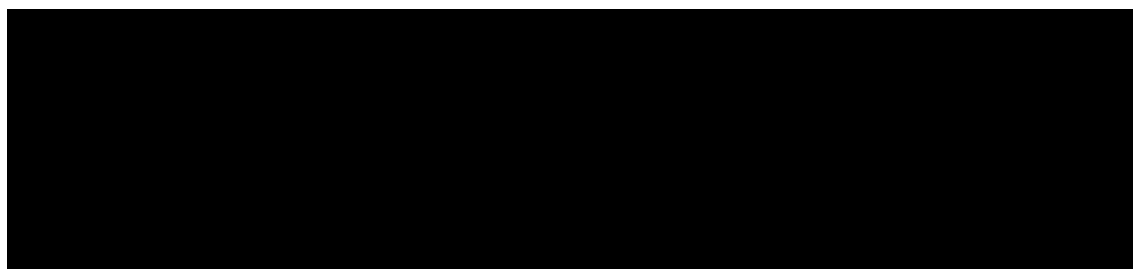


Figure IV. 36 - Zymography analysis of MMP-2 and MMP-9 activity in MCF-10A CM. **A.** Zymogram gelatine gel of media of MCF-10A cells treated with 0, 10^{-8} and 10^{-6} M of NE. The bands were identified by their molecular weight. **B.** Relative quantity of MMP-9 and MMP-2 activity was obtained through band volume using Image Lab. The values were normalized with the intensity of 0 M band. n=1.

In order to assess if CM of BrCa cells treated with NE affected OC MMPs expression, BrCa CM with and without OC contact were analysed.

MCF-7 CM was analysed before and after OC culture, MCF-7 gel presents 4 bands, representing the 2 MMPs in their pro and active forms (Figure IV.37A). The intensity profile of the 2 MMPs is similar with the different treatments. 0 and 10^{-8} M of NE show similar activities in the four forms and 10^{-6} M of NE has a lower intensity than the other conditions (Figure IV.37B). In the gel of OCs treated with MCF-7 CM, as seen in Figure VI.17, it is impossible to distinguish between the two forms of MMP-9 (Figure VI.37C). The intensity of the bands is similar with every NE treatment (Figure VI.37D).

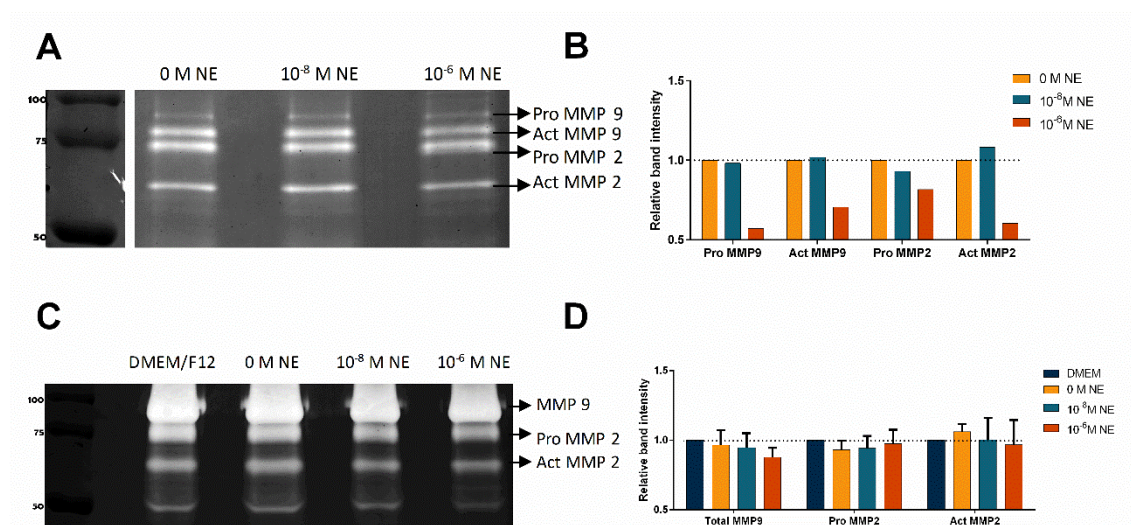


Figure IV. 37 - Zymography analysis of MMP-2 and MMP-9 activity in MCF-7 CM before and after OC contact. Zymogram gelatine gel of media of MCF-7 CM before (A) and after (B) OC contact. The bands were identified by their molecular weight. Relative quantity of MMP-9 and MMP-2 activity of MCF-7 CM before (C) and after (D) OC contact was obtained through band volume using Image Lab. The values were normalized with the intensity of 0 M band (n=1 condition).

In Figure VI.38A the gel for BT-474 CM before OC contact was analysed and also shows the four bands, with a low intensity for pro-MMP-9 band. The lane for 10^{-8} M of NE shows less activity for all MMPs and although 10^{-6} M of NE has lower pro MMP9 band intensity, when compared with 0 M of NE, the other 3 MMP forms have higher intensity in this condition (Figure VI.38B).



Figure IV. 38 - Zymography analysis of MMP-2 and MMP-9 activity in BT-474 CM before OC contact. **A.** Zymogram gelatine gel of media of BrCa cells treated with 0, 10^{-8} and 10^{-6} M of NE. The bands were identified by their molecular weight. **B.** Relative quantity of MMP-9 and MMP-2 activity was obtained through band volume using Image Lab. The values were normalized with the intensity of 0 M band (n=1 condition).

SK-BR-3 cells, which represent HER2 enriched subtype, present only 1 form of each MMP with a low intensity in Pro MMP-9 bands, before contacting with OCs (Figure IV.39A). The bands of 10^{-6} M present the lowest intensity of both MMPs (Figure VI.39B).

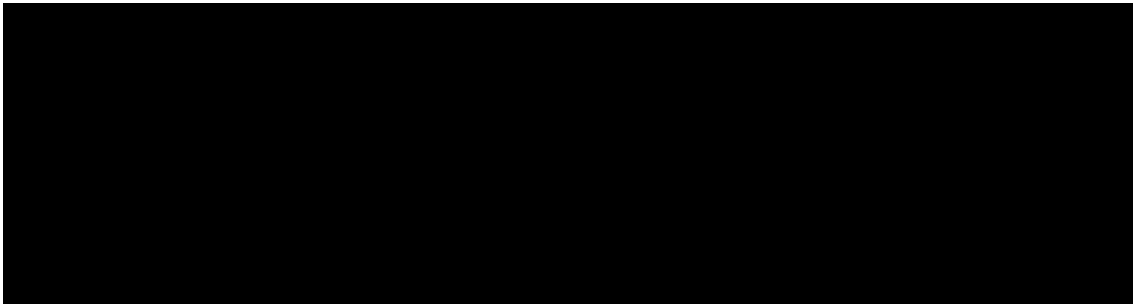


Figure IV. 39 - Zymography analysis of MMP-2 and MMP-9 activity in SK-BR-3 CM before OC contact. **A.** Zymogram gelatine gel of media of BrCa cells treated with 0, 10^{-8} and 10^{-6} M of NE. The bands were identified by their molecular weight. **B.** Relative quantity of MMP-9 and MMP-2 activity was obtained through band volume using Image Lab. The values were normalized with the intensity of 0 M band (n=1 condition).

MDA-MB-468 also shows 4 bands (Figure IV.40A). With 0 M presenting the highest intensity on all 4 forms of MMPs, followed by 10^{-6} M of NE with intermediate intensity and 10^{-8} M showing low activities (Figure IV.40B).

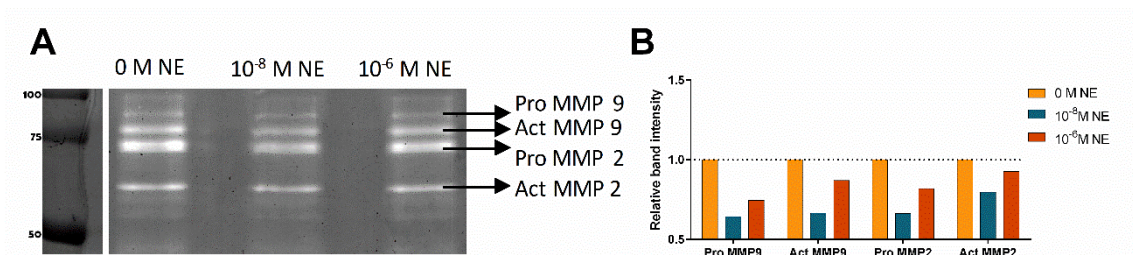


Figure IV. 40 - Zymography analysis of MMP-2 and MMP-9 activity in MDA-MB-468 CM before OC contact. **A.** Zymogram gelatine gel of media of BrCa cells treated with 0, 10^{-8} and 10^{-6} M of NE. The bands were identified by their molecular weight. **B.** Relative quantity of MMP-9 and MMP-2 activity was obtained through band volume using Image Lab. The values were normalized with the intensity of 0 M band (n=1 condition).

In the Figure VI.41A the MMP bands presented on each treatment in MDA-MB-231 CM before contacting with OCs, show similar intensities, excluding the pro-MMP-9 band (Figure VI.41B). This is most likely due to the artifact present on 0 M of NE band. The CM of OCs treated with MDA-MB-231 treated with NE CM, showed that 10^{-6} M of NE decreased the activity of Active MMP-2 (Figure VI.41D).

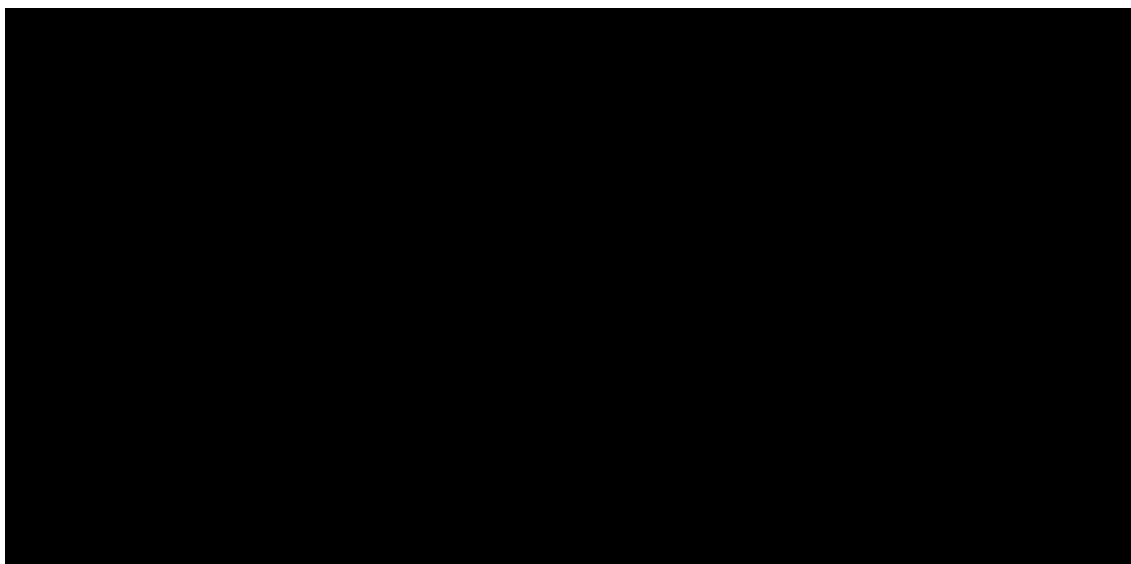


Figure IV. 41 - Zymography analysis of MMP-2 and MMP-9 activity in MDA-MB-231 CM before and after OC contact. Zymogram gelatine gel of media of MCF-7 CM before (A) and after (B) OC contact. The bands were identified by their molecular weight. Relative quantity of MMP-9 and MMP-2 activity of MCF-7 CM before (C) and after (D) OC contact was obtained through band volume using Image Lab. The values were normalized with the intensity of 0 M band (n=1 condition).

Pro MMP-9 shows a strong intensity in all treatment with Bo-1833 cells before contacting with OCs (Figure VI.42A). 10^{-8} and 10^{-6} M of NE showed the highest and the lowest intensity, respectively, in all MMPs of the Bo-1833 gel (Figure VI.42B). Lastly, zymogram from media of OC treated with CM of Bo-1833 treated with NE was performed, and 0 M of NE has a slight increase band intensity for active and pro MMP-2 (Figure VI.42D).

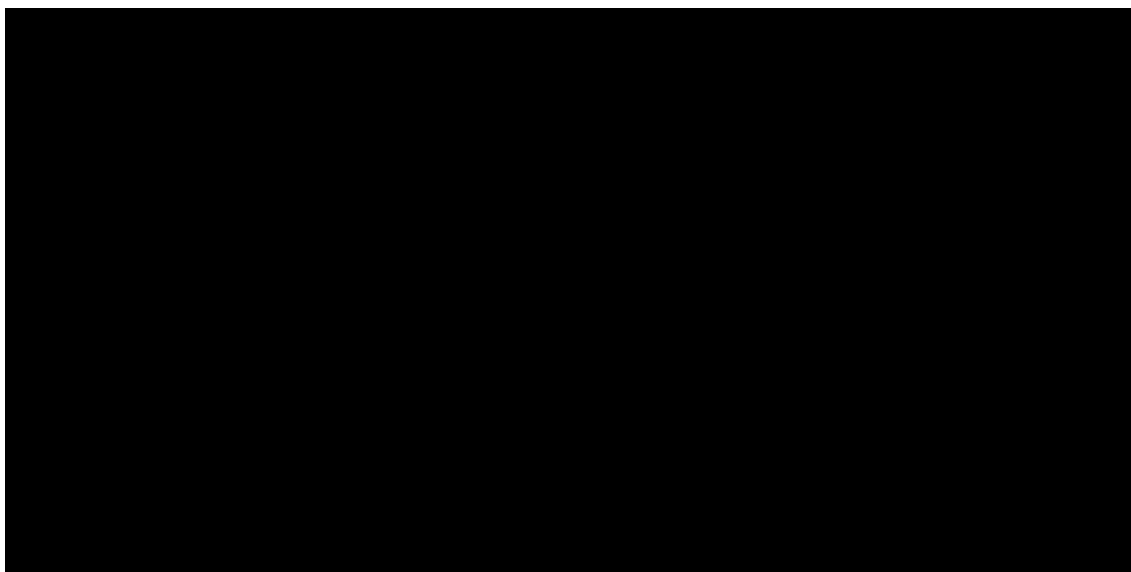


Figure IV. 42 - Zymography analysis of MMP-2 and MMP-9 activity in Bo-1833 before and after OC contact. Zymogram gelatine gel of media of MCF-7 CM before (A) and after (B) OC contact. The bands were identified by their molecular weight. Relative quantity of MMP-9 and MMP-2 activity of MCF-7 CM before (C) and after (D) OC contact was obtained through band volume using Image Lab. The values were normalized with the intensity of 0 M band (n=1 condition).

Since only one sample per condition was tested, for BrCa CM before OC contact, no statistical analysis can be made, however, it is possible to observe that the MMP profile between BrCa cell lines without contacting OCs and MCF-10A cell line is very different. While most BrCa cell lines express the 2 forms of MMP-2 and -9, MCF-10A only expresses pro MMP-2. However, it is possible to observe between cell lines there are different MMP profiles, with SK-BR-3 showing the bigger difference when compared with the other BrCa cell lines (Figure VI.39A). This once more highlights that BrCa subtypes have different characteristics that may differentiate their behaviour in bone.

Regarding the MMP expression of OC treated with BrCa CM, the only difference was a decrease in active MMP-2 activity in MDA-MB-231 CM when 10^{-6} M of NE was added (Figure VI.41D). This is a very similar result as OCs directly treated with NE (Figure VI.17.B). However, the fact that there are no differences seen in MMP-9 bands may be due to the fact that there is an excess of sample quantity.

Although zymography is an important tool to observe the presence or absence of MMP in media samples, only a semi-quantitative analysis can be made. Moreover, in order to see the two MMP-2 bands, it is impossible to differentiate both bands of MMP-9. Therefore, more sensitive methods, such as ELISA or western blotting analysis must be performed to evaluate the activity of MMPs in these samples.

Concluding remarks and Future perspectives

The link between SNS and bone metastasis progression allowed to understand that there are a lot of variables implied in the metastatic process that remain poorly understood and that should be explored to develop potential specific therapies for bone metastatic BrCa. Although the role of OBs has been explored, the underlying mechanisms of OCs in BrCa-induced bone metastasis, specifically the involvement of SNS in these processes, remain largely unknown. Therefore, the effects of the adrenergic activation on the major intervenient of bone mass loss were evaluated.

Since α - and β -ARs are present in both breast and bone tissue and seem to have an important role to play in stimulating BrCa metastasis to bone, a characterization of the adrenergic profile was performed using distinct BrCa cell lines representing each BrCa subtype. The analysis shows that β 1- and β 2-ARs were mainly expressed by triple-negative cells. Regarding α 2-ARs, α 2C-AR subtype was expressed by luminal cell lines while α 2A-AR was expressed in BT-474 and triple-negative cells. Although no α 1-AR gene was expressed in qRT-PCR analysis, the unspecific α 1 antibody marked every cell line, showing strong staining in SK-BR-3 and BT-474 cell lines (HER2⁺ and luminal B subtype, respectively). In addition, the synthesizing enzymes (e.g. TH and PNMT) are mostly expressed in luminal and HER2 enriched cells. Importantly, in order to confirm the results obtained in this study, other methods to evaluate the presence of these receptors need to be performed, such as the use of more specific antibodies for immunofluorescence analysis and ARs protein quantification by western blotting.

OCs express ARs and SNS activation has been shown to affect bone microenvironment through bone loss, by directly affecting bone resorption in mice [118]. In humans, a study described that 10^{-6} M of NE caused a inhibition of proliferation on OC precursor cells (FLG 29.1) and increased OC maturation and TRAP activity [145]. Therefore, the direct effects that NE has on OC differentiation and activity were evaluated.

The treatments of NE on OCs suggest that SNS activation has a direct effect in OC differentiation and activity. Treatments on early or late differentiation show an increase of OC number with $n \geq 8$ when cells are treated with 10^{-8} M of NE. The highest doses however, cause the inverse effect, decreasing the number of cells, almost to control levels. OC area, however, almost seems unaffected by NE treatments. OC activity also seems to be affected by NE, since increasing doses caused a trend towards an inhibition of matrix resorption, that was more evident with the highest dose. Of note, this decrease was in accordance with the result of TRAP activity assay.

Some studies have explored the effects of SNS activation on bone remodelling diseases, which leads to an increase in incidence of fractures and decrease of bone mass [64, 146]. These studies mainly focus on the role of OBs, suggesting that NE is released in bone and mainly binds to their β 2-AR, inhibiting bone formation and promoting bone resorption, via RANKL expression [126, 147]. The preliminary results of this study, however, suggest that the effect of SNS on OC activity is the opposite, when NE was added, bone resorption was inhibited. These contradictory results might be related with different methods, since these studies usually use AR genetic ablation on mice. Moreover, since there is a great variability between donors, the number of donors tested will need to be enlarged, to increase statistical power.

Interestingly, our studies also demonstrated that, when OCs were treated with BrCa cells CM collected after NE treatment, their morphology and resorbing activity was affected. In what regards OC differentiation, NE treatment of BrCa cells caused different effects on OC number and area, depending on the BrCa subtype and differentiation stage:

1) in early OC differentiation, just by adding the CM of BrCa cells, the OC number decreased in most cell lines and was almost always accompanied by an increase of cell area. Only MCF-7 CM caused a global increase of OC numbers. Also, in most BrCa CM, NE only slightly affected OC number, however, 10^{-6} M treatment of MCF-7 CM caused a pronounced decrease of OC numbers;

2) in the late OC differentiation, contrarily to treatments in early differentiation, the number of OCs treated with MCF-7 CM decreases. In BT-474, MDA-MB-468 and MDA-MB-231 CM, OC number is increased, which is the opposite to what happens during early differentiation. CM of SK-BR-3 and Bo-1833 cell lines have similar OC numbers as control conditions. Area measurements increase in MCF-7 CM but decrease in the other BrCa cells CM. Moreover, NE treatments seem to affect late differentiation more than early differentiation. NE caused an OC number increase when treated with CM of SK-BR-3 and Bo-1833, and a decrease with CM of BT-474 and MDA-MB-468. In treatments of MDA-MB-231 CM, only 10^{-6} M of NE caused a decrease of OC number triggered by the presence of MDA-MB-231. Treatment of MCF-7 cells cultured with 10^{-6} M of NE increased area of OCs, just as the effects caused in early differentiation. Area measurements of all other cell lines depict the same subtile profile: a small increase with 10^{-8} M and a small decrease when 10^{-6} M is added.

Nevertheless, these results were obtained from one donor and need further confirmation using more donor samples.

In what regards OC resorption activity, our data suggests that when BrCa CM was added, OC resorptive activity was enhanced in all BrCa subtypes, when compared to their control media, which is in accordance with the literature [140-142]. Additionally, the resorbing activity of OCs treated with CM from luminal and HER2-enriched cells treated with NE was similar to their respective CM without NE. On the other hand, triple-negative cells treated with increasing NE doses caused a gradual decrease in OC activity. These results suggest that the involvement of NE/SNS on bone resorption induced by BrCa is dependent on the BrCa subtype, which might be explained by distinct adrenergic profiles observed earlier, namely the expression of ARs and enzymes by each subtype.

As future perspectives, it is proposed to discover which AR is being, in fact, activated by the different NE concentrations, in order to know which pathways are being activated to spread SNS response. Furthermore, it would be interesting to perform a screening to determine which proteins are being expressed/modulated before and after NE treatment by BrCa cells, that might have an impact on OC activity. Moreover, regarding the resorption activity of OCs, an important characteristic is the depth of the resorptive events. Therefore, if one could quantify this measure and other properties of the events caused in the matrix, more information could be found that help to explain OC behaviour.

Different time points and duration of NE treatment on BrCa cells can also be performed to evaluate how the effects of acute and chronic stress alter cell behaviour. Additionally, the adrenergic signalling pathways expressed by each BrCa subtype can be inhibited by siRNA, and the importance that each pathway has on bone microenvironment could be explored when the CM is administered to the OCs. Prospectively, it would be interesting to establish a co-culture of human OBs and OCs to better mimic bone microenvironment. Treatments with the same NE conditions, with and without BrCa contact would be administered to understand if the effects caused were maintained or shifted by the presence of OBs.

It would also be interesting to assess the SNS profile of human biopsies of both primary and bone metastasis tumours to compare to the *in vivo* data. Quantification of catecholamine levels in stressed women with or without BrCa metastasis could also be performed, as a method to link SNS activation with metastasis progression.

The sample size used for these studies was small, and the variability of results from human donors is larger than working with cell lines, therefore the sample number needs to be increased. Prospectively, attempts can be made to decrease the variability

through reduction of donor's diversity by limiting characteristics such as age, race and blood type.

Overall, the preliminary results of this study suggest that SNS activation has an effect on OC maturation and function. And CM of BrCa cells previously treated with NE also alter OC morphology and activity, indicating that SNS can modulate BrCa metastasis by affecting the function of OCs.

References

1. Siegel, R.L. et al. (2017) Cancer Statistics, 2017. *CA Cancer J Clin* 67 (1), 7-30.
2. Richie, R.C. and Swanson, J.O. (2003) Breast cancer: a review of the literature. *J Insur Med* 35 (2), 85-101.
3. A Frank, G. et al. (2013) WHO Classification of tumors of the breast, 2012.
4. Senkus, E. et al. (2015) Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 26 Suppl 5, v8-30.
5. La Vecchia, C. et al. (2010) Cancer mortality in Europe, 2000-2004, and an overview of trends since 1975. *Ann Oncol* 21 (6), 1323-60.
6. Edge SB, B.D., Compton CC, Fritz AG, Greene FL, Trotti A. (2010) AJCC cancer staging manual, 7th edition. France: Springer.
7. Pandey, P.R. et al. (2010) Role of myoepithelial cells in breast tumor progression. *Frontiers in bioscience : a journal and virtual library* 15, 226-236.
8. Stewart BW, W.C. (2014) World Cancer Report 2014. Lyon, France: International Agency for Research on Cancer.
9. McPherson, K. et al. (2000) ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics. *Bmj* 321 (7261), 624-8.
10. LT, A. (2017) An Overview of Breast Cancer Epidemiology, Risk Factors, Pathophysiology, and Cancer Risks Reduction. *MOJ Biol Med* 1 (4).
11. Polyak, K. (2011) Heterogeneity in breast cancer. *The Journal of Clinical Investigation* 121 (10), 3786-3788.
12. Holliday, D.L. and Speirs, V. (2011) Choosing the right cell line for breast cancer research. *Breast Cancer Res* 13 (4), 215.
13. Dai, X. et al. (2015) Breast cancer intrinsic subtype classification, clinical use and future trends. *Am J Cancer Res* 5 (10), 2929-43.
14. Inic, Z. et al. (2014) Difference between Luminal A and Luminal B Subtypes According to Ki-67, Tumor Size, and Progesterone Receptor Negativity Providing Prognostic Information. *Clinical Medicine Insights. Oncology* 8, 107-111.
15. Seal, M.D. and Chia, S.K. (2010) What is the difference between triple-negative and basal breast cancers? *Cancer J* 16 (1), 12-6.
16. Sabatier, R. et al. (2014) Claudin-low breast cancers: clinical, pathological, molecular and prognostic characterization. *Mol Cancer* 13, 228.
17. Dias, K. et al. (2017) Claudin-Low Breast Cancer; Clinical & Pathological Characteristics. *PLOS ONE* 12 (1), e0168669.
18. Prat, A. et al. (2015) Clinical implications of the intrinsic molecular subtypes of breast cancer. *Breast* 24 Suppl 2, S26-35.
19. Yersal, O. and Barutca, S. (2014) Biological subtypes of breast cancer: Prognostic and therapeutic implications. *World J Clin Oncol* 5 (3), 412-24.

20. Prat, A. and Perou, C.M. (2011) Deconstructing the molecular portraits of breast cancer. *Molecular Oncology* 5 (1), 5-23.
21. Malhotra, G.K. et al. (2010) Histological, molecular and functional subtypes of breast cancers. *Cancer Biol Ther* 10 (10), 955-60.
22. Chambers, A.F. et al. (2002) Metastasis: Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer* 2 (8), 563-572.
23. Lambert, A.W. et al. (2017) Emerging Biological Principles of Metastasis. *Cell* 168 (4), 670-691.
24. Leber, M.F. and Efferth, T. (2009) Molecular principles of cancer invasion and metastasis (review). *Int J Oncol* 34 (4), 881-95.
25. Suva, L.J. et al. (2011) Bone metastasis: mechanisms and therapeutic opportunities. *Nat Rev Endocrinol* 7 (4), 208-18.
26. Sahai, E. (2007) Illuminating the metastatic process. *Nat Rev Cancer* 7 (10), 737-749.
27. Gupta, G.P. and Massagué, J. (2006) Cancer Metastasis: Building a Framework. *Cell* 127 (4), 679-695.
28. Paget, S. (1889) The distribution of secondary growths in cancer of the breast. 1889. *Cancer Metastasis Rev* 8 (2), 98-101.
29. Lee, Y.T. (1983) Breast carcinoma: pattern of metastasis at autopsy. *J Surg Oncol* 23 (3), 175-80.
30. Kozlow, W. and Guise, T.A. (2005) Breast cancer metastasis to bone: mechanisms of osteolysis and implications for therapy. *J Mammary Gland Biol Neoplasia* 10 (2), 169-80.
31. Arto, A. et al. (1985) Significance of estrogen and progesterone receptors, disease-free interval, and site of first metastasis on survival of breast cancer patients. *Cancer* 56 (7), 1696-1700.
32. Clark, G.M. et al. (1987) Survival from first recurrence: relative importance of prognostic factors in 1,015 breast cancer patients. *J Clin Oncol* 5 (1), 55-61.
33. Hess, K.R. et al. (2003) Estrogen Receptors and Distinct Patterns of Breast Cancer Relapse. *Breast Cancer Research and Treatment* 78 (1), 105-118.
34. Soni, A. et al. (2015) Breast cancer subtypes predispose the site of distant metastases. *Am J Clin Pathol* 143 (4), 471-8.
35. Kast, K. et al. (2015) Impact of breast cancer subtypes and patterns of metastasis on outcome. *Breast Cancer Res Treat* 150 (3), 621-9.
36. Savci-Heijink, C.D. et al. (2015) Retrospective analysis of metastatic behaviour of breast cancer subtypes. *Breast Cancer Res Treat* 150 (3), 547-57.
37. Kennecke, H. et al. (2010) Metastatic behavior of breast cancer subtypes. *J Clin Oncol* 28 (20), 3271-7.
38. Smid, M. et al. (2008) Subtypes of breast cancer show preferential site of relapse. *Cancer Res* 68 (9), 3108-14.
39. Feng, X. and McDonald, J.M. (2011) Disorders of bone remodeling. *Annu Rev Pathol* 6, 121-45.

40. Croucher, P.I. et al. (2016) Bone metastasis: the importance of the neighbourhood. *Nat Rev Cancer* 16 (6), 373-86.
41. Chen, Y.C. et al. (2010) Breast cancer metastasis to the bone: mechanisms of bone loss. *Breast Cancer Res* 12 (6), 215.
42. Nakamura, H. (2007) Morphology, Function, and Differentiation of Bone Cells.
43. Weilbaecher, K.N. et al. (2011) Cancer to bone: a fatal attraction. *Nat Rev Cancer* 11 (6), 411-25.
44. Boyle, W.J. et al. (2003) Osteoclast differentiation and activation. *Nature* 423 (6937), 337-42.
45. Kleinbans, C. et al. (2015) Comparison of osteoclastogenesis and resorption activity of human osteoclasts on tissue culture polystyrene and on natural extracellular bone matrix in 2D and 3D. *J Biotechnol* 205, 101-10.
46. Sisay, M. et al. (2017) The RANK/RANKL/OPG system in tumorigenesis and metastasis of cancer stem cell: potential targets for anticancer therapy. *Onco Targets Ther* 10, 3801-3810.
47. Sterling, J.A. et al. (2011) Advances in the biology of bone metastasis: how the skeleton affects tumor behavior. *Bone* 48 (1), 6-15.
48. Mundy, G.R. (2002) Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat Rev Cancer* 2 (8), 584-93.
49. Le Pape, F. et al. (2016) The role of osteoclasts in breast cancer bone metastasis. *Journal of Bone Oncology* 5 (3), 93-95.
50. Ren, G. et al. (2015) Bone metastasis and the metastatic niche. *J Mol Med (Berl)* 93 (11), 1203-12.
51. Eleftheriou, F. (2016) Role of sympathetic nerves in the establishment of metastatic breast cancer cells in bone. *J Bone Oncol* 5 (3), 132-134.
52. Coleman, R. (2016) Bone targeted treatments in cancer The story so far. *Journal of Bone Oncology* 5 (3), 90-92.
53. Coleman, R. (2011) The use of bisphosphonates in cancer treatment. *Ann N Y Acad Sci* 1218, 3-14.
54. Coleman, R.E. et al. (2011) Breast-Cancer Adjuvant Therapy with Zoledronic Acid. *New England Journal of Medicine* 365 (15), 1396-1405.
55. (EBCTCG), E.B.C.T.C.G. (2015) Adjuvant bisphosphonate treatment in early breast cancer: meta-analyses of individual patient data from randomised trials. *The Lancet* 386 (10001), 1353-1361.
56. Hadji, P. et al. (2016) Adjuvant bisphosphonates in early breast cancer: consensus guidance for clinical practice from a European Panel. *Ann Oncol* 27 (3), 379-90.
57. Stopeck, A.T. et al. (2010) Denosumab compared with zoledronic acid for the treatment of bone metastases in patients with advanced breast cancer: a randomized, double-blind study. *J Clin Oncol* 28 (35), 5132-9.
58. Powe, D.G. et al. (2010) Beta-blocker drug therapy reduces secondary cancer formation in breast cancer and improves cancer specific survival. *Oncotarget* 1 (7), 628-38.

59. Ganz, P.A. et al. (2011) Examining the influence of beta blockers and ACE inhibitors on the risk for breast cancer recurrence: results from the LACE cohort. *Breast Cancer Res Treat* 129 (2), 549-56.
 60. Barron, T.I. et al. (2011) Beta blockers and breast cancer mortality: a population- based study. *J Clin Oncol* 29 (19), 2635-44.
 61. Melhem-Bertrandt, A. et al. (2011) Beta-Blocker Use Is Associated With Improved Relapse-Free Survival in Patients With Triple-Negative Breast Cancer. *Journal of Clinical Oncology* 29 (19), 2645-2652.
 62. Eleftheriou, F. (2005) Neuronal signaling and the regulation of bone remodeling. *Cell Mol Life Sci* 62 (19-20), 2339-49.
 63. Ma, Y. et al. (2011) beta2-Adrenergic receptor signaling in osteoblasts contributes to the catabolic effect of glucocorticoids on bone. *Endocrinology* 152 (4), 1412-22.
 64. Takeda, S. et al. (2002) Leptin regulates bone formation via the sympathetic nervous system. *Cell* 111 (3), 305-17.
 65. Lueboonthavatchai, P. (2007) Prevalence and psychosocial factors of anxiety and depression in breast cancer patients. *J Med Assoc Thai* 90 (10), 2164-74.
 66. Compas, B.E. et al. (1999) Adjustment to breast cancer: age-related differences in coping and emotional distress. *Breast Cancer Res Treat* 54 (3), 195-203.
- adrenoceptors.
- Cancer Chemotherapy and Pharmacology 58 (1), 50.
68. Eleftheriou, F. (2015) Chronic stress, sympathetic activation and skeletal metastasis of breast cancer cells. *Bonekey Rep* 4, 693.
 69. Chida, Y. et al. (2008) Do stress-related psychosocial factors contribute to cancer incidence and survival? *Nat Clin Pract Oncol* 5 (8), 466-75.
 70. Spiegel, D. and Giese-Davis, J. (2003) Depression and cancer: mechanisms and disease progression. *Biol Psychiatry* 54 (3), 269-82.
 71. Armaiz-Pena, G.N. et al. (2009) Neuroendocrine modulation of cancer progression. *Brain, Behavior, and Immunity* 23 (1), 10-15.
 72. Antoni, M.H. et al. (2006) The influence of bio-behavioural factors on tumour biology: pathways and mechanisms. *Nature Reviews Cancer* 6, 240.
 73. Thaker, P.H. and Sood, A.K. (2008) Neuroendocrine influences on cancer biology. *Semin Cancer Biol* 18 (3), 164-70.
 74. Thaker, P.H. et al. (2013) Importance of adrenergic pathways in women's cancers. *Cancer Biomark* 13 (3), 145-54.
 75. Cole, S.W. et al. (2015) Sympathetic nervous system regulation of the tumour microenvironment. *Nat Rev Cancer* 15 (9), 563-72.
 76. Insel, P.A. (1993) Adrenergic receptors, G proteins, and cell regulation: implications for aging research. *Exp Gerontol* 28 (4-5), 341-8.
 77. McEwen, B.S. (2002) Sex, stress and the hippocampus: allostasis, allostatic load and the aging process. *Neurobiol Aging* 23 (5), 921-39.

96. Drell, T.L.t. et al. (2003) Effects of neurotransmitters on the chemokinesis and chemotaxis of MDA-MB-468 human breast carcinoma cells. *Breast Cancer Res Treat* 80 (1), 63-70.

97. Lang, K. et al. (2004) Induction of a metastatogenic tumor cell type by neurotransmitters and its pharmacological inhibition by established drugs. *Int J Cancer* 112 (2), 231-8.

98. Sloan, E.K. et al. (2010) The sympathetic nervous system induces a metastatic switch in primary breast cancer. *Cancer Res* 70 (18), 7042-52.

99. Madden, K.S. et al. (2011) beta-Adrenergic receptors (beta-AR) regulate VEGF and IL-6 production by divergent pathways in high beta-AR-expressing breast cancer cell lines. *Breast Cancer Res Treat* 130 (3), 747-58.

-adrenoceptor blocker therapy improve cancer survival?

Findings from a population-based retrospective cohort study. *British Journal of Clinical Pharmacology* 72 (1), 157-161.

101. Campbell, J.P. et al. (2012) Stimulation of host bone marrow stromal cells by sympathetic nerves promotes breast cancer bone metastasis in mice. *PLoS Biol* 10 (7), e1001363.

102. Mulcrone, P.L. et al. (2017) Skeletal Colonization by Breast Cancer Cells Is Stimulated by an Osteoblast and beta2AR-Dependent Neo-Angiogenic Switch. *J Bone Miner Res* 32 (7), 1442-1454.

-adrenoceptors on tumor cells play a critical role in stress-enhanced metastasis in a mouse model of breast cancer. *Brain, behavior, and immunity* 57, 106-115.

104. Sorlie, T. et al. (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* 98 (19), 10869-74.

105. Kang, Y. et al. (2003) A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* 3 (6), 537-549.

106. Mehta, G. et al. (2012) Opportunities and challenges for use of tumor spheroids as models to test drug delivery and efficacy. *Journal of Controlled Release* 164 (2), 192-204.

107. Imamura, Y. et al. (2015) Comparison of 2D- and 3D-culture models as drug-testing platforms in breast cancer. *Oncol Rep* 33 (4), 1837-43.

108. Singh, M. et al. (2016) Three-Dimensional Breast Cancer Models Mimic Hallmarks of Size-Induced Tumor Progression. *Cancer Res* 76 (13), 3732-43.

109. Oridate, N. et al. (1996) Reconstituted basement membrane (Matrigel): a useful semisolid medium for growth of tumor cell colonies. *In Vitro Cell Dev Biol Anim* 32 (4), 192-3.

110. Ivascu, A. and Kubbies, M. (2007) Diversity of cell-mediated adhesions in breast cancer spheroids. *Int J Oncol* 31 (6), 1403-13.

111. Végh, A.M.D. et al. (2016) Part and Parcel of the Cardiac Autonomic Nerve System: Unravelling Its Cellular Building Blocks during Development. *Journal of Cardiovascular Development and Disease* 3 (3), 28.

112. Bonnet, N. et al. (2008) Adrenergic control of bone remodeling and its implications for the treatment of osteoporosis. *J Musculoskelet Neuronal Interact* 8 (2), 94-104.

113. Fonseca, T.L. et al. (2011) Double disruption of alpha2A- and alpha2C-adrenoceptors results in sympathetic hyperactivity and high-bone-mass phenotype. *J Bone Miner Res* 26 (3), 591-603.

114. Rains, S.L. et al. (2017) Beta-adrenergic receptors are expressed across diverse cancers. *Oncoscience* 4 (7-8), 95-105.
115. Liu, D. et al. (2015) A Her2-let-7-AR circuit affects prognosis in patients with Her2-positive breast cancer. *BMC Cancer* 15, 832.
116. Powe, D.G. et al. (2011) Alpha- and beta-adrenergic receptor (AR) protein expression is associated with poor clinical outcome in breast cancer: an immunohistochemical study. *Breast Cancer Res Treat* 130 (2), 457-63.
117. Park, M.S. et al. (2014) The antihypertension drug doxazosin inhibits tumor growth and angiogenesis by decreasing VEGFR-2/Akt/mTOR signaling and VEGF and HIF-*Oncotarget* 5 (13), 4935-4944.
118. Aitk β -Adrenoreceptor ligands regulate osteoclast differentiation in vitro by direct and indirect mechanisms. *Archives of Biochemistry and Biophysics* 482 (1), 96-103.
119. Hamajima, K. et al. (2018) Suppression of osteoclastogenesis via β -adrenergic receptors. *Biomedical Reports* 8 (5), 407-416.
120. Lutgendorf, S.K. et al. (2011) Social isolation is associated with elevated tumor norepinephrine in ovarian carcinoma patients. *Brain Behav Immun* 25 (2), 250-5.
121. Lutgendorf, S.K. et al. (2009) Depression, social support, and beta-adrenergic transcription control in human ovarian cancer. *Brain Behav Immun* 23 (2), 176-83.
122. Sommer, C. et al., Ilastik: Interactive learning and segmentation toolkit, 2011 IEEE International Symposium on Biomedical Imaging: From Nano to Macro, 2011, pp. 230-233.
123. Carpenter, A.E. et al. (2006) CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biology* 7 (10), R100.
124. Muschter, D. et al. (2015) Sympathetic Neurotransmitters Modulate Osteoclastogenesis and Osteoclast Activity in the Context of Collagen-Induced Arthritis. *PLOS ONE* 10 (10), e0139726.
125. Costa, L. et al. (2008) Impact of skeletal complications on patients' quality of life, mobility, and functional independence. *Support Care Cancer* 16 (8), 879-89.
126. Eleftheriou, F. et al. (2005) Leptin regulation of bone resorption by the sympathetic nervous system and CART. *Nature* 434 (7032), 514-20.
127. Grässel, S. (2014) The role of peripheral nerve fibers and their neurotransmitters in cartilage and bone physiology and pathophysiology. *Arthritis Research & Therapy* 16 (6), 485.
128. Merrild, D.M.H. et al. (2015) Pit- and trench-forming osteoclasts: a distinction that matters. *Bone Research* 3, 15032.
129. Ek-Rylander, B. et al. (1994) Dephosphorylation of osteopontin and bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase. Modulation of osteoclast adhesion in vitro. *J Biol Chem* 269 (21), 14853-6.
130. Jones, G.T. (2014) Matrix metalloproteinases in biologic samples. *Adv Clin Chem* 65, 199-219.
131. Mosig, R.A. et al. (2007) Loss of MMP-2 disrupts skeletal and craniofacial development, and results in decreased bone mineralization, joint erosion, and defects in osteoblast and osteoclast growth. *Human molecular genetics* 16 (9), 1113-1123.

132. Kuchuk, I. et al. (2013) Incidence, consequences and treatment of bone metastases in breast cancer patients-Experience from a single cancer centre. *J Bone Oncol* 2 (4), 137-44.
133. Jensen, A.O. et al. (2011) Incidence of bone metastases and skeletal-related events in breast cancer patients: a population-based cohort study in Denmark. *BMC Cancer* 11, 29.
134. Fata, J.E. et al. (2000) The Osteoclast Differentiation Factor Osteoprotegerin-Ligand Is Essential for Mammary Gland Development. *Cell* 103 (1), 41-50.
135. Rucci, N. and Teti, A. (2010) Osteomimicry: how tumor cells try to deceive the bone. *Front Biosci (Schol Ed)* 2, 907-15.
136. Santini, D. et al. (2011) Receptor activator of NF- κ B (RANK) expression in primary tumors associates with bone metastasis occurrence in breast cancer patients. *PLoS One* 6 (4), e19234.
137. Santini, D. et al. (2011) Expression pattern of receptor activator of NF κ B (RANK) in a series of primary solid tumors and related bone metastases. *J Cell Physiol* 226 (3), 780-4.
138. Dent, R. et al. (2009) Pattern of metastatic spread in triple-negative breast cancer. *Breast Cancer Res Treat* 115 (2), 423-8.
139. Gong, Y. et al. (2017) Impact of molecular subtypes on metastatic breast cancer patients: a SEER population-based study. *Scientific Reports* 7, 45411.
140. Clohisy, D.R. et al. (1996) Human breast cancer induces osteoclast activation and increases the number of osteoclasts at sites of tumor osteolysis. *Journal of Orthopaedic Research* 14 (3), 396-402.
141. Aukes, K. et al. (2017) Breast cancer cell-derived fibroblast growth factors enhance osteoclast activity and contribute to the formation of metastatic lesions. *PLOS ONE* 12 (10), e0185736.
142. Grano, M. et al. (2000) Breast Cancer Cell Line MDA-231 Stimulates Osteoclastogenesis and Bone Resorption in Human Osteoclasts. *Biochemical and Biophysical Research Communications* 270 (3), 1097-1100.
143. McCawley, L.J. and Matrisian, L.M. (2000) Matrix metalloproteinases: multifunctional contributors to tumor progression. *Mol Med Today* 6 (4), 149-56.
144. Stankovic, S. et al. (2010) Activity of MMP-2 and MMP-9 in sera of breast cancer patients. *Pathol Res Pract* 206 (4), 241-7.
145. Frediani, U. et al. (1996) Catecholamines modulate growth and differentiation of human preosteoclastic cells. *Osteoporos Int* 6 (1), 14-21.
146. Ducy, P. et al. (2000) Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell* 100 (2), 197-207.
147. Kajimura, D. et al. (2011) Genetic determination of the cellular basis of the sympathetic regulation of bone mass accrual. *J Exp Med* 208 (4), 841-51.

Annexes

Table A.1 - Primers used in qRT-PCR analysis.

Name	Genbank accession no.	Sequence (5'-3')	Length
Beta-2-microglobulin (B2M)	NM_004048.2	Fw 5'- GATGAGTATGCCTGCCGTGT -3' Rv 5'- TCGGGCATCTTCAAACCTCC -3'	105
Adrenoreceptor alpha 1A (ADRA1A)*	AY389505.1	Fw 5'-TCATGTACTGCCGCGTCTAC-3' Rv 5'-GGGCGTTTTTCCGATGGATG-3'	120
Adrenoreceptor alpha 1B (ADRA1B)	NM_000679.3	Fw 5'-CTCTACCGCTTGGCTCCTTGT-3' Rv 5'-GGAGCATGGGTAGATGATGGG-3'	116
Adrenoreceptor alpha 1D (ADRA1D)**	NM_000678.3	Fw 5'-TCTCCCGTGAGAAGAAAGCG-3' Rv 5'-CGGGAACAAGGAGCCGAG-3'	107
Adrenoreceptor alpha 2A (ADRA2A)*	NM_000681.3	Fw 5'-ATCCTGGCCTTGGGAGAGAT-3' Rv 5'-TCTCAAAGCAGGTCCGTGTC-3'	161
Adrenoreceptor alpha 2B (ADRA2B)*	NM_000682.6	Fw 5'-AGTGCAAGCTCAACCAGGAG -3' Rv 5'- GCGCAGGTAGACAAGGATCA-3'	95
Adrenoreceptor alpha 2C (ADRA2C)*	NM_000683.3	Fw 5'- CTGGTCATGCCCTTCTCGTT-3' Rv 5'- TGCAAAACAGCACATCGAGC-3'	100
Adrenoreceptor beta 1 (ADRB1)	NM_000684.2	Fw 5'- ACCCCAAGTGCTGCGATTTCTG -3' Rv 5'- GCTCGCAGCTGTCGATCTTCTT -3'	153
Adrenoreceptor beta 2 (ADRB2)	NM_000024.5	Fw 5'- GGACTTCCATTGATGTGCTGT -3' Rv 5'- GTCAGCAGGCTCTGGTACTTG -3'	112
Adrenoreceptor beta 3 (ADRB3)	NM_002686.4	Fw 5'- CTCGACGGGGCTTCTTGG -3' Rv 5'- GAGGCCAGAGGTTTTCCACA -3'	90
phenylethanolamine <i>N</i> -methyltransferase (PNMT)	NM_002686.4	Fw 5'-TCGCCACCGGTGAAGTG-3' Rv 5'-GGTGATGTCCTCAAAGTGGCT-3'	101
Tyrosine hydroxylase (TH)*	NM_199292.2	Fw 5'- GGCCGTGCAGCCCTACCAAG -3' Rv 5'- ACGGAGAAGGGGCGCTGGAT -3'	120
Dopamine beta-Hydroxylase (DBH)	NM_000787.3	Fw 5'- CTCCGGGATCCACATCTTCG -3' Rv 5'- GCGGATCTCCTGGAAGTGAG -3'	142

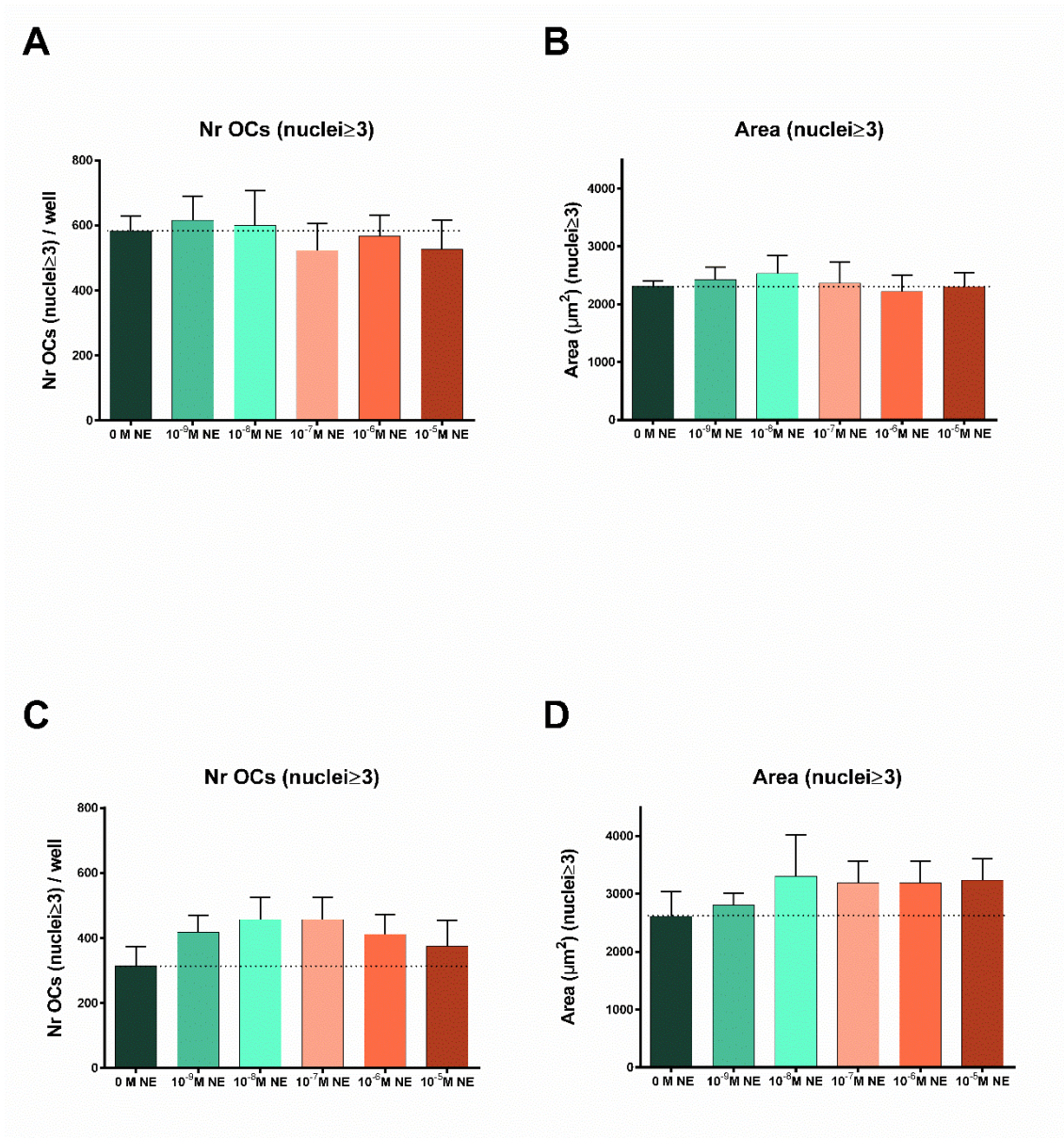


Figure A. 1 – Effects of direct NE treatment on OCs at early (A-B) and late (C-D) differentiation stages.

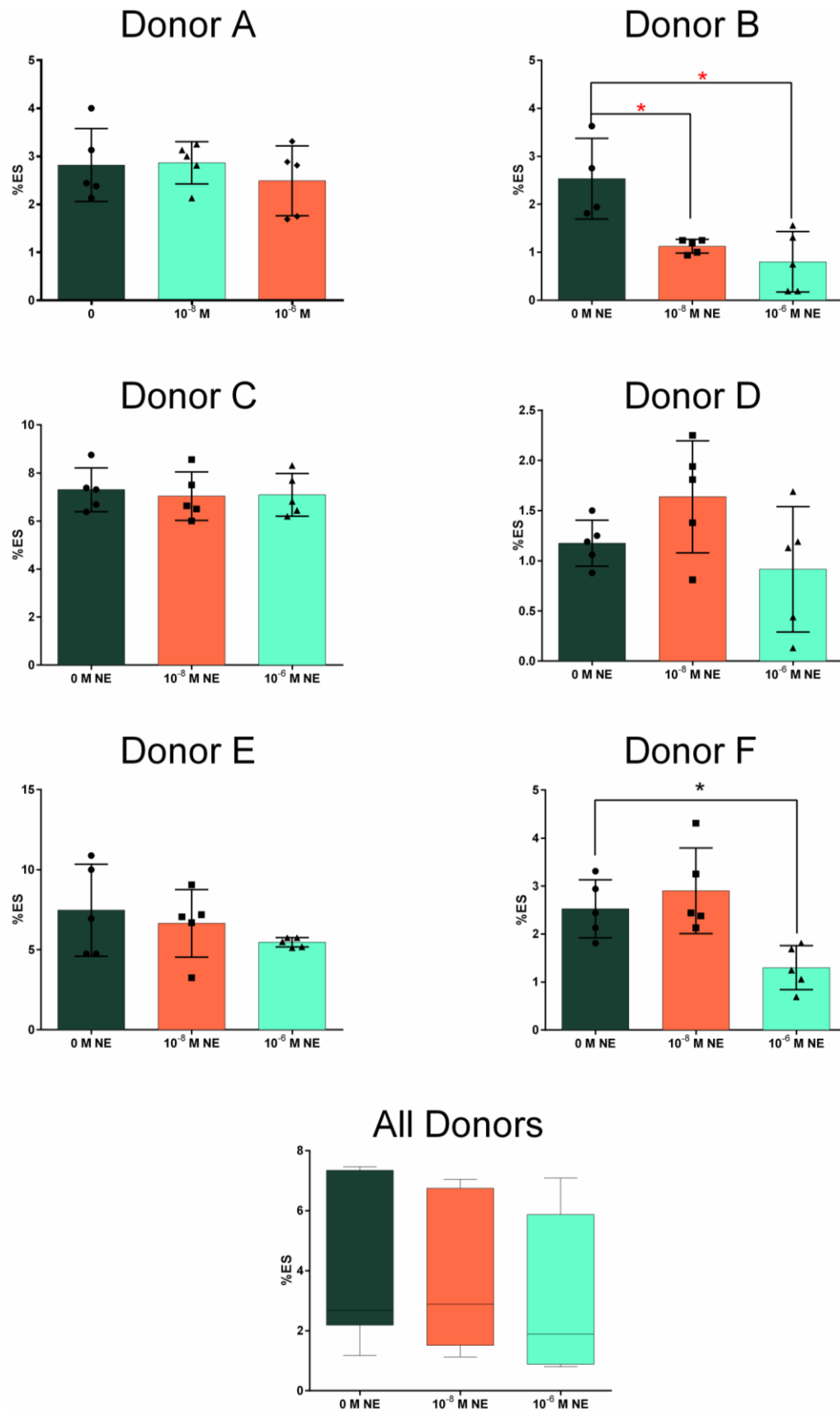


Figure A. 2 - Direct effect of NE treatment on OC activity of all 6 donors. Resorptive activity was quantified by the percentage of eroded surface (ES) per total bone surface. OCs were treated with 0, 10^{-8} and 10^{-6} M of NE. Data is expressed as mean±SD from donor A to donor F graph. The mean of 5 bone slices of each condition from each donor was grouped and data is expressed as min to max n=6 (5 bone slices per condition).

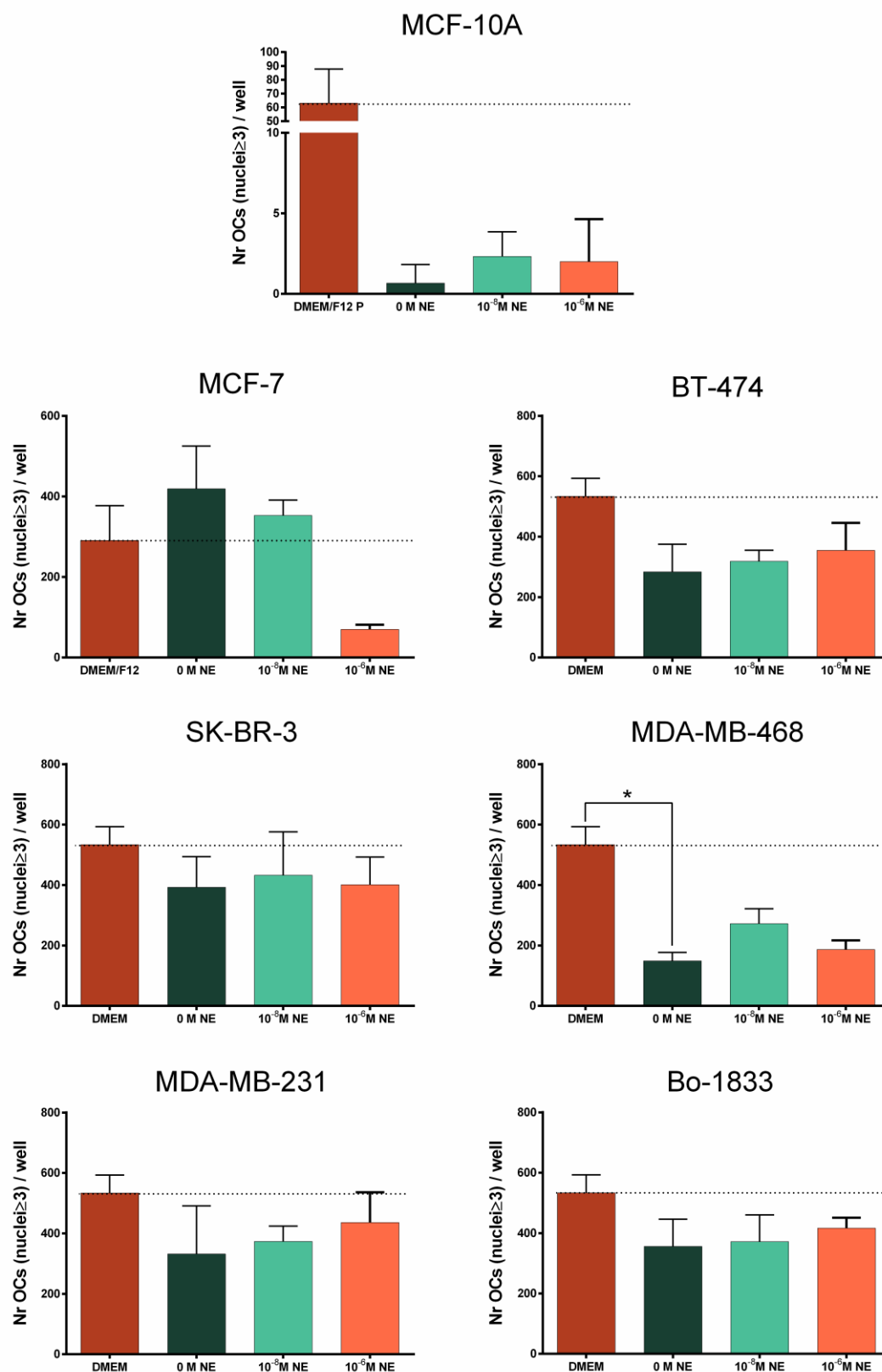


Figure A. 3 – Effects of BrCa cells treated with NE CM on OCs number at early differentiation stage.

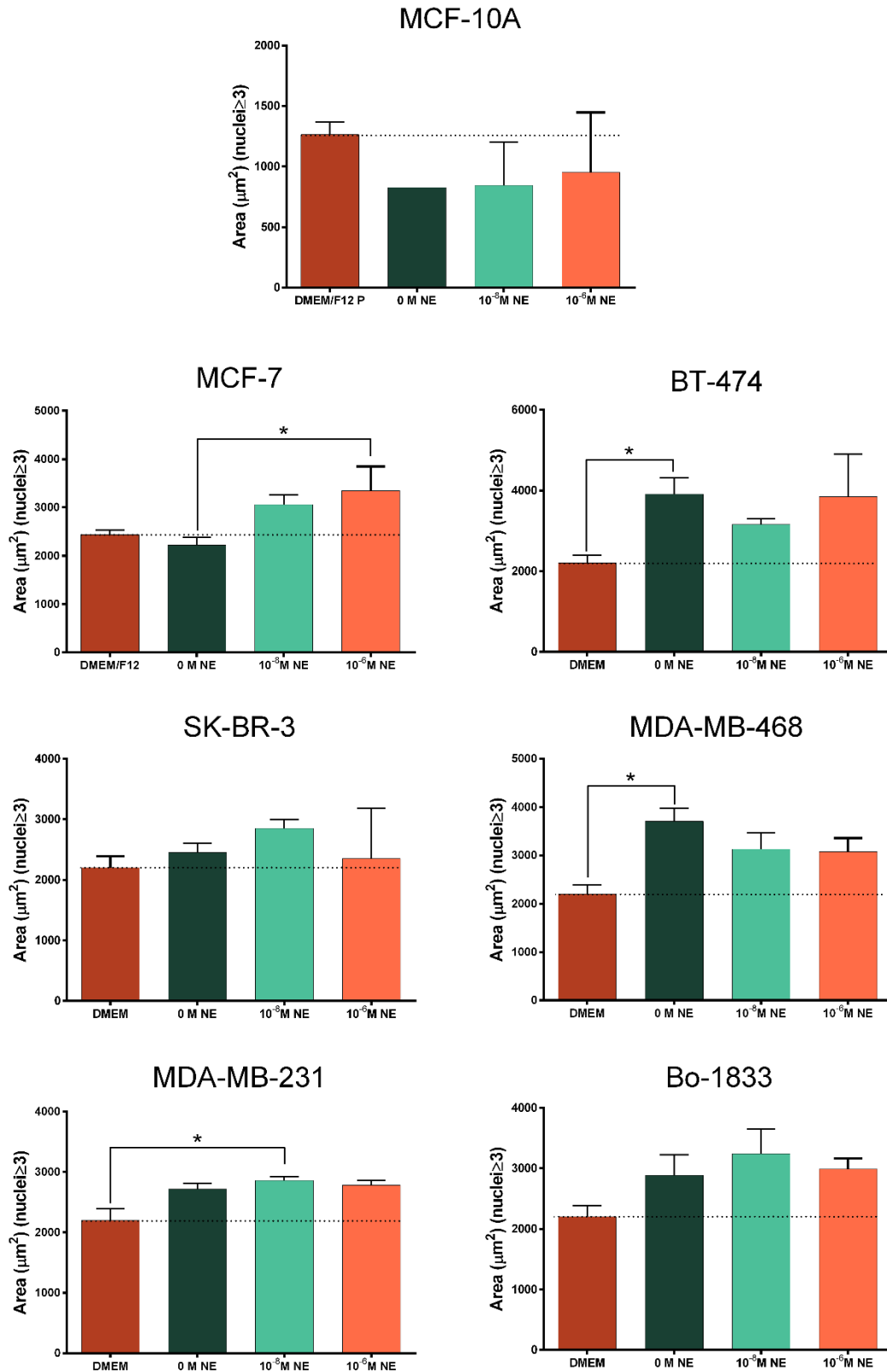


Figure A. 4 – Effects of BrCa cells treated with NE CM on OCs area at early differentiation stage.

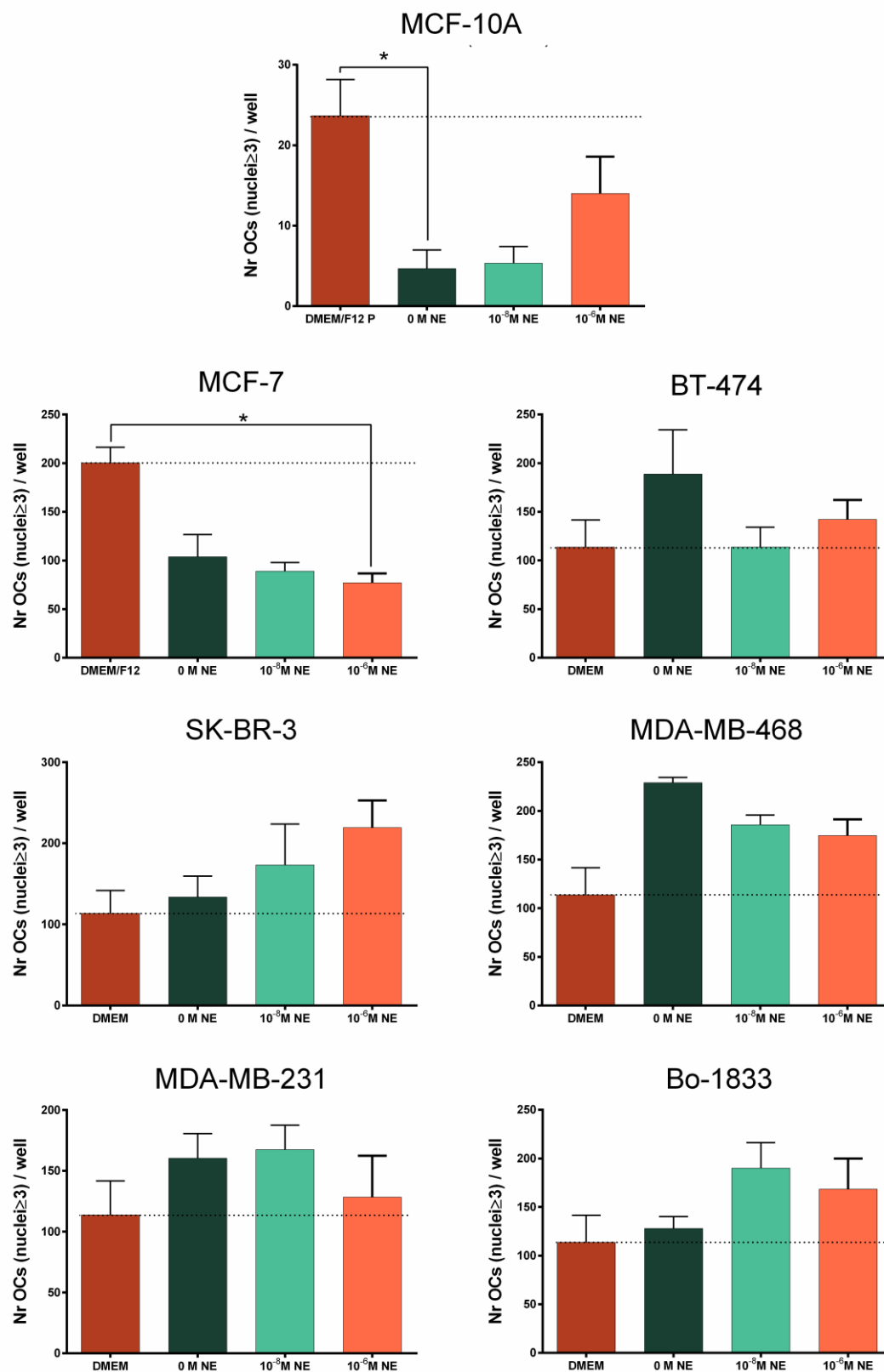


Figure A. 5 – Effects of BrCa cells treated with NE CM on OCs number at late differentiation stage.

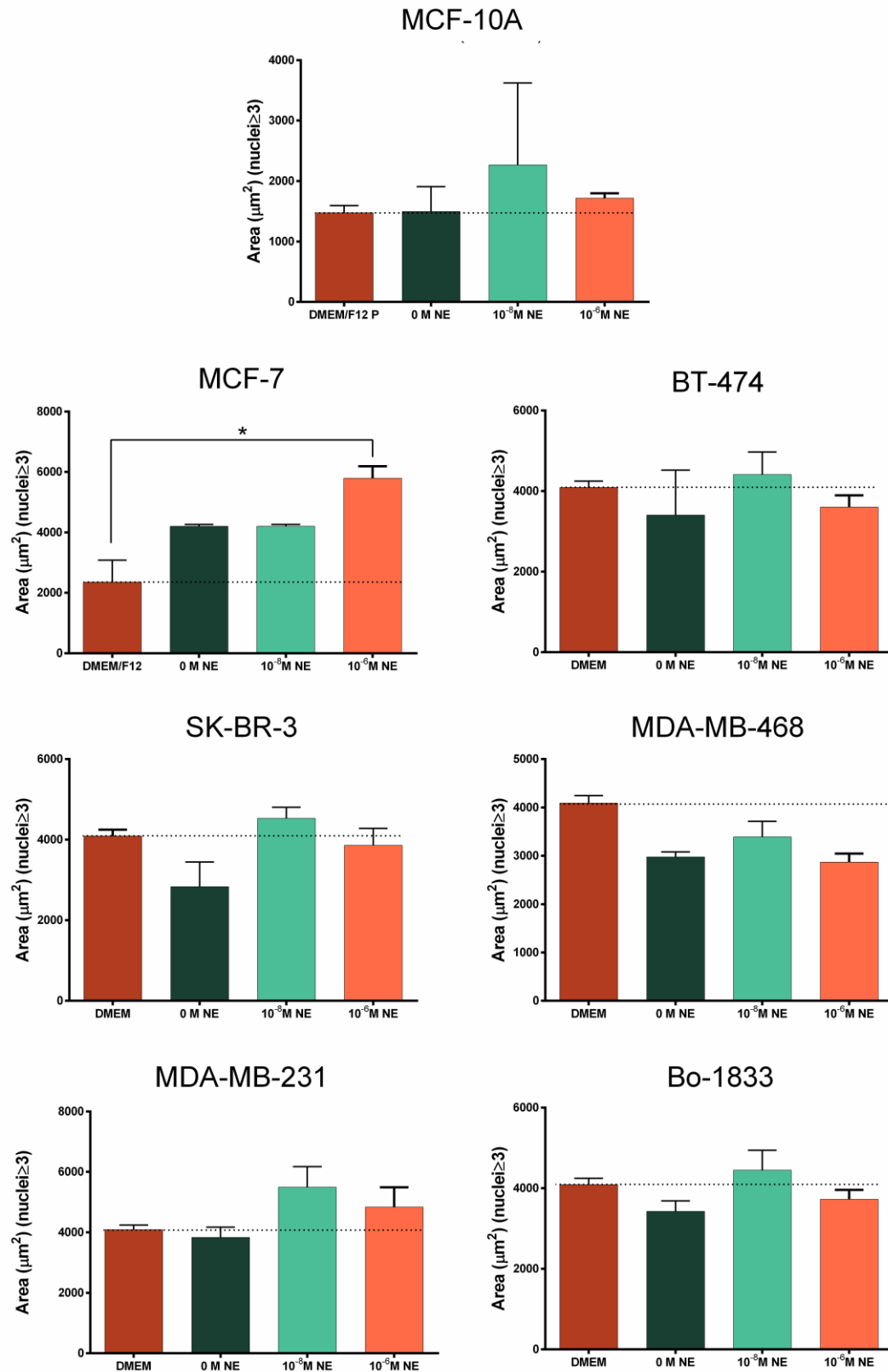


Figure A. 6 – Effects of BrCa cells treated with NE CM on OCs area at late differentiation stage.