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DEPARTAMENTO DE BIOTECNOLOGÍA



**THE ROLE OF THE LONG NON-CODING RNA *NEAR1*  
IN BREAST CANCER DISSEMINATION TO THE BRAIN.**

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## Abstract

Metastatic breast cancer is an incurable disease, which carries a particularly poor prognosis when it occurs in the brain. Brain metastasis most commonly arises from ER<sup>-</sup>/PR<sup>-</sup>/Her2<sup>-</sup> (triple negative or TNBC) malignancies. Long non-coding RNAs (lncRNAs) represent a vast and largely unknown region of the human genome and are emerging as key mediators of cancer biology. Some lncRNAs play a key role in metastasis. Using bioinformatic predictions and analysis of clinical datasets, we identified a short-list of lncRNAs associated with the metastatic progression of TNBC and selected *NEAR1* as the strongest candidate based on the differential expression between our two cell lines: the parental MDA-MB-231, a broadly studied TNBC model which does not metastasise to the brain, and the MDA-MB-231BR sub-clone, selected for its ability to form brain metastasis *in vivo* (100% rate). *NEAR1* was the most up-regulated lncRNA and higher expression of this lncRNA is associated with poorer prognosis in Overall and Distant Metastasis Free Survival, according to clinical data. The aim of this project is to characterize the role of *NEAR1* in tumour dissemination to the brain. To do so, we have measured the effects of silencing this lncRNA on cell proliferation, metastatic potential and adhesion to endothelial brain hCMEC/D3 cells. The results show that *NEAR1* might have a significant role in cell proliferation after 7 days which correlates with results obtained when its migration potential was measured. *NEAR1* does not have any significant role in brain endothelial cell adhesion. These results indicate that, although *NEAR1* over-expression correlates with worse overall survival and has an effect in proliferation and migration, further studies are needed to understand its role in tumour invasion and brain colonisation

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## Introduction.

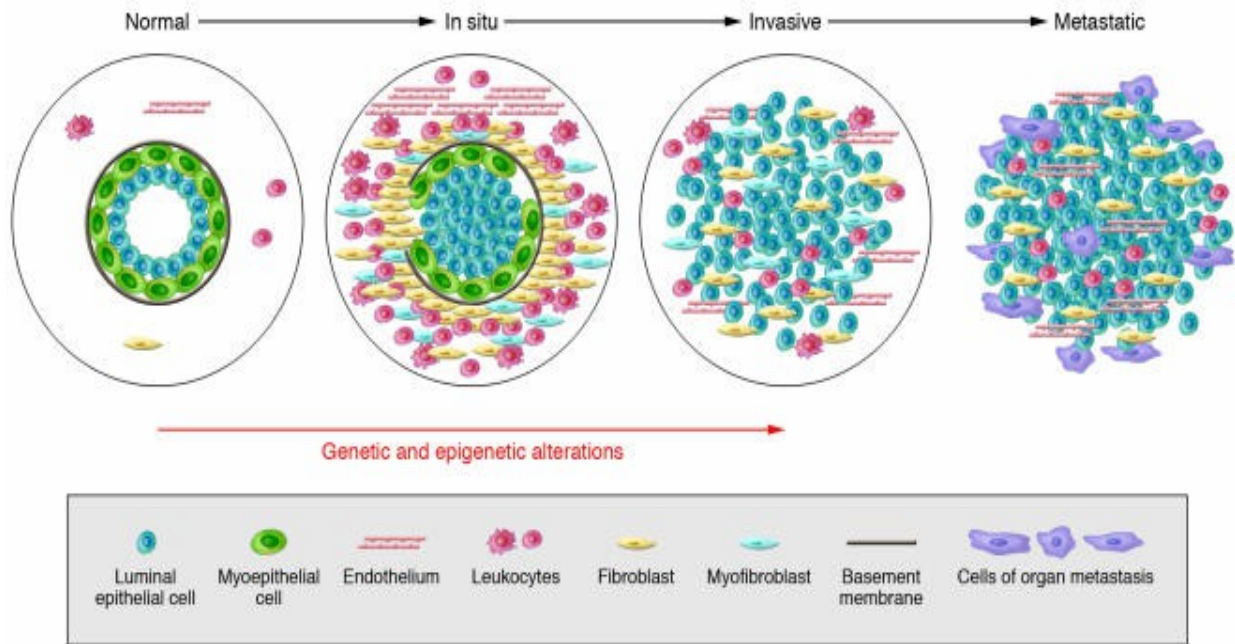
### Breast cancer

Breast cancer (BC) is not a single disease, but rather is composed of distinct subtypes associated with different clinical outcomes. Understanding this heterogeneity is key for the development of targeted preventive and therapeutic interventions.

BC is the result of an accumulation of a large number of individual genetic mutations that together, they alter elements of the complex internal signalling system of a single breast cell. Recurrent division of this cell results in the formation of a colony of aberrant cells which disrupted regulation makes them more prone to accumulate other mutations to eventually develop BC. Although tumour initiation and progression are predominantly driven by acquired genetic alterations, recent data implicate a role for micro-environmental and epigenetic changes as well, e.g. female sex hormones (oestrogen and progesterone) control the rate of mitosis and therefore influence the rate that mutations occur.

There are several types of tumours that may develop within different areas of the breast. Most of them, are benign (fibrosis is only the formation of scar-like connective tissue) and might induce lumpiness or breast pain with no further complications.

The natural development of BC involves progression through defined pathological and clinical stages, starting with ductal hyperproliferation, which evolves into Ductal Carcinoma *In situ* (DCIS), a non-invasive BC, whose origin relies inside the milk ducts of the breast and does not invade surrounding fatty and connective tissue (most common form of non-invasive breast cancer. This DCIS is, simultaneously, thought to be the precursor of Invasive Ductal Carcinomas (IDC), when the tumour cells penetrate the wall of the duct, invading the fatty tissue of the breast (most common type of BC, 80% of diagnosed patients) and finally into metastatic disease, the stage where the disease has spread to distant sites beyond the axillary lymph nodes (Figure 1).<sup>4</sup>



Polyak K. 2007

Figure 1. Hypothetical model of BC tumour progression through NCI, IDC and metastatic carcinoma. Normal breast ducts are composed of the basement membrane and a layer of luminal epithelial and myoepithelial cells. Cells that form the stroma include various leukocytes, fibroblasts, myofibroblasts and endothelial cells. In NCI, the myoepithelial cells are epigenetically and phenotypically altered and their number decreases, because of degradation of the basement membrane. At the same time, the number of stromal fibroblasts, myofibroblasts, lymphocytes and endothelial cells increase. Loss of myoepithelial cells and basement membrane results in IDC, where tumour cells can invade surrounding tissues and migrate to distant organs, eventually leading to metastases.

### BC subtype classification

BC classification into clinically relevant subtypes has always been an important task for therapeutic decision. Accumulating evidence has indicated that histopathological and biological characteristics exhibit distinct behaviours that lead to different treatment responses. Consequently, they should be given different therapeutic strategies.

First categorisations were based in traditional clinicopathological variables (tumour size, grade and nodal involvement), together with the presence or absence of classical immunohistochemistry (IHC) reception markers such as oestrogen receptor (ER),

progesterone receptor (PR) and over-expression of human epidermal growth factor receptor 2 (HER2); those who lack all three markers are denominated triple negative breast cancer (TNBC).

In general terms, those cancers that express either ER, PR and/or HER2 are targetable with therapies directed against these receptors, whilst TNBC treatment remains restricted to traditional chemotherapy.<sup>68</sup>

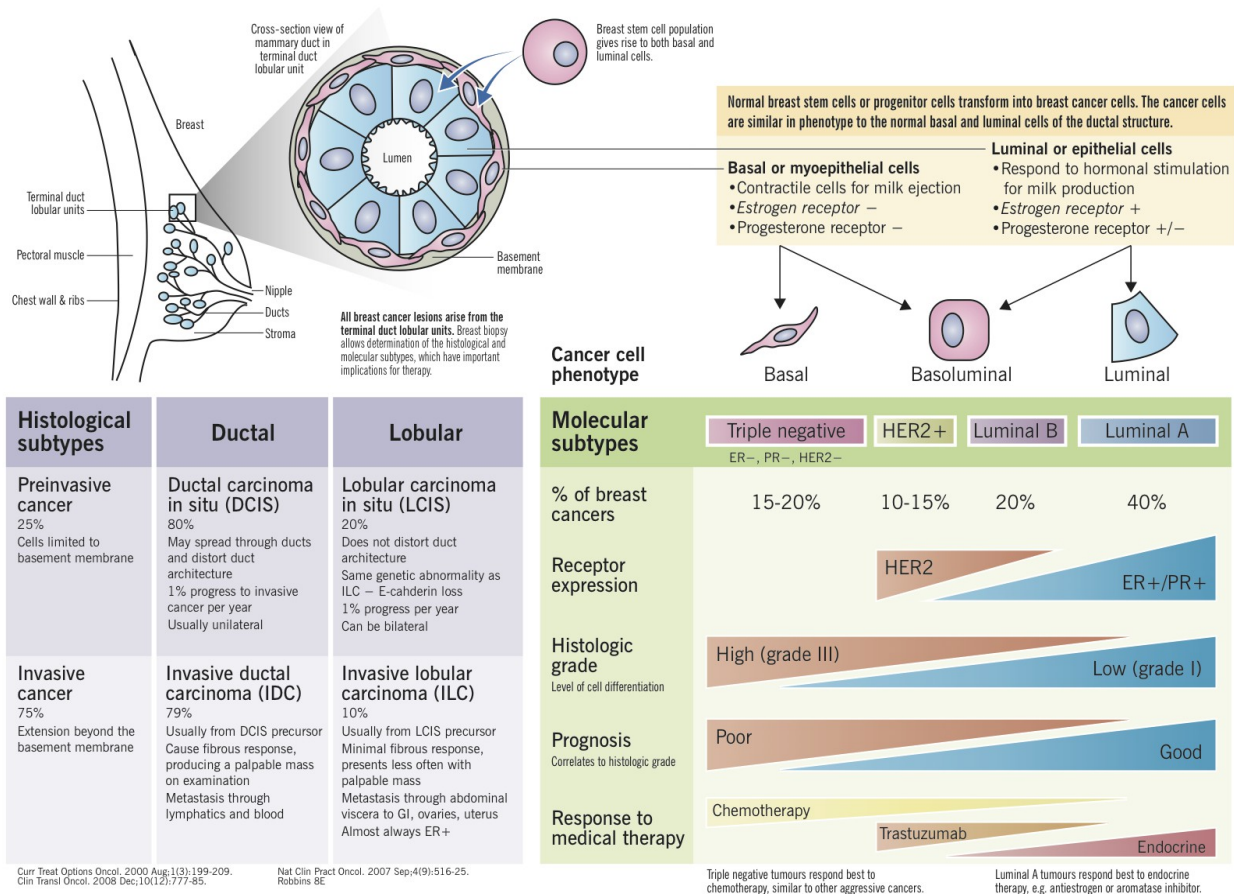
Currently, this classification has evolved as a consequence of expression array analysis techniques, which lead to the classification of BC according to four main intrinsic molecular subtypes according to patterns of gene expression (Figure 2):

- **Luminal A** subtype is hormone-receptor positive (ER<sup>+</sup> and/or PR<sup>+</sup>), HER2 negative (HER2<sup>-</sup>) and has low levels of the protein Ki-67, which helps in tumour growth control. Luminal A are considered low-grade, tend to grow slowly and have the best prognosis. They represent 54.3% of all patients.
- **Luminal B** subtype is also hormone-receptor positive (ER<sup>+</sup> and/or PR<sup>+</sup>) and either HER2<sup>+</sup> or HER2<sup>-</sup> with high levels of Ki-67 protein. Luminal B tend to grow slightly faster and their prognosis is worse and in terms of treatment, the approach varies from luminal A.
- **HER2+** subtype is hormone-receptor negative (ER<sup>-</sup>,PR<sup>-</sup>) and over-express HER2 and, with it, other genes in the same amplicon such as *GRB7* and *PGAP3*; between 40% and 80% of these tumours harbour TP53 mutation. Although they carry a poor prognosis, they are sensitive to anthracycline and taxane-based neoadjuvant chemotherapy, as well as Trastuzumab, a monoclonal antibody against HER2 (some HER2+ tumours are Trastuzumab resistant).
- **Basal-like** subtype is ER<sup>-</sup>, PR<sup>-</sup>, HER2<sup>-</sup> (it accounts for 60% to 90% of TNBC cases) and show expression profiles that mimic the basal epithelial cells of other parts of the body and breast myoepithelial cells (hormone-receptor and HER2 negative expression pattern, proliferation related genes and high expression of basal markers such as keratins 5, 6, 14, 17 and EGFR). This tumours follow an aggressive clinical

course, a lower disease-specific survival and higher risk of local and regional relapse. The metastasis pattern also show a tendency towards visceral organs (and bone) and less lymph node involvement. Given their triple negative receptor status, this tumours are not amenable to conventional targeted therapies, leaving chemotherapy the only option. <sup>41</sup>

## Breast cancer pathogenesis and histologic vs. molecular subtypes

Eric Wong and Jenna Rebelo



Erik W. 2012

Figure 2. Schematic representation of breast anatomy. Histological and molecular and molecular classification of BC. The mammary gland is composed of lobules (glandular milk-producing structures) which drain into a system of ducts, which are connected to the nipple. Between them, the breast contains fat and connective tissue. BC arises from the terminal duct lobular units and when normal breast cells transform into cancer cells, they conserve similar phenotype as the ductal structure that they developed from, giving them the characteristics for their histological classification. However, molecular BC subtypes are more complex and not only stand for their IHC



features but for the presence/absence of signature receptors. Dividing BC in these four subtypes has allowed to assign each one of them an estimated level of aggressiveness, prognosis prediction and response and/or future resistance to medical therapy.

## **Incidence worldwide**

BC is the leading cause of cancer-related death among females and the second most common worldwide.<sup>96</sup>In 2012, more than 1.67 million new cancer cases were diagnosed (25% of all cancers). This incidence varies up to 10-folds across the world regions, with rates ranging from 27/100,000 in Middle Africa and Eastern Asia to 92/100,000 in Northern America and Western Europe. Despite mortality rates between world regions may differ up to 4-fold increased in less developed countries (from 6 per 100,000 in Eastern Asia to 20 per 100,000 in Western Africa)<sup>45</sup> (Figure 3B), there is only a slight difference in terms of incidence between High Income Countries (HICs, 883,000) and less developed regions (794,000) being higher in the first group (Figure 3A), which can be explained by the efficiency of BC screening programs and a higher prevalence of the known risk factors.

## **Risk factors**

The first risk factors of developing BC starts with the fact of being a woman. In fact, only in 2017, the American Cancer Society, estimated 252,710 new cases of invasive breast cancer would be diagnosed among women and 2,470 cases in men. In addition, 63,410 cases of *in situ* breast carcinoma would be diagnosed among women. Approximately 40,610 women and 460 men were estimated to die from breast cancer that year.<sup>1</sup>

Other risks include age (2 out of 3 invasive BC are detected in women 55 or older), overweight (obese women, BMI > 25, have higher risks of developing BC and increased risk of recurrence), ethnicity (African American women are more likely to develop more aggressive tumours that start at younger ages), smoking and alcohol, sedentary lifestyle and diet.

There is a specific group of risk factors that have in common that they are all caused by longer exposure to oestrogen due to changes in lifestyle. This, includes: late pregnancy and

decreasing number of children (women who haven't had a full-term pregnancy or have their first child after 30 have higher risks of BC), breastfeeding reduces the risks (specially when it is done for more than a year), use of hormonal contraceptives (for an extended period and/or early on age) or Hormonal Replacement Therapy (HRT) in menopausal women (oestrogen-only HRT increases BC risk 10% only when used more than 10 years but, combination oestrogen-progesterone HRT increases the risk up to 75% probability). Also, established hormonal factors like starting menstruation at a younger age and/or going through menopause later in life.

The last risk factor is genetic predisposition. Between 5% and 10% of cancer cases are hereditary, specially mutations in *BRCA1* and *BRCA2* DNA repairing genes (women with a mutation in one of those genes or both, increase their risk up to an 80% during their lifetimes and the tumour associated tends to start at younger ages and occur in both breasts. Other commonly BC related genes are *ATM*, *BARD1*, *BRIP1*, *CDH1*, *CHEK2*, *MRE11A*, *MSH6*, *NBN*, *PALB2*, *PTEN* (mutations in this gene increase risk up to 85%), *RAD51C*, *RAD50* and *TP53* (the cancer risk in women with mutations in this gene is up to nearly 100%, and in men is 73%).<sup>2</sup>

## **Diagnostic**

BC is sometimes found after first symptoms appear, but many women with BC show no symptoms. In that matter, early detection is key, and that is achieved with regular BC screening programs.

According to the American Cancer Society's recommendations, clinical and/or self breast examination has not shown any significantly change in finding BC, nevertheless, they help in rising awareness on women getting familiar with their breasts and any possible change.

Regular mammography screening can prevent BC deaths by detecting solid tumours at an early stage when treatment is more effective (relative reductions in BC death are reduced between 13% and 25%). It is recommended in most HICs. However, many studies reviewed the evidence on mammography screening, concluded that harms, (undetected cancers, false

positives and over-diagnosis) outweighed benefits(Using UK population data of 2007, for 1,000 women invited to biennial mammography screening for 20 years from age 50, only between 2 and 3 women are prevented from dying of BC while 200 tumours diagnosed were false positives).

When screening is recommended, the age range differs between countries from 40 to 74 years and the recommended interval varies from 1 to 3 years.<sup>56</sup>

Women at high risk (25% or greater based in family history), that have themselves (or a first-degree relative) a known *BRCA1/BRCA2* mutation, a known risk-increasing disease (Li-Fraumeni or Cowden syndromes) or having a personal history of BC are recommended to supplement mammography with other imaging modalities that have demonstrated better results on detecting mammographically occult cancers. Magnetic Resonance Imaging (MRI) has a higher sensitivity for malignancy (84.6%) than mammography (38.6%) or ultrasound (39.6%). Further, the use of MRI in combination with mammography has a higher sensitivity (92.7%) than the use of ultrasound as an adjunct to mammography (52%).<sup>66</sup>

## **Treatment**

The treatment of BC patients can be personalised by combining structural and functional information from imaging with immunohistochemical markers and gene expression to make a personalised treatment planning and response assessment.

The primary means of local and regional breast cancer treatment remains surgical intervention. During the first half of the 20th century, women diagnosed with BC were commonly treated by radical mastectomy. However, since breast-conserving surgery (BCS) was shown to offer women the exact same long-term survival as those who had a mastectomy, lumpectomy, the removal of the tumour and a small cancer-free margin of healthy tissue combined with radiation therapy is generally the recommendation to downstage the primary tumour and make breast conservation possible.<sup>66</sup> The lymph nodes will then need to be evaluated. Most patients with invasive cancer will have either a sentinel lymph node biopsy or an axillary lymph node dissection. <sup>2</sup>

Radiation therapy has shown to reduce the risk of BC recurrence compared with no radiation therapy. However, it does not lengthen women's lives. This therapy has a role in the regional control of nodal disease in many patients with node-positive stage II or stage III BC or with high-risk factors for local-regional recurrence (lymphovascular invasion, young age, high grade tumours or hormone receptor- negative BC). As the primary risk area for regional recurrences is the supra-clavicular and high axillary region, radiation is usually directed to this areas. This radiation can be given before surgery (neoadjuvant radiation therapy) to shrink the tumour to a smaller size to make it easier to remove or when surgery is not possible, or after surgery (adjuvant radiation therapy).<sup>2</sup>

Chemotherapy is mostly used and shows best results when used in combination with other drugs, either before surgery (neoadjuvant chemotherapy) to shrink the tumour in case of locally advanced BC, and also to test its response and study a different approach if a response is not shown. Also, after surgery (adjuvant chemotherapy) to kill any remaining malignant cells and prevent further relapses. This treatment is generally recommended for patients with disease at high risk of recurrence or advanced BC (ER<sup>-</sup>/PR<sup>-</sup>, HER2<sup>-</sup>, large HER2<sup>+</sup> and lymph node-positive BC). RNA-based genomic testing might be considered for patients with ER<sup>+</sup> or lymph node-negative tumours to estimate the risk of a distant recurrence as well as determine if they will benefit from chemotherapy.

Endocrine treatment is usually considered a standard choice for patients with advanced ER<sup>+</sup> BC and non life-threatening disease, or for older patients who are unfit for aggressive chemotherapy regimens.<sup>63</sup> The effectiveness of this treatment relies in the presence of oestrogen (ER<sup>+</sup>) and/or progesterone (PR<sup>+</sup>) receptors, which are about 60-75% of BC cases. Hormonal therapy can be very efficient, unfortunately, the most efficacious combination of hormonal therapies and chemotherapy has yet to be determined, and resistance to endocrine therapy occurs in most patients. Table 1 summarises the main specific drugs for chemoprevention in BC and their molecular targets, including Aromatase, Tamoxifen and cytokines, the most broadly used in oncological medicine.<sup>63</sup>

Table 1. Main molecular targets and specific hormonal therapy drugs used in hormone-receptor positive BC.

Molecular target	Main drugs available
COX-2	<sup>1</sup> Celecoxib, <sup>1</sup> non-steroidal anti-inflammatory drugs (NSAID), <sup>2</sup> rofecoxib
EP	<sup>1</sup> ONO-8711
HDAC	<sup>1</sup> Suberoylanilide hydroxamic acid (SAH)
Aromatase	<sup>2</sup> Anastrozole, <sup>2</sup> exemestane, <sup>2</sup> letrozole
ER- $\alpha$	<sup>2</sup> SERMs (tamoxifen, raloxifene)
ER- $\beta$	<sup>1</sup> Resveratrol, <sup>2</sup> TAS-108
PPAR $\gamma$	<sup>1</sup> Pioglitazone, <sup>1</sup> rosiglitazone
RAR	<sup>1</sup> 9- <i>cis</i> -retinoic acid (9-CRA)
RXR	<sup>1</sup> Bexarotene
HMGCoA reductase	<sup>1</sup> Statins
TGF- $\beta$	<sup>1</sup> CDDO
VEGF / VEGF receptor	<sup>2</sup> Bevacizumab

Modified from [12]. <sup>1</sup>Preclinical Use, Isolated Experiences or Experimental Use; <sup>2</sup>clinical Use. COX=cyclooxygenase; EP=EP type prostaglandin E<sub>2</sub> receptor; ONO-8711=a type of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) receptor EP<sub>1</sub>-selective antagonist; HDAC=histone deacetylase; ER=estrogen receptor; SERMs=selective estrogen receptor modulators; TAS-108=(7 $\alpha$ )-21-[4-[(diethylamino)methyl]-2-methoxyphenoxy]-7-methyl-19-norpregna-1,3,5(10)-trien-3-ol 2-hydroxy-1,2,3-propanetricarboxylate; PPAR $\gamma$ = peroxisome proliferator-activated receptor gamma; RAR=retinoic acid receptor; RXR=retinoid X receptor; HMGCoA=3-hydroxy-3-methylglutaryl-coenzyme A; TGF- $\beta$ =transforming growth factor beta; CDDO= synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid; VEGF= vascular endothelial growth factor.

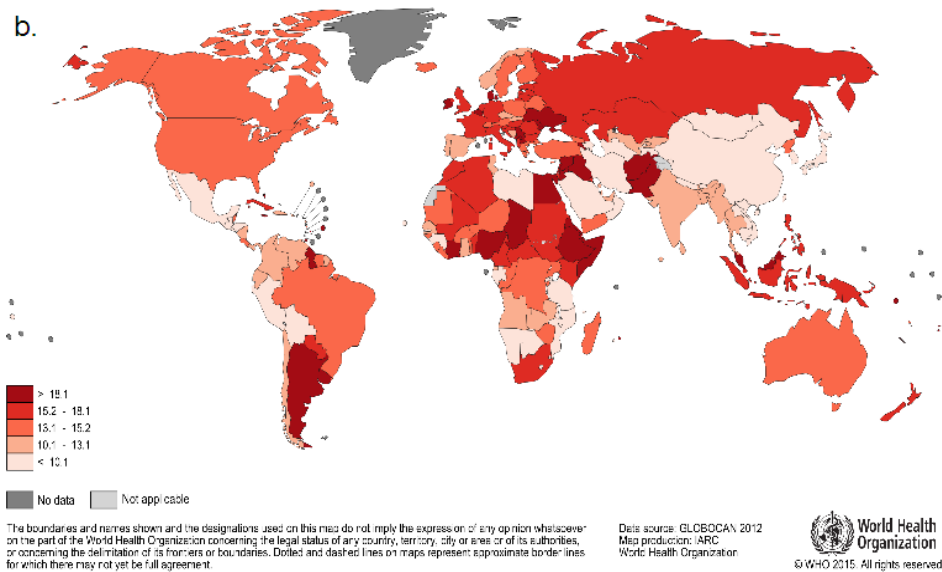
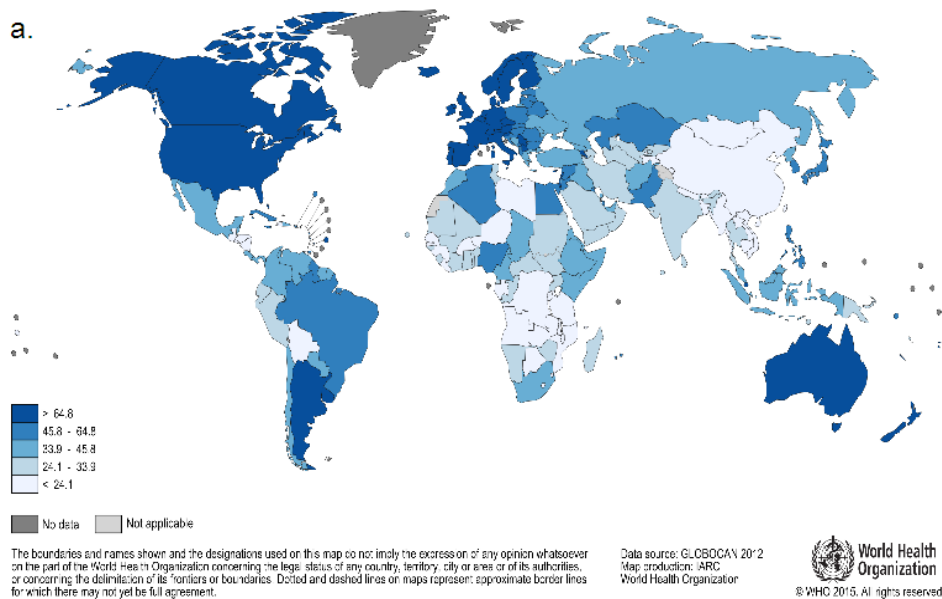
Lumachi et al. 2011

Targeted therapy is another option to prevent BC patients from the side effects that systemic chemotherapy therapy carries with it, due to its unspecificity. On the contrary, targeted therapy acts against specific genes, proteins or the tissue environment. For example, the most common used targeted drug in HER2<sup>+</sup> BC tumours is Trastuzumab (or Herceptin), a monoclonal antibody against HER2 which and is recommended to treat non-metastatic early-stage BC that express that protein. Currently, patients with stage I to stage III BC, are recommended to receive trastuzumab in combination with chemotherapy from 6 months to a year. Although this treatment and other HER2 targeted therapies are associated with highest efficacy, only patients with the highest levels of HER2 (20% of all BC patients) show a significant response. Furthermore, many patients with high HER2 levels, despite receiving this treatment, can present relapse after 8 months.<sup>61</sup>

Although BC has historically been considered immunologically silent, several preclinical and clinical studies suggest that immunotherapy can represent a new approach for the

treatment of some patients.<sup>105</sup> At the present time, there are 23 active clinical trials on BC immunotherapy.

Despite this vast availability, there still are patients with ER<sup>+</sup>/PR<sup>+</sup> BC refractory to endocrine therapy, about 15% of the total BC patients are TNBC and targeted therapeutic options remain quite limited. New therapeutic strategies for BC are needed to improve clinical outcomes, particularly those with advanced disease.



GLOBOCAN, 2012 (IARC)

Figure 3. Statistics of incidence and mortality in BC worldwide, 2012. Whilst the incidence rate remains similar between HICs and low developed countries thanks to implantation of screening programs (A), the mortality rate is higher in the second group (B)

### **Triple Negative Breast Cancer**

Triple Negative Breast Cancer (TNBC) is the most aggressive form of BC manifestation. Although they comprise a very heterogeneous group, it is defined by the absence of all of both hormone receptors (ER<sup>-</sup>/PR<sup>-</sup>) and HER2<sup>-</sup> and represents approximately 15% to 25% of all BC cases. It is believed that most TNBC are of the basal-like subtype.

As a consequence of this poor differentiation, TNBC is most aggressive of all types of BC. They show high rates of proliferation, show a shorter median time from relapse to death and lack of currently identified specific treatment targets, reducing the standard treatment options to cytotoxic chemotherapy (adding taxanes appears to improve outcomes, as well as platinum-containing regimens) and joining clinical trials. However, the lack of prospective randomized data prevents medical oncologists to develop an optimal standard-of care regimen in patients with invasive TNBC.<sup>102</sup>

Clinical data have shown that TNBC is more likely to invade adjacent connective tissue. Furthermore, patients with recurring and/or metastatic TNBC are more likely to develop visceral and brain metastases.<sup>1</sup> Five-year survival studies published in 2007 of more than 1,600 women found that TNBC had about a 20% increased risk of death, which became stable after that period.

### **Brain metastasis in TNBC**

Breast cancer brain metastasis (BCBM) is also known as BC in stage IV, which means that has spread beyond the breast and invaded nearby lymph nodes and migrated to other organs through blood and/or lymphatic systems. Distant metastasis are the responsible for more

than the 90% of BC-related death. In BC, it primarily metastasises to the bone (70%), lung (15%), liver (12%) and brain (3%).<sup>2</sup>

Despite its low incidence, progression in the central nervous system (CNS) has become the major life-limiting problem. BC is the second most common cancer associated with brain metastasis in HIC countries and, as the OS of an advanced BC patient increases, the incidence of brain metastasis rises as well.

The standard treatment for BCBM is still restricted to local treatment with a combination of surgery and radiotherapy. However, systemic therapy may still be an option to suppress disease further progression. The prognosis of these patients is still very poor, with median survival times of 4 to 6 months with brain irradiation treatment and only 2 months without it, where they are limited to palliative care.<sup>7,94</sup>

Moreover, retrospective studies have demonstrated that clinically, patients with TNBC have different proportions in distant metastasis destinations and this data is highly remarkable in brain. The incidence of brain metastasis changes from a 3% BC in general, but when TNBC patients were evaluated, they have found that this rate increases up to 36.4%.<sup>14</sup>

Many studies have investigated cancer cell intrinsic mechanisms alone or their interaction with extrinsic micro-environmental factors that enhance the metastatic potential of primary tumour cells. It is known that the metastatic cascade comprises a series of steps to accomplish invasion, migration, dissemination and colonisation of distant organs. However, and the regulation underneath this process still remains incomplete.<sup>24,30,35</sup>



## Long non-coding RNAs

Since the human genome was sequenced, one of the most unexpected discoveries was to realise that it only encodes about 20,000 protein-coding genes, being less than 2% of the total and the majority of the remaining sequence was considered transcriptional noise. Later on, with transcriptome sequencing technologies, this 90% was found to be actively transcribed. In fact, it was more complex than expected, showing extensive anti-sense, overlapping and non-coding RNA expression. More than that, this “dark matter” was discovered to play a major biological role in cellular development and metabolism. Specifically, long non-coding RNAs (lncRNAs) have been shown to play a major role in developmental and tissue specific expression patterns, immune response, as well as influence a high number of human diseases, including cancer.<sup>94</sup>

lncRNAs are defined as transcripts ranging in length from 200nt to ~100 Kb that lack significant open reading frames (ORFs). Many identified lncRNAs are transcribed by RNA polymerase II and are polyadenylated, but there might be some exceptions. Generally, lncRNA expression levels are lower than protein-coding genes and tend to be under weaker evolutionary constraint.<sup>19</sup> However, there is a 3% of lncRNAs that appear to have originated more than 300 million years ago and can be found between species. Despite RNAs in general and non-coding RNAs in particular need less sequence conservation to maintain their function compared to proteins, there is high sequence conservation of lncRNA promoters (higher than protein promoters), which correlates with the hypothesis that regulation of lncRNA expression is important.

Many lncRNAs carry features reminiscent of protein-coding genes, such as 5' cap and alternative splicing. In fact, many lncRNA precursor genes have two or more exons and about 60% of lncRNAs have polyA tail. In fact, lncRNAs are distinguished from other non-coding RNAs for being located within 10Kb of protein-coding genes and many lncRNAs are anti-sense to coding genes or intronic.<sup>29</sup> They are preferentially enriched in the chromatin and nucleus of the cell, although they can be found in different cellular compartments including the cytoplasm.

LncRNAs have been generally thought to be unstable due to their low expression levels but recent studies indicate that only a minority (29%) of characterised lncRNAs are unstable with half-lives below 2h, while the 62% are extremely stable with half-lives of more than 12h.

While other non-coding RNAs, like miRNAs, have been heavily studied and are well understood for their function in gene regulation in protein translation, little is known about lncRNAs. Although several thousand lncRNAs are transcribed, relatively few have been functionally well characterised.<sup>19</sup>

### **The role of lncRNAs in cancer**

Accumulating studies in a variety of cancers report that aberrant lncRNA expression may be a major contributor to tumour initiation and progression.<sup>42,85</sup> As a result of advances in cancer transcriptome profiling and crescent evidences supporting lncRNA function, a number of differentially expressed lncRNAs have been associated with cancer. The lncRNA *PCGEM1* induces tumorigenesis and progression via *RhoA* up-regulation and oncogene regulation in epithelial ovarian and prostate cancer.<sup>13</sup>

As previously mentioned, lncRNAs regulate a range of biological functions in normal cells and, the disruption of some of these functions, such as genomic transcriptional regulation and imprinting play a critical role in cancer development. The expression of *H19* is high during embryo development but is down-regulated in most tissues shortly after birth with the exception of skeletal tissue and cartilage. Loss of imprinting and a subsequent strong expression when is needed has been documented in oesophagus, colon, liver, bladder and hepatic human cancers.<sup>31</sup>

Further, several studies have suggested that lncRNAs have potential roles as diagnostic and prognostic markers in cancer. Several investigations on large clinical cancer samples have demonstrated that specific lncRNAs such as *HOTAIR* and *GAS5* can influence the outcomes of radiotherapy and act as a valuable prognostic biomarker. In fact, with the exploration going to a deeper and finer status, the role of lncRNAs in the formation of tamoxifen resistance is an object study. In BC, lncRNA *BCAR4* in a tamoxifen-sensitive ER<sup>+</sup> BC cell line blocked the

anti-proliferative effects of tamoxifen, increasing resistance. Accordingly, the effects of other lncRNAs were also studied (lncRNAs *ROR*, *UCA1*, *CCAT2* among others), increasing the list of tamoxifen-resistant regulators.<sup>99</sup>

### **The role of lncRNAs in metastasis**

Understanding metastasis as a multi-step process that requires the participation of transcriptional and translational regulation over time in response to distinct changes in the extracellular environment, is key to comprehend that such a complex series of events imply that not only genetic alterations are responsible for inducing cancer spread, but additional factor must regulate this intricate process. In parallel, an increasing number of studies report that lncRNAs are some of the most differentially expressed transcripts between primary and metastatic cancers. *MALAT1* was initially found over-expressed in lung cancer metastases and correlates with deeper tissue invasion, higher histological grade and shorter metastasis-free survival (MFS). *HOTAIR* over-expression occurs in around 30% breast neoplasms and is strongly associated with metastatic progression by shortening MFS independently of tumour size, stage and molecular subtype.

Although the lncRNAs function may vary extensively in structure and activity between cell types and cancers, their principal function is to physically interact with epigenetic complexes, recruiting them to specific *loci*.

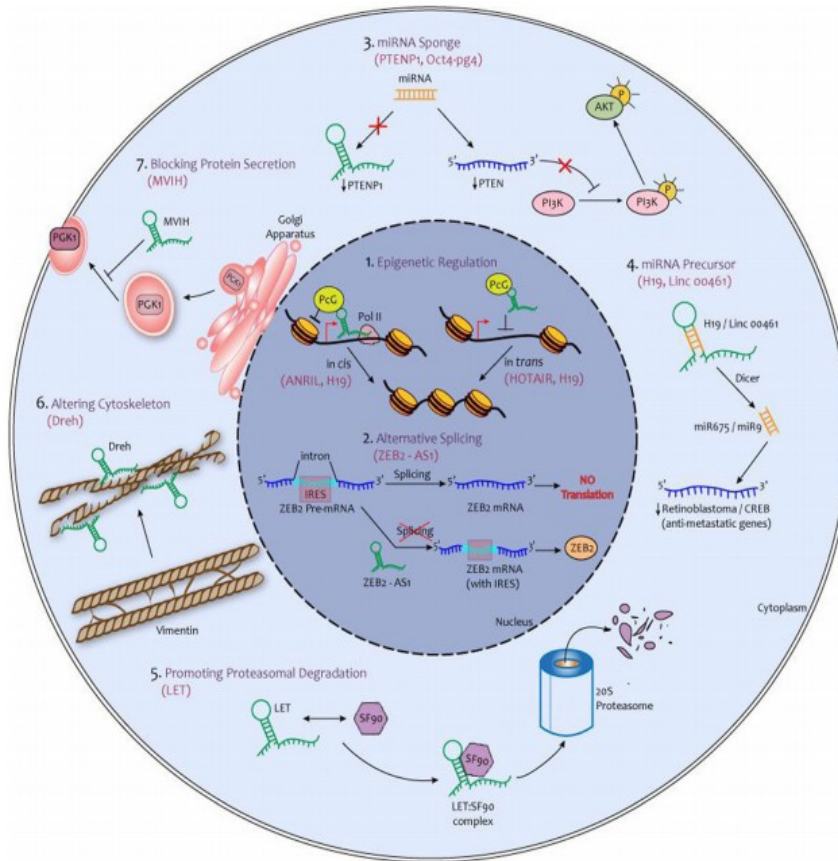
Figure 4 illustrates the mechanisms by which lncRNAs regulate metastatic cancer progression. lncRNA can play an important functional role in epigenetic regulation through interaction with the polycomb repressive complexes (PRC1 and PRC2) or regulation of alternative splicing in the nucleus. In the cytoplasm, they can function as miRNA precursors that are known to have a key role in cancer progression, interacting with proteins and promote its degradation or binding to cytoskeletal proteins and directly alter their structure or also by altering the micro-environment.<sup>15</sup>

## The role of lncRNAs in BC

Despite growing knowledge regarding the molecular mechanisms of lncRNA functions in malignancy, the mechanism of action of most lncRNAs in BC remains unclear.

lncRNAs are involved in mammary gland development (*PINC* is involved in cell survival and cell cycle progression and has been shown to inhibit mammary cell differentiation), as well as BC evolution. Recent studies in BC tumorigenesis have revealed that the lncRNA *ANCR* suppresses tumour migration and invasion via degradation of *EZH2*. On the other side, *CCAT2* promotes BC proliferation and tumour formation through the WNT signalling pathway.

Regarding BC clinical outcome, many lncRNAs have shown to have a role in further classification inside a given BC subtype, e.g. studies targeting lncRNAs as molecular markers for DCIS characterisation. In addition, a number of lncRNAs have been shown to predict prognosis, survival, relapse probability or treatment resistance. In those cases, it has been shown that lncRNA expression signature is independent of age and cancer subtype. According to these discoveries, different lncRNA-based therapies can be designed, each one of them following a specific strategy based on lncRNA function.<sup>88</sup>



Crea et al. 2014

Figure 4. Summarised mechanisms by which lncRNAs can promote cancer metastasis. (1) Epigenetic regulation via interaction with PcG proteins and (2) interruption of alternative splicing are nuclear processes, while (3) acting as miRNA sponge or (4) miRNA precursor, (5) promoting proteasomal degradation, (6) cytoskeletal degradation and (7) blockage of protein secretion are carried out and/or interact with the different cell compartments and cytoplasm.

## Hypothesis

Based on the rationale exposed in the introduction, we conclude that many lncRNAs involved in BC are yet to discover, with special emphasis in lncRNAs which might have a key role in BCBM. This project tries answer the following questions:

1. Which lncRNAs are functionally relevant in BC metastasis to the brain?
2. What role does the chosen lncRNA play in metastatic progression of our TNBC brain metastatic cell line?

That discovery would help us, not only understand the process that an invasive cell goes through and therefore translate this knowledge to clinically relevant strategies like early prevention, design of personalised treatments, etc.

## Aim of the study

To test this hypothesis, we will:

1. Elaborate a short-list of lncRNAs potentially involved in TNBC metastasis to the brain. They will be carefully selected with the combined use of bioinformatic prediction tools and the analysis of clinical datasets, together with literature validation of the results obtained with previously established criteria.
2. Measure differential expression of the selected lncRNAs between two TNBC cellular models: parental MDA-MB-231 and MDA-MB-231BR subclone, which differs from the parental line for its ability to form brain metastasis *in vivo* (100% rate).
3. The most relatively up-regulated lncRNA in MDA-MB-231BR will be selected for further characterisation. For that purpose we will:
  - 3.1 Study the effect of lncRNA over-expression in the prognostic of BC patient cohorts.
  - 3.2 Perform functional studies by modulating its expression and measuring the effects of this manipulation on TNBC metastatic potential: cell proliferation, migration and brain endothelial adhesion.

## **Materials and Methods**

### **Cell lines and culture methods.**

Human breast cancer cell lines MDA-MB-231 and MDA-MB-231BR (basal type, TNBC) were gently given by Nicola R. Sibson (University of Oxford). They were maintained in Dulbecco's modified Eagle's medium (DMEM 1X; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% Pen-Strep (10,000Units/ml Penicillin, 10,000µg/ml; Gibco) and 2mM L-Glutamine (Sigma). They were incubated at 37°C and 5% CO<sub>2</sub> and media was changed after 2 days. Experiments and cell passages were carried out from an 80% confluence T-75 flask and prior to that, cells were washed with Hanks' Balanced Salt Solution (HBSS; Sigma) modified without calcium chloride and magnesium sulfate, detached with 0.25% Trypsin-EDTA (Gibco) and centrifuged for 5min, at 1500rpm, 4°C.

Brain microvascular endothelial hCMEC/D3 cells were grown in Endothelial Basal Medium-2 (EMB-2; Lonza) medium supplemented with 2.5% v/v FBS, 0.04% v/v Hydrocortisone, 0.1% v/v Ascorbic Acid, 0.025% v/v VEGF, 0.1% v/v hFGF-B, 0.025% v/v R3-IGF-1, 0.025% v/v hEGF, 0.1% v/v GA-1000 (EGM™-2 MV Microvascular Endothelial SingleQuots™ Kit; Lonza Walkersville, MD USA) in a previously type I collagen (0.1% solution in 0.1M acetic acid) coated T-75 flask. They were incubated at 37°C and 5% CO<sub>2</sub> and media was changed after 2 days. Cells were washed with Hanks' Balanced Salt Solution (HBSS; Sigma) modified without calcium chloride and magnesium sulfate, detached with 0.25% Trypsin-EDTA (Gibco) and centrifuged for 5min, at 1500rpm, 4°C.

### **RNA isolation.**

MDA-MB-231 and MDA-MB-231BR cells (250,000/well; total volume 2ml) were seeded for 48 hours in a 6-well plate. After that, the Rneasy® Plus Mini Kit (Qiagen) was used for total RNA isolation according to manufacturer's protocol.

### **LncRNA selection and clinical databases.**

In order to elaborate a short-list of candidate lncRNAs to measure differential expression levels between cell lines, lnc2cancer1.0 database <sup>74</sup> was used. A total of 111 lncRNAs shown association with breast cancer, therefore a posterior screening was carried out, in which only the lncRNAs that followed those three criteria were selected: 1) Expressed in BC and/or TNBC, 2) Up-regulated in BC compared to normal tissue, 3) Positive association with metastasis. Other valuable but not discriminative aspects considered were: studies in MDA-MB-231 and/or MDA-MB-231BR cells, brain metastasis association in other tumours, prognosis prediction and Epithelial-Mesenchymal Transition (EMT).

For clinical data of prognosis based on a given lncRNA expression, Kmpot database was used for breast cancer, selecting the following parameters: best cut-off in Overall Survival (OS) and Distant Metastasis-free Survival (DFMS), only JetSet probes and compared ER+ to ER-subtypes.

### **Reverse transcription-PCR (RT-PCR) and quantitative PCR (qPCR).**

Pure RNA isolated from MDA-MB-231 and MDA-MB-231BR (1µg/10µl) was subjected to RT-PCR analysis in 20µl reaction mixture. High capacity cDNA reverse transcription kit (Applied Biosystems 4368814) and the reaction was performed at 25°C for 10min, followed by 37°C for 120min and 85°C for 5min. The resulting cDNA was diluted 1:100 for qPCR analysis. For qPCR, Taqman™ Universal PCR Master Mix (ThermoFisher 4304437) together with Taqman® Gene Expression Assays Human probes were used according to manufacturer's protocol. A total of 16 lncRNA Human probes were used: *FAM225A* (Hs03681728\_s1), *loc286437* (Hs03296631\_m1), *LINC00261 (NEAR1)* (Hs03679073\_m1), H19 (Hs00399294\_g1), *SOX2OT* (Hs00415716\_m1), *CCAT1* (Hs04402620\_m1), *CCAT2* (Hs04403001\_s1), *linc-ROR* (Hs04332550\_m1), *MALAT1* (Hs00273907\_s1), *MIAT* (Hs03300285\_g1), *PCAT18* (Hs03300285\_g1), *ANRIL* (Hs03300540\_m1), *PCAT6* (Hs01054758\_g1), *FOXCUT* (Hs04407100\_g1), *HOTAIR* (Hs03296631\_m1) and *HPRT1* (Hs028006951\_m1) as housekeeping gene. <sup>23,103</sup>The qPCR reaction was performed in an optimised for lncRNA 40-



cycle program of 95°C for 15sec, followed by 60°C for 1min using MJ Opticon Monitor™ Analysis Software.

### **Fractionation assay for *NEAR1* localisation.**

From an 80% confluence cell sample re-suspended in 1ml HBSS, RNA was isolated from each nuclear and cytoplasmic fraction using the PARIS™ Kit (Invitrogen AM1921) according to manufacturer's protocol. After that, qPCR was carried out to validate its localisation, with Taqman® Gene Expression Assays Human probes *HPRT1* and *GAPDH* (Hs02786624\_g1) as cytoplasmic controls and *MALAT1* as nuclear.

### **siRNA reverse-transfection for *NEAR1* silencing.**

MDA-MB-231 and MDA-MB-231BR were seeded in 6-well (250,000/well; total volume 2ml), 24-well (350,000/well; total volume 500µl) or 96-well plates (2,500/well; total volume 100µl) after transfection and incubated for 48h. Knock-down of *NEAR1* was performed using the reverse-transfection method of delivery. The small interference RNA (siRNA) and lipid complex is prepared using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Three different dicer-substrate siRNAs against *NEAR1* were purchased from Integrated DNA Technologies; siRNA1 (hs.Ri.LINC00261.13.1), siRNA2 (hs.Ri.LINC00261.13.2) and siRNA3 (hs.Ri.LINC00261.13.3). siRNA against *HPRT1* was used as positive control (HPRT-S1 DS) and a Scrambled with sequence Sense: rCrGrUrUrArArUrCrGrCrGrUrArUrArArUrArArUrArCrGrCrGrUAT Anti-sense: rArUrArCrGrCrGrUrArUrArCrGrCrGrArUrUrArArCrGrArC was used as negative control. Final siRNA concentration was 20nM diluted in Reduced Serum Medium (Opti-MEM 1X; Gibco).

### **MTT assay for cell proliferation.**

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay was used as an indirect method to evaluate the effect of *NEAR1* silencing on proliferation. MDA-MB-231 and MDA-MB-231BR were transfected with negative control and siRNA3 (hs.Ri.LINC00261.13.3) and seeded in a 96-well plate (2,500/well; total volume 100µl) for 1, 3, 5 and 7 days. After incubation, cells were incubated for 1h with 60µl of fresh media or Dimethyl Sulfoxide (DMSO; Sigma). 10% MTT dye (5mg/ml) were added to the media and cells were incubated at 37°C for 3h. Upon completion, media was discarded and 50µl DMSO was added and gently mixed to homogenize during 10min. Absorbance was measured in a plate reader using Optima Software (BMG Labtech). Spectrophotometer multichromatic filters set at 570nm and 680nm.

### **Wound-healing assay for cell migration.**

MDA-MB-231BR cells were transfected with negative control and siRNA1 (hs.Ri.LINC00261.13.1), siRNA2 (hs.Ri.LINC00261.13.2) and siRNA3 (hs.Ri.LINC00261.13.3) and seeded in a 24-well (350,000/well; total volume 500µl) for 18h until the surface is covered with a monolayer. After incubation, a longitudinal scratch was made using a sterile P20 pipette tip. Pictures (Data Cell; Olympus and QiCam; Qimaging with Image Pro-Plus 7.0 Software at 4X; PhL filter and light at 2.5) were taken at the same 3 different points along the wound immediately after wounding (0h) and 4h, 8h, and 24h until it was completely closed. The images obtained were analysed using the macro "Wound Healing Tool" from ImageJ software package.

### **Cell adhesion assay in static conditions.**

The adhesion assay was carried out using the Cytoselect™ Tumor-endothelium adhesion Assay (Cell Biolabs, INC) with protocol optimisation to breast cancer cell lines. <sup>46</sup> hCMEC/D3 cells were seeded (35,000/well; total volume 100µl) in a previously collagen coated (60µl at R/T for 1h) 96-well plate for 24h until the surface is covered with a mono-layer. After

incubation, media from treatment cells was removed and cells were gently washed with HBSS and we added 25pg/ml of TNF $\alpha$  (10 $\mu$ g/ml) and incubated for 24h.

Wild-type and transfected with negative control and siRNA1 (hs.Ri.LINC00261.13.1), siRNA2 (hs.Ri.LINC00261.13.2) and siRNA3 (hs.Ri.LINC00261.13.3) MDA-MB-231BR cells were seeded in a 6-well (250,000/well; total volume 2ml) for 18h. After incubation, harvest cancer cells. Wild-type cell suspension was counted 0, 1,000, 2,500, 5,000, 10,000, 25,000, 50,000 and 100,000 cells in serum-free media for calibration curve and 0.25x10<sup>5</sup> cells/ml of NC, siRNA1, siRNA2 and siRNA3. A 1X dilution of Cytotracker™ Solution (500X) was prepared and added to a final concentration of 2X to 500 $\mu$ l of media. Different cancer cell solutions were re-suspended in 125 $\mu$ l serum-free growth media and add 125 $\mu$ l of this Cytotracker solution and incubated for 1h.

After incubation, media was removed from hCMEC/D3 cells and washed twice and 200 $\mu$ l of different marked cancer cell samples were added to each well already containing the endothelial mono-layer and incubated for 90min. After that, cells were washed and lysed according to manufacturer's protocol.

Fluorescence was measured in a plate reader using Optima Software (BMG Labtech). Fluorometer filters set at 485nm excitation and 520nm emission.

### **Statistical analysis.**

All statistical analyses were performed using GraphPad Prism 7.04. Results are presented as mean  $\pm$  standard deviation of the mean. Relative gene expression of lncRNAs among the cell lines was calculated using the 2<sup>- $\Delta\Delta$ Ct</sup> method as described in Schmittgen & Livak (2008).<sup>47</sup> Significant differences among groups for the levels of expression of the different lncRNAs were evaluated using one-way ANOVA. For MTT and wound-healing assays, two-way ANOVA was used for evaluation. For every test, most robust multiple comparisons tests were used and the significance level considered was 95% (p=0.05). Every analysis was run using the statistical program Graphpad Prism 7.

## Preliminary results

### Breast Cancer cell lines.

Under standard cell culture conditions, there were no apparent differences in cell morphology of MDA-MB-231 and MDA-MB-231BR when compared to each other in two dimensions on the bottom of cell culture flasks (Figure 5). However, in terms of growth, based on microscopical observations, MDA-MB-231BR cells showed higher rates, supporting the prediction that this cell line has an enhanced proliferative capability compared to the parental cell line.

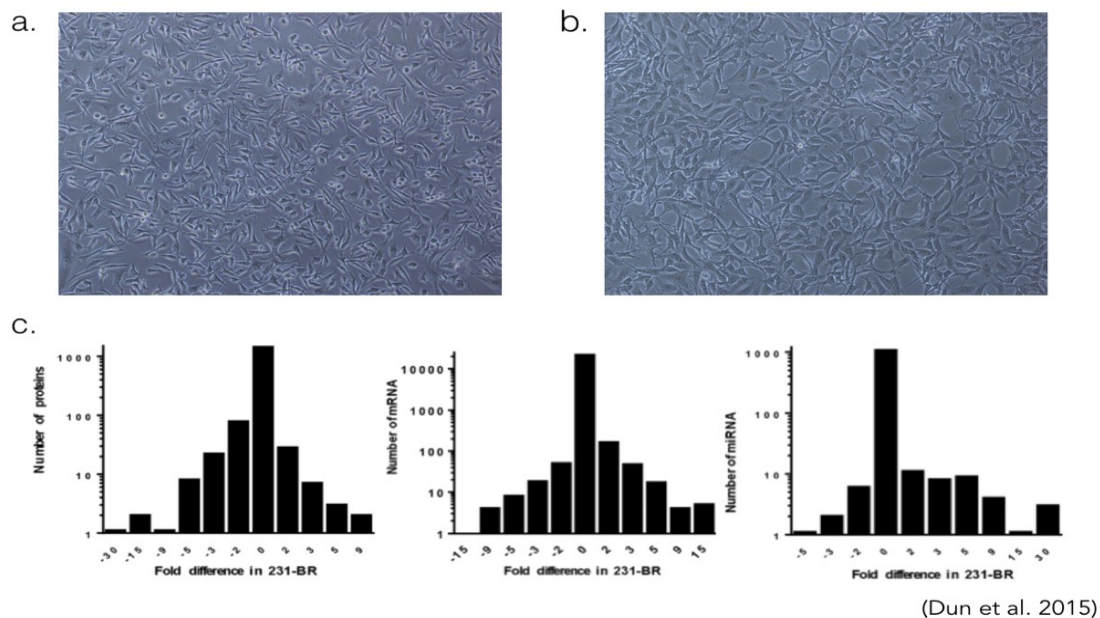


Figure 5. Cell morphology of cell lines grown in two-dimensional culture. Both MDA-MB-231 (A) and MDA-MB-231 (B) demonstrate a similar elongated and spindly appearance under inverted microscope (4X). (C) Study made by Dun et al in 2015 which compares number of proteins, mRNAs and miRNAs from MDA-MB-231 and MDA-MB-231BR. From all the quantified transcripts by SILAC, more than 90% are shared by both cell types. In conclusion, both cell lines share high genetic similarity and differences in MDA-MB-231BR can be explained by differential expression between them.

## Differential expression of the selected short-list of lncRNA between BC cell lines.

A total number of 16 lncRNAs were selected using the criteria previously described. Table 2 lists the final short-list with the criteria they were following and other relevant information that might be relevant for their selection.

Then, the expression level of each lncRNA in both cell lines was measured by qPCR and compared. As our interest lies in lncRNAs that might drive the metastatic capability in BC, the expression levels of MDA-MB-231 were normalised to 1 to measure the expression fold changes of each lncRNA in MDA-MB-231BR compared to its parental. Only two lncRNAs (*linc00261*, also known as NEPC Associate lncRNA 1, or *NEAR1* from now on, and *FAM225A*) exhibited a higher differential expression ( $p\_value < 0.0001$ ). (Figure 6A)

Table 2. Final short-list of the selected 16 lncRNAs for differential expression studies and which criteria do they match (lnc2cancer database).

FINAL PROPOSED SHORTLIST OF lncRNAs BASED ON lnc2Cancer DATABASE AND VALIDATED IN LITERATURE						
lncRNA	cancer type	upregulated	cell line MDA-MB-231	metastasis	poor prognosis	brain met
MALAT1	TNBC	x	x	x		x
linc00261	gastric	x		x		
MIAT	glioblastoma	x		x	x	
PCAT18		x		x		
CDKN2B-AS1 (ANRIL)	TNBC	x	x	x	x	
ENST00000425295 (PCAT6)	TNBC	x		x		
HOTAIR	TNBC	x	x	x	x	x
TCONS_00011636 (FOXCU1)	BLBC	x	x	x	x	
ENSG00000231528 (FAM225A)	TNBC	x	x (+ MDA-MB-231BR)	x	x	x
LOC286437 (NR_039980.1.1)	TNBC	x	x (+ MDA-MB-231BR)	x	x	x
H19	BC	x	x	x	x	x
ENSG00000242808 (SOX2OT)	TNBC	x	x	x	x	
CCAT1	BC	x		x	x	
ENSG00000281881 (SPRY4-IT1)	ER BC	x	x	x	x	
ENSG00000280997 (CCAT2)	BC	x	x	x	x	
ENSG00000258609 (linc-ROR)	TNBC	x	x	x	x	

***NEAR1* is constantly up-regulated in MDA-MB-231BR and correlates with worse prognosis in BC and other hormone-resistant tumours.**

In order to select the best candidate to continue with functional studies, both up-regulated lncRNAs *NEAR1* (relative expression in MDA-MB-231BR =  $1.93 \pm 0.07$ ;  $p\_value < 0.0001$ ) and *FAM225A* (relative expression in MDA-MB-231BR =  $2.46 \pm 0.20$ ;  $p\_value < 0.0001$ ) (Figure 6A) were validated by another independent qPCR. The results showed that the expression of *NEAR1* (relative expression in MDA-MB-231BR =  $1.69 \pm 0.69$ ) is more stable than the expression of *FAM225A* (relative expression in MDA-MB-231BR =  $1.38 \pm 0.30$ ) between replicates and remains up-regulated from MDA-MB-231. (Figure 6B)

Moreover, the clinical significance of both lncRNAs over-expression was determined using the publicly available database Kmpplot.com which has a background set of gene expression data and survival information of 1,809 patients from GEO (Affymetrix HGU133A and HGU133+2 microarrays). When introduced our two candidate genes, the Kaplan Meier curves obtained showed that *NEAR1* higher expression correlates with worse prognosis in BC. The effect of *NEAR1* over-expression significantly decreases OS between ER<sup>+</sup> (Figure 7A)  $n = 209$ ;  $p\_value = 0.78$ ; HR = 1.11 (0.52 – 2.37) and ER<sup>-</sup> (Figure 7B)  $n = 152$ ;  $p\_value = 0.018$ ; HR = 2.35 (1.13 - 4.88) tumour patients. The same trend can also be observed in DFMS after 18 months between ER<sup>+</sup> (Figure 7C)  $n = 718$ ;  $p\_value = 0.65$ ; HR = 0.8 (0.31 - 2.07) and ER<sup>-</sup> (Figure 7D)  $n = 181$ ;  $p\_value = 0.26$ ; HR = 1.65 (0.7 – 3.82) tumour patients.

In terms of *FAM225A* over-expression, the outcome of the patients does not change significantly neither in OS nor DFMS when compared between ER<sup>+</sup> and ER<sup>-</sup> (data not shown).

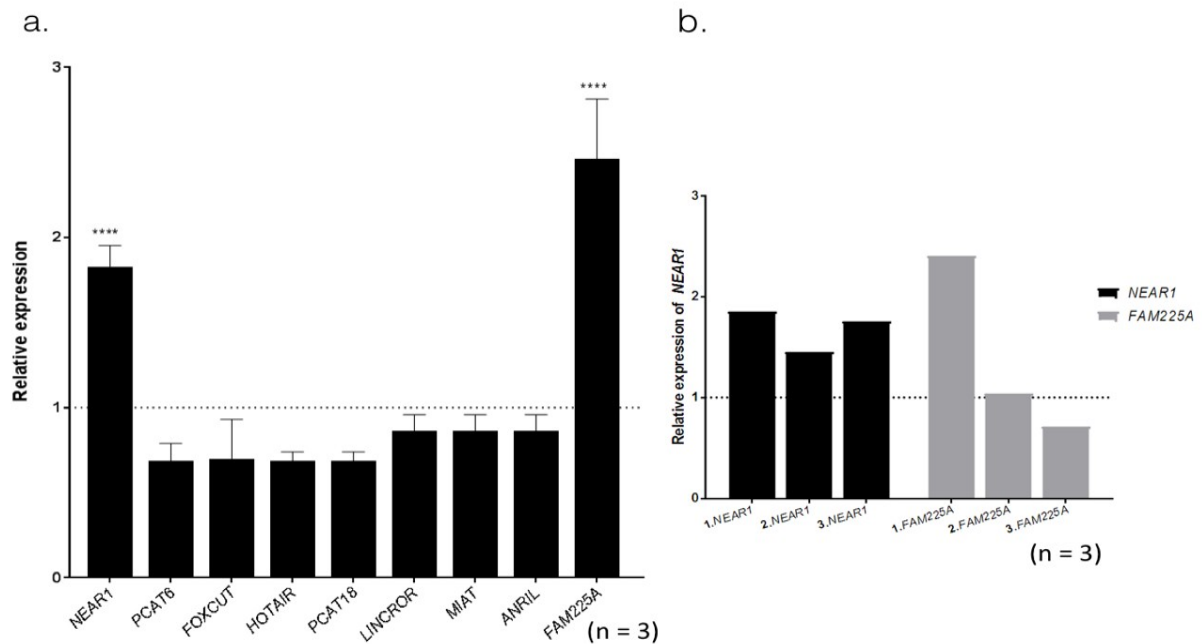


Figure 6. Differential expression between MDA-MB-231 and MDA-MB-231BR of the selected short-list of lncRNAs. RNA from both cell lines was extracted after 48h and relative expression was measured by qPCR using TaqMan assay expression probes. Relative expression of each lncRNA in MDA-MB-231 was normalised to 1 and relative fold change in MDA-MB-231BR was compared ( $2^{-\Delta\Delta Ct}$ ). (A) 9 out of 16 lncRNAs from the short-list are represented, being significantly up-regulated only *NEAR1* (relative expression =  $1.93 \pm 0.07$ ; \*\*\*\*  $p$ \_value < 0.0001) and *FAM225A* (relative expression =  $2.46 \pm 0.20$ ; \*\*\*\*  $p$ \_value < 0.0001). One-way ANOVA (Pearson coefficient of correlation, R square = 0.8727) with Sidak's multiple comparisons test. (B) qPCR validation for the up-regulated lncRNAs *NEAR1* and *FAM225A*. Out of 3 different experiments, the expression of *NEAR1* is more stable between replicates (relative expression =  $1.69 \pm 0.69$ ) than the expression of *FAM225A* (relative expression in MDA-MB-231BR =  $1.38 \pm 0.30$ )

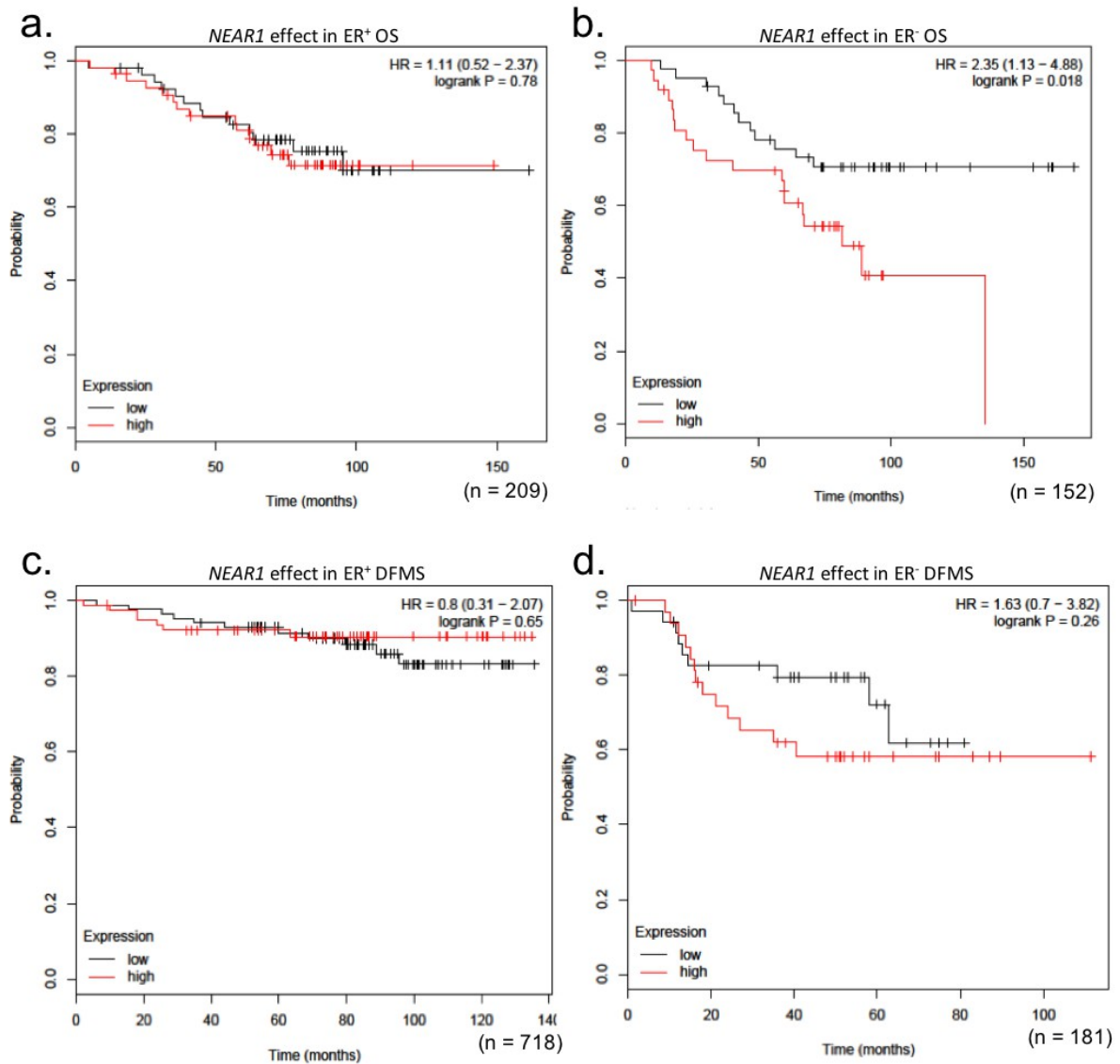


Figure 7. *NEAR1* higher expression correlates with worse prognosis in BC. The effect of *NEAR1* over-expression is significantly worse in terms of OS between ER<sup>+</sup> (A) p<sub>value</sub> = 0.78; HR = 1.11 (0.52 - 2.37) and ER<sup>-</sup> (B) p<sub>value</sub> = 0.018; HR = 2.35 (1.13 - 4.88) tumours. The same trend can also be observed in DFMS after 18 months between ER<sup>+</sup> (C) p<sub>value</sub> = 0.65; HR = 0.8 (0.31 - 2.07) and ER<sup>-</sup> (D) p<sub>value</sub> = 0.26; HR = 1.65 (0.7 - 3.82) tumours. Kmplot.com



## Functional studies on *NEAR1*. Results.

### ***NEAR1* is located in the cytoplasm and can be knocked-out with high efficiency.**

In order to assess *NEAR1* sub-cellular localisation and approach the its optimal silencing method, RNA fractionation assays <sup>15</sup>were carried out. *NEAR1* was found to be mainly cytoplasmic ( $90\% \pm 0.2$ ;  $p\_value < 0.0001$ ) (Figure 8A). This data indicates that this transcript's is likely located within the cytoplasm and its main function could take place there. This results also indicate that an effective silencing can be successfully reached by siRNAs.

To investigate whether *NEAR1* might have an important role in the development of brain metastasis, functional studies were carried out in both MDA-MB-231 and MDA-MB-231BR cell lines. For that purpose, 20nmol DsiRNAs were used to silence *NEAR1* by reverse transfection. The expression of *NEAR1* was determined by qPCR after 2 and 7 days after knock-down (Figure 8).

After 2 days, a knock-down of  $62.98\% \pm 2.07$  with siRNA1,  $67.14\% \pm 1.06$  with siRNA2 and  $57.46\% \pm 1.23$  with siRNA3 was achieved in MDA-MB-231 compared to negative control and  $74.84\% \pm 1.19$  with siRNA1,  $73.72\% \pm 0.58$  with siRNA2 and  $56.34\% \pm 1.37$  with siRNA3 in MDA-MB-231BR (Figure 8B), which suggests that siRNA1 and siRNA2 were more effective in silencing after 48h ( $p\_value < 0.0001$ ).

After 7 days, the percentage of knock-down was reduced to  $44.21\% \pm 2.81$  with siRNA1,  $65.54\% \pm 1.08$  with siRNA2 and  $56.95\% \pm 2.64$  with siRNA3 was in MDA-MB-231 compared to negative control and  $42.95\% \pm 0.4$  with siRNA1,  $15.27\% \pm 1.28$  with siRNA2 and  $52.08\% \pm 1.07$  with siRNA3 in MDA-MB-231BR (Figure 8C). This results show a drastic decrease of the knock-down potential of siRNA1 and siRNA2, specially in MDA-MB-231BR after 7 days ( $p\_value < 0.0001$ ). Consequently, only siRNA3 was used for cell proliferation (MTT) studies due to its silencing capability over 50%, needed to obtain significant conclusions.

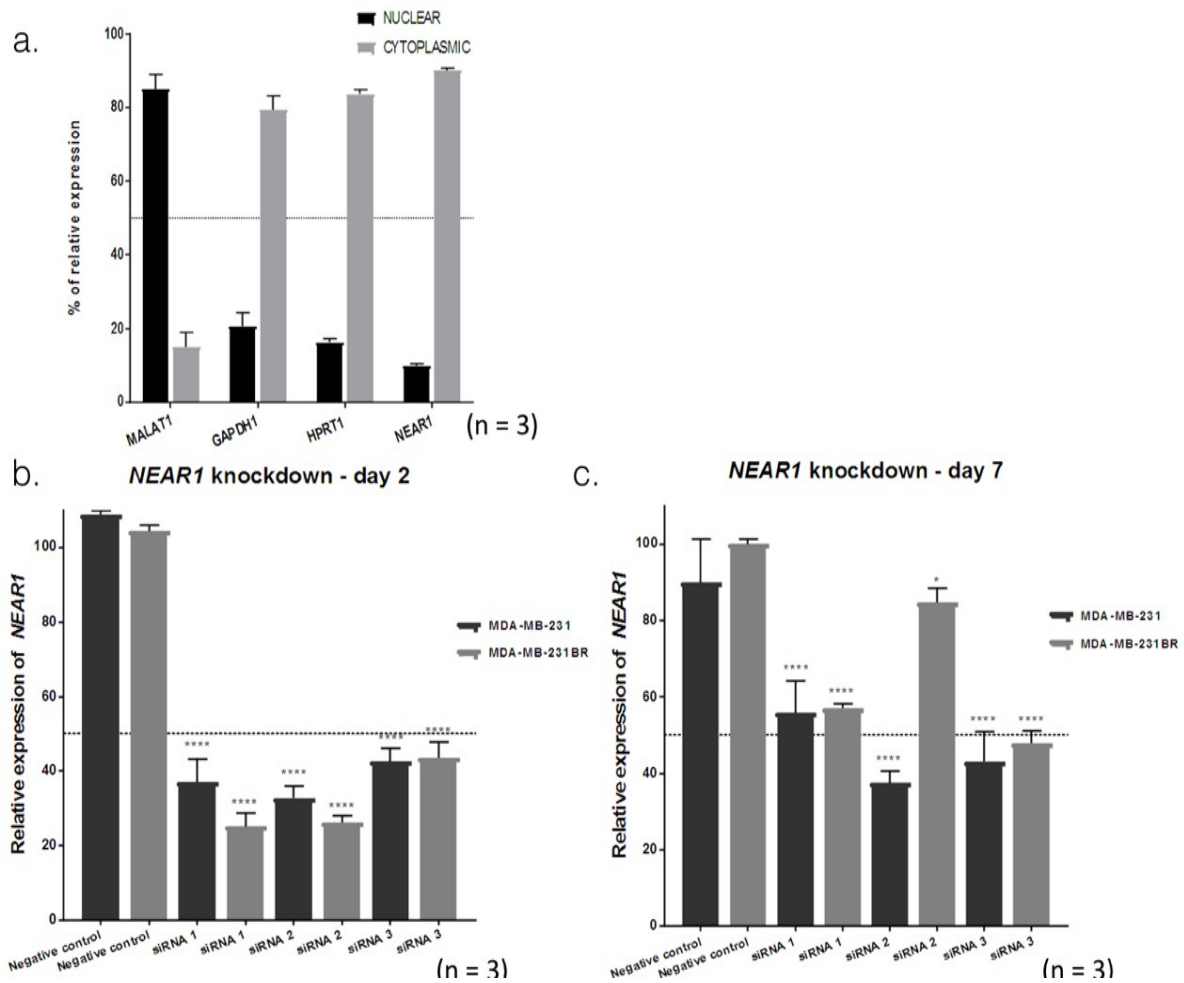


Figure 8. *NEAR1* expression is predominantly cytoplasmic and can be efficiently knocked-down up to 7 days with siRNAs. (A) qPCR validation of fractionation assays in MDA-MB-231BR demonstrated that 90% of *NEAR1* is localised in the cytoplasm ( $p_{\text{value}} < 0.0001$ ). Unpaired t-test (Pearson coefficient of correlation, R square = 0.9999). qPCR results from reverse-transfection with designed siRNAs which specifically target *NEAR1*, show that (B) After 2 days, a knock-down of  $62.98\% \pm 2.07$  with siRNA1,  $67.14\% \pm 1.06$  with siRNA2 and  $57.46\% \pm 1.23$  with siRNA3 was achieved in MDA-MB-231 compared to negative control and  $74.84\% \pm 1.19$  with siRNA1,  $73.72\% \pm 0.58$  with siRNA2 and  $56.34\% \pm 1.37$  with siRNA3 in MDA-MB-231BR (\*\*\*\*  $p_{\text{value}} < 0.0001$ ). One-way ANOVA (Pearson coefficient of correlation, R square = 0.9893) with Sidak's multiple comparisons test. (C) After 7 days, knock-down percentage is reduced to  $44.21\% \pm 2.81$  with siRNA1,  $65.54\% \pm 1.08$  with siRNA2 and  $56.95\% \pm 2.64$  with siRNA3 was in MDA-MB-231 compared to negative control and  $42.95\% \pm 0.4$  with siRNA1,  $15.27\% \pm 1.28$  with siRNA2 and  $52.08\% \pm 1.07$  with siRNA3 in MDA-MB-231BR (\*  $p_{\text{value}} = 0.0274$ ; \*\*\*\*  $p_{\text{value}} < 0.0001$ ). One-way ANOVA (Pearson coefficient of correlation, R square = 0.9588) with Sidak's multiple comparisons test.

### ***NEAR1* does not show any significant role in cell proliferation.**

To evaluate the effect of *NEAR1* on proliferation of MDA-MB-231BR cells, all siRNAs were used for cell proliferation analysis by performing a time-point MTT assay at day 1, 3, 5 and 7. Although MTT assay is not a standard method of measuring cell proliferation, MTT staining correlates with cell number. However, knowing the initial number of living cells and by measuring cell viability throughout the days, this technique can give us an estimation of cell proliferation.

Since only siRNA3 had shown a significant (>50%) silencing effect after 1 week, only that data is shown. As shown in Figure 9, *NEAR1* knock down does not show any significant difference between siRNA3 and NC until day 7, when *NEAR1* knock-down appear to have a proliferative effect on MDA-MB-231BR compared to NC (siRNA 3 normalised cell count =  $20.89 \pm 4.23$ ; NC normalised cell count =  $15.89 \pm 3.21$ ) ( $p\_value = 0.0112$ )

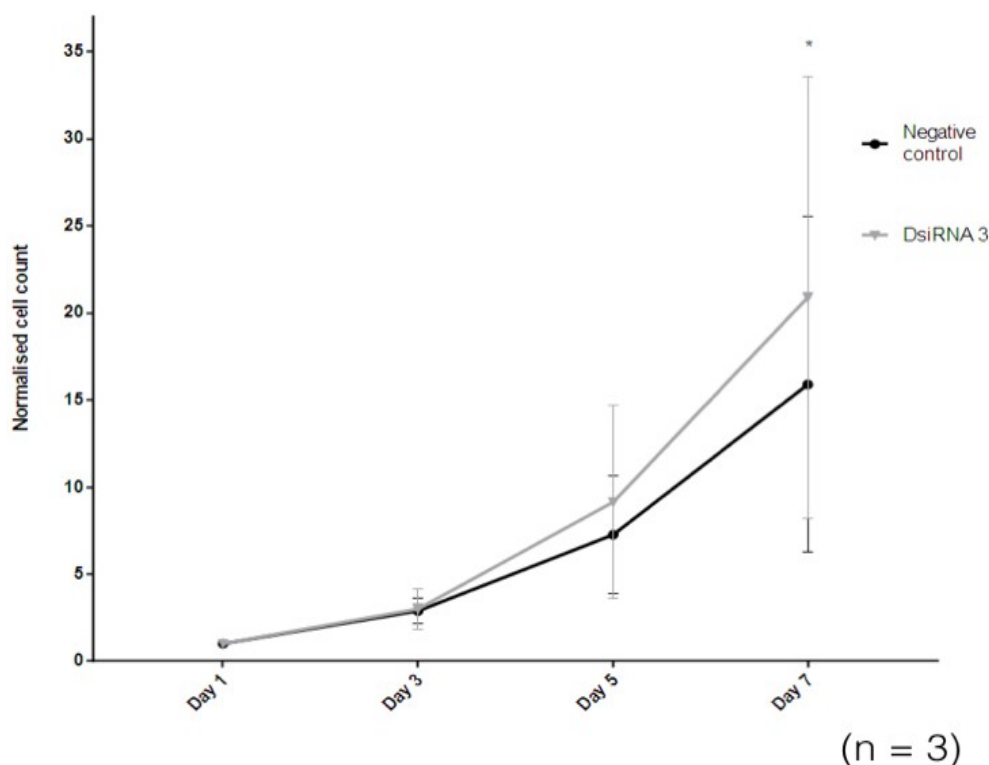


Figure 9. *NEAR1* knock-down does not significantly affect MDA-MB-231BR proliferation. MTT assays performed with siRNA3 were not significant until day 7 compared to NC (siRNA3 normalised cell count =  $20.89 \pm 4.23$ ; \*  $p\_value = 0.0112$ ). Moreover, silencing of *NEAR1* appears to increase cell proliferation. Two-way ANOVA ( $\alpha = 0.05$ ) with Tukey's multiple comparisons test.

### ***NEAR1* might have a significant role in the migration capacity of MDA-MB-231BR cells.**

In order to assess whether *NEAR1* has any key role in MDA-MB-231BR metastatic capacity, I tested their migration capacity by carrying out a wound-healing assay. This assay provides an *in vitro* system to compare the invasion ability of this metastatic cell line transfected not to express *NEAR1* in comparison to the NC transfection control.

Variability between replicates prevent the differences to be significant when compared in a 2-way ANOVA with Dunnett's multiple comparisons test (Figure 10B). However, when compared and analysed the biological replicates within the same experiment, the results show significant differences from 8 hours since the wound in siRNA3 (% of gap area =  $64.85 \pm 1.24$ ;  $p\_value = 0.0205$ ) and being the most significant reduction of migration at 24h (% of gap area =  $14.89 \pm 1.47$  with siRNA1,  $p\_value = 0.0012$ ;  $8.99 \pm 1.40$  with siRNA2 and  $9.14 \pm 2.63$  with siRNA3,  $p\_value < 0.0001$ ). (Figure 10C). Collectively, these results show at least a trend that *NEAR1* has a role in the migration capacity of MDA-MB-231BR and, although a decrease of migration was expected with *NEAR1* silencing, our results in BC correlate with the proliferation rates previously obtained.

### **The molecular pathway of *NEAR1* getting into the BBB cannot be explained by its role in adhesion to endothelial brain cells.**

The only way for tumour cells to access the brain is by hematogeneous metastasis. To do so, they have to cross the BBB before arresting in micro-vessels to form their pre-metastatic niche. With the aim of providing answers about how MDA-MB-231BR interacts with brain endothelial cells as a first step of BBB interaction and see whether *NEAR1* plays any role in this process, I carried out a static adhesion assay of transfected MDA-MB-231BR cells to hCMEC/D3 (Figure 11A). This human cerebral endothelial cell line has been extensively characterised for brain endothelial phenotype and is a model of human BBB function.

For the experiment, wild type and *NEAR1* silenced MDA-MB-231BR cells were seeded on to a hCMEC/D3 mono-layer under two different conditions, being the untreated a representation of normal brain and stimulated with TNF $\alpha$  as an inflammatory condition. It is

well known that metastatic cells can mimic trans-endothelial migratory mechanisms like leukocytes. Moreover, an inflammatory environment fosters proliferation, survival and migration of tumour cells.<sup>97</sup>

The results (% of MDA-MB-231BR cells adhered in normal conditions:  $43.81 \pm 5.60$  with NC,  $40.86 \pm 5.27$  with siRNA1,  $50.27 \pm 6.70$  with siRNA2 and  $41.56 \pm 7.05$  with siRNA3; % of MDA-MB-231BR in inflammation condition:  $53.92 \pm 6.69$  with NC,  $46.53 \pm 3.71$  with siRNA1,  $54.33 \pm 7.46$  with siRNA2 and  $50.52 \pm 8.76$  with siRNA3), do not show any significant differences neither between the transfection control and after *NEAR1* silencing within the same condition, nor between same silencing samples when compared normal to inflammation.(Figure 11B)

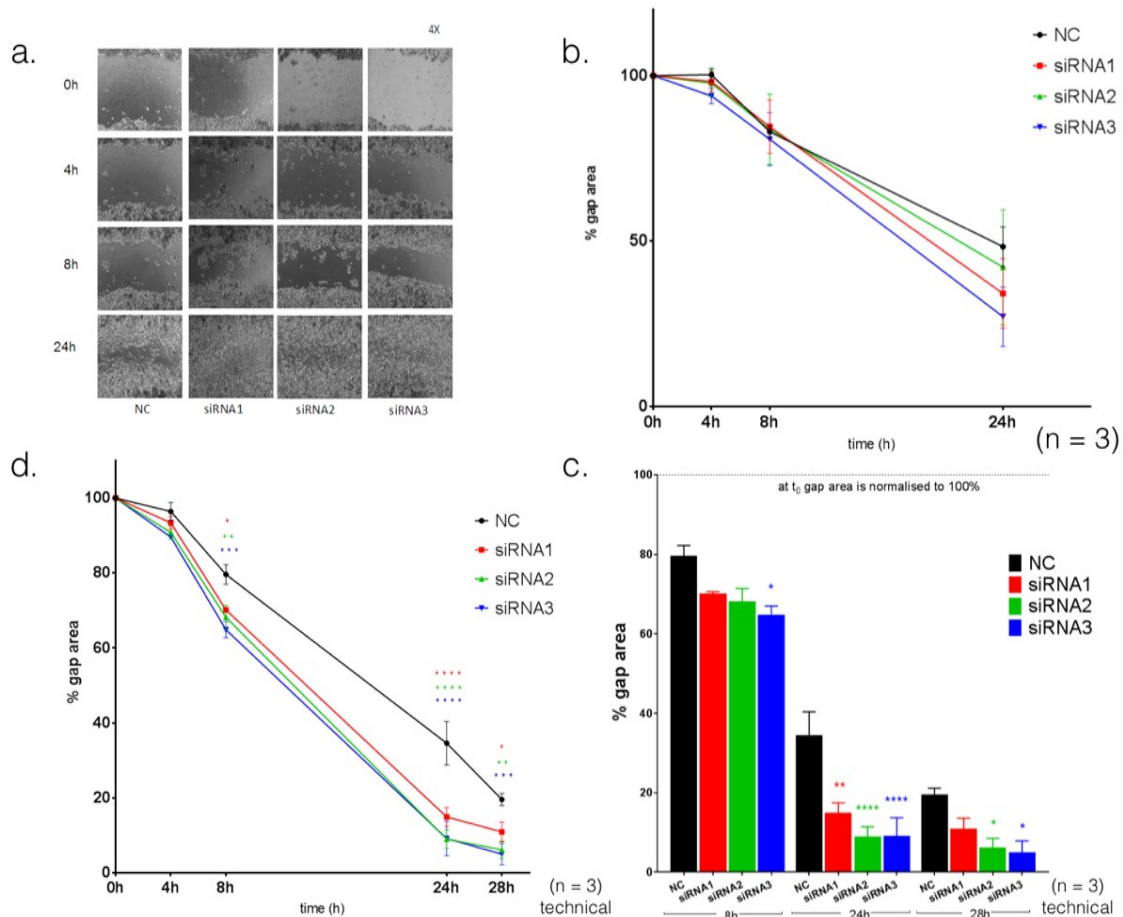


Figure 10. *NEAR1* knock-down might significantly affect the migration capacity of MDA-MB-231BR. (A) Representative images for wound-healing assay. Images taken at 4X and PhL filter and analysed with ImageJ. (B) MTT results of all replicates merged do not show any significant differences among

time-points. Two-way ANOVA ( $\alpha = 0.05$ ) with Dunnett's multiple comparisons test. However, (D, C) within each sample, significant differences in healing can be observed, from 8h in siRNA3 (% of gap area =  $64.85 \pm 1.24$ ; \*  $p\_value = 0.0205$ ) reaching its maximum at 24h (% of gap area =  $14.89 \pm 1.47$  with siRNA1, \*\*  $p\_value = 0.0012$ ;  $8.99 \pm 1.40$  with siRNA2 and  $9.14 \pm 2.63$  with siRNA3, \*\*\*\*  $p\_value < 0.0001$ ). Two-way ANOVA ( $\alpha = 0.05$ ) with Dunnett's multiple comparisons test (B) One-way ANOVA (Pearson coefficient of correlation, R square = 0.9768) with Sidak's multiple comparisons test.

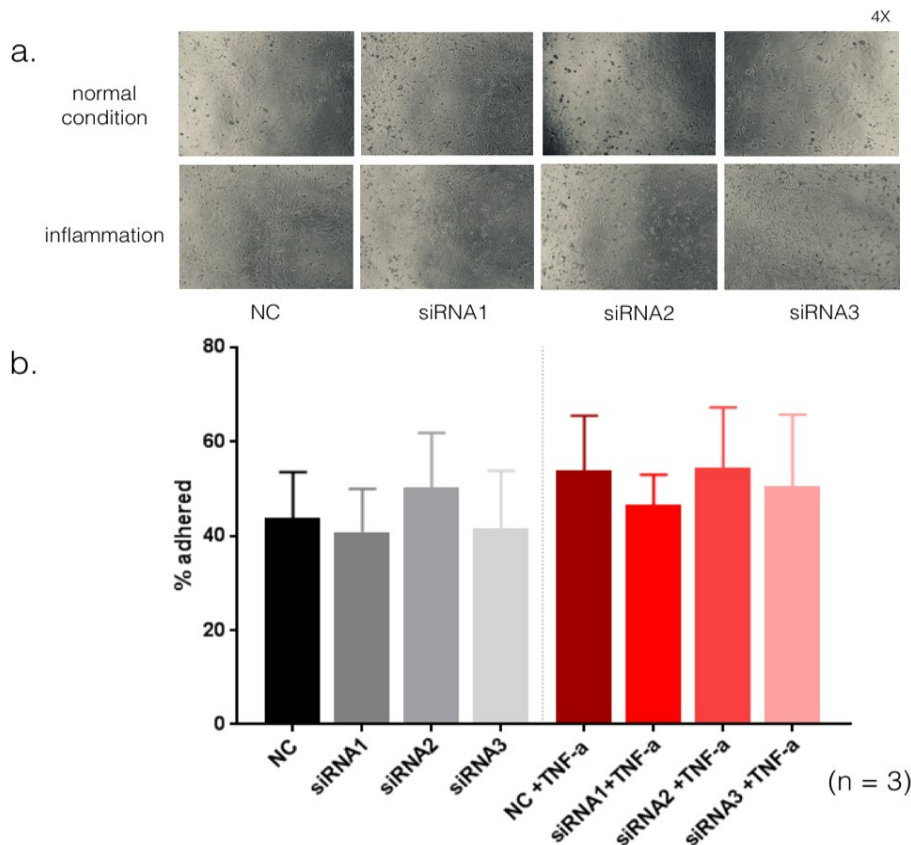


Figure 11. *NEAR1* knock-down does not significantly affect MDA-MB-231BR adhesion to brain endothelial cells. Adhesion assays in static conditions were carried out in both wildtype and *NEAR1*-silenced MDA-MB-231BR cells, representing two scenarios: normal brain conditions and inflammation by adding 0.25pg/ml of TNF $\alpha$ . (A) Represents the total number of MDA-MB-231BR cells (rounded) adhered to hCMEC/D3 (monolayer) before being washed. Inverted microscope, 4X. (B) Comparison between transfected MDA-MB-231BR for *NEAR1* KD (% cells adhered in normal conditions:  $43.81 \pm 5.60$  with NC,  $40.86 \pm 5.27$  with siRNA1,  $50.27 \pm 6.70$  with siRNA2 and  $41.56 \pm 7.05$  with siRNA3; % of cells in inflammation condition:  $53.92 \pm 6.69$  with NC,  $46.53 \pm 3.71$  with siRNA1,  $54.33 \pm 7.46$  with siRNA2 and  $50.52 \pm 8.76$  with siRNA3). One-way ANOVA (Pearson coefficient of correlation, R square = 0.0333) with Dunnett's multiple comparisons test.

## Discussion

The aim of this project was to discover at least one highly up-regulated lncRNA in our TNBC brain metastatic cell line and perform some functional studies that might help us understand how this lncRNA can promote BC progression and migration to the brain.

For that purpose, two TNBC cell line models were used. Parental MDA-MB-231, one of the most studied TNBC cell lines, it is well characterised and does not metastasise to the brain, and the MDA-MB-231BR sub-clone, selected for its ability to metastasise *in vivo* with a 100% rate.

To elaborate a short-list of candidate lncRNAs for further studies, BC databases and literature was screened to select a final number of 16 lncRNAs which followed the previously established three criteria: expressed in BC and/or TNBC, up-regulated in BC *versus* normal tissue and positive association with metastasis. Then, the expression level of all 16 lncRNAs was measured and compared between both cell lines and the results showed that *NEAR1* was the most up-regulated in the most stable manner between replicates. *NEAR1* had also a clinical relevance, since there has been proven that its over-expression correlates with worse prognosis in terms of OS and DFMS in ER<sup>-</sup> BC patients.

Once determined that *NEAR1* would be the selected candidate for further studies we decided to modulate *NEAR1* expression in both cell lines by silencing it using the retro-transcription method. As we demonstrated that *NEAR1* is mostly found in the cytoplasmic fraction, three siRNAs were tested to *NEAR1* knock-down. It was efficiently silenced for, at least a week, thus further studies were able to be performed.

MTT assays were carried out in both cell lines to measure the effects of *NEAR1* knock-down in cell viability during a 7-day time-point as an estimation of tumour proliferation. The results showed that siRNA3 silencing increased significantly cell proliferation from day 7 in MDA-MB-231BR cells. If we considered that *NEAR1* might have a role in increasing BC cells proliferation rate, we would have expected that its knock-down resulted in lower cell viability after 7 days and obtained the opposite, which could be explained by *NEAR1* having a homoeostatic role that we would need further studies.

To determine the role of *NEAR1* in cell migration, wound-healing assays were performed in transfected MDA-MB-231BR cells. This technique has a high variability between replicates because the measurements are taken in percentage of remaining area compared to an initial wound done by manually scratching with a pipette on a cell mono-layer, so more reliable results are obtained when compared replicates within a same experiment. In this case, *NEAR1* knock-down was expected to have a role in incrementing cell migration, which is translated in smaller gap areas. Consequently, *NEAR1* knock-down should result in less migration, therefore wider gap area. The results were, once again, contrary as expected, but not surprisingly since they correlate with previous proliferation results.

Another hallmark in MDA-MB-231BR cells migration to the brain would be the interaction between the circulating invasive cells and the BBB to trespass from the blood system to the CNS. For that, their first step is to adhere to brain endothelial micro-vessels and to discover if *NEAR1* might have a role in this process we carried out an adhesion assay in static conditions. Both wild-type and transfected MDA-MB-231BR cells were exposed to a mono-layer of hCMEC/D3 in both normal conditions and recreating inflammation to mimic the micro-environment. Although there is a trend of *NEAR1* affecting adhesion in a promising way, the differences are not significant enough to consider that it has a key role in cell adhesion to the BBB.

## Conclusions

The main conclusions from this study are as follows:

1. *NEAR1* is constantly up-regulated in MDA-MB-231BR, which represent a cellular model of TNBC.
2. This lncRNA has also been found up-regulated in other hormone-resistant tumours like NEPC.
3. Higher expression levels of *NEAR1* in ER<sup>-</sup> BC patients are significantly correlated with worse prognosis in terms of OS and follow the same trend on DMFS.



4. *NEAR1* is located in the cytoplasm and the transfection efficiency of *NEAR1* knock-down is significant in both cell lines for, at least, a week, with >50% *NEAR1* reduced expression after 48h, being siRNA2 the most effective in both cell lines in a short-term manner but losing silencing efficiency after 7 days. A week after transfection, only siRNA3 achieves >50% silencing potential in MDA-MB-231BR cells.
5. *NEAR1* silencing with siRNA3 increases significantly cell proliferation from 7 days after transfection in MDA-MB-231BR cells.
6. *NEAR1* silencing might have a significant role in cell migration. The results obtained were not significant between replicates. However, reducing variability between experiments resulted in a significant decrease in the time needed to heal a wound, that starts at 8h since wound with siRNA3 and observing highest results at 24h with all siRNAs in MDA-MB-231BR cells.
7. *NEAR1* silencing does not show any significant difference in brain epithelial cell adhesion, neither compared to NC nor between same conditions in normal when compared to an inflammation scenario.

In conclusion, although *NEAR1* does not appear to be a key regulator in BCBM when analysing the selected cancer progression hallmarks for this study, clinical results of prognosis in BC patients demonstrate that there is a correlation between the over-expression of *NEAR1* in those patients and a critically observable worse prognosis. That means that it might have a role in other functions yet to be discovered.

Further studies are needed to elucidate how *NEAR1* might work alone or by interacting with other cellular or environmental components to decrease survival and affect tumour progression in BC and other hormone-receptor cancers like NEPC.

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