Thesis

The role of NMDA receptors in the aggressive phenotype of Inflammatory Breast Cancer (IBC).

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List of abbreviation

ANG2 – Angiopoietin-2

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BBB – blood brain barrier

BCBM – Breast Cancer to Brain Metastasis

CA^{2+}– Calcio

C-Met – tyrosine-protein kinase Met

CNS – Central nervous system

COX-2 – Prostaglandin-endoperoxide synthase 2

CSF1 – colony stimulating factor 1 receptor

EAAt2 – Excitatory amino acid transporters

EGFR – epidermal growth factor receptor

EMT– Epithelia to Mesenchymal Transition

ER – Estrogen Receptors

EZH2 – enhancer of zeste 2 polycomb repressive complex 2 subunits

Glu – Glutamate

GSTA5 – Glutathione S-Transferase Alpha 5

HBEGF – Heparin Binding EGF Like Growth Factor

HER2+ – human epidermal growth factor receptor 2 enriched

HER2- – human epidermal growth factor receptor 2 low expression

HR+ – presence of hormone receptors

HR – low expression of hormone receptors

IBC – Inflammatory Breast Cancer

IL-6 – Interleukin-6

MAPK – mitogen-activated protein kinase

mGluR – metabotropic glutamate receptors

mTOR – mammalian target of rapamycin (mTOR)

NMDA – N-methyl-D-aspartate

NMDAR – N-methyl-D-aspartate Receptor

NMDAR1 – N-methyl-D-aspartate Receptor, subunit 1

NMDAR2 – N-methyl-D-aspartate Receptor, subunit 2

Non-IBC – Non – Inflammatory Breast Cancer

NST – tumors as invasive non-special types

PDGFRA – platelet-derived growth factor receptor alpha

PI3K – Phosphatidylinositol 3 kinase

PDL1 – Programmed death-ligand 1

PR – Progesterone Receptor

SNAIL – Zinc finger protein SNAI1

TGF – Transforming Growth Factor

TGLI1 – truncated glioma-associated oncogene homolog 1

TNBC – Triple Negative Breast Cancer

TN-IBC – Triple Negative Inflammatory Breast Cancer

TWIST 1 / 2 – Twist-related protein 1 / 2

VEGF – Vascular endothelial growth factor

ZEB 1 / 2 – Zinc finger E-box-binding homeobox 1 / 2

ZO – zonula occludens

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Abstract:

Inflammatory Breast Cancer (IBC) is the most aggressive and lethal type of breast cancer. In most cases, the presence of a solid tumor is replaced by swelling, redness, and skin changes, which results in the misdiagnosis of an infection during the invasion and migration phase. This type of cancer blocks blood and lymphatic vessels under the breast's skin, causing local inflammation and rapid metastasis. Furthermore, IBC has a 73% incidence of brain metastasis as compared to other cancer types like a adenocarcinomas of breast, lung 40%, and kidney 24% cancers (Bos et al., 2009; EMELINE et al., 2012) Previous research shows how tumor cells from different breast cancer subtypes (HR- / HER2-) and (HR- / HER2+) have a high incidence of crossing the Blood-Brain Barrier (BBB) due to its high expression of glutamate receptors such as N-methyl-D-aspartate receptors specifically (NMDAR1 and NMDR2) subunits. However, little is known of the role that these glutamatergic ionotropic receptors have in the development and/or progression of IBC cell lines (SUM149PT and SUM190PT). Therefore, this research has several objectives, the first of which is to provide a new understanding of the cellular and molecular action that NMDA receptors (NMDAR) have on IBC. The second goal is to detect, characterize, and quantify NMDAR expression in IBC cell lines employing Western blot analysis, qRT-PCR, and immunofluorescence to identify their presence in IBC and be able to associate it with phenotypes such as migration and proliferation in quite aggressive cell lines. This study will also examine the effects of inhibition of NMDAR in IBC cell models dose-response curves of the experimental drugs (memantine and dizocilpine) in 2D cultures. In order to achieve this, we conducted several experiments using Dose-response curves and generated 2D cultures to find the drug concentration that inhibits cell viability by 50% (IC50) for each of the inhibitors (memantine and dizocilpine). Functional assays (Wound Healing Assay and 3D colony formation) were performed to measure

cell migration and proliferation following each drug treatment. Findings on our first set of experiments revealed the preponderance and quantification of each of the cell lines under study (Non-IBC and IBC) through relative mRNA and protein abundance expression. The NMDAR sub-units were found in areas internal to the cell (endoplasmic reticulum, Golgi apparatus, perinuclear membrane), thus establishing new knowledge at the cellular and molecular level of NMDAR in cancer lines, especially in IBC. During this first discovery, the presence of NMDAR subunits gave IBC (Triple-negative and HER2-enriched) cell lines the ability to survive in a brain microenvironment after breast metastasis. On one hand, our second data set showed a significant decrease during migration and proliferation in IBC cell lines after dizocilpine treatment, on the other hand, memantine treatment had no effect during dose-response tests. We also found that the absence of the NMDAR's ligand glutamate and the presence of dizocilpine affects cell migration. The present findings demonstrate that the presence of NMDAR is essential for the pro-oncogenic characteristics of IBC cell lines (SUM149PT and SUM190PT) and could emerge as a new study for alternative treatment routes for patients with inflammatory breast cancer.

Dedication

I dedicate my dissertation work to my family (*Neida, Sergio Fran)* and friends specially to *Xavier* who supported me in each of the most difficult moments of my life.

Acknowledgment:

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Chapter I: General Introduction

"Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we can fear less"
Marie Curie (1867 – 1934)

1. Introduction:

1.1 Breast cancer

Breast cancer is one of the most common cancers globally, with an estimated 1.6 million cases (about 12% incidence in women and 1% in men) (Vermeulen, Van Deurzen, Schroder, Martens, & Van Diest, 2020), followed by prostate and lung cancer (Ferlay et al., 2015). Moreover, the mortality files of the demographic registry of Puerto Rico showed that breast cancer is the main cause of death from cancer in women, with an 18% incidence rate. Factors such as hereditary conditions, genetic mutations in BRCA 1/2 (Y. Song et al., 2020; Thorat & Balasubramanian, 2020) sedentary lifestyles (Nilsson et al., 2019), obesity, alcohol consumption, hormonal therapies, and dysbiosis of gut microbiota are risk factors of breast cancer. However, the frequency or relationship between these factors to the breast cancer diagnosis remains unknown (Chen et al., 2019). Breast cancer detection is often achieved through routine mammography exams. Routine manual examinations can detect the formation of a solid mass or lump in the areas of the nipple, breast, or armpits. Depending on the mass's location, a biopsy can corroborate the diagnosis (Maumy et al., 2020). Breast cancer is classified according to the tumor's histopathology, growth patterns, and stage determination/grade (severity determination). The histopathological classification of breast cancer subtypes is Luminal (A and B), Basal-like, and HER2 enrichment (Table1).

SUBTYPES	CHARACTERISTICS
LUMINAL A (HR+/HER2-)	The most common type of breast cancer. This type presents the most favorable prognosis and is usually receptive to hormonal therapy (estrogen and progesterone receptors). Low grade of breast cancer, 90% survival rate in a period of 5 years, has an incidence of 50% compared to the other subtypes.
LUMINAL B (HR+/HER2+)	Usually receptive to hormonal therapy (estrogen and progesterone receptors) and anti-HER2. It is more aggressive than luminal A. Luminal-B breast cancer patients may benefit from chemotherapy and hormonal therapy, and HER2-targeted therapy. 40% survival rate in a period of 5 years, has a frequency of 15% compared to other subtypes.
BASAL-LIKE (HR-/HER2-)	Lacks expression of estrogen receptor (ER), progesterone receptor (PR), and HER2 protein, with low or no response to molecular and hormonal target therapy. 0% survival rate in a period of 5 years, has a frequency of 10% -20% compared to other subtypes. .
HER2-ENRICHED (HR-/HER2+)	These cancers are treated with drugs that target a HER2 protein. 31% survival in a period of 5 years, has a frequency of 15% compared to other subtypes.

Table 1. *Description and characteristics of the different subtypes of cancer*. (HR +) High expression of estrogen and progesterone hormone receptors; (HR-) Low or weak expression of estrogen and progesterone hormone receptors; (HER2 +) High presence of human epidermal growth factor 2; (HER2-) Low presence or expression of human epidermal growth factor 2. Information taken from American Cancer Society webside (Trop et al., 2014)

Other detection and diagnosis of breast cancer are done through hormone receptor markers, proliferation markers like Ki-67, and gene expression classification such as PAN50, Oncotype DX, and MammaPrint (the most robust clinical trials to classify breast cancer subtypes to improve diagnosis and treatment options) (Creighton, 2012). Another way to classify breast cancer is through its diagnostic (in situ or invasive). Carcinoma *in situ* is one of the earliest stages of breast cancer and constitutes the phase when the cancerous cells have not left the mammary ducts. During this stage, it is suggested to extract the tumor and preserve the breast tissue (Chan et al., 2020). *Invasive* carcinoma has a 70 - 80% incidence in ductal breast cancers. This statistic considers several histopathological subtypes, which classify most tumors as invasive non-special types (NST—less homogeneous histological tumors both at the level of histological and molecular formation) (Rivera & Hannoun-Lévi, 2019; Shea, Koh, & Tan, 2020; Toğaçar, Ergen, & Cömert, 2020; Weigelt, Geyer, & Reis-Filho, 2010).

It is important to emphasize that some breast cancer therapies include radiation therapy, endocrine therapy (tamoxifen, oophorectomy), HER2 targeted therapies (trastuzumab), and angiogenesis inhibitors (Bevacizumab, sorafenib, sunitinib) (Jones, Haykowsky, Swartz, Douglas, & Mackey, 2007) (see Table 2 to see types of specific therapies). Despite this variety, the strenuous study of breast cancer and its treatment/therapeutic alternatives, inflammatory Breast cancer (IBC) is one of the rarest and most lethal is found among them due to the difference in symptoms and not-effective treatments.

1.2 Inflammatory Breast Cancer (IBC)

Inflammatory Breast Cancer (IBC) is the most aggressive subtype among invasive breast cancers. This cancer frequently occurs in younger women, representing 1% to 6% of cases in the United States and 10% in the Middle East/northern Africa, according to the American Cancer Association (ACS) statistics and previous data (Dobiasova & Mego, 2020). Clinically, IBC is characterized by changes in the skin, including redness, swelling, and pain in the breasts, as well as swollen glands near the neck or armpits. The lifespan of a patient diagnosed with IBC is five to eight years in comparison with other types of carcinomas of breast cancer 10 years or older (Rosenbluth & Overmoyer, 2019). Statistical studies of non-metastatic IBC in populations have shown the molecular distribution of the different subtypes that may be part of the IBC. It has been found that a large number of IBCs have luminal-B (51%) and HER2-enriched (28%) subtypes, followed by Basal-like (20-40%) and Luminal A (7%) subtypes (Cakar et al., 2018; Singh et al., 2020; Trop et al., 2014) see (Table 1) for details . According to the website of the *American Cancer Society*, there is a multimodal approach of treatments for IBC, consisting of preoperative chemotherapy for tumor shrinkage, total breast mastectomy, radiotherapy, chemotherapy, and targeted therapy generally targeting the HER2 subtypes.

After an initial treatment with Taxane, which can help reduce the size of the tumor and prevent cell spread, as is known, the different treatment alternatives depend on the molecular basis/histology of the cancer type. In this case, some of the most frequent treatments are mentioned according to the subtype of cancer, starting with the subtypes with the presence of hormone receptors (HP) where 60-70% prevalence is found. Premenopausal patients tend to develop this subtype of breast cancer where hormonal therapy is the most appropriate treatment (Tamoxifen,

letrozole, anastrozole, and exemestane) which decreases the production of hormones in the ovaries (Barzaman et al., 2020).

As a second instance, people who develop HER2+ (prevalence of ~20%) are given standard treatment with monoclonal antibodies which is considered appropriate. Typically, antiHER2 monoclonal antibodies such as trastuzumab, and pertuzumab bind to different sites of HER2. On the other hand, ado-trastuzumab emtansine as antiHER2 monoclonal antibody conjugated with mertansine (a microtubule inhibitor) is administered to these patients. For patients diagnosed with triple-negative phenotypes (HR-/HER2-) chemotherapy therapies are the most common. On occasions, these chemotherapies can be combined with bevacizumab (antibody against vascular endothelial growth factor). It should be noted that other therapeutic options have been used in IBC depending on the diagnostic study. As mentioned above, IBC is a very heterogeneous cancer subtype and is mostly diagnosed based on its clinical features. An imperative quest to treat IBC patients with triple-negative phenotypes is urgently emerging. Therefore, new treatments (Phase II and III Clinical Trials - Table 2.) have been focused more at the molecular level to combine the already established chemotherapies (Tufano, Teplinsky, & Landry, 2020).

BIOMARKER	THERAPEUTICS OPTIONS
TUMOR-ASSOCIATED **- HER2**	antiHER2
RELATED GENETIC **- ERBB2 / HER2**	Potential target for HR+ IBC HER2
RELATED GENETIC **- VEGF-D**	Angiogenesis inhibitors (Bevacizumab)
RELATED GENETIC **- PDGFRA**	PDGFRα inhibitor (BLU-285), monoclonal antibodies (olaratumab)
RELATED GENETIC **- CYCLIN E**	CDK inhibitors – targeting cyclin E/ CDK2 complex (dinaciclib)
RELATED GENETIC **- EZH2**	Radiosensitizers (CDK inhibitors)
AFFECTED PATHWAYS **- HER/PI3K/MTOR PATHWAY**	mTOR inhibitor (everolimus)
TUMOR MICROENVIRONMENT **- CSF1**	antiCSF1
TUMOR MICROENVIRONMENT **- IL-6**	anti-Il-6
TUMOR MICROENVIRONMENT **- PDL1**	anti-PDL1

Table 2. *Biomarkers and therapies* for tumors and tumor microenvironment, and pathways in IBC (Phase II and III Clinical Trials). (Hosonaga, Saya, & Arima, 2020, (Tufano et al., 2020)

1.3 Inflammatory Breast Cancer and metastatic to brain cancer

To quote a recent study, "*cancer metastasis is a complex disease, arising from a growing tumor from which cells escape to other parts of the body"* (Suhail et al., 2019). Cells in the primary organ begin a transition from epithelial to mesenchymal tissue (EMT), losing polarity, adhesion, and generating tissue degradation to achieve the migration and invasion process (Suhail et al., 2019). When the cancer cells reach the brain, they adhere or interact with astrocytes and glia,

forming a solid mass, and causing motor deficits and various neurological problems. For this reason, it is estimated that the interaction between cancer cells and neurons/gliomas is essential for the maintenance and growth of the cancerous environment and the regulation of the tissue (Koniali et al., 2020; Venkatesh et al., 2019). One of the mechanisms by which metastasized cancer cells successfully survive within the brain environment is via tumor-associated astrocytes' feedback. The cytokines activation and protein upregulation create a favorable environment for cancer cells in the brain. The adequate adaptation of these cancer cells promotes transcription factors such as c-Met and TGLI1 (transcription factor) that are highly involved in oncogenesis, angiogenesis, and oncogenic phenotypes (migration and invasion)(Venkatesh et al., 2019; Xing et al., n.d.). Interestingly, IBC has a 73% incidence to metastasize to the brain. Unfortunately, patients diagnosed with metastatic breast cancer to the brain only survive approximately two years after diagnosis (Chopra & Davies, 2020) (**Figure 1)**. Generally, when the cancer cells with mesenchymal phenotype reach the brain, they mostly stay in areas such as the hippocampus, prefrontal, and occipital regions (Kim, Kim, & Kim, 2018). Studies show the basal-like and HER2-enriched subtypes of breast cancer are more prone to develop brain metastases thanks to the disruption of the BBB involving microenvironmental niche–dormant cell interactions, neuroinflammatory cascades, and neovascularization (↑VEGF: vascular endothelial growth factor; ↑Ang2: angiopoietin2; ↓ZO: zonula occludens) (Custódio-Santos, Videira, & Brito, 2017; da Silva, Cardoso Nunes, Izetti, de Mesquita, & de Melo, 2020; Jin et al., 2018; Kuksis et al., 2021). Furthermore, patients diagnosed with brain metastasis with triple-negative phenotype have worse prognosis and survival rates (6 months of survival) than patients with HER2+ phenotypes thanks to the targeted therapies available for HER2-positive breast cancer (18 months of survival) (Dawood et al., 2008; Nam et al., 2008). Some of the treatments available for patients with brain-

metastasized breast cancer are strictly localized surgery, total brain radiotherapy, and stereotactic surgery (Mills et al., 2020).

Despite the abundant evidence that is found on breast cancer to brain metastasis, a certain relationship that explains this link is not known. Being able to relate/associate the reason for the concurrence of metastatic breast cancer to the brain, is of the utmost importance for new treatments in affected patients related to this diagnosis.

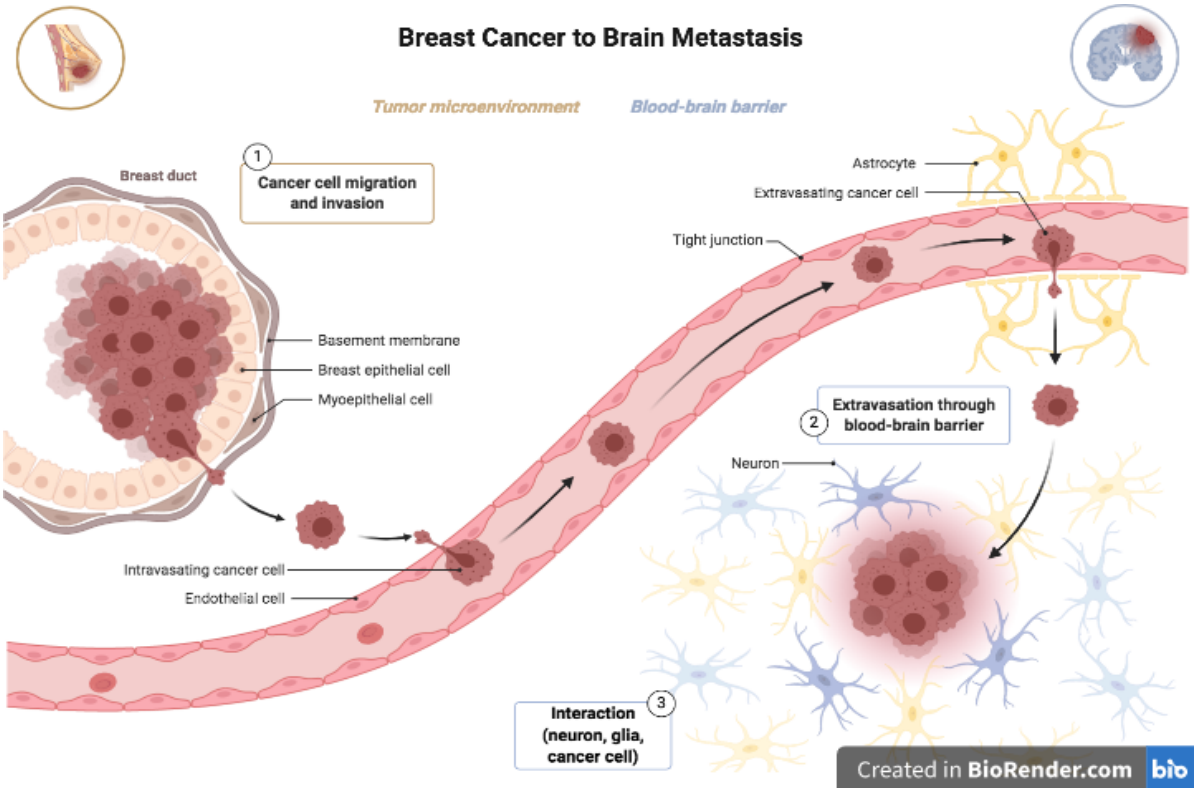


Figure 1. *Process of breast cancer metastasizing to the brain* **1**. During the migration and invasion stage, the epithelium cells lose polarity and cell junctions such as claudin, occluding, JAM, adherents' junctions, GAB junctions. During this process, an increase in factors such as (SNAIL, ZEB1 / 2, Twist1 / 2) can be observed, which influence the inhibition of epithelial phenotype that leads to the acquisition of mesenchymal phenotype (expressing N-cadherin, metalloproteinases) which allow the migration and metastasis of tumor cells to secondary organs, **2**. In the extravasation process, the cancerous or mesenchymal cell crosses the BBB increasing factors such as COX-2, EGFR, HBEGF

to change the permeability BBB (pericytes, abluminal basal lamina, perivascular astrocytes, and microglia) **3.** During the interaction between the cancer cell and astrocytes, produces the activation of anti-apoptotic genes (MAPK, GSTA5, Twist1) and growth factors (TGF) that help cancer cells survive and proliferate in the brain. Created with BioRender.com

1.4 Glutamate receptors (NMDAR) and Inflammatory Breast Cancer

Glutamate (Glu) is an excitatory amino acid (EAA) (Jewett & Thapa, 2020). It is found abundantly in the body, primarily in the nervous system, where it acts as a neurotransmitter. Glu works primarily as a neuroreceptor. It binds to 3 very important ionotropic receptors for neuron membrane depolarization (AMPA, Kainate, and NMDAR). On one hand, ionotropic glutamate receptors outside the nervous system (NMDA and AMPA) have a more prominent role during the molecular analysis in the glioma expression when compared with metabotropic glutamate receptors (mGluR).(Iwagaki & Miles, 2011; Lumeng J. Yu, Brian A. Wall, Janet Wangari-Talbot, 2017; Prickett & Samuels, 2012). On the other hand, it has been found that NMDA receptors are mostly related to excitotoxicity, regulation of development, nerve regeneration through induction of LTP, differentiation neuronal (Chakraborty, Murphy, & Coleman, 2017; Elke Muth-Köhne, Pachernegg, Karus, Faissner, & Hollmann, 2010; Kolls & Meyer, 2013), learning, and memory, and have been related to cancer cell progression in the brain (Hosonaga, Saya, & Arima, 2020; Li & Hanahan, 2013; Prickett & Samuels, 2012; Wright-Jin & Gutmann, 2019; Zhou & Danbolt, 2014). The NMDARs are a heterodimeric receptor with a channel permeable to Ca^{2+} ions. Normal Ca^{2+} levels lead to Calmodulin activation, and Calmodulin-dependent kinase produces an increase of kinases activity (pathways related to cell proliferation and growth cascades).

As mentioned above, cancer cells survival and proliferation within the brain mainly maintains calcium arrest and autocrine release of glutamate. It is suggested that cancer cells entering the brain leads to an increase in EAA transporter 2 (EAAT2) and NMDAR expression, reducing the concentration of Glu. The cancer cells take Glu and Ca^{2+}, creating an optimal environment for tumor growth and proliferation via ionic channel activation (Hosonaga et al., 2020; Venkataramani et al., 2019). It is also worth noting that a variety of cancer tumors secrete glutamate, causing increased Ca^{2+} permeability within normal cells activating Akt's signal, ERK/MAP, and PKA pathways (Corsi, Mescola, & Alessandrini, 2019).

Cancer cells have been found to secrete Glu for autocrine regulation, which in turn increases NMDAR activation and keeps the calcium flow active. The permeability of calcium within the cell and the decrease transport system of EAAT1, end in cell death through excitotoxicity in the neurons. Studies show that minimal mutations in NMDARs (NMDAR1 and NMDAR2 subtypes) can increase Ca^{2+} permeability inside the cell, thus creating a more favorable environment for cancer cells through signals of proliferation or protein synthesis **(Figure 2)** (Prickett & Samuels, 2012)

Analysis of expression for NMDAR in different cancer cell lines showed the presence of NMDAR1 and NMDR2 subunits (Abdul & Hoosein, 2005; Bunney, Zink, Holm, Billington, & Kotz, 2017; Gorska-Ponikowska, Perricone, Kuban-Jankowska, Lo Bosco, & Barone, 2017; Seifabadi et al., 2017). This data revealed the expression and importance of NMDAR in the cancer cell will be essential for the survival and proliferation of cancer in the established microenvironment. The signaling and localization of NMDARs beyond neuronal cells associated with breast cancer have not been studied in depth. These receptors are mainly located outside of

the neural membrane (synaptic and extrasynaptic areas) for neuronal depolarization and interaction with glia and astrocytes. Studies have shown, however, that an abnormal localization might be essential for carcinogenesis or maintenance of cancer cells within some types of cancer. These misallocation in cancer cells are found in breast cancer (T47D, MDA-MB-231), and neuroblastoma (SK-NBE). Associated with the cytoplasmic space, the location of these receptors enables a variety of oncogenic signaling pathways such as protein synthesis, proliferation, and cell maintenance (Glebov, 2020; Stepulak et al., 2005). Few studies have mentioned the location of these receptors in the cytoplasmic space. Nevertheless, it is mentioned that when NMDARs are found in the cytoplasm, they are associated with membranous organelles such as the endoplasmic reticulum and the Golgi apparatus (López-Menéndez et al., 2019).

Despite this information, little is known about the expression profile of NMDAR in IBC. Our present research study focused on determining the expression, localization, and cellular function of NMDARs receptors in *in vitro* cell model of IBC. NMDARs are essential for various cognitive processes within the CNS like learning and memory. Dysfunction in NMDAR1 and NMDAR2 subunits can cause severe cognitive problems, encephalitis, multiple sclerosis, Alzheimer's disease, among other neurodegenerative diseases (Corsi et al., 2019; Marttinen, Kurkinen, Soininen, Haapasalo, & Hiltunen, 2015; Suleman & Javed, 2018; Q. Wang, Zhao, & Han, 2015; R. Wang & Reddy, 2017). Moreover, this NMDAR subtype dysfunction is also involved in oncogenic phenotypes such as migration, proliferation, and invasion in metastatic breast cancer to the brain, indicating that it is of great interest for therapeutic targets to treat cancer (Dai, Xiang, Li, & Bai, 2016; Hanahan & Weinberg, 2011). The IBC is the most complicated cancer subtype due to highly heterogeneous pathological characteristics and behavior. It has been found that much of the metastasis from the breast to the brain is caused by breast tumors with the molecular

subtypes of triple-negative and HER2-enriched (Tang, Wang, & Bourne, 2008). After an extensive revision of the literature, we found no evidence of NMDARs' presence in IBC breast cancers. Previous studies show, however, that MDA-MB-231 and SK-BR3 (triple-negative and HER2-enriched phenotypes) express NMDAR (Bunney et al., 2017; Koiri, 2015; Seidlitz, Sharma, Saikali, Ghert, & Singh, 2009; Zeng et al., 2019). Our focus on NMDAR detection is on IBC cell lines (SUM149PT and SUM190PT). These cell lines are characterized by triple-negative (TN-IBC) and HER2-enriched phenotypes (HER2+), respectively, which are highly associated with brain metastases.

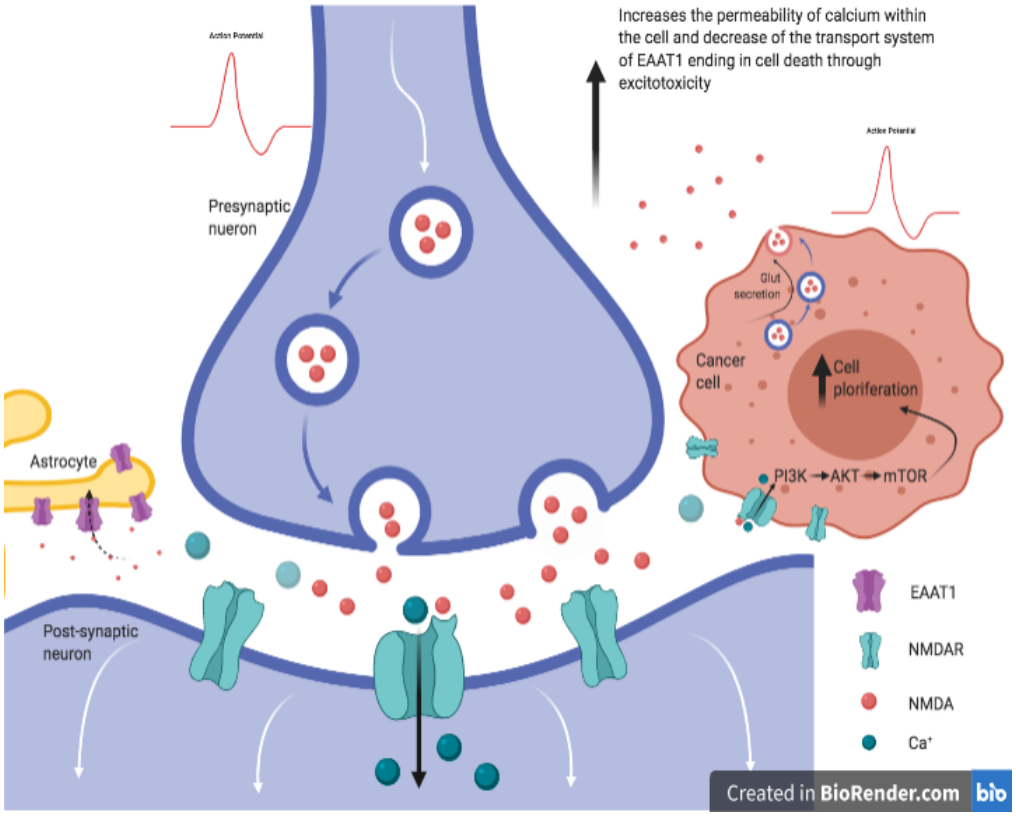


Figure 2. *Relationship between neuronal system (neuron/astrocyte) and cancer cell.* During calcium sequestration by a cancer cell, the active pathways such as PIK3, AKT, mTOR, ERK, Calmodulin and Calcineurin produce cell proliferation. Meanwhile, the cancer cell increases glutamate secretion simultaneously to produce constant depolarization of the neuron. This constant depolarization produces an increase of calcium concentrations, which then causes constant feedback to the activation of the proliferation and depolarization. Created with BioRender.com

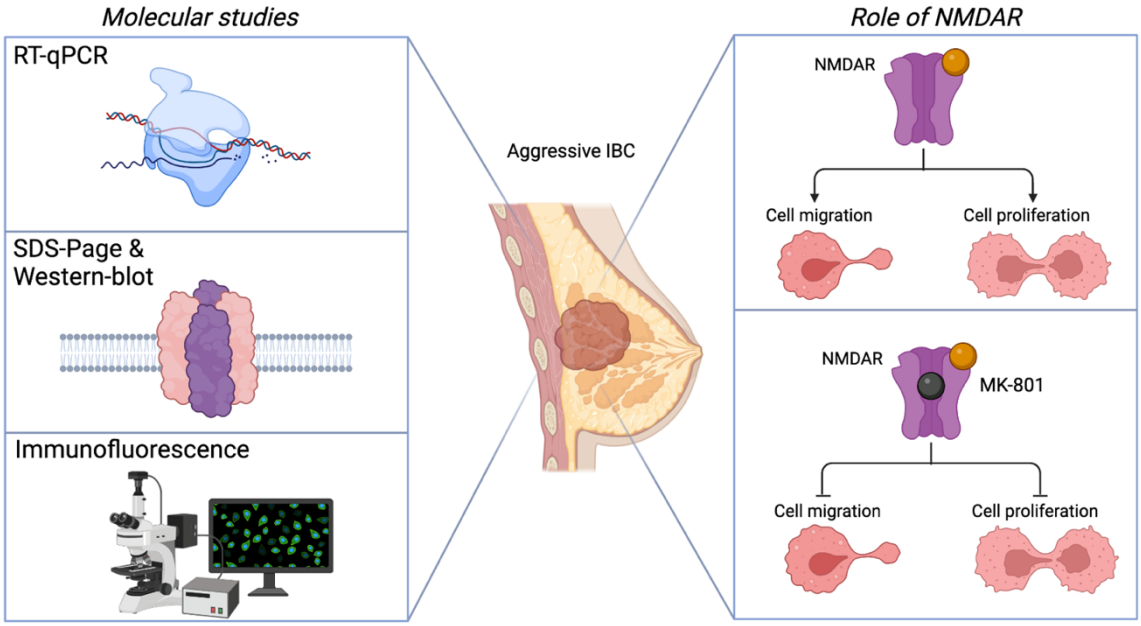
1.5 General Objective

The main objective of this master's thesis is to present evidence of the presence and possible roles of glutamate ionotropic NMDA receptors in inflammatory breast cancer (IBC). **Figure 3** summarizes the experimental design carried out for this research work.

To accomplish this objective, the following goals were developed:

1. **Identify the presence of NMDAR1 and NMDAR2 expression in IBC cell models.**
    a. We aimed to identify and compare the expression and location of the subunits of NMDAR1 and NMDR2B receptors in different breast cancer cell lines, emphasizing IBC cell lines SUM149PT and SUM190PT.
    b. We hypothesized that NMDARs subunits will be detected in the membrane of IBC cell lines.
2. **Determine the role of NMDAR on migration and proliferation of IBC cells**
    a. Using NMDAR selective antagonists memantine and dizocilpine (MK-801), we studied the effects of blocking the excitatory actions of NMDAR in the aggressive phenotypes (migration and proliferation) of IBC cells.
    b. We hypothesized that the inhibition of NMDAR will significantly decrease the migration and proliferation of IBC cells.

A.



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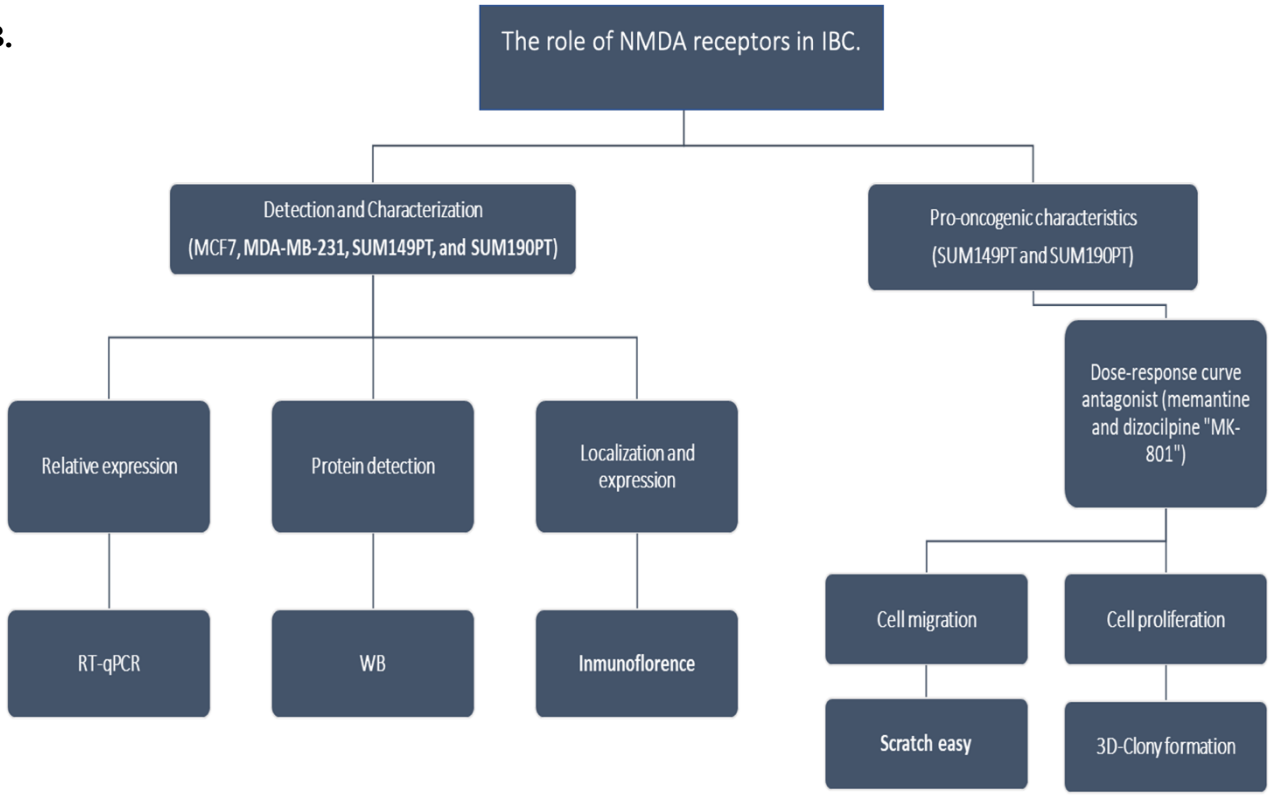


Figure 3. ***Research project summary.*** **A.** Graphic abstract B. Diagram and summary of experimental design. Research strategy for the characterization and detection of the role of NMDARs in aggressive phenotypes of IBC.

Chapter II:

Identification of NMDAR1 and NMDAR2 expression in IBC cells.

2.1 Introduction and rationale

Inflammatory breast cancer (IBC) is currently the most aggressive subtype of breast cancer, causing around 2-4% of deaths in women and 3% of deaths in men in the United States (Faldoni et al., 2020; Nofal & Yousef, 2019). IBC is characterized by a rapid onset of skin changes that underly the breast, including edema, redness, and swelling-that exhibit a wrinkled and orange-peel appearance of the skin defined as "peau d'orange" (Fernandez et al., 2013). Around 40-50% of IBCs are classified under two breast cancer phenotypes: the first, triple-negative breast cancer (TNBC) where they show a low presence of hormone receptors such as progesterone/estrogen receptors (HR-) and low presence of Human Epidermal growth factor Receptor (HER2-). And the second, amplification of Human Epidermal growth factor Receptor (HER2+).

The molecular subtypes TNBC and HER2+ can result in the enhanced probability that IBC cells metastasize to the brain by crossing the blood-brain barrier (BBB) (Weil, Palmieri, Bronder, Stark, & Steeg, 2005). Research studies have revealed that IBC has a 73% probability of metastasizing to the brain, limiting the patient's life expectancy of two years after diagnosis (Rosenbluth & Overmoyer, 2019). Moreover, recent research studies have shown that glutamate receptors are present in breast cancer cells that have metastasized into the brain, presumably increasing their survival rate in a synaptic environment (MA Gillentine, LN Berry, RP Goin-Kochel, MA Ali, J Ge, D Guffey, JA Rosenfeld, V Hannig, P Bader, M Proud, M Shinawi, BH Graham1, A Lin, SR Lalani, J Reynolds, M Chen, T Grebe, CG Minard, P Stankiewicz, AL Beaudet & Schaaf, 2017). The NMDA receptors (NMDAR), a type of ionotropic glutamate receptors, have been shown to be associated with oncogenic phenotypes in a wideset of cancer cell lines such as MCF7 and MDA-MB-231 (breast cancer lines) (Abdul & Hoosein, 2005; Alzahrani, 2019; Bunney et al., 2017;

Gorska-Ponikowska et al., 2017; Magaway, Kim, & Jacinto, 2019; Marqui, 2015; Seifabadi et al., 2017). NMDAR disturbs mammalian target of rapamycin (mTOR) pathways which are involved in regulating cell growth, survival, and migration (Alzahrani, 2019; Magaway et al., 2019; Marquard & Jücker, 2020; Murugan, 2019; Pedrosa, Mustafa, Soffietti, & Kros, 2018). Activated NMDAR triggers a transient Ca^{2+} influx, promoting a downstream signaling cascade which subsequently activates signals pathway abilities to control cell growth, survival, migration, and transcription **(Figure 4)** (Burket, Benson, Tang, & Deutsch, 2015; Koiri, 2015).

So far, it has been established that IBC has very few targets to treat, especially the Triple-negative and HER2-enriched subtypes. Meanwhile, even though NMDAR is associated with a variety of oncogenic phenotypes, it has not been studied in IBC. The discovery of NMDAR could act as a novel target for therapeutic purposes for patients with IBC diagnosis so that NMDAR in IBC could open a field of research for IBC patients.

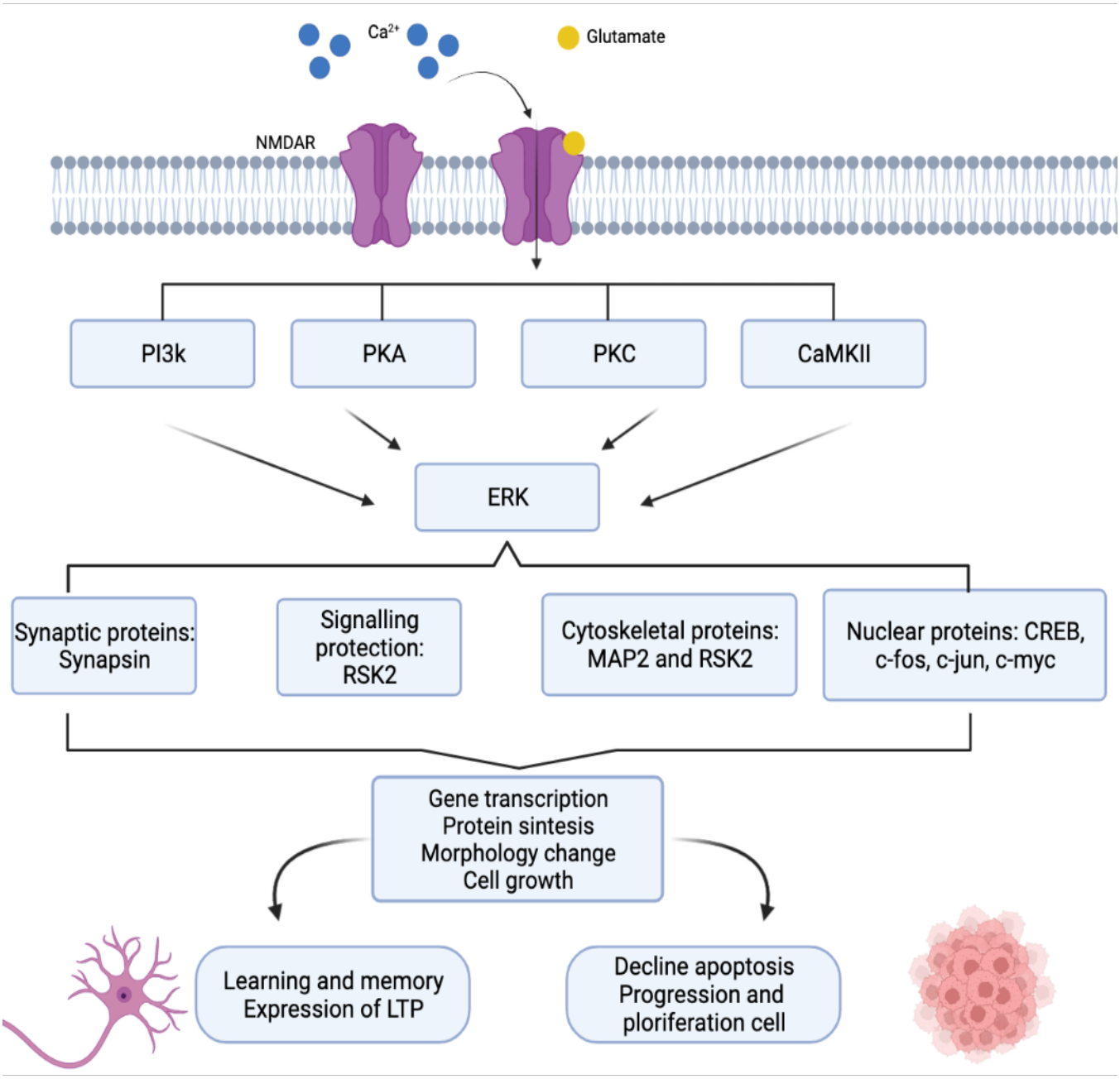


Figure 4. *Signaling pathways for NMDARs*. mTOR is the major cell growth regulatory pathway activated by ERK signaling. This is affected by the deregulation in the constitutive entrance of calcium through the NMDAR. Calcium continually influx activates several signaling pathways that activate and dephosphorylate kinases thereby causing an increase in regulation of mTOR (increased RSK2, MAP2, and nuclear proteins). The high regulation of mTOR keeps the signaling pathways active that increase the process of protein synthesis, survival, and proliferation in cancer lines. This process maintains the survival of the cancer cell. Created with BioRender.com

2.2Methods:

2.2.1 Cells lines: Non-IBC cell lines MCF7 (estrogen receptor-positive), and MDA-MB-231 (triple-negative) cell lines were bought from ATTC® company. For Inflammatory Breast Cancer, SUM149PT (triple-negative) and SUM190PT (HER2+) were acquired from BIOIVT elevating science®. These breast cancer cell lines were cultured and maintained under the conditions depicted in Table 3. The different cell cultures were kept growing for 2-4 days depending on the cell line. Cultures with less than 5 passages were used.

CELL LINE	GROWING MEDIUM	COMPANY
MCF7	• Dulbecco's Modifield Eagle's Medium – high glucose (DMEM) • Fetal Bavine Serum • Penicillin Streptomycin (Pen Strep)	• Sigma®; Ref D6429 • J.C. Gonzalez®; Ref HFBS001 • Gibco®; Ref 15140
MDA-MB-231	• RPMI 1640 • Fetal Bavine Serum • Penicillin Streptomycin (Pen Strep)	• CORNING®; Ref 10-041-CV • J.C. Gonzalez®; Ref HFBS001 • Gibco®, Ref 15140
SUM149PT	• Nutrient Mixture F-12 Ham • Fetal Bavine Serum • Insulin • Penicillin Streptomycin (Pen Strep) • Hydrocortisone	• Sigma®; Ref N6658 • J.C. Gonzalez®; Ref HFBS001 • MilliporeSigma®; Ref 19278 • Gibco®, Ref 15140 • Sigma®; Ref H0888-1G
SUM190PT	• Nutrient Mixture F-12 Ham • Fetal Bavine Serum • Insulin • Penicillin Streptomycin (Pen Strep) • Hydrocortisone	• Sigma®; Ref N6658 • J.C. Gonzalez®; Ref HFBS001 • MilliporeSigma®; Ref 19278 • Gibco®, Ref 15140 • Sigma®; Ref H0888-1G

Table 3. *Reagents used for the different cell culture media (MCF7, MDA-MB-231, SUM149PT and SUM190PT).* All culture media were kept in a refrigerator to maintain stability.

2.2.2 RT-qPCR: To determine the expression levels of NMDAR, a search of transcriptional genes for isoforms **(Table 4)** of human NMDAR1 and NMDAR2 was carried out in genome resources in NCBI. An alignment of nucleotides and proteins was achieved through the JALVIEW 2.11.1.3 program in order to find differences between isoforms and thus be able to design primers pairs. The primers used for RT-qPCR analysis of NMDAR2B were Forward (5'-CGTAGACCTGACCGACATC-3') and Reverse (5'-TTCTCCCAAGGTGCAGGTA-3'). The primers used for NMDAR1 were Forward (5'-CCATGCGCCTGCTGACGCT-3') and Reverse (5'-GGAGGTGGCATTGAGCTGA-3'). For the RT-qPCR experiment, we extracted between 300 - 400 ng/ul of the total RNA for the different cells lines MCF7, MDA-MB-231, SUM149PT, and SUM190PT, using the RNeasy mini kit (QIAGEN). The concentration and purity of RNA was measured using a nanodrop spectrophotometer (A260 / A280). After conducting these procedures, the total RNA was calculated and used for 20µl (1µM/µl) a reverse transcription reaction for the synthesis of cDNA using iScriptTM reverse transcription supermix for the RT-qPCR kit from BIO-RAD. qRT-PCR was performed 20µl of reaction using PowerUp SYBR Green Master Mix from Thermo Fisher (according to company protocol). Each assay consisted of its technical and biological triplicate n=9. GAPDH was used as a housekeeping gene control within each of the samples analyzed, Forward (5‘-ACCCAGAAGACTGTGGATGG-’3) and Reverse (5'-TCAGCTCAAGGGATGACCTTG-’3). After each experiment, a 3% agarose gel was run to verify the sizes of the amplified sequences. All reactions were prepared with 1µM of cDNA and 700 nM for each of the primers. The parameters for the thermal cyclin were polymerase activation and DNA denaturation: 95ºC for 2min; for the amplification it was used (denaturation: 95C for 15 sec; Annealing/Extension + Plate Read: 58C for 30sec, Cycles: 40, Melt-curve analysis: 95C for 5sec).

Name	Isoform	Primers
GRIN2A	**NM_000833.5:465-4859** Homo sapiens glutamate ionotropic receptor NMDA type subunit 2A (**GRIN2A**), transcript variant 2, mRNA **NM_001134407.3:571-4965** Homo sapiens glutamate ionotropic receptor NMDA type subunit 2A (**GRIN2A**), transcript variant 1, mRNA **NM_001134408.2:210-4055** Homo sapiens glutamate ionotropic receptor NMDA type subunit 2A (**GRIN2A**), transcript variant 3, mRNA	**FP: 5′ CATCTTGGGCATTCATGGG 3′** **RP: 5′ AGGGAGAAGACATGCCAGT 3′**
GRIN2B	**NM_000834.5:709-5163** Homo sapiens glutamate ionotropic receptor NMDA type subunit 2B (**GRIN2B**), mRNA	**FP: 5′ CGTAGACCTGACCGACATC 3′** **RP: 5′ TTCTCCCAAGGTGCAGGTA 3′**
GRIN2C	**NM_000835.6:158-3859** Homo sapiens glutamate ionotropic receptor NMDA type subunit 2C (**GRIN2C**), transcript variant 1, mRNA **NM_001278553.1:411-3032** Homo sapiens glutamate ionotropic receptor NMDA type subunit 2C (**GRIN2C**), transcript variant 2, mRNA	**FP: 5′ CAGTGTGGGTGATGATG 3′** **RP: 5′ ATGGGCACTGAGTTGTTGAA 3′**
GRIN2D	**NM_000836.4:507-4517** Homo sapiens glutamate ionotropic receptor NMDA type subunit 2D (**GRIN2D**), mRNA	**FP: 5′ TTCAGCTACGACCTCTACCT 3′** **RP: 5′ GACGGAGAAGTCCACGATCT 3′**
GRIN1	**NM_001185091.2** Homo sapiens glutamate ionotropic receptor NMDA type subunit 1 (GRIN1), transcript variant **GluN1-5b**, mRNA **NM_001185090.2** Homo sapiens glutamate ionotropic receptor NMDA type subunit 1 (GRIN1), transcript variant **GluN1-3b**, mRNA	**FP: 5′AGGAACTATGAAAACCTCG 3′** **RP: 5′ AGCATCGCGGCTGCGCGGT 3′**
GRIN1	**NM_007327.4** Homo sapiens glutamate ionotropic receptor NMDA type subunit 1 (GRIN1), transcript variant **GluN1-1a**, mRNA **NM_021569.4** Homo sapiens glutamate ionotropic receptor NMDA type subunit 1 (GRIN1), transcript variant **GluN1-2a**, mRNA	**FP: 5′ AGACACAGTGCTGCCGCGA 3′** **RP: 5′ TCAGCTCTCCCTATGACGG 3′**
GRIN1	NM_001185091.2 GluN1-5b NM_007327.4 GluN1-3b NM_021569.4 GluN1-1a NM_001185090.2 GluN1-2a **NM_000832.7** Homo sapiens glutamate ionotropic receptor NMDA type subunit 1 (GRIN1), transcript variant **GluN1-4a**, mRNA	**FP: 5′ CCATGCGCCTGCTGACGCT 3′** **RP: 5′ GGAGGTGGCATTGAGCTGA 3′**

Table 4. *Isoforms obtained from NCBI for the NMDR1 and NMDR2 receptors.*

2.2.3 Western Blots: Cell lines- To prepare protein extracts of (MCF7, MDA-MB-231, SUM149PT, and SUM190PT), the cells were grown to 80-90% confluency in growth medium according to the distributor's specifications (See Table 3). Cells were briefly washed with 1x Phosphate Buffer Saline (PBS) and lysed on ice using 1x Cell Lysis Buffer (9803S, Cell Signaling Technology) for 10 minutes. The cell lysates were centrifuged at 10,000 rpm for 20 minutes at 4ºC. The supernatant containing the protein extract was collected for further analysis. The protein concentration of all samples was determined using BCA method (Pierce™ BCA Protein Assay kit from thermoscientific REF.23227). Then, an SDS-PAGE was run using 25-50 μg at 80 volts, 30-40 Amp for 80 min of protein extract in a 4-15% polyacrylamide gel (Mini-Protean® TGX ™ Gel; BioRad) and transferred to a PVDF membrane for 7-10 minutes (high molecular weight transfer protocol). The membranes were blocked in 5% fat-free milk in (1x TBST - Tris-buffered saline and 0.1% of Tween 20) for 1 day at 20°C in the shaker and followed by incubation with the primary-antibody for 1 day at 20°C in the shaker. The primary antibodies used were NMDAR1 from Thermo Fisher and Abcam, (1:400 NDMAR1 Polyclonal PA3-102- Rabbit/IgG), (1:1000 GluR1 Monoclonal Antibody (MA5-27694)-Mause/IgG1), and (1:1000 Recombinant anti-NMDAR1 antibody Monoclonal (ab109182)- Rabbit/IgG1); primary antibody NMDAR2B (1:500 Phospho-NMDAR2B (Tyr1336) Polyclonal Antibody PA3-105-Rabbit/IgG), (1:500 NMDAR2B Monoclonal Antibody (NR2B) MA1-2014 - Mouse / IgG1), and (1:400 Anti-NMDAR2B antibody [NR2B] Monoclonal (ab28373) - Mouse /IgG1); GAPDH (1:1000 GAPDH mAB 2118S – Rabbit/IgG). After the incubation, the membranes were washed three times for 5 minutes in 1x TBST and incubated with secondary antibodies (1:1000 IgG anti-mouse 5415S) (1:1000 IgG anti-rabbit 7074P2). The membranes were developed using the ECL detection reagent from BioRad

(500µL of Super Signal™ west dura luminol/enhancer and 500µL Super Signal™ west dura stable peroxide) by the Azure® Imaging Biosystem.

2.2.4 Immunofluorescence: MCF7, MDA-MB-231, SUM149PT, and SUM190PT were grown on coverslips for 2 - 3 days and then fixed with 4% paraformaldehyde for 10min at room temperature. After three washes of 5 minutes with 1% PBS, the cells were permeabilized with 0.1 Tween20 diluted in 1% PBS for 5 min, blocked with 1% BSA/PBS/0.05% Tween20 for 30min at room temperature. The slides were incubated with NMDAR2B-antibody (1:250 in 1% BSA/PBS/0.05% Tween20, PA3-105 Thermofisher) and NMDAR1-antibody (1:250 in BSA/PBS/Tween20, PA3-102 Thermofisher) overnight at 4^0C. Slides were incubated with Goat anti-Rabbit IgG (H+L) Superclonal™ Secondary Antibody, Alexa Fluor® 488 conjugate (Product # A27034) at a dilution of 1:2000 for 1 h at room temperature. Nuclei were stained with SlowFade® Gold Antifade Mountant with DAPI (Thermo Fisher Product # S36938). F-actin were stained with Rhodamine Phalloidin (Thermo Fisher Product # R415, 1:2000). The stains were corroborated by fluorescence microscope with 40X and oil immersion objectives of 100X. Finally, the images were processed in a confocal microscope latest generation Nikon A1R laser at the Institute of Neurobiology in Old San Juan.

2.2.5 Confocal procedure: The confocal microscope is a Nikon A1R laser with Galvano and resonant scanner speeds, four lasers (403, 489, 561, 638), four fluorescence detectors (425-475, 500-550, 570- 620, 663-738), transmitted light detector (TD), and spectral detector. The Nikon Eclipse Ti inverted microscope at the facility has six objectives, low magnification is dry (4x, 10x,

20x) and high magnification are oil immersion (40x oil, 60x oil, 100x oil). The laser confocal microscope system uses the NIS Elements Advance Research 4.5 acquisition and analysis software. The images were processed in the same way and at the same intensity for each of the cell lines. Wavelength channels of (DAPI Em: 400nm; Alexa Fluor® 488. Em: 495nm; Rhodamine Phalloidin - Em: 505nm) were verified. Four random images were collected for each cell line (MCF7, MDA-MB-231, SUM149PT and SUM190PT), which were representative of each slide during the preparation of the immunostaining. In it, the location of each of the intensities was verified (DAPI: blue, Rhodamine: red, Alexa Fluor® 488: green)

2.2.6 Statistical analysis: Statistical significance one-way ANOVA or t-test were performed using GraphPad Prism 9. For 3D colony formation assays, quantile analysis was performed to eliminate extreme values in the data through Excel. Analysis was performed in technical triplicate and biological triplicate for each of the experiments.

2.3 Results and discussion

Our objective was to measure the expression level and cellular localization of NMDAR1 and NMDAR2 receptors in the IBC cell lines SUM149PT and SUM190PT. In previous studies, the expression of NMDAR has been found in different types of cancer. We used RT-qPCR, Western blots, and Immunofluorescence analyses to verify the expression of NMDAR1 and NMDAR2 subunits in different non-IBC (MCF7, MDA-MB-231) and IBC cell lines (Sum149PT and Sum190PT).

2.3.1 Relative quantification of NMDAR1 and NMDR2B subunit expression in IBC cell lines by RT-qPCR:

We examined the relative expression of the different isoforms of two different NMDA receptor subunits (NMDA2A-D, NMDA1a-b) in different cancer cell lines (MCF7, MDA-MB-231, SUM149PT, and SUM190PT). Expression of mRNA levels of the receptor subunits was normalized and compared to GAPDH, a housekeeping gene. Of all the primers that were designed, only (GRIN2B and GRIN1a) were amplified in our control cell line (MCF7). Therefore, only these primers (GRIN2B and GRIN1a) were verified in all cell lines **(Figure 5)**. Results showed that IBC cell lines have significantly less expression of NMDAR2B with respect to GAPDH compared to the other non-IBC cell lines (Ordinary One-way ANOVA p-values <0.05; p= 0.0008; **Figure 5B).** On the other hand, the relative expression for the NMDAR1 subunit tends to be more over-expressed in triple-negative cancer lines MDA-MB-231 and SUM149PT (Ordinary One-way ANOVA p-values<0.05; p= 0.0006; **Figure 5A**).

Within the different biological and technical triplicates made in this analysis, the Fold Change does not show significance for NMDAR2B (Ordinary One-way ANOVA p>0.05; p=0.08 **Figure 5D)**. The fold change for the NMDAR1 subunit was higher in cell lines (MCF7 and SUM190PT) and lower in cell lines with triple-negative phenotypes (MDA-MB-231 and SUM149PT) (Ordinary One-way ANOVA p<0.05; p=0.0037 **Figure 5D).** Meanwhile, the fold change trend for the NMDAR2B subunit was higher in SUM149PT lines (triple-negative and IBC) than in other cell lines. When confirming the amplification of each of the triplicates, no secondary band was observed (**Figure5C).** Non-IBC cell lines MCF7 and MDA-MB-231 were taken as controls (because they have been previously published in other studies) for comparative analysis of IBC cell lines (SUM149PT and SUM190PT).

Some of these results corroborate that non-IBC and IBC breast cancer cells contain genetic material to produce possible NMDARs, which in previous research have been studied in colon, pancreas, and lung cancer. These results may set a precedent for the genotypic characteristics of NMDARs for further analysis such as an increase in expression after having metastasized in the brain.

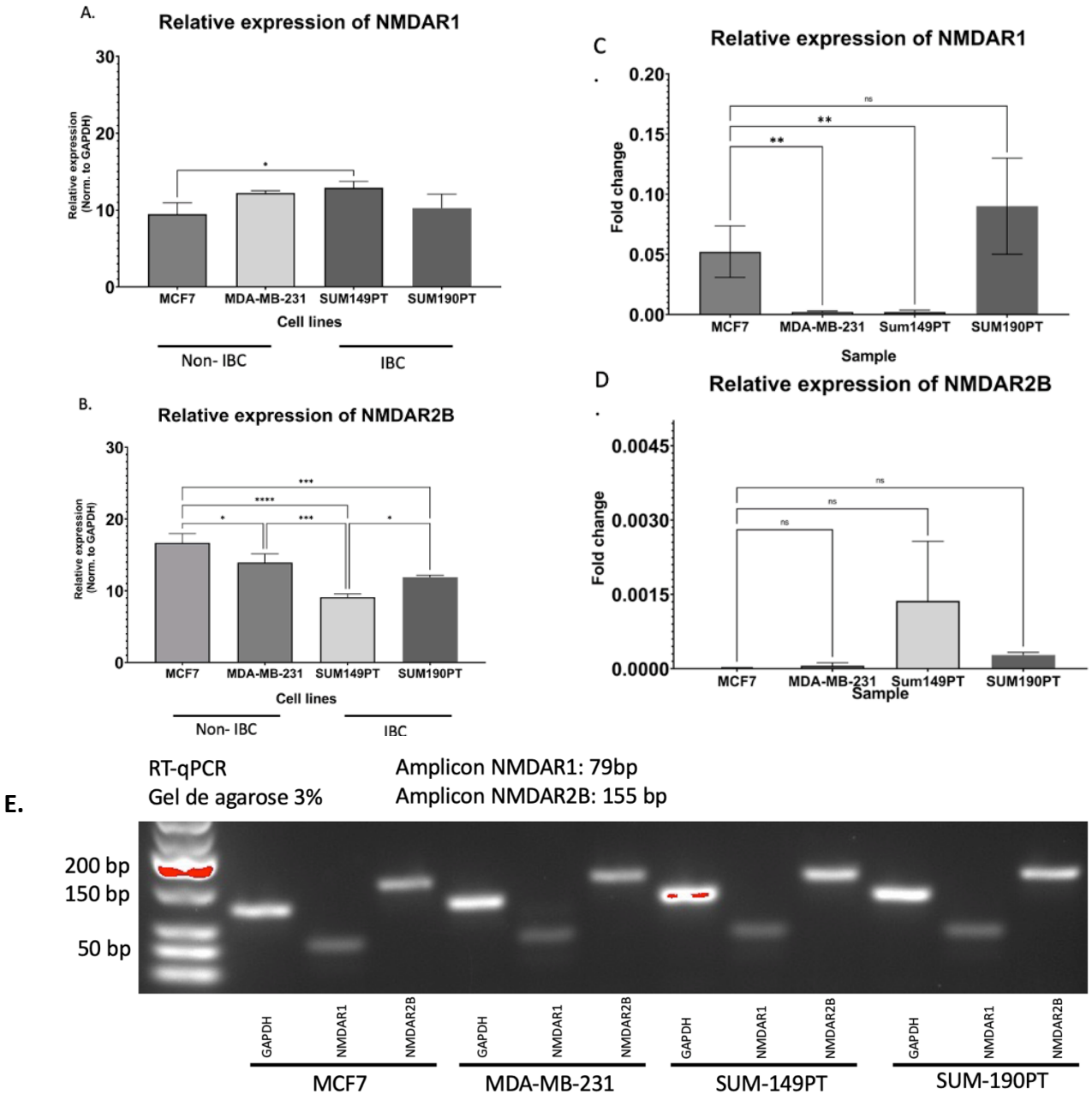


Figure 5. *Relative expression for NMDAR subunits*. A-B Relative expression with GAPDH was verified for each of the tissues studied (n = 9). C-D. Relative expression (Fold Change) comparison of four breast cancer cell lines (MCF7, MDA-MB-231, SUM149PT and SUM190PT). E. To confirm the amplification of the different primers in the different cell lines (MCF7, MDA-MB-231, SUM149PT, SUM190PT) during the RT-qPCR amplification, an agarose gel was carried out. The

Relative expression of NDMAR1 p-values of <0.05 (p= 0.0006); For the NMDAR2B p-values of <0.05 (p= 0.0008) with standard deviation. The Fold change NMDAR1 p-values of <0.05 (p= 0.0037); The NMDAR2B p-values of <0.05 (p= 0.08) were considered statistically significant and are indicated by asterisks (*).

2.3.2 Protein quantification of NMDAR1 and NMDR2B subunits in IBC cell lines:

After detection of the relative expression at the mRNA level, verification at the protein abundance was performed. During these experiments, relative expression analysis was performed with respect to a housekeeping gene or calibrator gene (GAPDH). During the processing of the membranes, clearer bands are observed compared to the GAPDH control. This means that there is less abundance of the different subunits compared to the GAPDH scaffolding protein. On the other hand, similar protein levels for the NMDAR1 subunit were found in all cell lines except for the MCF7 cell line (low protein expression) (P<0.05; P= 0.0001; **Figure 6A**). The similar expression between cancer lines (MDA-MB-231, SUM149PT, and SUM190PT) is in accordance with the functional character of the NMDA receptor **(Figure 6 A).** The NMDAR1 subunit has been identified as a required unit for the different combinations of heterotetramers formation (Lau, Saha, Faris, & Russek, 2004), for the NMDA2B subunit, different protein levels were found per cell line. For example, a significantly higher expression (P<0.05; P= 0.0002) of NMDAR2B subunits was observed for IBC cell lines when compared to non-IBC cell lines (**Figure 6B**). The NMDA2B subunit is responsible for the electrophysiological characteristic of the receptor and the regulation of phosphorylation which mediates a variety of functions in the cell (Delawary et al., 2010). Furthermore, NMDAR2 can be replaced by another isoform (NMDAR2 A-D) or it can be replaced

by another subunit such as NMDAR3. Previous research showed, during several meta-analyses of patients with breast cancer, that the subgroup of patients with HER2+ expression has an incidence of brain metastasis of 31% and 32% for patients with triple-negative phenotypes (Kuksis et al., 2020, 2021), opening the possibility for novel therapeutic targets like NMDAR. There are few therapies for patients with IBC and for patients with brain metastasis from breast cancer (BCBM) with these molecular phenotypes (Bailleux, Eberst, & Bachelot, 2021). Therefore, the presence of these subunits in these cancer lines is of great importance to open new fields of research in IBC and BCBM.

It is worth mentioning that during the study of receptor expression (NMDAR1 and NMDAR2), expression of various protein of different sizes (37 - 150 kd) was found with the different antibodies used **(Figure 14)**. However, the bands analyzed for this work were of the sizes provided by the suppliers' SDS page and the cited paper (100 kDa for NMDAR1 and 160 kDa for NMDAR2B). The other sizes could be assumed as previously established, NMDAR1 many splice variants and partly matches our NCBI sequence search. Similarly, for the NMDAR2 subunit, four different NMDAR2 subunits (A-D) are known, each with their respective variants (Laurie, Bartke, Schoepfer, Naujoks, & Seeburg, 1997; Yi, Zachariassen, Dorsett, & Hansen, 2018). This exposure can be justified by a fragment of conserved sequence which would explain the multiple bands during the Western Blot. It has been found that part of the investigations associated with the presence of this receptor carried out a knockout (focusing on bands associated with ~160kDa) of the receptor in xenograft and cell cultures (neurons and cancer cells) (Akerman, Allen, Sylvain, & North, 2011; Morelli et al., 2019; Zeng et al., 2019).

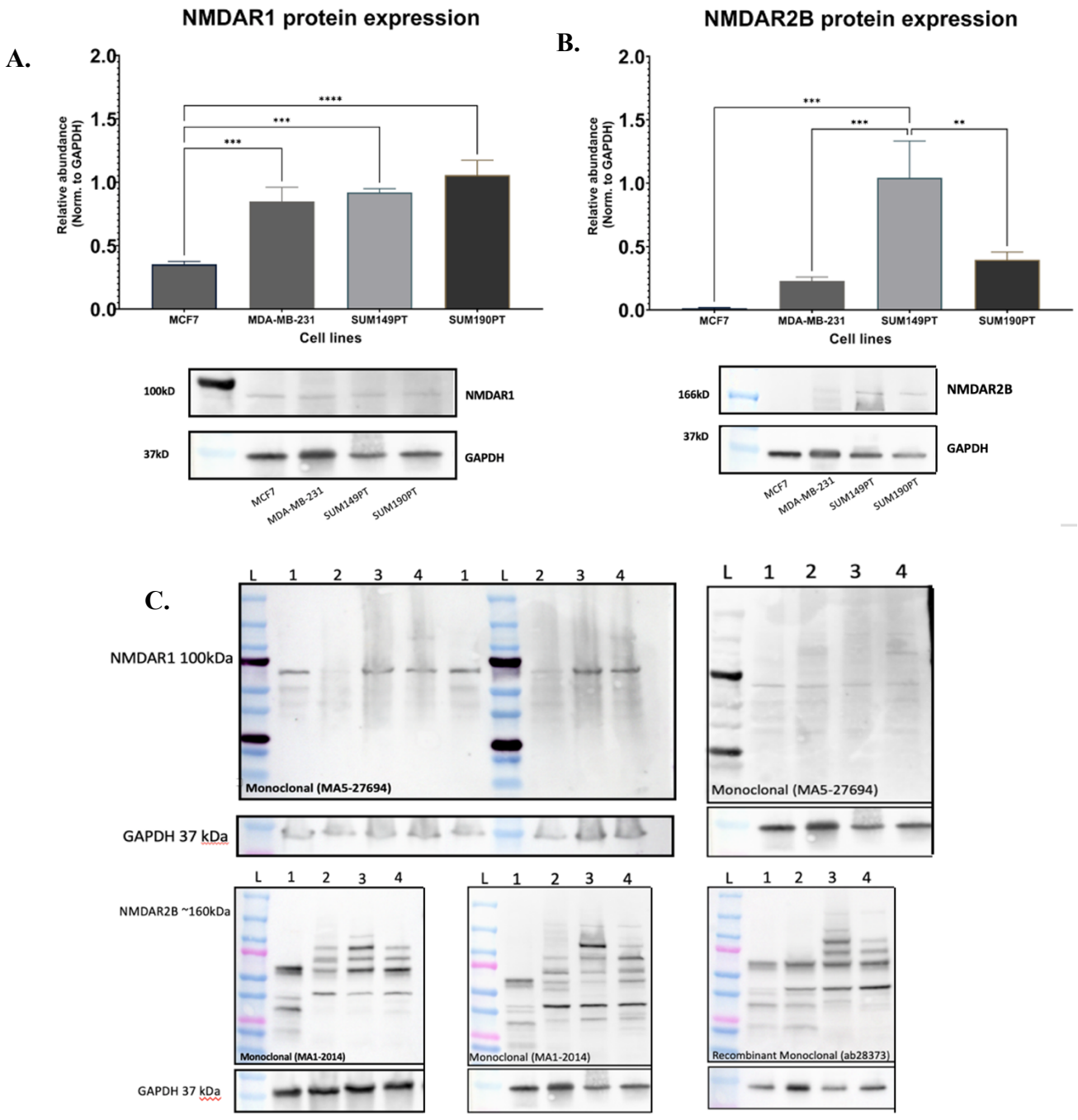


Figure 6. *Protein expression for NMDAR subunits*. **A-B** Protein expression comparison of four breast cancer cell lines (MCF7, MDA-MB-231, SUM149PT and SUM190PT). It is worth noting that the expression with GAPDH was verified and normalized for each of the tissues studied. **C.** Membranes used for protein analysis, each of the lanes is marked as follows: (L) Ladder Dual Color (1) MCF7, (2) MDA-MB-231, (3)SUM149PT, (4)SUM190PT. The NDMAR1 p-values of <0.05 (p= 0.0001) n=3; For the NMDAR2B p-values of <0.05 (p= 0.0002) n=3 was considered statistically significant and are indicated by asterisks (*).

2.3.3 Novel finding of NMDA localization through immunofluorescence in cancer lines:

The NMDAR signaling in the nervous system is orchestrated by glutamate binding and pore opening for calcium regulation, ending with neuronal membrane depolarization. On the other hand, it has been found that, in tissues outside the nervous system, where the cell membrane has low polarizing activity, the activity of NMDARs can be continuous, such as some types of cancer like pancreatic and breast cancer (Bunney et al., 2017; Glebov, 2020; Malsy et al., 2015). NMDARs outside the central nervous system have been reported to support cells during development and assist other oncogenic features (Chakraborty et al., 2017). During this work, we wanted to observe the localization of the NMDAR subunits in different lines of breast cancer. As mentioned before, the localization of NMDARs in cancer has hardly been studied, meaning that their study is of great importance.

In the first data set, staining of the NMDAR2B subunit is observed in the confocal images. The location of the subunit in all cell lines is detailed. The presence of the subunit in the MCF7 and SUM190PT cell lines is also more diffuse or homogeneous, which is more related to the cell membrane. On the contrary, in cell lines MDA-MB-231 and SUM149PT, the location of this subunit is more detailed in the cytoplasm or perinuclear area **(Figure 7).** In the second data set, NMDAR1 subunit staining is present in all cell lines. These images demonstrate that the NMDAR1 subunit is highly localized in areas of the cytoplasm in all cell lines **(Figure 8).**

The presence of functional receptors was found in the different breast cancer cell lines under study (MCF7, MDA-MB-231, SUM149PT, and SUM190PT). This type of analysis increases the research field in breast cancer in general, thus showing the presence and location in cell lines that have not been studied for this receptor NMDAR2B **(Figure 7)** and NMDAR1 **(Figure 8).** The third column details the location of NMDAR2B and NMDAR1. In this column, we can also detail

a slight stippling in the different cell lines. However, in SUM149PT cell lines for both NMDA receptors more density can be seen in perinuclear areas or membranous areas located in the cytoplasm. Meanwhile, as can be seen in the comparison between groups (NMDAR2 vs. NMDAR1), the stippling of NMDAR2 is a little more dispersed and that of NMDA1 is more localized in the cytoplasm. In the last column of the figures, the location of the structural protein b-actin is shown in red. At the moment, the presence in the membrane and cytoplasm of each of the cell lines was observed for both NMDAR subunits, thus associating the oncogenic characteristics, and assumed in previous studies. Few studies have found the location of this receptor in cancer lines. However, this immunofluorescence/staining process showed the existence of NMDAR in regions such as the cytoplasm located in membranous organelles of the cell such as endoplasmic reticulum, Golgi apparatus, endosome, the cell surface, and perinuclear region (Jiménez-González, Ogalla-García, García-Quintanilla, & García-Quintanilla, 2019). Finally, the non-specific binding of the primary antibodies was verified (NMDAR1 and NMDAR2B) during the incubations of the controls. Supplementary **(Figures 15 and 16),** for instance, evidences the absence of signal for NMDAR antibodies. The confocal images represent the confocal technician was requested to leave the same intensity parameters for each one of the images taken in each of the emission channels.

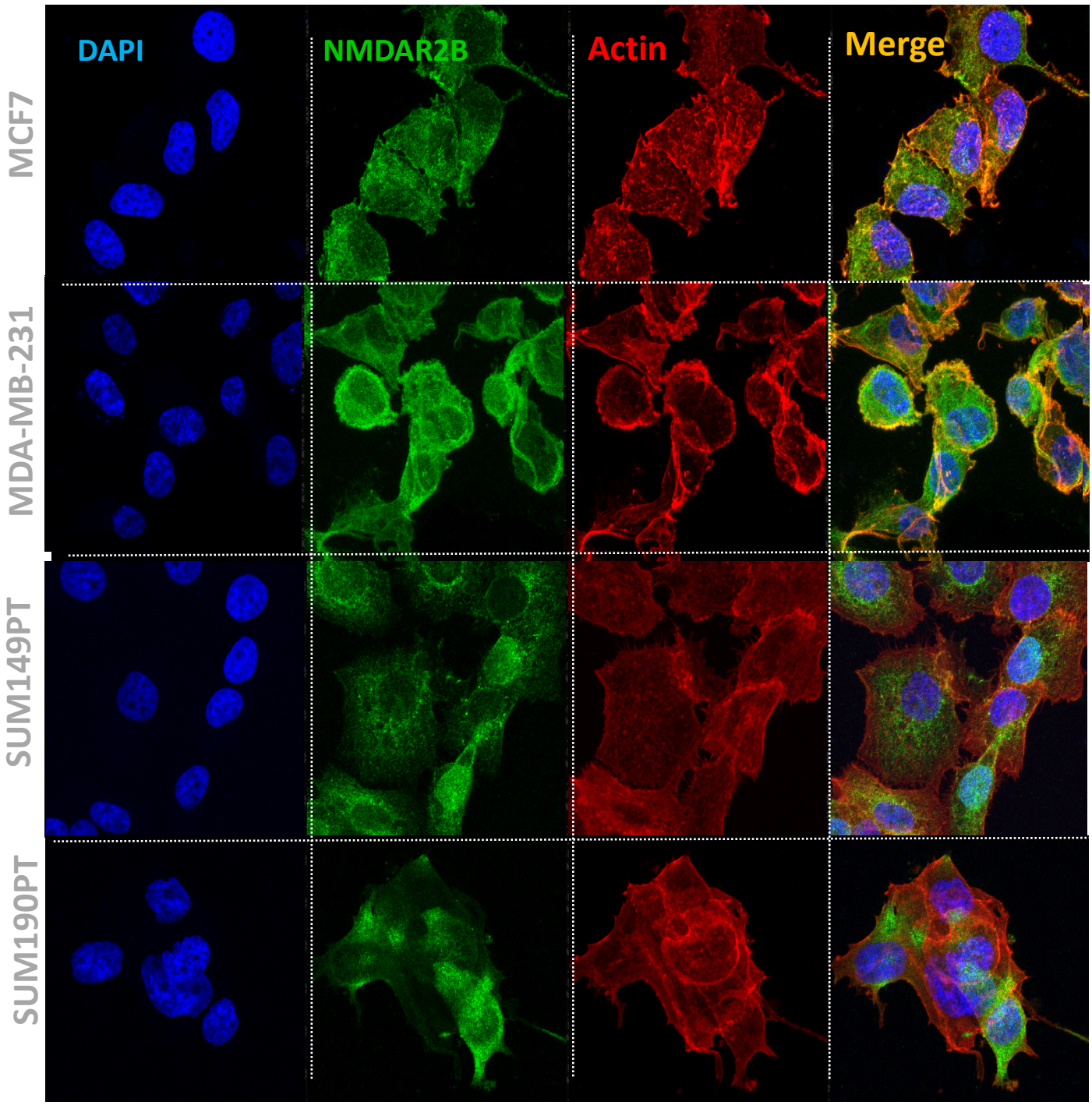

DAPI
NMDAR2B
Actin
Merge
MCF7
MDA-MB-231
SUM149PT
SUM190PT

Figure 7. *Localization and presence of NMDAR2 subunit in breast cancer cell lines* (MCF7, MDA-MB-231, SUM149PT, and SUM190PT). In the first column of images, we observe the location of the nucleus in the color blue. The second column details the location of NMDAR2B. Also, this column illustrates a slight stippling in the different cell lines. In SUM149PT cell lines for both NMDA receptors, more density can however be seen in perinuclear areas or membranous areas located in the cytoplasm. In the third column, the Actin staining is observed, which is a scaffolding protein, while the fourth column illustrates the superposition of the images. Photos are taken in a confocal microscope (100X), all fixation parameters, staining, sample preparation, and microscope settings were the same for all samples.

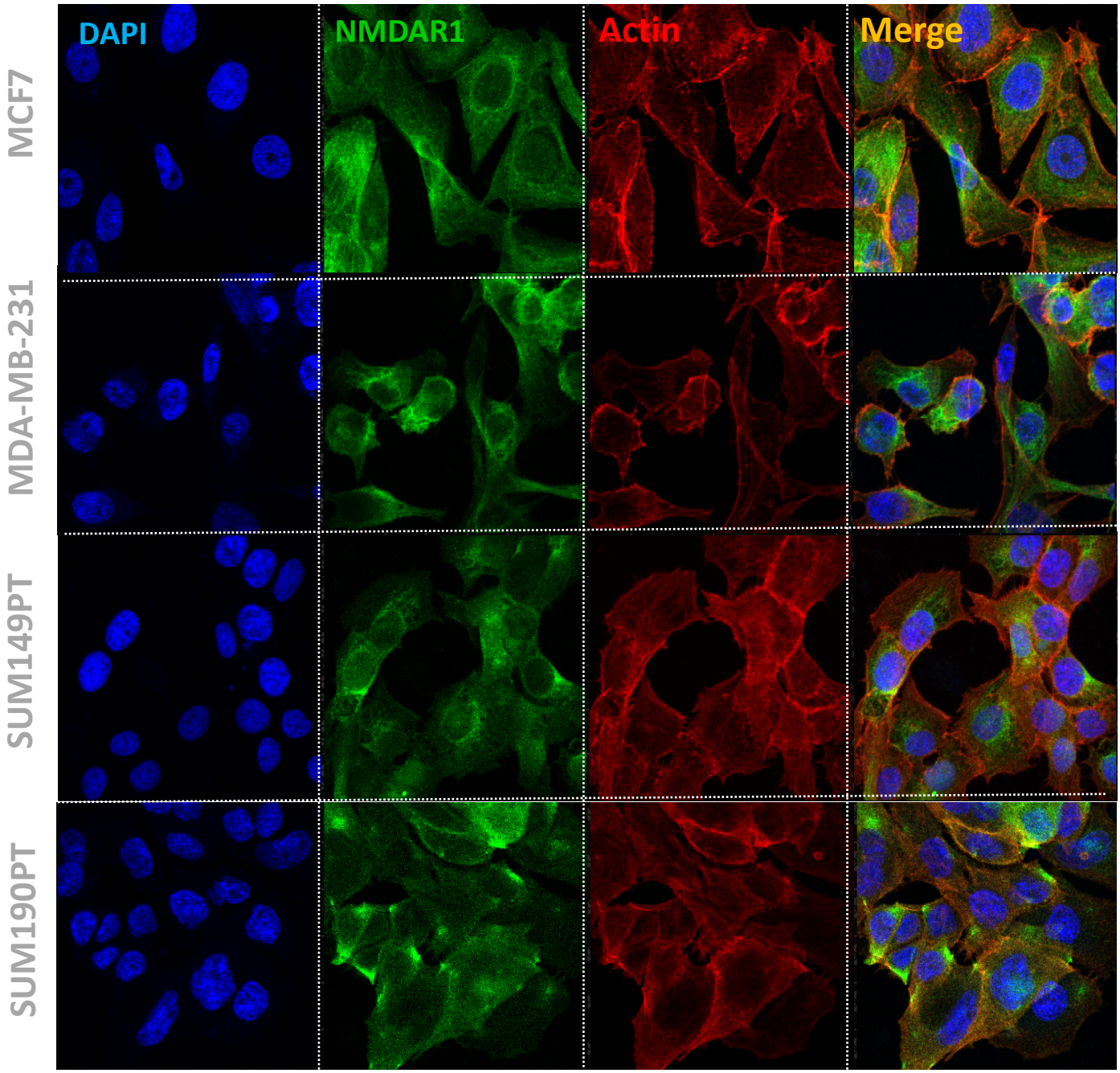

DAPI
NMDAR1
Actin
Merge
MCF7
MDA-MB-231
SUM149PT
SUM190PT

Figure 8. *Localization and presence of NMDAR1 subunit in breast cancer cell lines* (MCF7, MDA-MB-231, SUM149PT, and SUM190PT). In the first column of images, we observe the location of the nucleus in blue. In the Second column, we detail the location of NMDAR1. This column also demonstrates a slight stippling in the different cell lines. Nevertheless, in SUM149PT cell lines for both NMDA receptors~~,~~ more density can be seen in perinuclear areas or membranous areas located in the cytoplasm. Meanwhile, the third column displays the Actin staining, which is a scaffolding protein, whereas the fourth column is the superposition of the images. Photos are taken in a confocal microscope (100X), all fixation parameters, staining, sample preparation, and microscope settings were the same for all samples.

2.3.4 Discussion:

Recent studies have found the expression of NMDR1 and NMDAR2 in breast cancer cell lines such as MCF-7, MDA-MB-231, SKBR-3, and different glioma cell lines (Abdul & Hoosein, 2005; Zeng et al., 2019). However, no study has shown that these subunits are present in the IBC lines SUM149PT and SUM190PT. Consequently, the main objective of this study was to determine the expression of NMDAR1 and NMDR2B subunits in the IBC lines SUM149PT and SUM190PT, using as reference the cell line MDA-MB-231, a TNBC that expresses the NMDAR subunits, and MCF7, a commonly used breast cancer cell line that expresses NDMAR (Bunney et al., 2017; Seidlitz et al., 2009). We hypothesized that NMDAR subunits expression would be detected and localized in IBC cell lines. In order to conduct these experiments, we used western blots to verify the presence of NMDAR1 and NMDR2 proteins in the IBC cell lines (SUM149PT and SUM190PT) and compare it with other non-IBC (MCF-7 and MDA-MB-231) cell lines. Moreover, we quantified the gene expression by qRT-PCR to obtain the relative expression of NMDAR1 and NMDR2 subunits in the different IBC and non-BC cell lines. The expression of the different isoforms of each NMDAR were compared and measured by qRT-PCR. During these analyses, we identified the presence of NMDAR in IBC cancer lines. According to the literature, the NMDAR1 subunit is obligatory for the composition of the receptor. In our studies, we have found similar levels respectively between the experiments. Therefore, it is shown that the NMDAR1 subunit is present in IBC and is required for NMDAR functionality. On the other hand, it has been shown in the literature that the presence of NMDAR2 varies, either by different isoforms (NMDAR2 A-D) or by another subunit (NMDAR3). We were able to observe in our findings that the presence of NMAR2B varies between different cell lines at both the genomic level and at the protein level.

The increase expression of NMDAR can alter the morphology, oncogenic characteristics (migration, proliferation, and invasion), microenvironment interaction, and cellular metabolism (Du et al., 2019). Previous studies reported that an increase of interstitial fluid of a solid tumor increases the expression of NMDAR to maintain the constant autocrine regulation of Glu. (Li & Hanahan, 2013). Lastly, we discuss the quantification and characterization analysis of NMDARs in different cell lines through genomic and protein expression in different cell lines.

Inflammatory breast cancer (IBC) is one of the most aggressive cancers due to its lack of clinical treatment and is one of the least reported at a research level. For this reason, it is imperative to study a possible molecular route of therapy for these patients. Throughout this work, the importance of NMDARs during progression and oncogenic phenotypes in different types of cancer has been exposed, with a special focus on IBC. During this first aim, new knowledge has been provided and reported at the molecular and cellular level about NMDARs in both Non-IBC and IBC lines. Furthermore, it was possible to characterize the presence of NMDARs in different breast cancer lines through molecular analysis (RT-qPCR, western blot, and immunofluorescence); It was characterized cell lines, especially in IBC cell cultures, that were particularly involved in brain metastasis (SUM149PT and SUM190PT). From the characterization carried out in this research work, a difference in expression was found at the gene and protein levels, suggesting how NMDAR subunits in IBC cell lines can be associated with the aggressiveness of this type of cancer. Lastly, the integrity of the large portion of this receptor in different breast cancer cell cultures proposes a strong path of investigation for future therapeutic routes.

Chapter III:

To determine the active role of NMDAR in the oncogenic phenotypes of IBC cells

3.1 Introduction and rationale

In previous studies, treatment with NMDAR agonists such as D-Asp and D-Ser has shown to stimulate cellular proliferation in cancer cells (Abdul & Hoosein, 2005; Du et al., 2019). Other researchers have reported decreased cellular proliferation and spread of cancer tumors on the brain, thus helping the patient's cognitive health using NMDAR antagonists (memantine and dizocilpine "MK-801"), without side effects (X. Song et al., 2018). In other cancer research areas, NMDAR antagonists were shown to have positive effects on the pro-oncogenic phenotypes by diminishing migration, invasion, and proliferation. *We hypothesized that inhibition of NMDAR will significantly decrease migration and uncontrolled proliferation) of the IBC cells*. Using a NMDAR antagonist, we proposed to measure changes in the aggressive phenotypes mediated by NMDARs in IBC cells. For this purpose, using a cell viability assay, we generated dose-response curves for two NMDAR antagonists (in 2D and 3D cultures) to determine the IC50 for each of the drugs. We also performed two functional assays, Wound Healing, and 3D colony formation to measure the effects of the antagonists on the ability of IBC cells to migrate. These trials were carried out at different time points according to the functional assay (migration before cell doubling time - 19 hours for SUM149PT) and proliferation assay (3D colony formation assay - 10 days for both cell lines). A decrease was expected in migration and proliferation of IBC cell lines (SUM149PT, SUM190PT).

Dizocilpine (MK-801) and memantine are two small size antagonists used in different therapies related to NMDAR neuronal overexcitation. These two antagonists are used due to the difference in affinity according to the type of therapy. MK-801, with a high affinity, can remain bound to the receptor for a prolonged period. This drug is a neuroprotective agent in models of stroke, trauma, and parkinsonism, but can induce psychotic behavior and neuronal degeneration in some cases

(Kovacic & Somanathan, 2010). On the contrary, memantine has been more commonly employed for neurodegenerative diseases such as Alzheimer's due to its weak ionic bond (Johnson & Kotermanski, 2006; X. Song et al., 2018) **(Figure 9)**. These two antagonists are considered non-competitive NMDA blockers because they can enter the channel receptor and block current flow of calcium ions only after channel opening. Moreover, these two MNDAR antagonists have been shown to have antiproliferative and anti-invasive effects which, in turn, could lead to different effects on cascades of intracellular phosphorylation **(Figure 4)**.

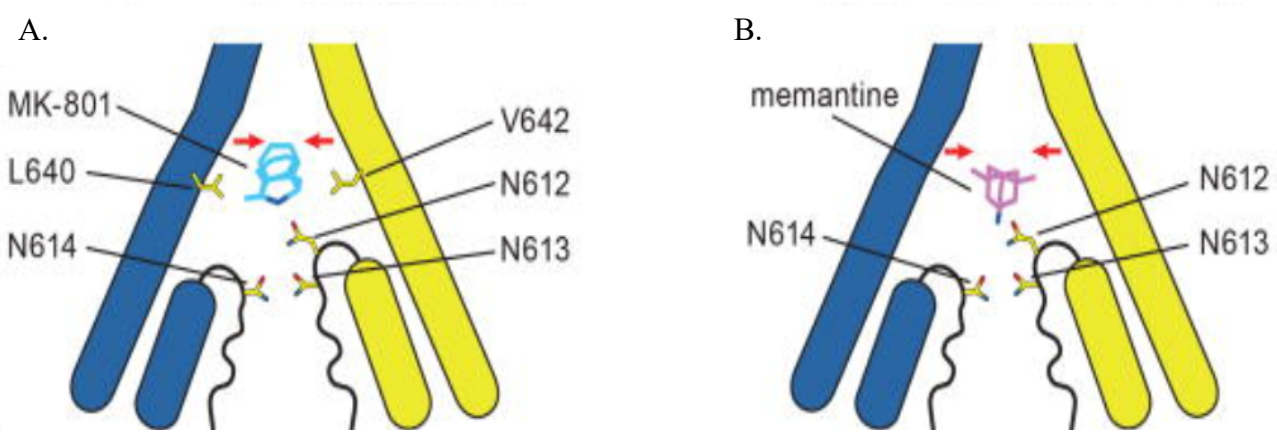


Figure 9. *Schematic representations of A. MK-801 and B. Memantine binding sites, respectively*. Both channel blockers induce channel closure (red arrows) while blocking the pore and adopting similar interactions with key asparagine residues. MK-801 binds in two-terminal amino acids and 2-fold related poses (2 conformational changes in the receptor), whereas Memantine binds in a single predominant pose (1 conformational change in the receptor) (Xianqiang Song, et, at; 2018).

3.2 Methods:

3.2.1 Cells lines: Human breast cancer cell lines culture and maintain with the conditions depicted in **(Table 5)**; For Inflammatory Breast Cancer, SUM149PT (triple-negative) and SUM190PT (HER2+) were acquired from BIOIVT elevating science®.

CELL LINE	GROWING MEDIUM	COMPANY
SUM149PT	• Nutrient Mixture F-12 Ham • *Nutrient Mixture F-12 Ham, Without L-glutamine • Fetal Bavine Serum • Insulin • Penicillin Streptomycin (Pen Strep) • Hydrocortisone	• Sigma®; Ref N6658 • Sigma®; Ref N4888 • J.C. Gonzalez®; Ref HFBS001 • MilliporeSigma®; Ref 19278 • Gibco®, Ref 15140 • Sigma®; Ref H0888-1G
SUM190PT	• Nutrient Mixture F-12 Ham • * Nutrient Mixture F-12 Ham, Without L-glutamine • Fetal Bavine Serum • Insulin • Penicillin Streptomycin (Pen Strep) • Hydrocortisone	• Sigma®; Ref N6658 • Sigma®; Ref N4888 • J.C. Gonzalez®; Ref HFBS001 • MilliporeSigma®; Ref 19278 • Gibco®, Ref 15140 • Sigma®; Ref H0888-1G

Table 5. *Reagents used for the different cell culture media (MCF7, MDA-MB-231, SUM149PT and SUM190PT).* All culture media were kept in a refrigerator to maintain stability. (*) Growth medium according to literature to have glutamate-free cultures and measure autocrine regulation.

3.2.2 Drugs:

For these experiments, specific antagonists for NMDAR, Memantine (M9292) and Dizocilpine (475878) were purchased from Sigma Aldrich. Both drugs were dissolved in water and stored at -20ºC in aliquots. Dizocilpine and Memantine have been prepared in a stock concentration of

75mM and 584.2mM, respectively. For each experiment performed, the drugs were diluted with a growth medium (**Table 5)** to obtain the concentration for each to the different cell cultures under study (SUM149PT and SUM190PT) (Bunney et al., 2017).

3.2.3 Dose-response curves:

We used monolayer growths (2D) to verify the concentration of NMDAR antagonists in serial dilutions between (12.5µM - 800 µM) in IBC cell lines (SUM149PT and SUM190PT). Cell viability was verified at various time points (24, 48, and 72 h) with the alamarBlue™ (from Thermo Fisher Scientific) Cell Viability Assay to generate a dose-response curve and calculate the IC50. Concentration parameters and time points were suggested in previous studies with different cells (North, Gao, Memoli, Pang, & Lynch, 2010). NMDAR antagonists (memantine and dizocilpine "MK-801") were prepared with Cell Culture Grade Water according to the manufacturer's instructions and previous studies (Valis et al., 2019). For the 2D culture system, cells were seeded in a 96 wells plate with 100 µl of the growth medium. After 24, 48, and 72 h of treatment, the medium was removed, 100 µl of 1:10 dilution of Alamar Blue Reagent were added, and the plate incubated for 2 h at 37°C. The intensity of the fluorescence of the dish was then measured (wavelength of 550nm of excitation and 585nm of emission) to quantify fluorescence in the Infinite® 200 PRO Microplate Readers (Tecan). Finally, the data was exported through excel and a dose-response curve (an XY table with the average of each of the samples with its standard deviation) was generated using GraphPad Prism. At this point, the concentration of 300 µM was determined from IC50 for MK-801 for the following functional assays. Subsequent functional trials with memantine were discarded because an IC50 was never obtained.

3.2.4 Wound Healing Assay (Cell motility and migration):

For these experiments, cell cultures with SUM149PT were grown in a 10mm 6-well plate to 100% confluence. The cell culture washed with HBSS 2-3 times were prepared to remove any type of sediment or previous growth medium. A scratch was carefully produced through the plate with a 10 µl tip. Subsequently, a volume of 2ml was applied with its respective treatment (MK-801, 300 µM). The area that remained free of cells during 0h and 19h was then quantified and compared using the ImageJ program. These images were taken at 1000X magnification by means of a light microscope through the NIS Elements Advance Research 4.5 acquisition and analysis software. The area data obtained in each of the experiments were tabulated and compared in Excel. Finally, the area closed after 19h by the cells was analyzed through statistical analysis and plotted.

3.2.5 3D Colony Formation for IBC cell lines: Proliferation and colony formation assay was performer through Matrigel in 24-well plate for SUM149PT and SUM190PT cell lines. At this point, 150 µl of Matrigel were incubated (30 min at 37 degrees) and 10,000 cells (2 h) were incubated for each treatment. A solution of 5% Matrigel with growth medium and 300 µM MK-801 was then applied to the different well cultures. The drug and the medium were changed every 2 days for a period of 10 days of the experiment. After this step was finalized, the area per pixel of approximately 60-70 colonies per well was counted for each treatment. The colony size was measured through ImageJ. Extreme values were checked using quartiles to verify their removal from the data. Finally, the data was statistically analyzed and plotted.

3.2.6 Statistical analysis: Statistical significance one-way ANOVA or t-test were performed using GraphPad Prism 9. For 3D colony formation assays, quantile analysis was performed to eliminate extreme values in the data through excel. Analysis was performed in technical triplicate and biological triplicate for each of the experiments.

3.3 Results:

Our objective for this aim was to determine if NMDARs were involved in the migration and proliferation phenotypes of IBC cell lines (SUM149PT and SUM190PT). In previous studies in different breast cancer cell lines (MDA-MB-231) and xenographs treatment with NMDAR antagonists decrease cellular proliferation. Using NMDAR antagonists (memantine and dizocilpine "MK-801"), we studied the changes in the aggressive phenotypes (migration and proliferation) of BC cells.

3.3.1 Inhibition of NMDA receptors in IBC cell lines:

A dose-response curve was performed for IBC cell lines SUM149PT and SUM190PT which exhibit triple-negative and HER2+ phenotypes associated with a high incidence of crossing the BBB and metastasizing to the brain. The MK-801 drug was found to have promising effects (decreased cell proliferation which is a vital indicator for viability assays) at a concentration of 300 μM. On one hand, for the drug memantine, IC50 was never reached. Cell viability in the presence of different concentrations of MK-801 was found to decrease for both IBC cell lines (n=9). For the MK-801 antagonist, the concentration of the different days (24, 48 and 72 hours) was averaged and the IC50 was determined to be 300μM. On the other hand, the use of the different concentrations of memantine had no effect on the decrease in cell viability.

The use of non-competitive antagonists MK-801 and memantine are associated with the successful blockage of the transmembrane domain of NMDARs. The mechanism of action of these drugs has been previously studied, establishing the comparative kinetics of both. MK-801, exhibits greater affinity due to its interaction with domains in the different subunits (NDMAR1/NMDAR2B), maintaining a greater time of synergism or binding specifying blocking of the NMDA receptor pore **(Figure 10A-B).** On the contrary, memantine, having weak binding to the ion channel and being associated with an active site of the Magnesium binding (ionic blocker of the NMDAR), causes changes in voltage that leads to the release of the receptor pore. This information is corroborated with increasing concentrations obtained for memantine **(Figure 10 C-D)**. Once this concentration was found for each of the IBC cell lines, it was decided to continue with the cell functional assays (3D colony formation and cell motility) using 300μM MK-801, because it showed an effect decreasing cell viability. However, this concentration is a little lower than what is generally administered for a neuroprotective role in different neuronal studies (Hargreaves, Hill, & Iversen, 1994; Woodruff et al., 1987).

MK-801 is a frequently used drug in the field of neuroscience and many of the mechanisms used for these drugs have already been studied (receptor blockade on the plasma membrane). Nevertheless, by detecting NMDAR in the cytoplasmic space of cancer cells, a very important question arises: how can these drugs have an effect inside the cell? How do these drugs cross the plasma membrane to block the receptors found inside the cell? Cancer research simplifies the reduced action of cell viability in the presence of NMDA antagonists. Little literature delves into the interaction of these antagonists in the membrane. During the search for an explanation for this phenomenon, it was found that MK-801 works partially as ketamine. As has been reported, MK-801 in an aqueous solution destabilizes the cell membrane, thus creating vesicles that introduce

the drug into the cell, releasing and release the drug, especially in the cytoplasm. As MK-801 does not have a specific bond with the phospholipids in the vesicles, it into the cytosol and finally binds to the internal receptors (Johnson & Kotermanski, 2006; Moring, Niego, Ganley, Trumbore, & Herbettet, 1994; Yang, Lee, & Sternson, 2015). Observing the decrease in cell viability with the specific NMDAR antagonist (MK-801), we corroborate part of the functionality of the NMDR in IBC lines (SUM149PT and SUM190PT).

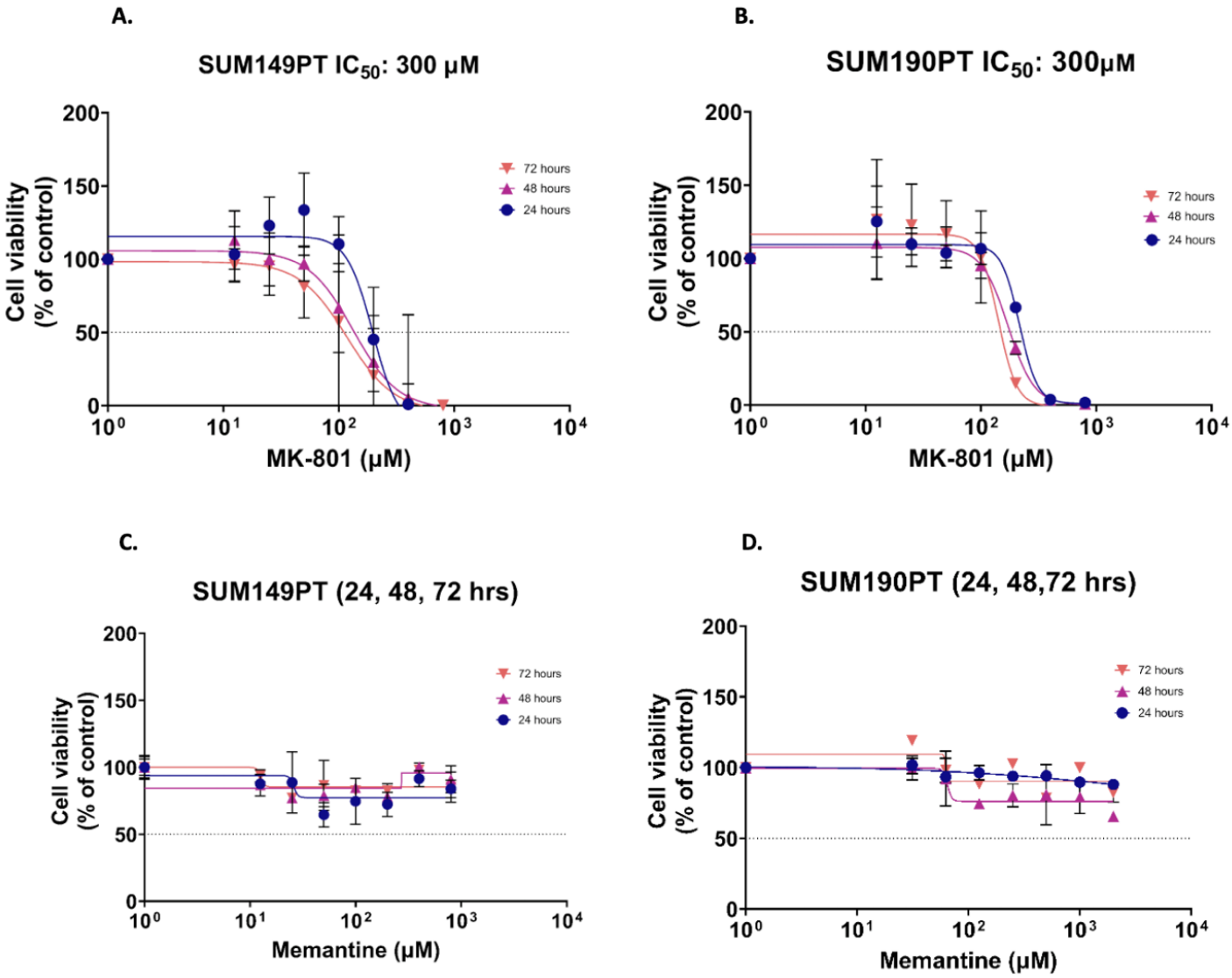


Figure 10. *MK-801 Dose-response curve for IBC cell lines*. Dose response curves for IBC cell lines SUM149PT and SUM190PT were made for NMDAR antagonists Memantine and Dizolcilpine/MK-801. A-B. Show decreased cell viability in IBC lines at high concentrations for Dizolcilpine/MK-801. A dose of 300 μM was determined as a half maximal inhibitory concentration (IC50) for subsequent assays. C-D. No decrease in cell viability is found for IBC with Memantine antagonists.

3.3.2 Effects of MK-801 on the migration and proliferation of IBC cell lines (SUM149PT and SUM190PT):

One of the functions of NMDAR in stages of brain development is the migration and distribution of neurons (Rzeski, Turski, & Ikonomidou, 2001). In cancer studies, however, it has been seen that it plays an important role in the progression, maintenance, and migration of a variety of cancer types such as breast cancer (Akerman et al., 2011; Chou, Tajima, Romero-Hernandez, & Furukawa, 2020; North et al., 2010). The elucidation of this receptor's role is of great importance because it indicates which oncogenic factors can be treated or related to NMDAR. We started by testing the 300μM concentration of MK-801 that was founded in the IC50 on cell migration phenotypes. A significant decrease (approximately 50%) in migration of SUM149PT cell lines was demonstrated in the presence of the antagonist of MK-801 compared to control ($p < 0.05$, $p = 0.0169$; **Figure 11 A-B**). It should be noted that there was no significant change in controls and vehicles ($p > 0.05$; **Supplementary Figure 17 A and C).**

We also carried out functional cell assays out as well as the 3D colony formation property which is a common assay of cell proliferation. One of the important characteristics of IBC is the high frequency of formation of small groups of tumor cells (tumor emboli) which are the cause of

obstructing lymphatic and milk ducts, causing its clinical classification (Arora et al., 2017). The presence of MK-801 significantly decreased (approximately 90%) the formation of colonies in both SUM149PT and SUM190PT, suggesting the high effectiveness in reducing tumor colonies ($p<0.05$, $p= <0001$; **Figure 12A-B)**. It should be noted that there was no significant change in controls and vehicles ($p>0.05$; Supplementary

Figure 18 A-B). These results hold great promise for treating two of the most concerning oncogenic factors for IBC (cell migration and tumor formation). It should be noted that another very important key element is the study of the invasion-like oncogenic property, since elucidating this can result in taking treatment measures in the event of a subsequent metastasis in the early detection of IBC.

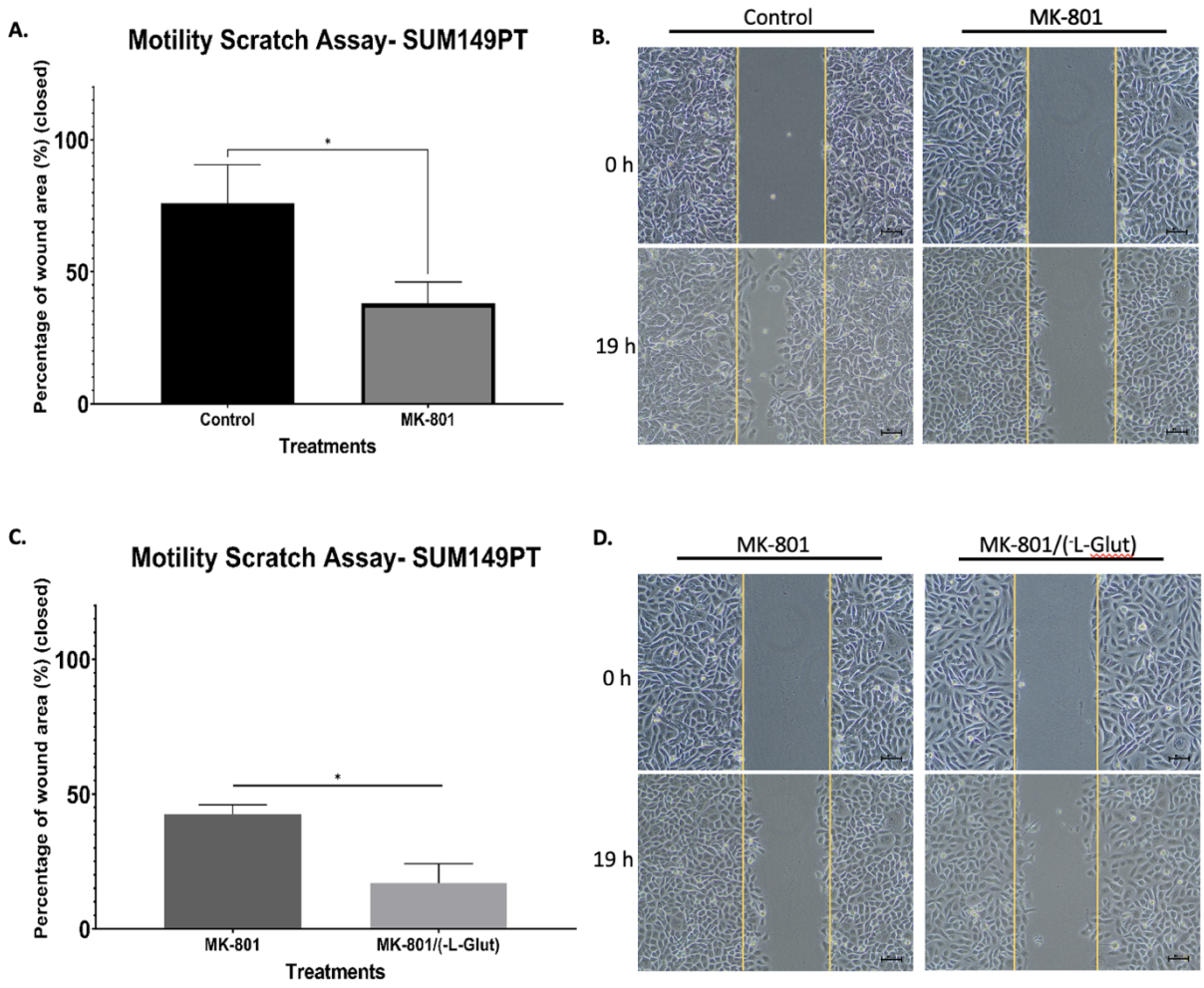


Figure 11. *Cell motility assay (*Wound Healing Assay*) for the SUM149PT cell line.* A-B. Representative images and statistical analysis (t-test, two-tail analysis) . Assay was done at 19 h under the effects of MK-801 at 300 µM as a non-competitive NMDAR antagonist. The p-values<0.05 (p= 0.0169) were considered statistically significant and are indicated by asterisks (*). C-D. Cell migration assays were performed with L-Glutamine-free medium (L-Glut) to see effects in the absence of the ligand. During these assays, the combination of the antagonist and the absence of the ligand have a synergistic response to decrease cell migration in SUM149PT cell lines. The p-values of <0.05 (p= 0.0449) were considered statistically significant.

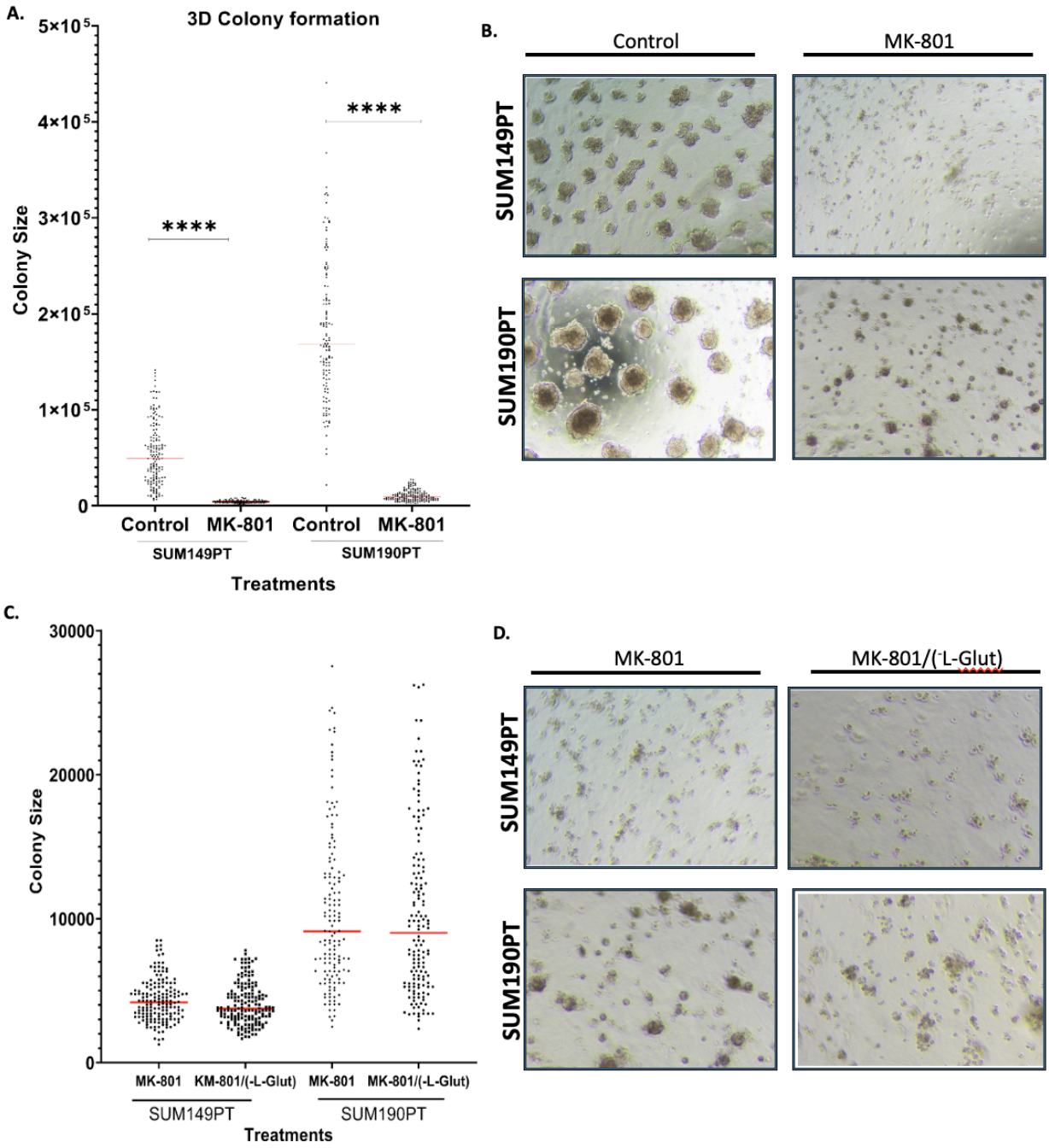


Figure 12. *MK-801 reduces colony formation for IBC cell lines SUM149PT and SUM190PT.* A-B. Quantification of colonies in triplicate for IBC cell lines SUM149PT and SUM190PT. ImageJ was used to quantify colony size. Approximately 60-70 colonies were quantified for each experiment where statistics were performed to eliminate extreme values. Significance was found for both cell lines under the 300 µM concentration of MK-801.

The p-values of <0.05 (p= <0001) were considered statistically significant and are indicated by asterisks (****). C-D. No significant change was observed during the absence of the ligand (L-Glutamine-free medium) under the effect of the antagonist during the 10 days of the experiment.

3.3.3 Importance of the Glutamate ligand on cell migration and proliferation of IBC cell lines:

Glutamate is one of the most abundant neurotransmitters in the nervous system and acts in excitatory transmission on neuroreceptors in the membrane, (Fouillioux, Contreras, Rivera, Terán, & Velasco, 2004; Li & Hanahan, 2013; Sexton et al., 2018) Sustained activation of NMDARs can cause excitotoxic cell death; therefore, much of the uptake/recycling of glutamate in the brain is through glia **(Figure 2)**.-Nevertheless, cancer cells lack functional uptake/recycling of glutamate, thus releasing it into the environment as product of cellular cystine uptake (Lyons, Chung, Weaver, Ogunrinu, & Sontheimer, 2007; Ye, Rothstein, & Sontheimer, 1999). Glutamate and NDMAR also plays an important role in neuronal migration during brain development but, in cancer cells, this activation involves migration, proliferation, and invasion. In our study, we observed the importance of the glutamate ligand precursor on different cell cultures of IBC cells through different functional assays of cell migration and proliferation. For these experiments, we used a glutamate-free medium and glutamate ligand precursor to verify the importance of the ligand in the different cell cultures (SUM149PT and SUM190PT).

Initially, we tested if there was any difference in colony formation during the (presence or absence) of the glutamate ligand **(Supplementary Figure 18B**) and no significant change was observed (p>0.05). Following this, the presence of the antagonist and the (presence or absence) of the

glutamate ligand were checked ($p>0.05$, **Figure 12C-D**) and no significant change was observed. This concludes that only the decrease in colony formation is associated with the blockade of the NMDA receptor through MK-801. Similarly, the importance of the ligand in the migration assays was observed. In this case, again no significant changes were observed between controls nor in the absence of glutamate ligand ($p>0.05$; Supplementary **Figure 17B-C**). However, in the presence of the antagonist (MK-801) and the absence of the ligand there was a significant decrease ($p>0.05$, $p= 0.0449$, **Figure. 11C**). This could indicate that the presence of glutamate acts mainly in cell migration activities or that other signaling pathways are occurring for examen AMPA receptors (Ishiuchi, 2009; Ribeiro, Custódio, & Santos, 2017)

3.3.4 Discussion

Glutamate receptors, especially NMDA, have been found to be a research route for alternative pathways in different types of cancer (Bastiaansen et al., 2020; Stepulak, Rola, & Polberg, 2014). In spite of this, in IBC, knowledge of this receiver is null. The data shows that the subunits (NMDAR1 and NMDAR2B) are in IBC cell lines (SUM149PT and SUM190PT). The use of specific antagonists for NMDAR in these cancer lines could provide a new field of study for alternative routes in therapy for this aggressive cancer subtype. During this second data set, we wanted to study the active role of NMDARs in IBC via specific NMDA antagonists such as Memantine and Dizocilpine. In our hypothesis, we established that the use of these antagonists would reduce oncogenic phenotypes such as cell migration and proliferation. MK-801 has been widely used for various uses such as neuroprotection, anticonvulsant, anesthetic, among others. Several cancer studies associate this drug as a possible alternative treatment for oncogenic phenotypes of cell migration, invasion, and proliferation. In this study, IBC cancer lines were subjected to the active ingredient of dizocilpine (MK-801) where decreased cell viability was demonstrated, with an IC50 of 300μM. This revealed the potential role of NMDARs in decreasing cell migration (approximately 50%) and proliferation (approximately 90%) in both cell lines (SUM149PT and SUM190PT). On one hand, for Memantine, being an antagonist with low binding affinity to the receptor, it did not show a decrease in cell viability or a half maximal inhibitory concentration (IC50). Due to this factor, functional experiments in cell cultures were not continued. The use of MK-801 as an alternate route and field of cancer research has been mentioned in several studies (Du et al., 2020). Part of this scientific contribution establishes that IBC cell lines (SUM149PT and SUM190PT) are affected by the specific antagonist of MK-801 (300μM An established concentration for neuroprotection) due to the presence of functional

NMDARs. For this finding, we have detailed that the concentration of MK-801 is lower in IBC cell lines (300uM) than in MCF7 and SKBR3 (600uM) breast cancer cell lines. On the other hand, a decrease in cell viability is observed with memantine at concentrations of 600uM in MCF7 and SKBR3 breast cancer lines but not in non-IBC cancer lines. (North et al., 2010). Glutamate is one of the excitatory neurotransmitters par excellences associated with NMDA, AMPA, Kianate, and mGluR receptors. Therefore, it is of great importance to know if this ligand can be involved in the activation of other possible routes (outside of NMDA). In our research, data set show thar glutamate-ligand is mostly associated with cell migration phenotypes, and this may be due to the fact that glutamate can bind to different receptors such as NMDA, AMPA, Kianate, and mGluR. Finally, the use of memantine as a therapeutic route in IBC lines was not very effective in none of the functional tests for IBC cell lines.

Chapter IV: General discussion:

4.Final Conclusions:

Inflammatory breast cancer (IBC) is known as one of the most aggressive and rare subtypes of breast cancer. The aggressiveness of this cancer subtype is due, in part, to the lack of established treatment and therapies for its molecular subtypes, such as triple-negative subtypes. Moreover, a high incidence (~70%) of breast cancer metastasized to the brain in hippocampus areas, specifically triple-negative and EHR2+ phenotypes, has been established. This diagnosis of metastasis reduces life expectancy by up to 2 years for patients with this type of medical evaluation. Despite targeted treatments, it is of great importance and urgency to examine alternative routes or mechanisms to assist these patients. It has been found that NMDA receptors play a relevant and substantial role in different types of cancer, such as pancreatic, lung and breast cancer. Despite being intimately involved with memory and learning in the brain, in cancer, NMDARs have been associated with the proliferation and maintenance of cancer cells. During this research work, we observed and detailed the expression and active role of NMDA receptors in IBC, especially in lines with phenotypes (triple-negative and HER2+) which are associated with metastasizing to the brain. This work was done in order to open a new field of research to help patients diagnosed with IBC and breast cancer metastasized to the brain.

During this research process, a new field of study began at both the molecular and cellular level on NMDR in breast cancer and IBC. By detailing the characterization and quantification of the expression of these receptors through various biomolecular analyses, we infer that NMDAR plays a role in the aggressive oncogenic phenotypes of IBC. These analyzes result in an increase in expression at the genomic and protein level in the IBC cancer lines. Similarly, it has been detailed that these receptors are located in strategic places for the cell. This work also shows that NMDA

receptors play an important role in both the migration and proliferation of IBC cancer lines, putting MK-801, a specific antagonist, as an alternative neuropharmacology pathway.

Finally, in this research process, after the detection and the role of NMDAR in IBC, the signaling pathway in the cancer-associated cascades remains incomplete (Figure 13), meaning that it still needs to be investigated further. NMDAR, being involved in processes such as protein synthesis, makes it an important agent for the in-depth study of new targeted therapy studies. The different signaling cascades involved in the process of protein synthesis, maintenance, and cell survival remain to be seen. Preliminary studies have shown a change in ERK phosphorylation during the presence of MK-801 (Paul, Nairn, Wang, & Lombroso, 2003). On the other hand, as part of the progress of the study of MK-801, the use in animal models would be imperative for the study of tumor progression and invasion/metastasis factors.

The information obtained in this research had the primary purpose of providing new information in the molecular scientific field on the functionality of NMDARs. This new gap gives rise to alternative routes to the development of targeted therapies and new research studies outside of the hormone receptors studied in breast cancer.

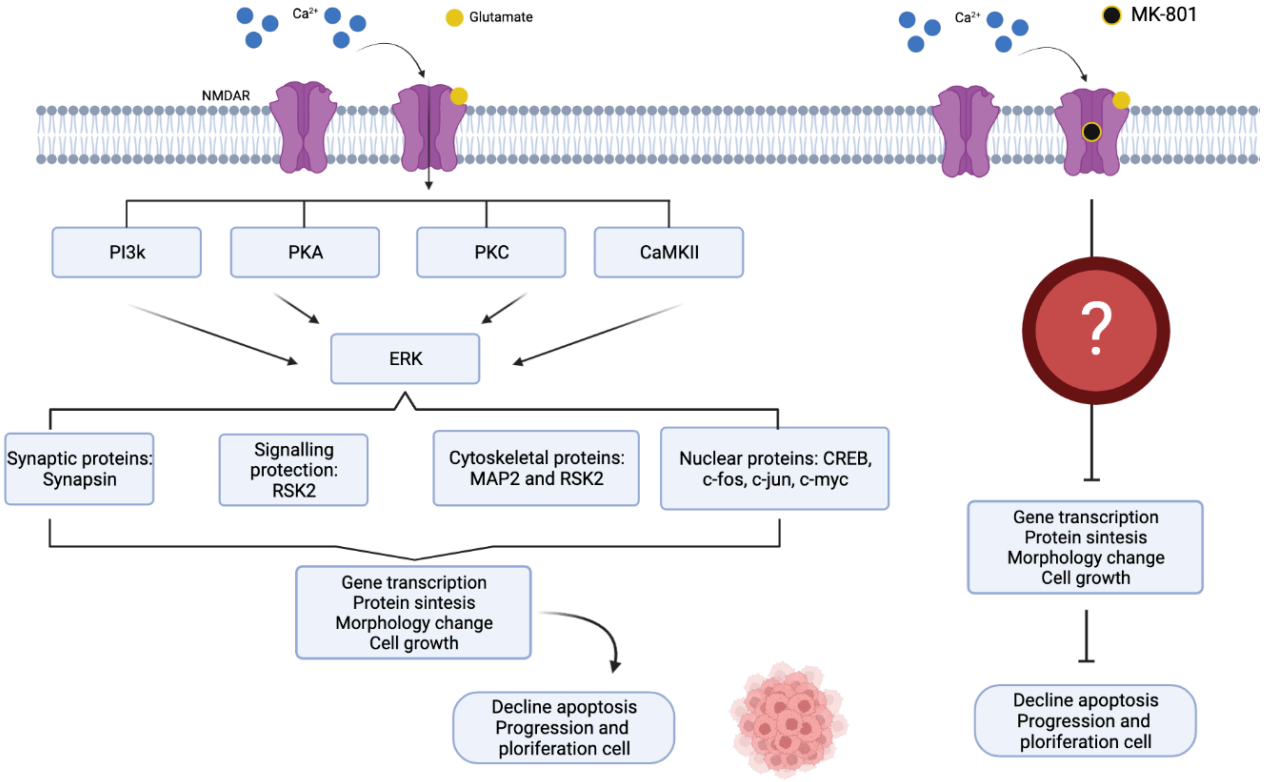
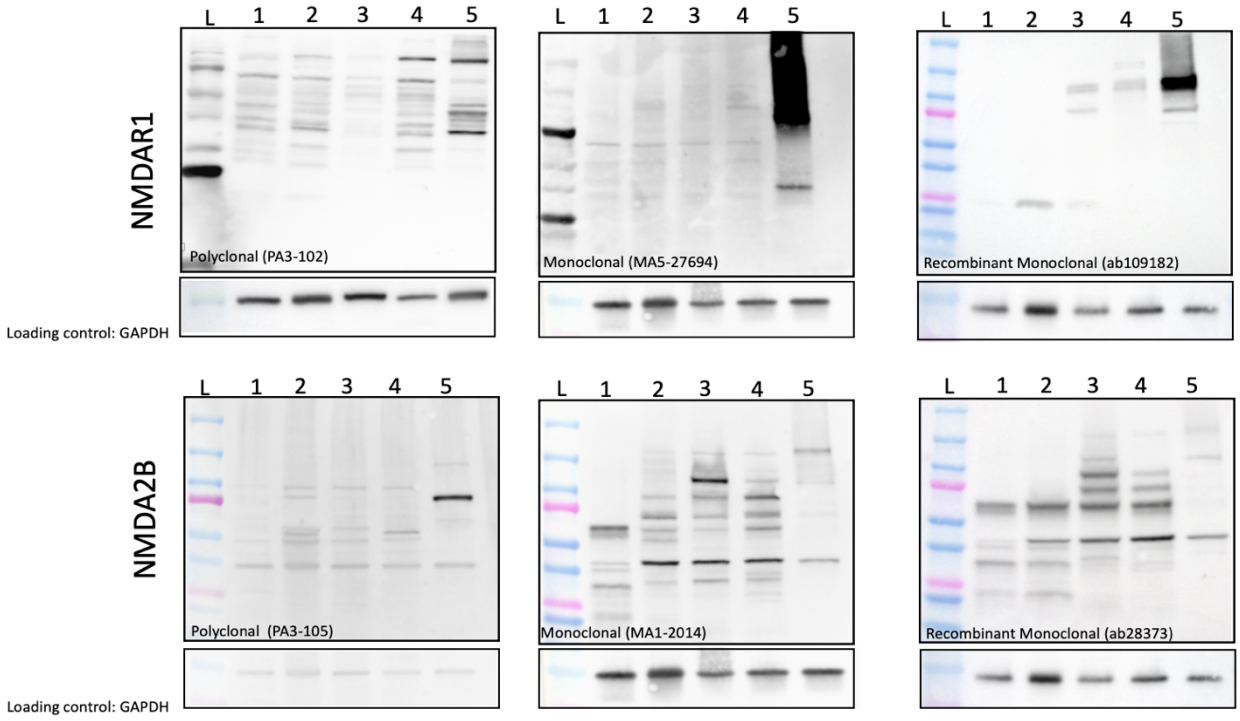


Figure 13. *Research model on signaling pathways or cascades involved in cancer cell progression*

Supplementary Data

(Supplementary Data)

Western Blot:



Sun-Unit	Antibody	The expected weights (kDa)
NMDAR1	Polyclonal PA3-102	120
	Monoclonal (MA5-27694)	60-100
	Recombinant Monoclonal (ab109182)	100-150
NMDAR2B	Polyclonal PA3105	180
	Monoclonal (MA1-2014)	160-180
	Recombinant Monoclonal (ab28373)	150-200

Figure 14. *Protein expression for NMDAR subunits with different antibodies*. Protein expression comparison of four breast cancer cell lines (MCF7, MDA-MB-231, SUM149PT and SUM190PT). In addition to this, the expression with GAPDH (37 kDa) was verified and normalize for each of the tissues studied. During this analysis, different antibodies were tested for the expression of NDMA subunits. Each of the lanes is marked as follows: (L) Ladder Dual Color (1) MCF7, (2) MDA-MB-231, (3)SUM149PT, (4)SUM190PT, (5) Hippocampus.

(Supplementary Data)

Immunofluorescence (controls)

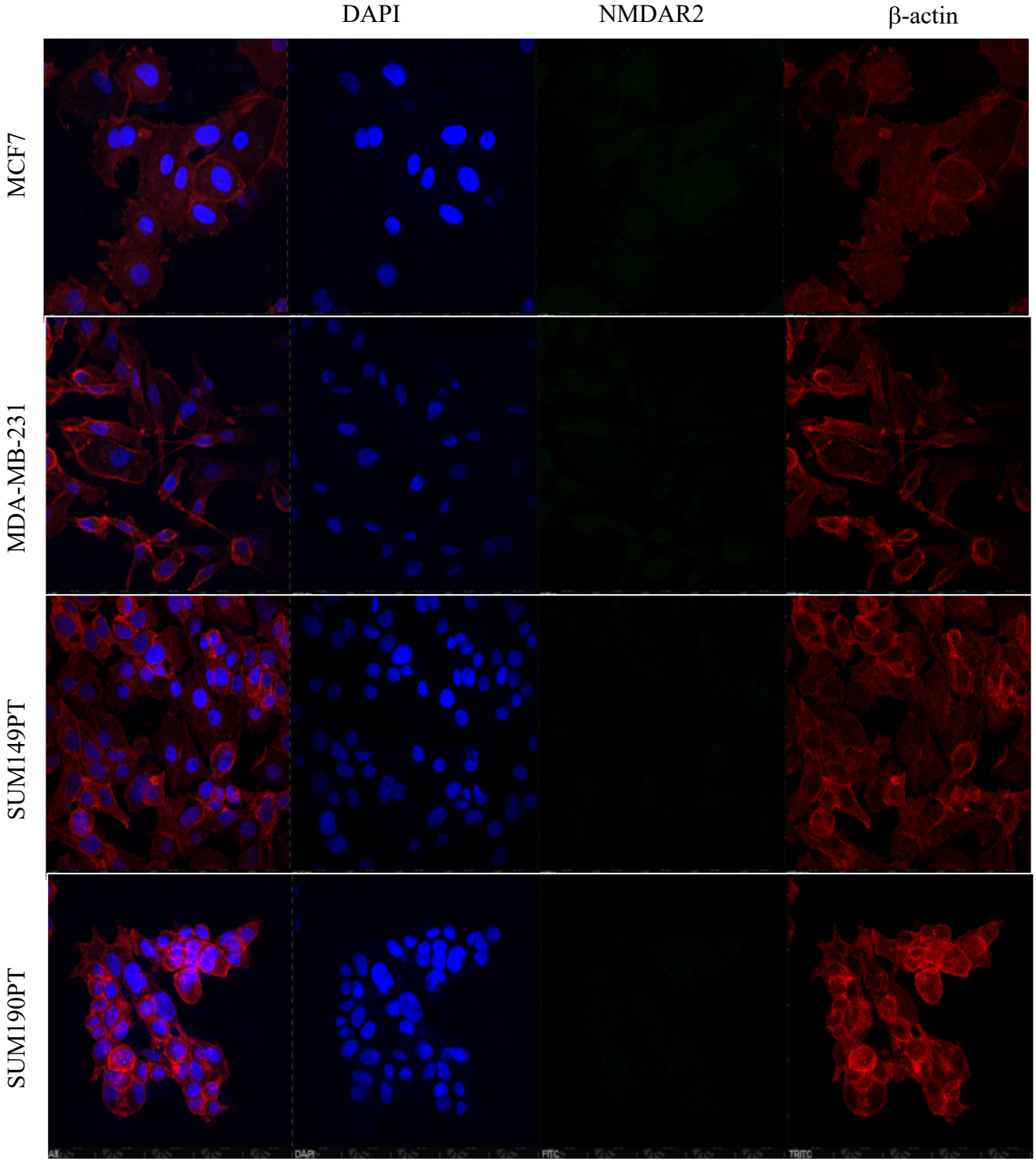


Figure 15. *Control of NMDAR2 subunit antibody specificity in breast cancer cell lines* (MCF7, MDA-MB-231, SUM149PT, and SUM190PT). Immunofluorescence staining for NMDAR2 protein; blue: nucleus, Green: NMDAR2, Red: actin. NMDAR2 absence staining revealed the antibody specificity of all cell lines. Photos are taken in a confocal microscope (60X), all fixation parameters, staining, sample preparation, and microscope settings were the same for all samples.

(Supplementary Data)

Immunofluorescence (controls)

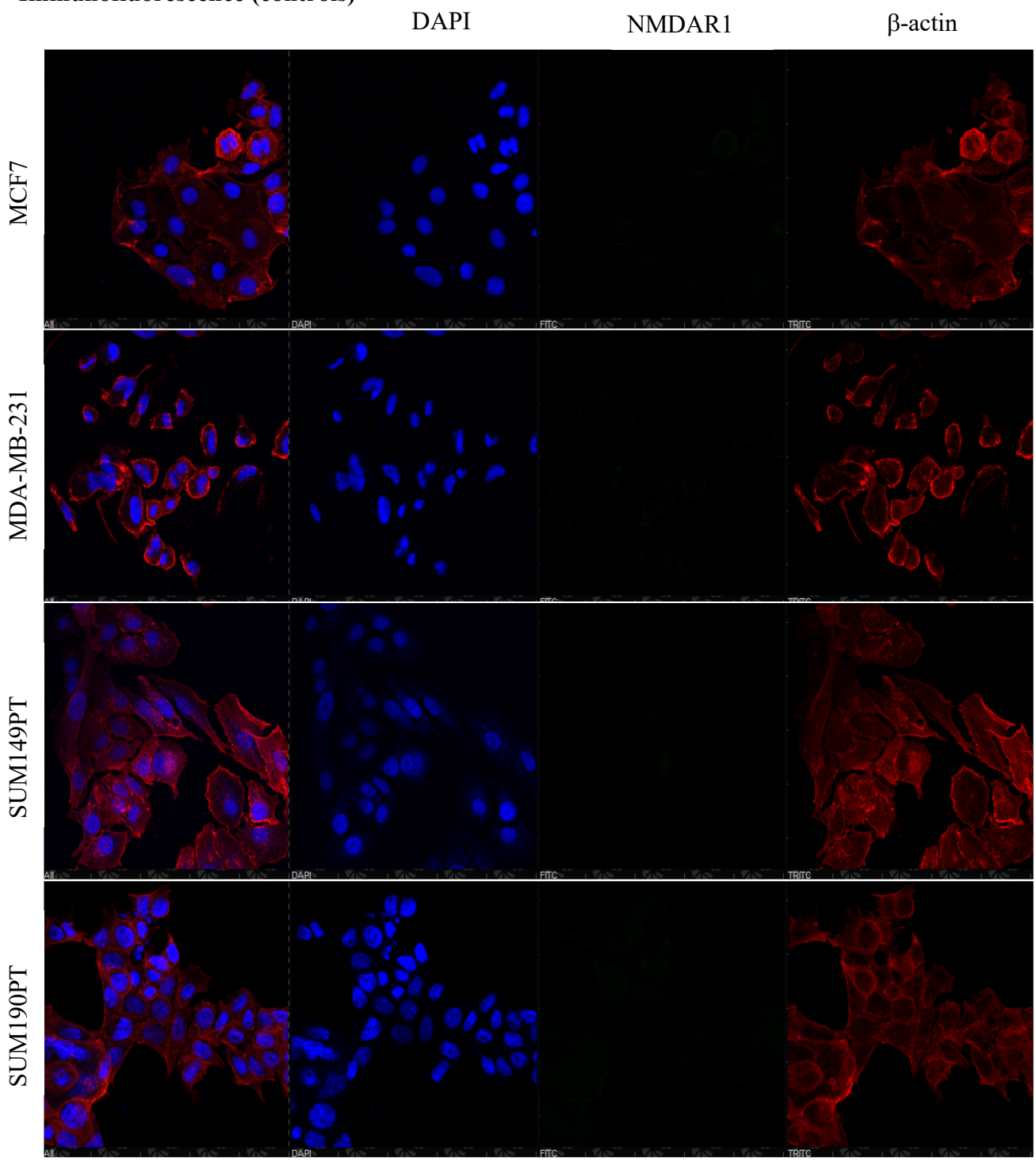


Figure 16. *Control of NMDAR1 subunit antibody specificity in breast cancer cell lines* (MCF7, MDA-MB-231, SUM149PT, and SUM190PT). Immunofluorescence staining for NMDAR2 protein; blue: nucleus, Green: NMDAR2, Red: actin. NMDAR2 absence staining revealed the antibody specificity of all cell lines. Photos are taken in a confocal microscope (60X), all fixation parameters, staining, sample preparation, and microscope settings were the same for all samples.

(Supplementary Data)

Wound Healing Assay **(controls)**

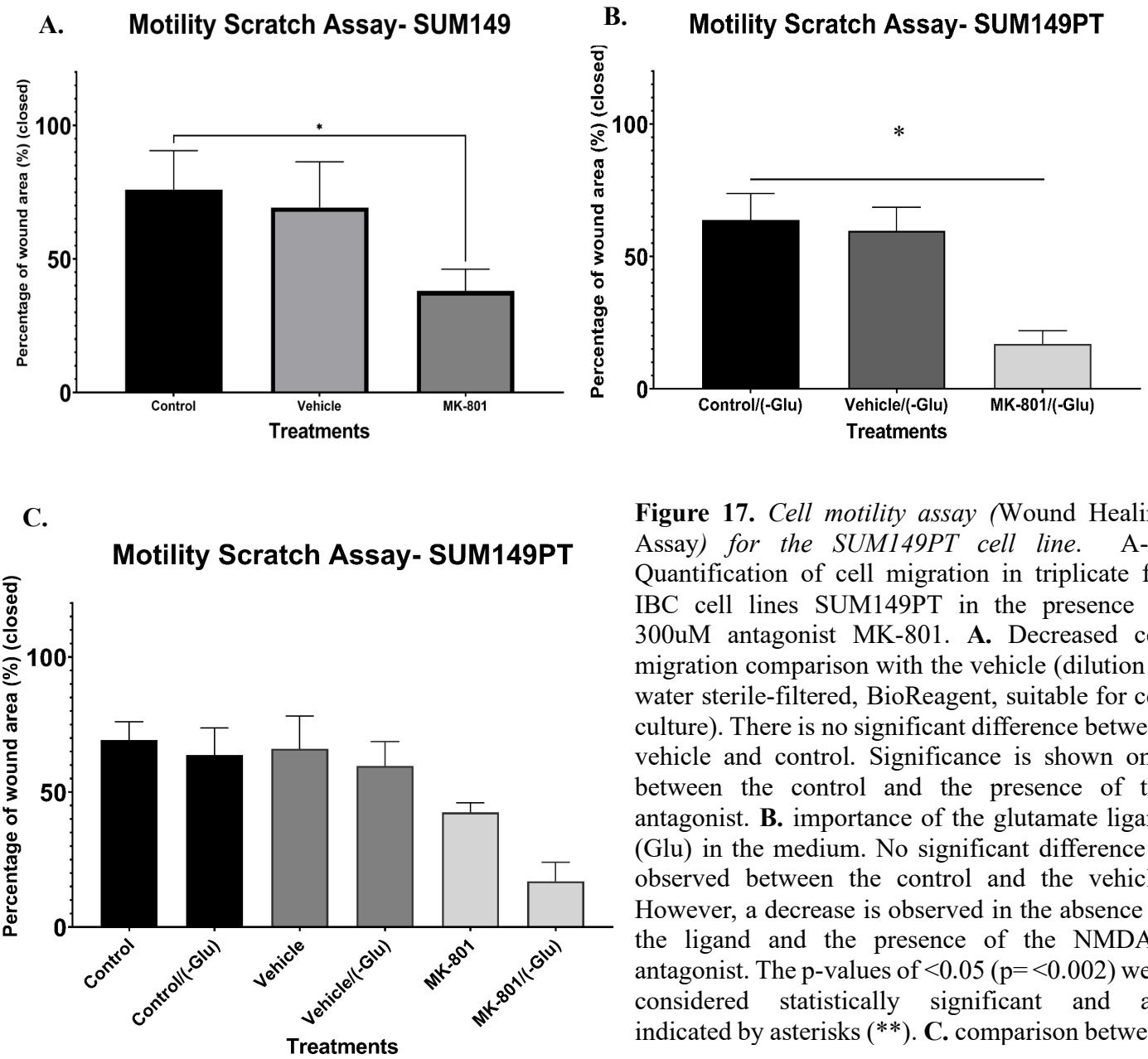


Figure 17. *Cell motility assay (*Wound Healing Assay*) for the SUM149PT cell line.* A-B. Quantification of cell migration in triplicate for IBC cell lines SUM149PT in the presence of 300uM antagonist MK-801. **A.** Decreased cell migration comparison with the vehicle (dilution in water sterile-filtered, BioReagent, suitable for cell culture). There is no significant difference between vehicle and control. Significance is shown only between the control and the presence of the antagonist. **B.** importance of the glutamate ligand (Glu) in the medium. No significant difference is observed between the control and the vehicle; However, a decrease is observed in the absence of the ligand and the presence of the NMDAR antagonist. The p-values of <0.05 ($p=<0.002$) were considered statistically significant and are indicated by asterisks (**). **C.** comparison between

Colony formation (controls)

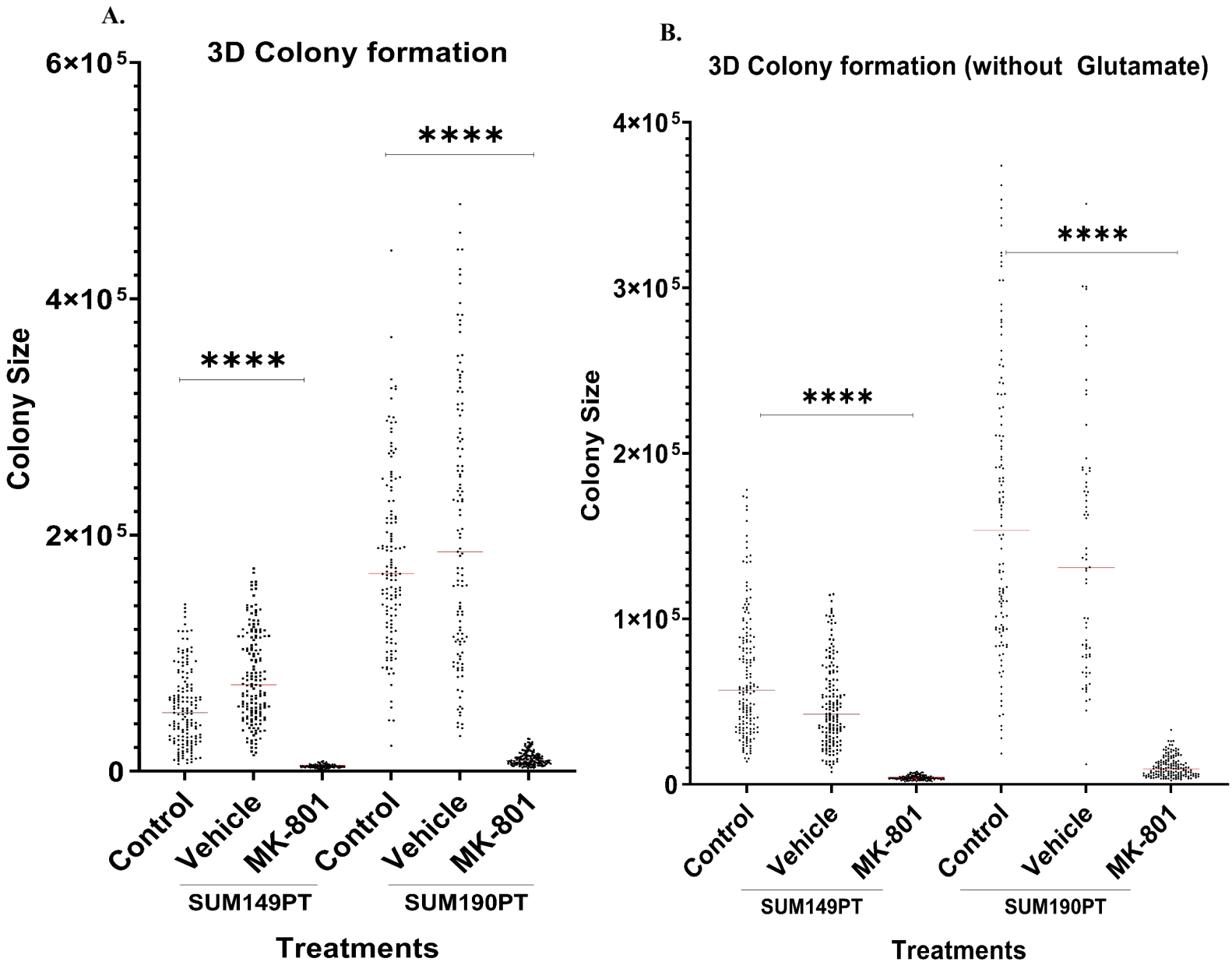


Figure 18. *MK-801 reduce colony formation for IBC cell lines (SUM149PT and SUM190PT*). A-B. Quantification of colonies in triplicate for IBC cell lines (SUM149PT and SUM190PT). ImageJ was used to quantify colony size. Approximately 60-70 colonies were quantified for each experiment where statistics were performed to eliminate extreme values. Significance was found for both cell lines under the 300 µM concentration of MK-801. The p-values of <0.05 (p= <0001) were considered statistically significant and are indicated by asterisks (****). No significant change was observed between vehicle and control.

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