# Adrenergic contribution to the stress induced carcinogenesis

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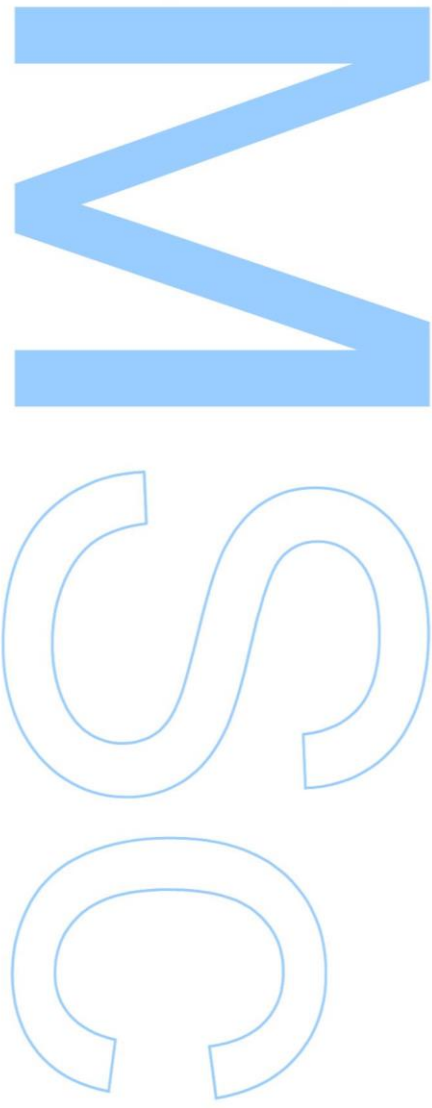
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Porto, 19 January 2019

Dany Silva

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# Resumo

O cancro da mama é a neoplasia maligna mais frequente em mulheres. Vários estudos demonstraram que o stress pode favorecer o aumento da incidência e mortalidade associado ao cancro. As catecolaminas (noradrenalina e adrenalina), liberadas sob condições de stress, podem ser responsáveis por este aumento de cancro associado ao stress. Estes mensageiros exercem os seus efeitos através de uma família de GPCR, denominada receptores adrenérgicos, apresentando três subfamílias distintas de recetores: $\alpha_1$, $\alpha_2$- e β-adrenoreceptores. Destes, os receptores β-adrenégicos parecem ser os mais envolvidos no cancro.

O presente trabalho teve como objetivo investigar os efeitos mediados pelos receptores β-adrenégicos em duas linhas celulares diferentes, as células MCF-10A, uma linha celular não tumorigénica e as células MCF-7, uma linha celular tumorigénica.

Tal como o stress, a menopausa também parece ser um fator de risco no desenvolvimento do cancro da mama. Durante a menopausa, em paralelo com a diminuição dos níveis de estrogênio, existe também um aumento na liberação de catecolaminas. Sabendo o impacto dos estrogénios no desenvolvimento e progressão do cancro da mama, neste estudo também foi investigado a influência dos estrogénios (17β-estradiol; principal forma ativa dos estrogénios) nos efeitos mediados pelos receptores β-adrenégicos.

Os resultados obtidos nestre trabalho apoiam a hipótese que as catecolaminas podem promover a tumorigénese sob condicções de stress ou em condições em que existe níveis elevados de catecolaminas plasmáticas, como o observado durante a menopausa. Esta conclusão é suportada pela observação de que a isoprenalina, um agonista dos receptores β-adrenégicos, aumentou o potencial tumorigénico das células MCF-10A, evidenciado pelo aumento da viabilidade celular, formação de colónias e capacidade de produzir adrenalina. O efeito da isoprenalina na indução de tumorigénese foi menos evidente nas células MCF-7, onde apenas foi observado um aumento na proliferação celular.

O estrogénio 17β-estradiol preveniu os efeitos tumorigénicos causados pela ativação dos receptores β-adrenégicos nas células não-tumorigénicas MCF-10A, efeito este mediado pelo receptor GPER. No entanto, observou-se ainda que, o 17β-estradiol, em células tumorigénicas, induziu um aumento da proliferação celular, o que sugere que, nas mulheres com elevado risco de já possuirem células tumorigénicas (como as células MCF-7), a administração de estrógenos poderá causar um aumento na incidência de cancro da mama em vez de ocorrer uma prevenção deste tipo de cancro.

Este estudo também mostrou que o propranolol, um antagonista dos receptores β-adrenérgicos, causou uma inibição acentuada da viabilidade / proliferação celular nas células MCF-7, não causando efeitos nas células MCF-10A. O efeito seletivo do propranolol nas células tumorigénicas poderá dever-se a uma influência na degradação de material obtido por autofagia (ou por processos idênticos). Esta ação seletiva do propranolol nas células tumorigénicas pode ser particularmente útil como uma nova intervenção farmacológica no cancro da mama.

**Palavras-chave:** cancro da mama, stress, catecolaminas, receptores adrenérgicos, GPER

# Abstract

Breast cancer is the most frequent malignancy in women. Several reports demonstrated that stress may favour the increase of cancer incidence and mortality. Catecholamines (noradrenaline and adrenaline) are released under stress conditions and may be responsible for this increase in cancer associated with stress. These messengers exert their effects through a family of GPCR, called adrenergic receptors (AR) which present three distinct subfamilies: $\alpha_1$, $\alpha_2$- and β-adrenoceptors. From these, the β-adrenoceptors (β-AR) appear to be most involved in cancer.

The present work aimed at investigate the β-AR mediated effects in two different cell lines, the MCF-10A cells, a non-tumorigenic cell line and the MCF-7 cells, a tumorigenic cell line.

As stress, menopause also seems to be a risk factor for breast cancer. During menopause there is also an increase in the release of catecholamines which is parallel with a decrease in oestrogen levels. Knowing the impact that oestrogens also has in the development and progression of breast cancer, the influence of 17β-estradiol (the main active from of oestrogens) in this pathway was also investigated.

The results presented here support the view that catecholamines may promote tumorigenesis under stress or under other conditions where high levels of plasmatic catecholamines are reached, such as those observed during menopause. This conclusion was supported by the observation that isoprenaline, a β-AR agonist, increased the tumorigenic potential of the non-tumorigenic MCF-10A cells, revealed by increased cell viability, colony formation and capacity to produce adrenaline. The effect of isoprenaline in inducing tumorigenesis was less evident in MCF-7 cells, where only an increase in cell proliferation was observed.

The oestrogen, 17β-estradiol prevented the tumorigenic effects caused by β-AR activation in non-tumorigenic MCF-10A cells, an effect mediated by the GPER. However, it was further observed that 17β-estradiol per se, was capable to induce cell proliferation in tumorigenic cells, suggesting that the administration of oestrogens in a population of women with high probability to already have tumorigenic cells (like the MCF-7 cells), an increase in incidence of breast cancer rather than a prevention of cancer may be observed.

This study also showed that propranolol, a β-AR antagonist, caused a marked inhibition of MCF-7 cell viability/proliferation while it did not affect MCF-10A cells. These selective effects in tumorigenic cells seem to be due to an influence in the degradation of material obtained by autophagy (or any identical processes). This selective action of propranolol in tumorigenic cells may be particularly useful as a new pharmacological intervention in breast cancer.

**Keywords:** Breast cancer, stress, catecholamines, adrenergic receptors, GPER

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# Abbreviations

| | |
|---|---|
| **3´MA** | 3´ methyladenine |
| **ANS** | Autonomic nervous system |
| **AR** | Adrenergic receptors |
| **AVOs** | Acidic vesicular organelles |
| **BARK** | β-adrenergic receptor kinase |
| **CNS** | Central nervous system |
| **CTR** | Control |
| **DMEM/F12** | Dulbecco's Modified Eagle's Medium/ F-12 |
| **DMEM-HG** | Dulbecco's Modified Eagle's Medium – high glucose |
| **EGF** | Epidermal growth factor |
| **EGFR** | Epidermal Growth Factor Receptor |
| **EPAC** | Exchange protein activated by adenylyl cyclase |
| **ER** | Oestrogen receptor |
| **FAK** | Focal adhesion kinase |
| **FBS** | Fetal bovine serum |
| **GPER** | G-protein-coupled oestrogen receptor 1 |
| **HER2** | Human epidermal growth factor receptor 2; |
| **HPLC-ECD** | High-performance liquid chromatography with electrochemical detection |
| **ISO** | Isoprenaline |
| **MDC** | Monodansylcadaverine |
| **MTT** | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide |
| **M-βCD** | Methyl-β-cyclodextrin |
| **PKA** | Protein kinase A |
| **PMA** | Phorbol 12-myristate 13-acetate |
| **PNMT** | Phenylethanolamine N-methyltransferase |
| **PR** | Progesterone receptor |
| **SERM** | Selective oestrogen receptor modulator |
| **TH** | Tyrosine hydroxylase |
| **β-AR** | β-adrenoreceptors |

# I. Chapter: Introduction

# 1. Introduction

## 1.1. Cancer

Cancers results from an abnormal and uncontrolled cell division. They differ from benign tumours from their ability to invade surrounding tissues and to originate secondary tumours at distant locations (Hanahan and Weinberg, 2011).

Most cancers fall into one of three main groups: carcinomas, sarcomas and leukemias, according to the type of cell from which they arise. From these, nearly 90% of human cancers, are carcinomas, malignancies of epithelial cells (Ding et al., 2013).

The first step in the development of cancer is the initiation phase. According to the genetic hypothesis for the origin of cancer, it involves the alterations or mutations of genes (e.g. in pro-oncogenic genes such as RAS and MYC or in tumor suppressor genes such as BRCA1, BRCA2 and TP53), which can lead to dysregulation of biochemical signalling pathways associated with cellular proliferation, survival and differentiation (Finver et al., 1988; Baker et al., 1990; Downward, 2003; Friedenson, 2007). These alterations may arise spontaneously or be induced by exposure to non-genotoxic and genotoxic carcinogens, such as radiation, hormones, chemicals, infections and hypoxia (Benigni et al., 2013; Cuninghame et al., 2014). While genotoxic carcinogens directly interact with DNA (as either parent chemicals or reactive metabolites) and induces DNA damage, non-genotoxic carcinogens have been associated, for instance, with changes in oxidative stress, modulation of metabolizing enzymes and disruption of the balance between proliferation and apoptosis (Benigni et al., 2013; Lee et al., 2013; Cuninghame et al., 2014).

The following steps of cancer formation involves i) the accumulation of actively proliferating preneoplastic cells (hyperplasia) ii) phenotypic changes (dysplasia) and further genotypic changes in cells, allowing for an increase in invasive and metastatic potential and iii) metastasis, which involves the spreading of cancer cells from the primary site to other parts of the body through the bloodstream or the lymph system (Lodish H, 2000).

## 1.2. Breast cancer

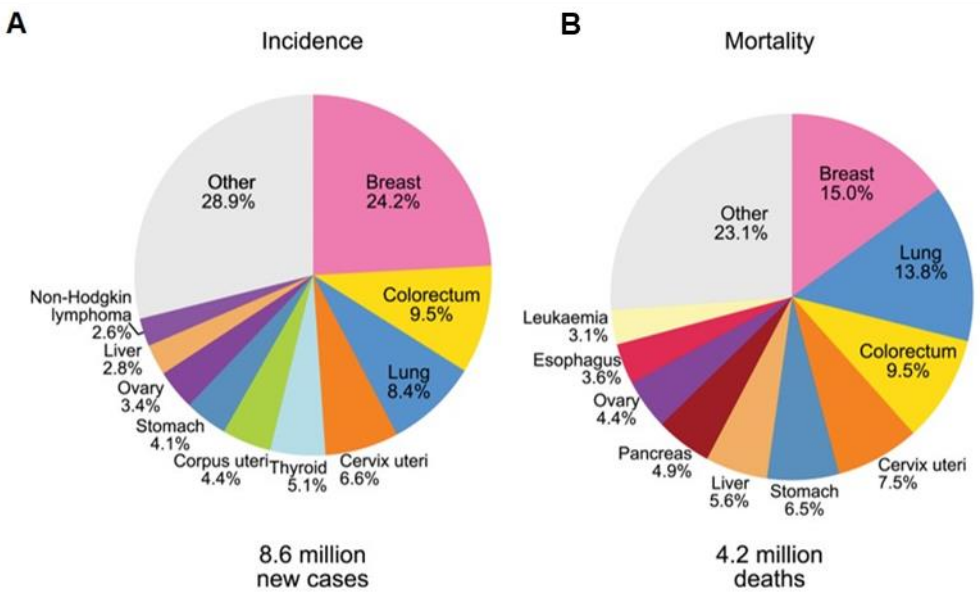
Breast cancer is, by far, the most common cancer in women (Bray et al., 2018). Estimations for 2018 showed that this disease accounted for almost one in four cancer

cases among women, with more than two million new cases worldwide (**Figure 1A**). It represents 15% of the overall female cancer associated mortality (**Figure 1B**), being the deadliest type of cancer, in women (Bray et al., 2018).

The incidence of breast cancer is globally increasing: 1.7 million cases in 2012 *vs* 2.1 million cases in 2018, showing a 25% increase, 5% per year (Ferlay et al., 2015; Bray et al., 2018). Most of the occurrences take place in developed countries where the higher incidence rates are associated with a higher prevalence of known risk factors for the development of this disease (Bray et al., 2018).

For many less developed countries, the incidence rates for breast cancer are also expected to increase, as a result of a more ‘‘westernised’’ lifestyle, coupled to a longer life expectancy (Forjaz de Lacerda et al., 2018).

In spite of the higher incidence of breast cancer cases in developed countries, the mortality is similar to that observed in developing countries (**Figure 1C, red bars**) (Bray et al., 2018). This was mainly achieved due to crucial improvements in diagnosis techniques (e.g. mammography) and therapies (e.g. anti-estrogenic therapies, chemotherapeutic agents, monoclonal antibodies), available in developed countries (Shulman et al., 2010; Bray et al., 2018). In fact, in these countries, the five-year survival rate (% of people with cancer in a treatment group who is alive five years after the diagnostic or treatment) of early stage breast cancer can reach values up to 80 to 90% (Ferlay et al., 2015).



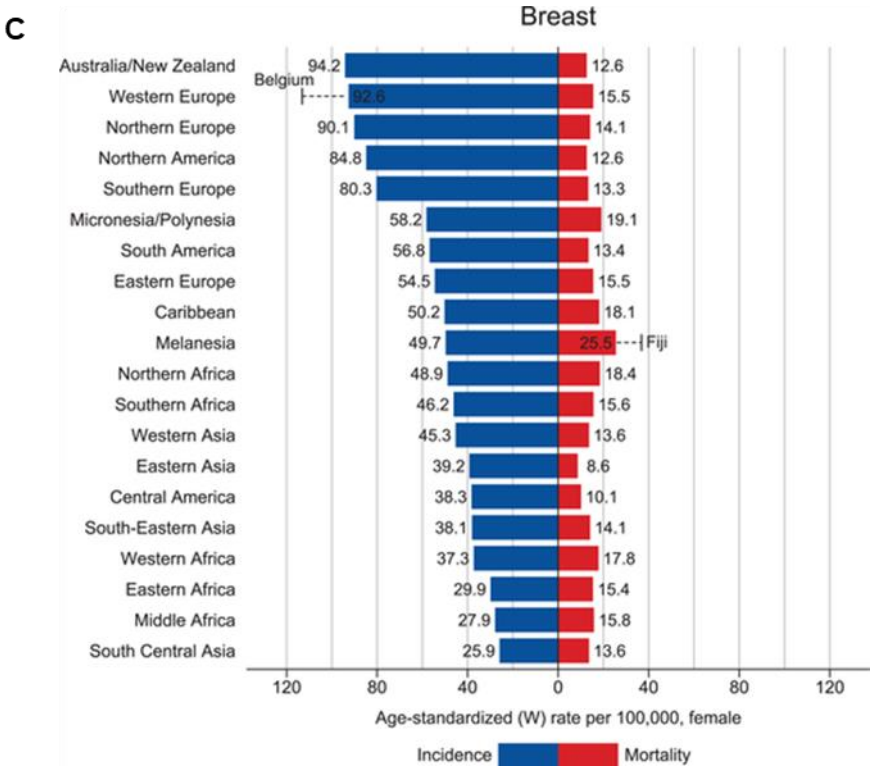



**Figure 1. Worldwide incidence (A) and mortality (B) of cancer in females in 2018.** C) Region-specific incidence and mortality age-standardized rates for cancers of the female breast in 2018 (Bray et al., 2018)

In Portugal, breast cancer incidence is similar to that observed in other developed countries. However, the mortality rate in Portugal is one of the lowest observed in Europe (16.6 per 100.000 women *vs* 21.8 per 100.000 women in Europe) (Ferlay et al., 2018). According to the projections published by Forjaz de Lacerda et al. (2018), in Portugal, the incidence of breast cancer is expected to continue to increase, especially in the North, where, in 2025, the incidence rate is expected to be approximately 1.2 to 1.3 times higher among northern women (268.5 per 100 000 woman-years), compared to the South (230.8) and the Centre (204.1), respectively. Contributions for this increase in incidence rates may be associated with a higher socio-economic status, which is known to be associated with the developing of breast cancer and to the adoption of a westernized lifestyle among Portuguese women, who are now more likely to have lower parity, delay childbearing and have increased usage of contraceptive methods (Parkin et al., 2001; Marques-Vidal et al., 2012; Forjaz de Lacerda et al., 2018).

## 1.3. Breast structure and the development of cancer

Breast malignancies arise from epithelial cells of the glandular milk ducts or lobules of the breast, mainly in the terminal duct lobular units (Harris et al., 2012).

The structure of the human breast and its association with the formation of several diseases, including tumours is shown in **Figure 2**. The glandular tissue consists of a branching ductual-lobular system (Geddes, 2007). It is made of 12–20 lobes, which are drained by collecting ducts that converge at the nipple in a radial arrangement (Wellings et al., 1975; Geddes, 2007). Each of the lobes are further divided in lobules, which in turn is made up of acini. The acini and the terminal duct form the secretory units of the mammary gland, the terminal duct lobular units, the main structures where breast carcinomas arise (Harris et al., 2012).

All these structures are also surrounded by the stroma, composed of varying amounts of fat, connective tissue, blood vessels, nerves and lymphatics (Harris et al., 2012).

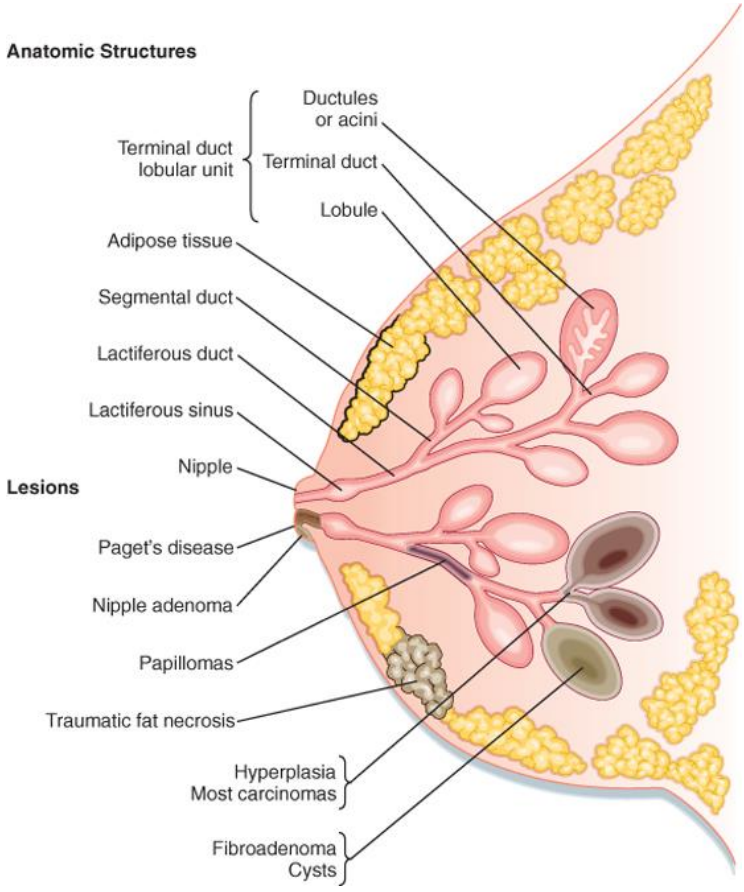



**Figure 2. Scheme depicting the structure of human breast and its association with the formation of several diseases, including tumours** (Koeppen, 2008)**.**

If cancerous cells are restricted to the mammary ductal–lobular system, tumours are classified as benign *in situ* carcinomas. If the neoplastic cells rupture the basal membrane (membrane that separate the epithelium from the stroma) and invade into the adjacent stroma, the tumour becomes malignant (Weigelt and Bissell, 2008).

Malignant breast cancers are very heterogeneous, exhibiting a wide range of morphological types, molecular profiles and clinical behaviours (Kleihues and Cavenee, 2000). Based on morphological characteristics (e.g. growth pattern, cytology, differentiation pattern), invasive breast cancers can be classified into at least 21 different subtypes, each of one showing particular prognostic and clinical characteristics (Kleihues and Cavenee, 2000; Weigelt and Bissell, 2008).

Oestrogens are steroid hormones and critical regulators of the normal breast development and function (Ascenzi et al., 2006). The effects of oestrogens are driven by the three known oestrogen receptors (ER): two are nuclear hormone receptor (ERα, ERβ) while the other one is a seven trans-membrane domain G-protein-coupled oestrogen receptor 1 (GPER). The ERα is the main receptor involved in the carcinogenic action of oestrogen (Lipovka and Konhilas, 2016), regulating cell proliferation, cancer metastasis and the anti-apoptotic effects of oestrogen (Lipovka and Konhilas, 2016).

It is well established that factors known to increased exposure to oestrogens over a woman's lifespan which occurs with early menarche, late menopause, hormone replacement therapy and obesity represents an increased risk for the development of breast cancer (Collaborative Group on Hormonal Factors in Breast, 2012; Dall and Britt, 2017).

Breast cancers are classified according to the ER molecular profiles into two major groups: ER positive and ER negative tumours (Perou et al., 2000; Cancer Genome Atlas, 2012). These two major groups can be further divided in five subgroups based on HER2 expression and other molecular characteristics (Perou et al., 2000; Cancer Genome Atlas, 2012). Subtypes based on this classification, as well as incidence rates and some therapeutic approaches, are summarised in **Table 1**.

**Table 1**. **Classification of breast cancer into several subtypes based on the molecular profiles.**

| Subtypes | Imunoprofile | Clinical characterisitcs | Prevalence |
|---|---|---|---|
| **Luminal A** | $ER^+$, $PR^{+/-}$, $HER2^-$ , Low Ki67 | Usually chemotherapy responsive | 40% |
| **Luminal B** | $ER^+$, $PR^{+/-}$, $HER2^+$, High Ki67 | Usually endocrine responsive, variable to chemotherapy. | 20% |
| **Basal** | $ER^-$, $PR^-$, $HER2^-EGFR^+$, High Ki67, | Endocrine nonresponsive, usually chemotherapy responsive | 15% |
| **Claudin-low** | $ER^-$, $PR^-$, $HER2^-$ E-cadherin, claudin-3, claudinin-4 and 7 low | Intermediate response to chemotherapy | 10% |
| **HER2** | $ER^-$, $PR^-$, $HER2^+$ | Trastuzumab responsive, chemotherapy responsive | 20-30% |

HER2, human epidermal growth factor receptor 2; PR, progesterone receptor; EGFR- Epidermal Growth Factor Receptor. From Holliday and Speirs (2011); Kittaneh et al. (2013); Dias et al. (2017)

Breast cancers responsive to ER are the most common type of breast cancer diagnosed (Lumachi et al., 2013), which reveals the role of oestrogens as one of the key factors in the initiation and progression of breast cancer.

The ER status is used in clinical practice to predict the response to adjuvant anti-estrogenic therapies (Harris et al., 2007). The development of therapies to target ER and its signalling pathway, such as the selective oestrogen receptor modulator (SERM), tamoxifen, or aromatase inhibitors not only led to a dramatic increase in the survival rate - 91% at 5 years post diagnosis and 80% at 15 years (Nasrazadani et al., 2018), but was shown to be highly effective in preventing ER+ breast cancers in high-risk women (Richardson et al., 2007; Vogel et al., 2010).

## 1.4. Risk factors for breast cancer: the role of stress

Aside from reproductive factors (e.g. early menarche, late menopause, low parity) breast cancer may be also associated with various others risk factors that may initiate or modify the process of neoplastic transformation of breast cells. Some of these factors may include family susceptibility (BCRA1; BCRA2 mutations), race (higher risk in Caucasian women), higher body mass index and behavioural factors, such as exposure to psychological stress (Friedenson, 2007; Chida et al., 2008; Gnerlich et al., 2011; Marques-Vidal et al., 2012).

Exposure to stress has long been associated with a negative impact on human health, especially if the stress is experienced chronically. Prolonged exposure to stress is known

to lead to maladaptive responses in various body organs and to pathophysiological mechanisms such as cardiovascular and autoimmune diseases, psychological disturbances and metabolic dysregulations (Dimsdale, 2008; Mariotti, 2015; Morey et al., 2015; Yaribeygi et al., 2017).

Stress is a complex process involving environmental and psychosocial factors which activates a cascade of information-processing pathways both in the central nervous system (CNS) and peripheral nervous system (Thaker et al., 2007).

To cope with stress situations, humans, like other organisms, activates mainly, two hormonal pathways: 1) the hypothalamic – pituitary – adrenal axis, resulting, mainly, in the released of glucocorticoids from the adrenal cortex and through 2) the sympathetic division of the autonomic nervous system (ANS), leading to an increase in circulating catecholamines (adrenaline and noradrenaline) (Goldstein, 2003; Zhou et al., 2016b; McEwen, 2017).

The efferent pathways of the ANS consist of two kinds of neurons that transmit impulses from the CNS to the peripheral target tissues. The preganglionic neuron originates in the CNS. The axon of this neuron travels to an autonomic ganglion, where it synapses with a postganglionic neuron by releasing acetylcholine and leading to a released of noradrenaline. Alternatively, acetylcholine from preganglionic neuron can also directly stimulate the adrenal medulla (acting as a post-ganglionic neuron) to induce the release of noradrenaline and adrenaline into the bloodstream (Fink, 2000).

The ANS response towards as a stressor occurs rapidly (Fink, 2000). It is mediated primarily by the sympathetic adrenomedullary system, where it requires the activation of the CNS from sympathetic neurons and release of catecholamines, and the subsequent adrenergic stimulation of the target organs (Thaker et al., 2007).

In general, adrenergic receptors activation leads to the effects commonly seen in the “fight-or-flight” response: pupil dilation, increased sweating, mobilization of energy by increasing glycogenolysis and lipolysis, increased heart rate and increased blood pressure (Tank and Lee Wong, 2015; Rabasa and Dickson, 2016; Zhou et al., 2016b). This response is considered to be an important survival mechanism, enabling a quick reaction to life-threatening situations (Rabasa and Dickson, 2016).

The synthesis of catecholamines occurs mainly in the brain, adrenal medulla and in some sympathetic nerve fibers (Wong et al., 2007; Daubner et al., 2011). **Figure 3** describes the steps leading to the conversion of the amino acid L-tyrosine into noradrenaline and adrenaline.

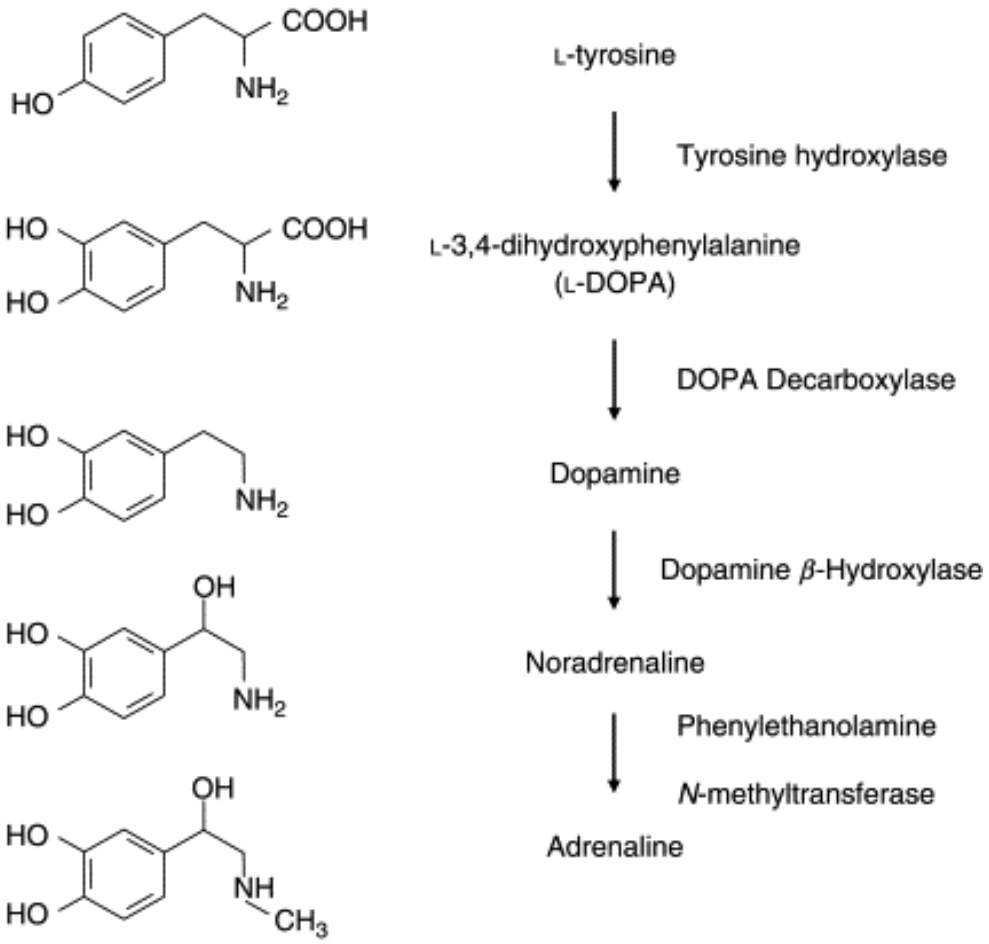



**Figure 3 Pathway for the biosynthesis of catecholamines.** The first reaction involves the conversion of L-tyrosine into L-DOPA by the tyrosine hydroxylase (TH) enzyme. L-DOPA is then converted into dopamine by the enzyme DOPA decarboxylase. Subsequently, dopamine can be converted into noradrenaline by the enzyme dopamine β-hydroxylase. The enzyme phenylethanolamine N-methyltransferase (PNMT), with S-adenosyl-L-methionine as cofactor can convert noradrenaline into adrenaline (Daubner et al., 2011)..

Adrenaline and noradrenaline exert their effects on peripheral tissues via interaction with a class of G protein-coupled receptors, called adrenoreceptors or adrenergic receptors (AR) (Alexander et al., 2017). This class can be subdivided into three pharmacological types: $\alpha_1$; $\alpha_2$; and β-adrenoreceptors (β-AR), which can be further classified in several subtypes ($\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1D}$; $\alpha_{2A}$, $\alpha_{2B}$, $\alpha_{2C}$; $\beta_1$, $\beta_2$, $\beta_3$, respectively), each of one presenting

different affinity for their natural ligands (noradrenaline and adrenaline) (Alexander et al., 2017).

Coupling to G proteins is assumed to be the main transduction system of ARs, although some cross-talk may occur with other transduction systems (Tilley, 2011; Song et al., 2018). **Table 2** shows the main transduction pathways for the several subtypes of ARs.

**Table 2. Main transduction mechanisms for the ARs subtypes** (Alexander et al., 2017)

| Ars | Primary transduction mechanisms | Secondary transduction mechanisms |
|---|---|---|
| $\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1D}$ | Gq/G11 family:<br>Phospholipase C stimulation<br>Calcium channel | Phospholipase A2 stimulation<br>Phospholipase D stimulation |
| $\alpha_{2A}$, $\alpha_{2B}$, $\alpha_{2C}$ | Gi/Go family:<br>Adenylate cyclase inhibition<br>Potassium channel<br>Calcium channel | Gs family:<br>Adenylate cyclase stimulation |
| $\beta_1$ | Gs family:<br>Adenylate cyclase stimulation | Gi/Go family:<br>Guanylate cyclase stimulation |
| $\beta_2$ | Gs family:<br>Adenylate cyclase stimulation | Gi/Go family:<br>Guanylate cyclase stimulation |
| $\beta_3$ | Gs family:<br>Adenylate cyclase stimulation | Gi/Go family:<br>Guanylate cyclase stimulation<br>Adenylate cyclase inhibition |

Over the last decades, successive studies have addressed the relationship between stress and its related messengers, namely catecholamines, and the development of various types of cancers, and identified psychosocial factors including stress, chronic depression and lack of social support as risk factors in cancer progression (Spiegel, 1994; Spiegel and Giese-Davis, 2003; Moreno-Smith et al., 2010; Campbell et al., 2012; Mariotti, 2015).

By using meta-analytic methods, Chida et al. (2008) further confirmed that stress-related psychosocial factors were associated with adverse effects on cancer incidence and that stressful life experiences were related to poor cancer survival and higher mortality (Chida et al., 2008). In line with these observations, increased in catecholamine levels in individuals who experience acute or chronic stress was reported (Rupp and Jacob, 1995; Schmidt and Kraft, 1996; Tang et al., 2013; Bastos et al., 2018), which further supports the association between stress and its adrenergic messengers (noradrenaline and adrenaline) and cancer progression.

In addition to the adrenaline and noradrenaline originated from the adrenal gland or from the adrenergic sympathetic nerve fibres, it was also found that some cancer cells may also be able to produce catecholamines (Wong et al., 2007; Shi et al., 2011). This appear to be the case of breast cancer cells (HER-2 overexpressing cells), where it was found that the β-adrenoceptor, in cooperation with the HER2 receptor, induces an autocrine adrenaline release and a strong mitogenic effect in these cells (Shi et al., 2011). The impact of catecholamines production within the tumor and their putative involvement in the control of tumorigenesis and immunosuppression of the tumor microenvironment are poorly understood.

## 1.5. Adrenergic stimulation and cancer

In addition to the classical role of adrenaline and noradrenaline in the “fight or flight” response, several studies showed that catecholamines have a role in promoting the progression of cancer (Shi et al., 2011; Coelho et al., 2017; Cui et al., 2019) which may be particularly relevant in conditions where a persistent adrenergic stimulus occurs due to the long-lasting elevation of plasma catecholamines such as in chronic stress.

Pre-clinical studies (*in vitro* and *in vivo* studies), showed that stress-activated pathways can lead to tumour proliferation, resistance to apoptosis, invasion, metastasis, increased angiogenesis and alteration in the immune responses and in the stroma microenvironment in several types of cancer, further supporting the link between stress and tumorigenesis (Moreno-Smith et al., 2010; Cole and Sood, 2012).

The effects of the adrenergic system in cancer appear to be mostly dependent on the β-AR (Coelho et al., 2017), where the presence of the $\beta_1$-AR and $\beta_2$-AR subtypes have been virtually described in all cancer types (Cole and Sood, 2012).

The first evidence for the role of β-AR, in cancer was found in a human lung adenocarcinoma cell line, in which, isoprenaline, a β-adrenergic agonist, increased proliferation of these cells (Schuller and Cole, 1989). Successive studies have then shown that activation of β-AR cause pro-tumorigenic effects (increased proliferation, invasion and angiogenesis, resistance towards apoptosis and impaired cellular immune response) in different types of cancer cell lines/tumours, including breast cancer (Schuller, 2010; Madden et al., 2011; Cole and Sood, 2012; Coelho et al., 2017).

Putative transduction mechanisms that couple activation of ARs to cellular pathways relevant for carcinogenesis have been put forward and are depicted in **Figure 4**. The model proposes that the transient increase of intracellular cAMP triggered by Gs

activation, activates two major biochemical effector systems: the protein kinase A (PKA) and the exchange protein activated by adenylyl cyclase (EPAC), leading to the activation of several transcription factors and important pathways in cancer (Cole and Sood, 2012; Coelho et al., 2017).

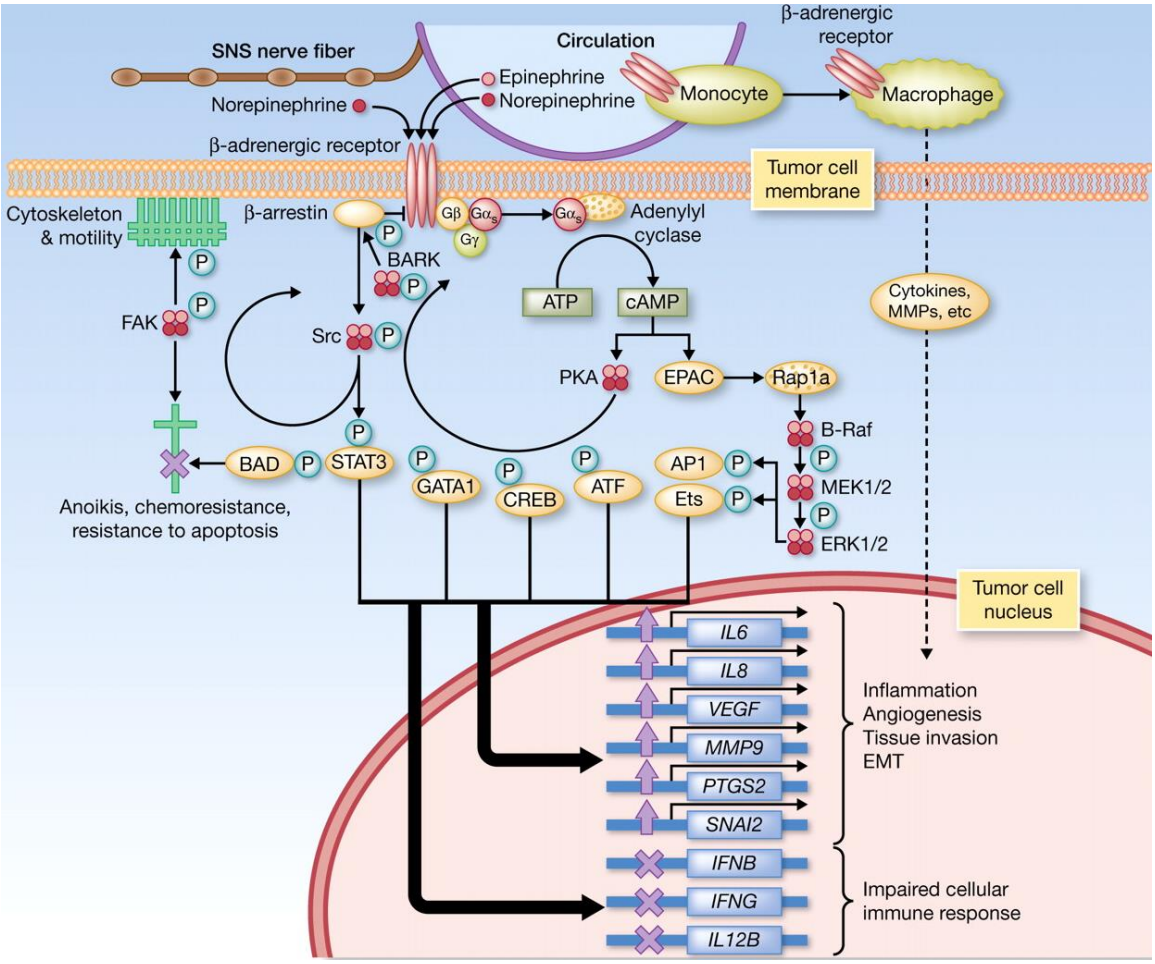



**Figure 4. ANS fight-or-flight stress responses releases catecholamines into tumor microenvironment.** Catecholamines bind to β-AR, resulting in the activation of PKA which phosphorylate multiple target proteins. This led to the activation of several transcription factors (e.g. transcription factors of the CREB/ATF and GATA families, β-adrenergic receptor kinase (BARK), STAT3, and the focal adhesion kinase (FAK)), leading to changes in cell trafficking and motility, as well as cellular resistance to apoptosis. The second biochemical effector system activated by the β-AR is the activation of EPAC by cAMP, resulting in the activation of the mitogen-activated protein kinase signalling pathway and downstream effects on diverse cellular processes. The general pattern of transcriptional responses induced by β-adrenergic signalling includes upregulated expression of metastasis-associated genes involved in inflammation, angiogenesis, tissue invasion, and epithelial-mesenchymal transition, and downregulated expression of genes facilitating antitumor immune responses (Cole and Sood, 2012).

A link between activation of AR and cancer is also corroborated by studies showing the impact of the clinical use of β-blockers in cancer incidence and mortality. Propranolol, a β-AR antagonist widely used to treat conditions such as hypertension, *angina pectoris*

and arrhythmias was associated with a significant reduction in tumour recurrence, longer disease-free intervals, reduced risk of metastasis and reduced risk of cancer death (e.g. breast, melanoma, ovarian, prostate cancer) (Powe et al., 2010; Barron et al., 2011; Childers et al., 2015). Moreover, emerging evidence reported that propranolol may have various mechanisms of action that can be particularly relevant in cancer treatment: it has anti-proliferative and anti-metastatic effects and it is able to modulate the tumour stroma (e.g. immune cells, cancer-associated fibroblast) (Campbell et al., 2012; Zhou et al., 2016b; Shaashua et al., 2017; Ashrafi et al., 2018; Chen et al., 2018; Nagaraja et al., 2018). All these characteristics make propranolol a potential candidate for drug repurposing in cancer.

## 1.6. Oestrogens, adrenergic stimulation and cancer

Chronic stress is not the only condition in which a sustained release of adrenergic messengers occur. Evidence exist supporting that the same may occur during menopause, particularly during hot flashes events (Kronenberg et al., 1984; Freedman, 2014). Although the endocrine physiology of the hot flashes is still not well understood, it is known to be mediated by an interrelation between adrenergic signalling and the decrease in oestrogen levels, observed during the menopause (Freedman, 2014).

A relationship between the occurrence of hot flashes and an increased incidence of breast cancer is still debatable (Huang et al., 2011; Chlebowski et al., 2018). A large-scale study has found that women presenting persistent vasomotor symptoms, which includes hot flashes and night sweats, are more likely to be diagnosed with breast cancer (Chlebowski et al., 2018) what brings additional evidence for the existence of a link between the hormonal disturbances underlying menopause (namely increase in catecholamine release and decrease of oestrogens) and the development of cancer. It should be kept in mind that a putative interaction between adrenergic signalling and oestrogen would not be exclusive of tumorigenesis. A relationship between oestrogens and adrenergic signalling is observed in the normal mammary gland where the β-AR levels fluctuate with the estrous cycle, being in a higher number during the proestrous and estrous cycle (Marchetti and Labrie, 1990). Interestingly, this association can also be found in mammary tumors where treatment of ovariectomized rats with 17β-estradiol caused a highly significant increase in β-AR levels and tumor growth (Marchetti et al., 1991).

Further studies are needed to clarify this relationship between oestrogens and adrenergic signalling. A better understanding of the mechanisms underlying the β-adrenergic influence in cancer and the influence of oestrogens in this pathway may offer new opportunities to identify possible pharmacological targets to treat breast cancer.

# II. Chapter: Aim

# 2. Aim

The present work aimed at further investigate the role of adrenergic signalling in tumorigenesis in breast cancer cells and how it can be modified by oestrogens and by alterations in cell metabolism that occurs during tumorigenesis. This study will focus on the effects mediated by β-AR in two different breast cell lines, in the MCF-10A cells, a non-tumorigenic cell line and in the MCF-7 cells, a tumorigenic cell line.

For these purposes, the effects of agonists and antagonists of β-ARs (isoprenaline and propranolol, respectively) in cellular viability/proliferation, a critical component in the carcinogenesis cascade were studied. Potential differences in adrenergic signalling in non-tumorigenic and tumorigenic cells were investigated in order to bring new contributions to better understand the claimed clinical efficacy of β-blockers, such as propranolol, in cancer, in particular breast cancer.

Knowing the impact that oestrogens also have in the development and progression of breast cancer and their relationship with the adrenergic stimulation, the influence of 17β-estradiol (the most potent and prevalent oestrogen) in this pathway and on the β-AR signalling was also investigated in order to better understand whether the adrenergic signalling is influenced by oestrogens and, if so, if this influence is altered along the tumorigenesis process.

# III. Chapter: Material and methods

# 3. Material and methods

## 3.1. Chemicals

Fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium – high glucose (DMEM-HG), supplemented L-Glutamine were obtained from Biochrom (Biotecnómica, São Mamede, Portugal). 4-Hydroxytamoxifen and phorbol myristate acetate were obtained from Tocris Bioscience (Biogen, Madrid, Spain). Goat anti-rabbit IgG conjugated with horseradish peroxidase (sc-2004) was from Santa Cruz Biotechnology Inc. (Frilabo, Maia, Porto, Portugal). Monoclonal anti-tyrosine hydroxylase antibody (ab137869) and the polyclonal anti-tyrosine hydroxylase antibody (ab112) were obtained from Abcam (Cambridge, UK). Trypsin/EDTA was obtained from Invitrogen (Alfagene, Carcavelos, Portugal). L-glutamine was purchase from Gibco (Biotecnómica, São Mamede, Portugal). Dulbecco's Modified Eagle's Medium/F-12 (DMEM/F12), (-)-Isoprenaline, (+)- propranolol, 17β-estradiol, methyl-β-cyclodextrin, penicillin/streptomycin mixture (10 000 $U.mL^{-1}$/10 $mg.mL^{-1}$, respectively), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), crystal violet solution, hydrocortisone, epidermal growth factor, human insulin, acridine orange, 3-methyladenine (3´MA), sodium pyruvate, Hoescht 33258 and monodansylcadaverine (MDC) were purchased from Sigma-Aldrich (Sigma-Aldrich-Química S.A., Sintra, Portugal).

## 3.2. Cells and culture conditions

The experimental work described in this dissertation was performed using two different cell lines, MCF-7, a tumorigenic ER+ breast cancer cell line and MCF-10A, a non-tumorigenic ER- cell line. Both cells lines were purchased from ATCC ®. All experiments were carried out in batches of cells with passages number lower than 35 in MCF-10A and lower than 50 in MCF-7 to avoid genotypic/phenotypic changes. The MCF-7 cells were routinely cultured in T25 $cm^2$ cell culture flask with DMEM-HG medium supplemented with 10% heat-inactivated FBS and 1% of a mixture of penicillin/streptomycin (10000 $U.mL^{-1}$/10 $mg.mL^{-1}$, respectively). MCF-10A cells were cultured in T75 $cm^2$ cell culture flaks with DMEM/F12 medium supplemented with sodium bicarbonate (1.2 $g.L^{-1}$), stable L-glutamine (2 mM), epidermal growth factor (EGF; 20 $ng.mL^{-1}$), human insulin (3.5 $\mu g.mL^{-1}$), hydrocortisone (0.5 $\mu g.mL^{-1}$), 10% heat-inactivated FBS, 1% of a mixture of penicillin/streptomycin (10000 $U.mL^{-1}$/10 $mg.mL^{-1}$,

respectively) and pH adjusted to 7.4. Both cells lines were maintained at 37ºC, in a humidified atmosphere to avoid medium evaporation, and with 5% $CO_2$ to allow optimum pH during culture. For cell culture maintenance, cells were split twice a week and kept below 90% confluence. For this purpose, culture medium was removed, and cells were subsequently washed with 100 mM PBS. Then, cells were treated with a trypsin solution (0.25% trypsin; 0.025% EDTA in PBS) and incubated for six minutes, at 37ºC, to allow the detachment of the cells. Cells were then resuspended in complete medium and allowed to attach in new cell culture flaks.

## 3.3. Determination of the optimal percentage of serum

Serum is a key component for growing and maintaining cells in culture. It is an important source of growth factors, proteins, hormones, minerals and adhesion factors which facilitates cell survival and growth (Van der Valk et al., 2010).

In order to study the effects of the treatments in MCF-7 and in MCF-10A cells, it was necessary to perform a dose-response curve by increasing the concentrations of FBS for each cell line. This allowed to i) decrease the influence of the FBS (it imposes strong mitogenic effects on cells) on the effects caused by each treatment and ii) to distinguish cell survival from cell proliferation in functional studies by selecting a serum concentration that did not affect cell viability. In MCF-10A, EGF and the human insulin supplements were also removed since they also induced a strong mitogenic effect in these cells.

The functional assays, in MCF-7 breast cancer cells, were performed in the absence of FBS, since MCF-7 cells continue to proliferate in the absence of FBS.

For the MCF-10A breast non-tumorigenic cells, the concentration of 4% FBS was chosen for the functional assays, since it was the only concentration of serum that induced a similar homogenous response through each experimental timepoint.

## 3.4. Cell treatment

Prior to every experiment, the MCF-7 and the MCF-10A cells were centrifuged at 1500 rpm, for five minutes at 20ºC, and viable cells were counted using the trypan blue dye exclusion method. MCF-7 and MCF-10A cells were then prepared using an initial cell density of $2.3 \times 10^4$ cells.mL$^{-1}$ and $4.0 \times 10^4$ cells.mL$^{-1}$, respectively, in complete medium

and incubated for 24h. After this period, MCF-7 cells were additionally incubated in serum-free medium for 24h to starve the cells from hormones and growth factors. Cells were then treated with increasing concentrations of the non-selective ER agonist, 17β-estradiol (1 - 100 nM), the non-selective β-AR agonist, isoprenaline (0.1 – 10 μM), the non-selective β-AR antagonist, propranolol (1 μM – 100 μM) or with the $\beta_2$-AR inverse agonist, ICI 118,551 (10 μM), alone or in combination. In some experiments, MCF-7 were also treated with a cholesterol depletion agent, methyl-β-cyclodextrin (1 - 1000 μM), alone or in combination with the compounds mention above. MCF-10A were also treated with 4-hydroxyamoxifen (1 -10000 nM), alone or in combination with isoprenaline. The concentrations for the agonists/antagonists were chosen based on the different affinities towards its target receptors (available in: International Union of Basic and Clinical Pharmacology/The British Pharmacological Society http://www.guidetopharmacology.org). Stock solutions of the compounds tested were prepared in DMSO or in water and freshly diluted to the final concentration in medium (DMEM-HG in MCF-7 cells and DMEM/F12 supplemented with 4% FBS in MCF-10A cells), immediately before the experiment. The appropriate control/solvent experiments were carried out using cells exposed to DMSO (0.1%). Cells were then incubated at 37ºC, up to 72h depending on the experimental assay.

## 3.5. MTT reduction assay

The MTT reduction assay was carried out in order to evaluate cell viability, after exposure to the compounds described before. This assay consists on the endocytosis of and subsequent reduction of the MTT reagent (yellow colored) into insoluble formazans crystals (purpled colored), that can be quantified by spectrophotometry. This step is exclusively carried out by living cells through NAD(P)H-dependent cellular oxidoreductase enzymes. Since inviable cells lose the ability to convert MTT into formazan crystals, this method allows for the quantification of viable cells.

This assay was carried out in the MCF-10A non-tumorigenic breast cells and in the MCF-7 breast cancer cells. To perform this assay, cells were seeded in 96-well plates (200 μL per well). After drug treatment, cells were incubated for 24, 48 and 72h. At the end of each timepoint, cell culture medium was removed and the MTT reagent (0.5 $mg.mL^{-1}$ in PBS, 100 μL) was added to the plates and incubated for three hours, protected from light. At the end of this incubation period, the MTT solution was removed and 100 μL DMSO were added to each well to dissolve the formazan crystals formed. Thereafter, absorbance was measured at 570 nm in an automated microplate reader (Sinergy HT,

Biotek Instruments Inc, Vermont, USA). Results are expressed as percentage of the respective control. All conditions were performed in parallel triplicates or sextuplicates.

## 3.6. Kinetic Label-Free Proliferation Assay

To characterize cell proliferation upon exposure to treatments, the number of cells was monitored using an automated label-free cell counting, as previously described (Clayton, 2017). This method uses defocus high contrast brightfield images, which causes a cell mediated-refraction of the light in order to produce a bright spot. The Gen5 Image Analysis Software can then accurately count the bright spots, enabling long-term proliferation studies in a label free manner.

This assay was initially carried out in the MCF-10A and in MCF-7 cells. Nevertheless, for the MCF-10A cells it was not possible to accurately quantify the number of cells at every time point due to their slow growing abilities, and due to the experimental methodology performed (the MCF-10A were cultured in 4% serum DMEM/F12 without supplements, which lead to some cell death).

To perform this assay, cells were seeded in 96-well plates (200 µL per well). After drugs treatment, four high contrast brightfield images, at the center of the well, were captured (Lionheart FX microscope, BioTek Instruments, Winooski, USA), for each time point (T0; T24, T48; T72h): two focused images were used for reference, and the other two defocused images for cell counting. The Gen5 Image Analysis Software was then used to perform the stitching and kinetic alignment of the two defocused images and to do the image pre-processing in order to obtain the best possible enhancement of contrast, reducing each cell to a single bright spot. Object masking thresholds were then set to identify each cell for counting. Plates were maintained at 37 ºC inside the Lionheart FX microscope chamber throughout the imaging steps. Results for the number of cells obtained at each time point were then normalized to the cell number achieved at T0, and the results were expressed as percentage of the respective control, for each time point. All conditions were performed in parallel, in triplicates or sextuplicates.

## 3.7. Colony formation assay

The colony formation assay was performed as described previously (Soares et al., 2014) with some modifications. MCF-10A cells were seeded in 12-well plates with an initial density of 1000 cells per well in complete medium and allowed to attach for 24h.

Thereafter, medium was replaced with fresh complete medium, in the absence or in the presence of the drugs. After 72h incubation, cells were re-treated with the appropriate drugs and allowed to continually grow for a total of seven days. In the end of the experiment, colonies were fixed with 4% (w/v) paraformaldehyde in PBS (five minutes), stained with crystal violet (0.5% v/v, in distilled water) for five minutes and rinsed twice with distilled water. Representative images of the colonies were taken using a digital camera. Afterwards, crystal violet was eluted with glacial acetic acid (10%) and absorbance at 600 nm was measured in an automated microplate reader (Sinergy HT, Biotek Instruments Inc, Vermont, USA). All the conditions were performed in parallel and in triplicates.

## 3.8. Protein expression by Western blot

To carry out this assay, MCF-10A cells were seeded in petri dishes of 60.1 $cm^2$ (10 mL per dish). After 24h incubation with the treatment, cell culture medium was removed, and cells were rinsed with ice-cold PBS. Total cell protein were extracted by adding 300 µL of lysis buffer with protease inhibitors 5 mL RIPA, 25 µL Na3VO4 200 mM, 25 µL NaF 1 mM, 100 µL PMSF 100 mM, 1 µL aprotinin (10 $mg.mL^{-1}$) and 1 µL of leupeptin (10 $mg.mL^{-1}$). Ceramic beads of 1.4 mm diameter were added each tube, and the samples were then subjected to two cycles of 15 seconds at 5800 rpm in the Precellys Evolution Homogenizer to enhance cell disruption (Bertin Instruments, France). Afterwards, the lysates were incubated on ice for one hour and then centrifuged at 20000 g for 45 minutes, at 4°C. To ensure the loading of the same amount of protein for all the samples, the total protein concentration in the supernatant was determined using the Bradford method (10 µL of each sample diluted 1:10 + 200 µL dye reagent diluted 1:5 and filtered 0,2 µm), using bovine-albumin as standard. Absorbance were measured at 570 nm in an automated microplate reader (Sinergy HT, Biotek Instruments Inc, Vermont, USA).

Afterwards, equal amounts of protein (20 µg) were heat-denatured through boiling at 70ºC for ten minutes in 6X sample buffer (0.35 M Tris-HCl pH 6.8, 4% sodium dodecyl sulfate (SDS), 30% glycerol, 9.3% dithiothreitol and 0.01% bromophenol blue). Proteins were subjected to a 10% SDS-PAGE (polyacrylamide gel electrophoresis) at 125 V for one hour with running buffer (25 mM Tris Base, 250 mM glycine, 0.1% SDS).

Proteins were next electrotransferred from gels onto pure nitrocellulose membranes at 25 V and 2.5 A, for three minutes using the Trans-Blot turbo Transfer System (Bio-Rad). Next, membranes were incubated under gentle agitation for one hour at room

temperature with 5% bovine-albumin in PBST (0.1% Tween 20 in PBS pH 7.4, filtered) in order to block additional reactive sites that might exist on the nitrocellulose membrane. Immunoblotting was then carried out overnight, at 4ºC, by probing the membranes with the primary antibody rabbit anti-tyrosine hydroxylase (ab137869- 1:5000; ab112- 1:500). By the end of this incubation period, nitrocellulose membranes were washed with PBST and incubated for one hour at room temperature, under gentle shaking, with the secondary antibody anti-rabbit IgG conjugated to horseradish peroxidase (1:5000).

The immunocomplexes formed were detected using an enhanced chemiluminescence system and a Novex ECL detection kit. The immunoblots were scanned using the BioRad ChemiDoc MP Imaging System and images were captured using Image Lab software.

## 3.9. Detection of catecholamines by HPLC-ECD

HPLC with electrochemical detection (HPLC-ECD) was used in order to evaluate the presence of catecholamines in MCF-10A and in MCF-7 cells.

To carry out this assay, both cell lines were seeded in petri dishes of 60.1 $cm^2$ (10 mL per dish). Upon 24h incubation in the absence or in the presence of isoprenaline (in 0% serum to minimize the influence of FBS), supernatants were collected and perchloric acid 2 M (1:10) was added. The samples were filtered (Spin X filters, Costar) using centrifugation (12000 g for five minutes) and frozen (-20ºC) until analysis. HPLC-ECD analysis were performed at the Laboratory of Clinical Chemistry of the Centro Hospitalar do Porto - Hospital de Santo António, with the collaboration of Dr. Henrique Reguengo. The concentration of catecholamines in the supernatants were calculated using noradrenaline and adrenaline standard solutions.

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## 3.10. Morphological transformation assay

To evaluate MCF-10A cell neoplastic transformation, a morphological transformation assay was carried out. MCF-10A cells were seeded in 24-well plates with an initial density of $1 \times 10^5$ cells per well in complete medium and allowed to reach confluency. Thereafter, medium was replaced with fresh complete medium, in the absence or in the presence of drugs (isoprenaline or PMA). Then, cells were incubated continually for one month. Cells were refed two times per week in the absence or in the present of drugs, and analysed for the presence of transformed foci, using a phase contrast microscope.

Occasionally, images were also taken with the Lionheart FX microscope. At the end of the experiment, cells were also stained with Hoescht 33258 (5 $\mu g.mL^{-1}$) to increase the detailed microscopic visualization and examination of foci.

## 3.11. Monodansylcadaverine and acridine orange staining

Acridine Orange staining was performed as described (Evangelatov et al., 2016). MCF-7 cells were seeded in 96-well plates (200 µL per well). After drugs treatments, MCF-7 cells were stained with 5 $\mu g.mL^{-1}$ of acridine orange in serum free-medium for 15 min at 37ºC, washed, and analysed on the Lionheart FX microscope. Data were analysed using Gen5 Image Analysis Software.

To visualized autophagic structures, the monodansylcadaverine (MDC; 50 µM) marker was used. Cells were treated as for acridine orange staining. Images were taken using the Lionheart FX microscope.

## 3.12. Data and statistical analysis

Data were firstly tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test), using the GraphPad Prism X V software package. Differences between treatments and controls were compared using one-way analysis of variance (ANOVA), followed by the *post-hoc* multiple comparisons Dunnett's *t* test, whenever applicable. In assays assessing the combination effects, differences between treatments were assessed using ANOVA, followed by *post-hoc* multiple comparisons Tukey's *t* test, whenever applicable or using a Student´s *t* test. A value of $p<0.05$ was considered to denote significant statistical differences.

# IV. Chapter: Results

# 4. Results

## 4.1. Effects of the β-AR activation in cell viability of the human breast non-tumorigenic cell line (MCF-10A)

To clarify the effects of the β-AR activation, a set of experiments was firstly performed to characterise the effects of the adrenergic ligands, on the viability of MCF-10A cells, using the MTT assay.

Isoprenaline and propranolol, a β-AR agonist and antagonist, respectively, were used to evaluate the effects of the β-AR activation in MCF-10A cell viability. Isoprenaline is a potent and full β-AR agonist: pKi values: i) $\beta_1$-AR: 6.6 - 7.0; ii) $\beta_2$-AR: 6.4 and iii) $\beta_3$-AR: 5.1 - 6.2 whereas propranolol is a β-AR antagonist: pKi values: i) $\beta_2$-AR: 9.1 – 9.5 and ii) $\beta_3$-AR: 6.3 - 7.2.

The effects of the adrenergic ligands in MCF-10A cell viability are shown in **Figure 5**. After 24h incubation, the β-AR agonist isoprenaline (0.1 – 10 μM) increased viability, of MCF-10A cells and the maximal increase was observed in cells treated with 0.1 μM isoprenaline (**Figure 5**). However, when cells were incubated with isoprenaline for 72h those effects were no longer observed: isoprenaline (0.1 – 10 μM) failed to alter viability of MCF-10A cells (**Figure 32A, Annex**).

The β-AR antagonist, propranolol (1 – 100 μM), *per se*, had no effect in MCF-10A cell viability, after a 24h incubation period (**Figure 6A**). Moreover, when combined with isoprenaline (0.1 μM), propranolol prevented the increase in MCF-10A cell viability caused by isoprenaline (**Figure 6B**).

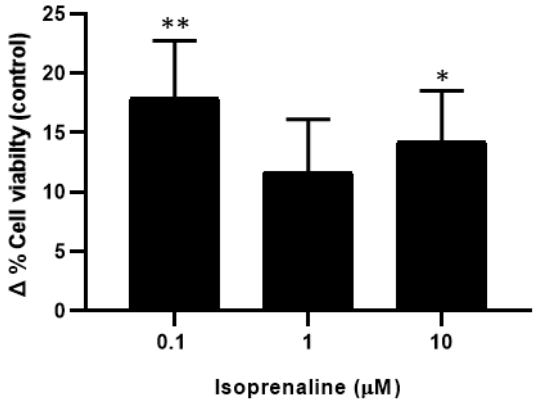



**Figure 5. Effects of the β-AR agonist isoprenaline on MCF-10A cell viability, after 24h of incubation.** Upon seeding, MCF-10A cells were incubated for 24h and subsequently treated with the indicated drugs for further 24h (T0). To evaluate cell viability, the MTT assay was used. A detailed description of the method can be found in the Materials and Methods section. Results were expressed as percentage of control and then as a difference between the effect caused by isoprenaline and the effect caused by the control. Results are presented as mean ± SEM, from 6-8 independent experiments. Significant differences from control: * $p<0.05$; ** $p<0.01$; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Dunnet´s *t* test.

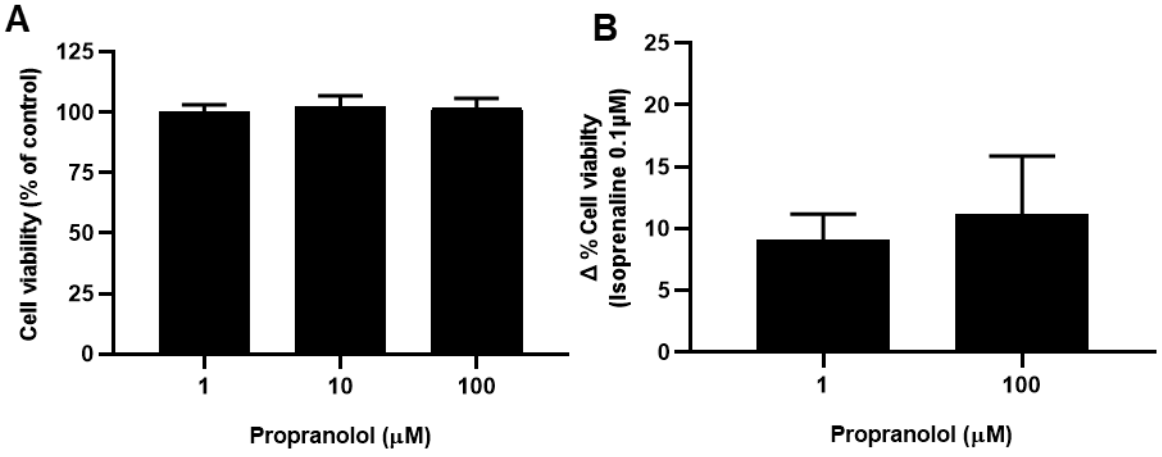



**Figure 6. Effects of the β-AR antagonist propranolol, alone (A) or in combination with isoprenaline (B), on MCF-10A cell viability, after 24h of incubation**. Upon seeding, MCF-10A cells were incubated for 24h and subsequently treated with the indicated drugs (T0) for further 24h. To measure the cell viability, the MTT assay was used. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of control in panel A and as a difference between the effect caused by isoprenaline 0.1 μM and the effect caused by the combined exposure of propranolol (1 and 100 μM) + isoprenaline in panel B. Results are presented as mean ± SEM, from 6-8 independent experiments.

## 4.2. Influence of 17β-estradiol in the β-AR-mediated effects in MCF-10A cell viability

To evaluate the influence of oestrogens in the effects mediated by the β-AR, 17β-estradiol (a full ER agonist; pKi values: i) ER-α: 9.8, ii) ER-β: 9.3, iii) GPER: 8.2 – 8.5) was used.

The effect of 17β-estradiol on MCF-10A cells viability is shown in **Figure 7A**: 17β-estradiol (1 – 100 nM), *per se*, did not changed cell viability either when cells were incubated for 24h (**Figure 7A**) or for 72h (**Figure 32B, Annex**).

The effects caused by exposure to 17β-estradiol and isoprenaline were only evaluated at 24h exposure, as no effects were observed for both drugs at 72h incubation (**Figure 32, Annex**).

As shown in **Figure 7B**, treatment of MCF-10A cells with 17β-estradiol, inhibited the increase in cell viability caused by isoprenaline. This inhibition was more evident for cells treated with 0.1 µM isoprenaline (**Figure 7B**) since it was at this concentration that isoprenaline induced a maximum increase in MCF-10A cell viability (**Figure 5A**).

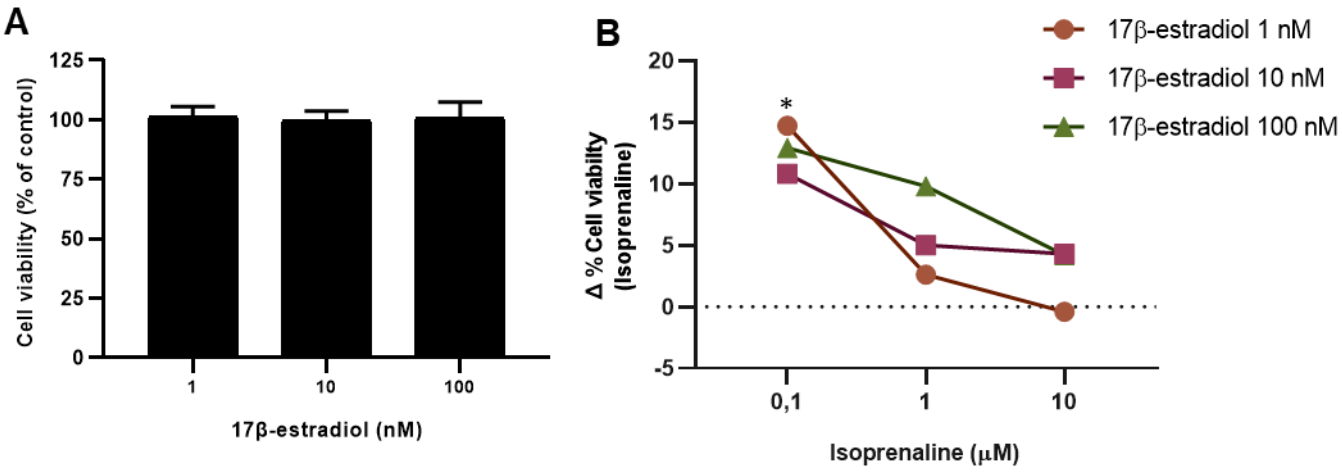



**Figure 7. Effects of the ER agonist 17β-estradiol, alone (A), or in combination with isoprenaline (B), on MCF-10A cell viability, after 24h of incubation**. Upon seeding, MCF-10A cells were incubated for 24h for adherence and subsequently treated with the indicated drugs (T0) for additional 24h. To evaluate cell viability, the MTT assay was used. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of control in panel A and as a difference between the effect caused by isoprenaline and the effect caused by the combined exposure of 17β-estradiol (1 – 100 nM) plus isoprenaline in panel B. Results are presented as mean ± SEM, from 6-8 independent experiments. Significant differences from isoprenaline 0.1 µM: * $p<0.05$; one-way analysis of variance (ANOVA), followed by post-hoc multi-comparisons Dunnet´s t test.

The non-tumorigenic MCF-10A cells are catalogued as being an ERα-; ERβ+ and a GPER+ cell line. To further explore which oestrogen receptors were involved in the modulation of the β-AR signalling, MCF-10A cells were treated with 4-hydrotamoxifen (4-OHT). In breast tissue, 4-OHT acts as an agonist of the GPER and as an antagonist of the ERα and of the ERβ (pKi values: i) ER-α: 9.9, ii) ER-β: 10.6 iii) GPER: 8.2 – 8.5). Therefore, if 17β-estradiol is preventing isoprenaline effects through the ERβ, combined treatment of 4-OHT and isoprenaline would still increase cell viability whereas if 17β-estradiol is acting through the GPER, the combined treatment would reduce the effects elicited by isoprenaline.

As shown in **Figure 8A,** treatment of MCF-10A cells with 4-OHT for 24h, *per se*, did not change cell viability. When treated in combination with isoprenaline (0.1 μM), 4-OHT reduce, in a concentration dependent-manner, the increase in MCF-10A cell viability caused by the β-AR agonist, isoprenaline (**Figure 8B**). These results support an involvement of the GPER as the oestrogen receptor responsible for the modulation of the β-AR mediated stimulation of MCF-10A cell viability.

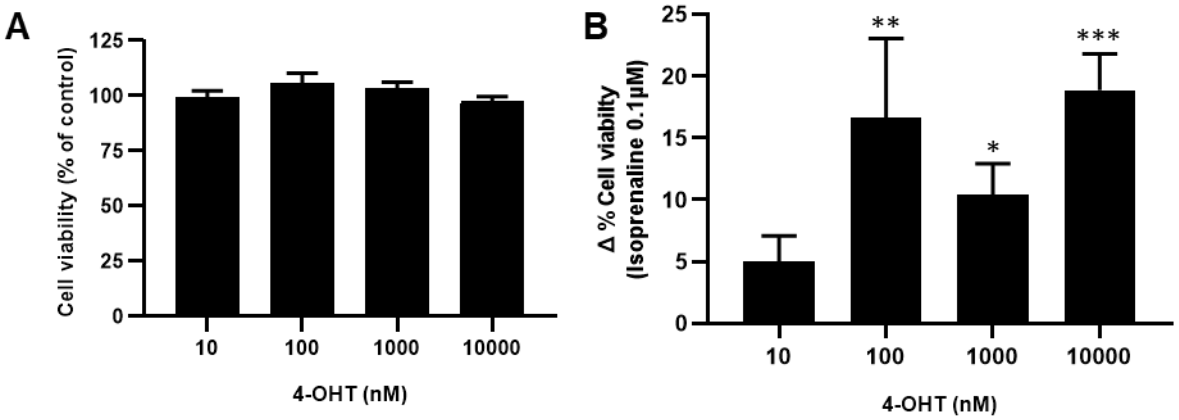
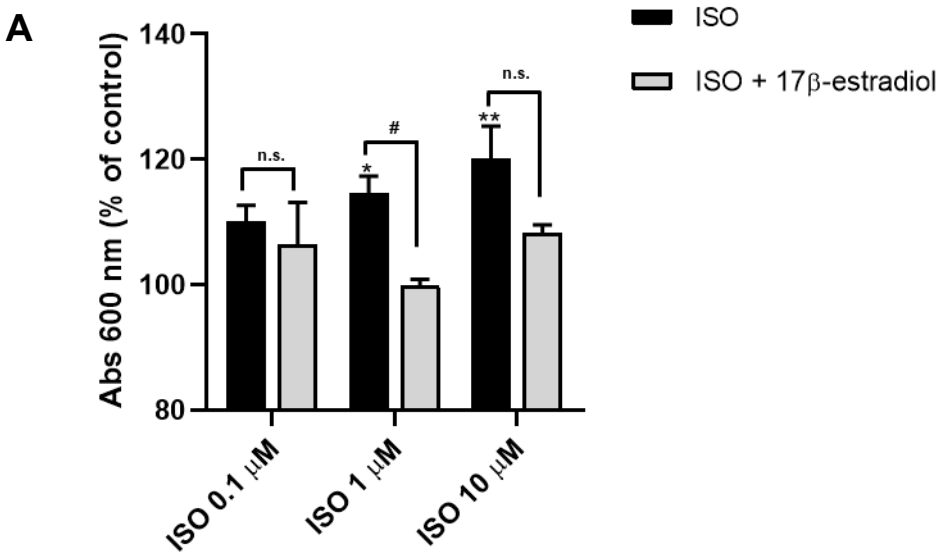



**Figure 8**. **Effects of 4-hydroxytamoxifen (4-OHT) and the combine exposure of the β-AR agonist isoprenaline with increasing concentrations of 4-OHT, on MCF-10A cell viability, after 24h of incubation**. Upon seeding, MCF-10A cells were incubated for 24h for adherence and subsequently treated with the indicated drugs (T0) for additional 24h. evaluate the cell viability, the MTT assay was used. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of control in panel A and as a difference between the effect caused by isoprenaline 0.1 μM and the effect caused by the combined exposure of 4-OHT (10 – 10000 nM) plus isoprenaline in panel B. Results are presented as mean ± SEM, from 3-7 independent experiments. Significant differences from isoprenaline 0.1 μM: * $p<0.05$, ** $p<0.01$, *** $p<0.001$; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons **Tukey's *t*** test.

## 4.3. Effects of the β-AR activation and the influence of 17β-estradiol in the clonogenicity of the MCF-10A cells

The colony formation assay was also used to further characterise the effects of the β-AR in MCF-10A cells and the influence of oestrogen receptors, in these effects.

**Figure 9** depicts the results obtained in the clonogenic assays carried out in the MCF-10A cells. Isoprenaline (0.1 – 10 μM), markedly increased the number of colonies formed, in a concentration dependent manner (**Figure 9A**). 17β-estradiol (100 nM), alone did not change the number of colonies formed (104.6 % ± 2.1; n=3). However, when combined with isoprenaline, 17β-estradiol reduced the increase in the number of colonies caused by isoprenaline (**Figure 9A**).



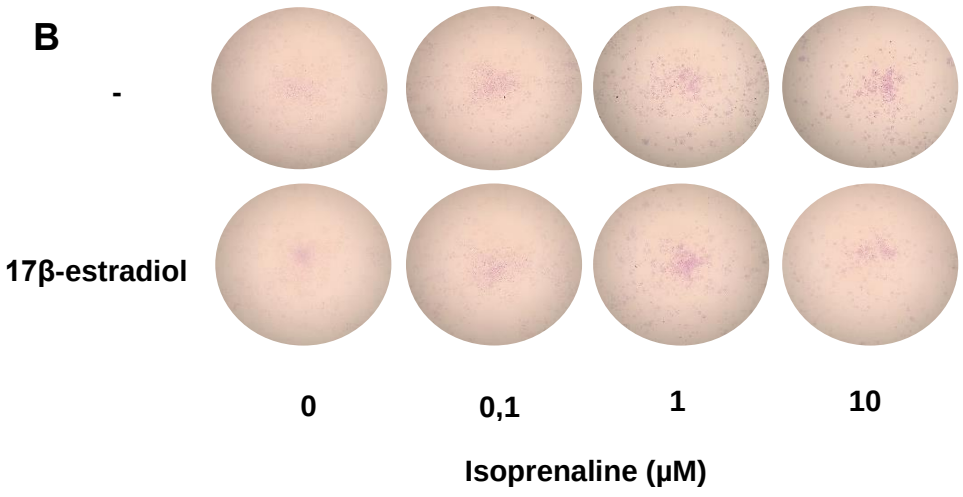



**Figure 9**. **Influence of 17β-estradiol on the effects caused by the β-AR agonist isoprenaline in MCF-10A clonogenicity, after 7 days of treatment.** Upon seeding, MCF-10A cells were incubated for 24h for adherence and subsequently treated with the indicated drugs (T0) for 7 days. A) Results for absorbance (λ=600 nm: Abs 600 nm), after crystal violet solubilization. Results are expressed as percentage of control and presented as mean ± SEM, from 3-4 independent experiments. B) Representative images of the colony formation assay in MCF-10A cells. A detailed description of the method can be found in the Materials and Methods section. Images were obtained using a digital camera. Significant differences from control: * p<0.05; ** p<0.01, one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Dunnet´s *t* test; Significant differences from isoprenaline treatment: $^{\#}$p<0.05; *Student´s t* test. ISO – isoprenaline; n.s – no significant differences (Student´s t test)

## 4.4. Effects of the β-AR activation on the expression pattern of the TH enzyme in MCF-10A cells

Previous work from our group has confirmed that breast cancer cells may acquire conditions to produce their own catecholamines during the tumorigenic transformation (manuscript under preparation). Western blot analysis was used to analyse the influence of the β-AR agonist, isoprenaline, in the expression pattern of enzyme TH (tyrosine hydroxylase), the rate-limiting enzyme in catecholamines biosynthesis, in the non-tumorigenic MCF-10A cells. The MCF-7 cell line was used a positive control.

As shown on **Figure 10A**, MCF-7 cells expressed the enzyme TH, revealed by the appearance of a band of the expected size (≈57-59 kDa).

In MCF-10A cells, a band, relative to the putative TH enzyme (immunoreactive to the monoclonal antibody raised against TH), is visible at around 40 kDa (**Figure 10A**). To ensure that this band was specific, a set of experiments was also performed using a different antibody, which confirmed the specificity of the MCF-10A band (data not shown).

Interestingly, although this enzyme is different from that found in MCF-7 cells, isoprenaline stimulation (1 μM), significantly increased its expression (**Figure 10B**).

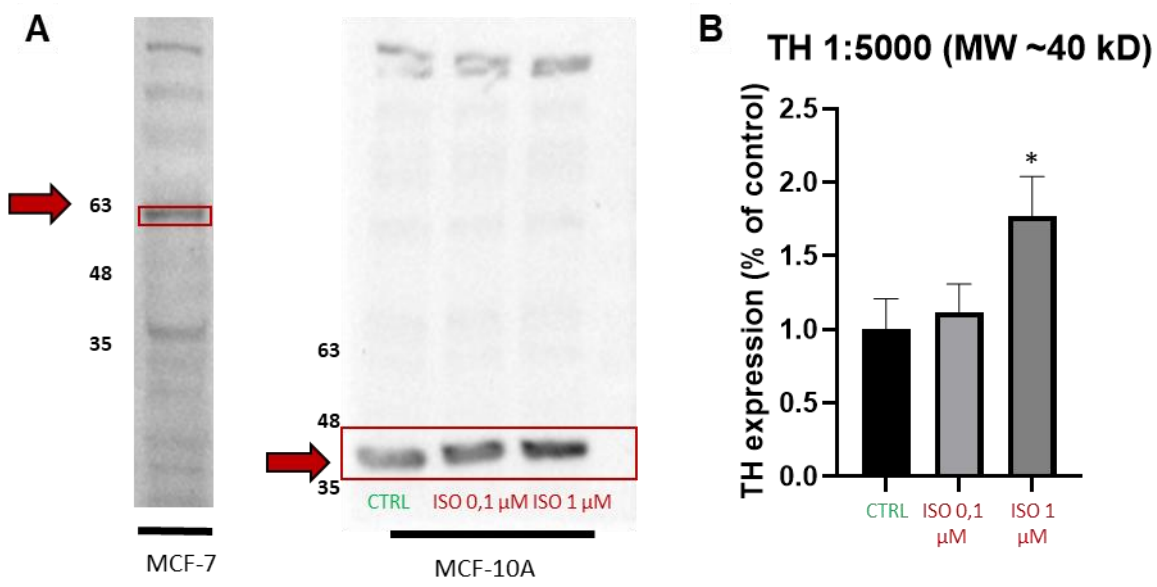



**Figure 10. Expression pattern of TH enzyme in MCF-10A cells, in the absence and in the presence of isoprenaline.** Upon seeding, MCF-10A cells were incubated for 24h for adherence and subsequently treated with the indicated drugs (T0) for additional 24h**. A)** Representative blots of the enzyme TH in MCF-10A cells in the presence of increasing isoprenaline concentrations. MCF-7 cells were used as positive control. **B)** Quantitative evaluation of TH protein expression in MCF-10A cells. Significant differences from the respective control: *p<0.05; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Dunnet´s *t* test; ISO – isoprenaline; CTRL – Control

## 4.5. HPLC-ECD detection of catecholamines in MCF-10A cells

Based on the results obtained in western blot, it is conceivable that MCF-10A cells may have some capacity to produce catecholamines as these cells express TH (**Figure 10**). HPLC-ECD was performed using the conditioned medium of MCF-10A cells, in the absence or in the presence of isoprenaline, to further confirm this hypothesis. The MCF-7 cell line was used a positive control.

As shown in **Table 3**, in conditioned medium of MCF-7 breast cancer cells, both noradrenaline and adrenaline were detected in significant amounts, especially adrenaline (about 10x higher than noradrenaline).

In the conditioned medium of MCF-10A cells, both noradrenaline and adrenaline were detected, suggesting that MCF-10A cells present catecholamines synthesis capacity (**Table 3**).

Treatment of MCF-10A cells with isoprenaline (0.1 µM), significantly changed the profile of catecholamines observed in their conditioned medium: noradrenaline was no longer detected whereas the presence of adrenaline increased 84% (**Table 3**).

**Table 3. Concentrations of catecholamines in conditioned medium of MCF-10A and of MCF-7 cells detected by HPLC-ECD.**

| | Noradrenaline (nM) | Adrenaline (nM) |
|---|---|---|
| **MCF-7** | 1.89 ± 0.34 | 11.02 ± 0.70 |
| **MCF-10A** | 1.26 ± 0.36 | 1.59 ± 0.46 |
| **MCF-10A + Isoprenaline 0.1 µM** | n.d | 2.94 ± 0.73 |

Values were obtained, using standard solutions for noradrenaline and adrenaline and are expressed in mean ± SEM from 3 independent experiments.

## 4.6. Effects of the β-AR activation in MCF-10A neoplastic transformation

The results presented to this point already support the hypothesis that catecholamines have a role in breast cancer development process. Moreover, we also attempted to further reveal the potential tumorigenic effects of catecholamines in the MCF-10A non-tumorigenic cell line, by using a neoplastic transformation assay, to directly imply catecholamines as tumorigenesis promoters.

The assay performed is a well-established method for characterizing the ability of transformed cells to continually grow independently of contact inhibition, after reaching confluency, which is a marker of neoplastic transformation (Yusuf and Frenkel, 2010). Phorbol 12-myristate 13-acetate (PMA) was used as a positive control for the neoplastic transformation (Park et al., 2004).

Preliminary results showed that continuous exposure during one month to PMA, induced the formation of structures within the confluent monolayer compatible with described transformed foci (**Figure 34, Annex**). Staining with Hoescht 33352 revealed that these structures were composed of cells, which further supports this possibility.

Treatment of MCF-10A with isoprenaline (0.1 – 10 µM) for 30 days, however, did not cause any formation of transformed foci (**Figure 34, Annex**). It would be interesting, in future works, to increase the incubation time of the assay.

## 4.7. Effects of the β-AR activation in cell viability of the human breast tumorigenic cell line (MCF-7)

In the present study, the MCF-7 cell line, a human breast cancer cell line, was used to characterize the β-AR-mediated effects in a tumorigenic cell model. The first approach performed was to characterise the effects of the β-adrenergic ligands (agonist: isoprenaline and antagonist: propranolol) in MCF-7 cell viability, using the MTT assay.

As shown in **Figure 11A**, the β-AR agonist isoprenaline (0.1 – 10 μM) did not change cell viability after a 24h incubation period. Longer incubation times were tested to assess if any effects would be detected in more critical conditions for viability, namely those created by longer incubation times points (up to 72h). When the incubation time was increased to 72h, isoprenaline led to a significant decrease in cell viability, at the maximum concentration tested (10 μM) (**Figure 11B**).

The β-antagonist propranolol (1 – 100 μM) led to a dramatic decrease in viability of MCF-7 cells, in a concentration- and time-dependent manner (**Figure 12A and 12B**, for 24h and 72h, respectively**)**.

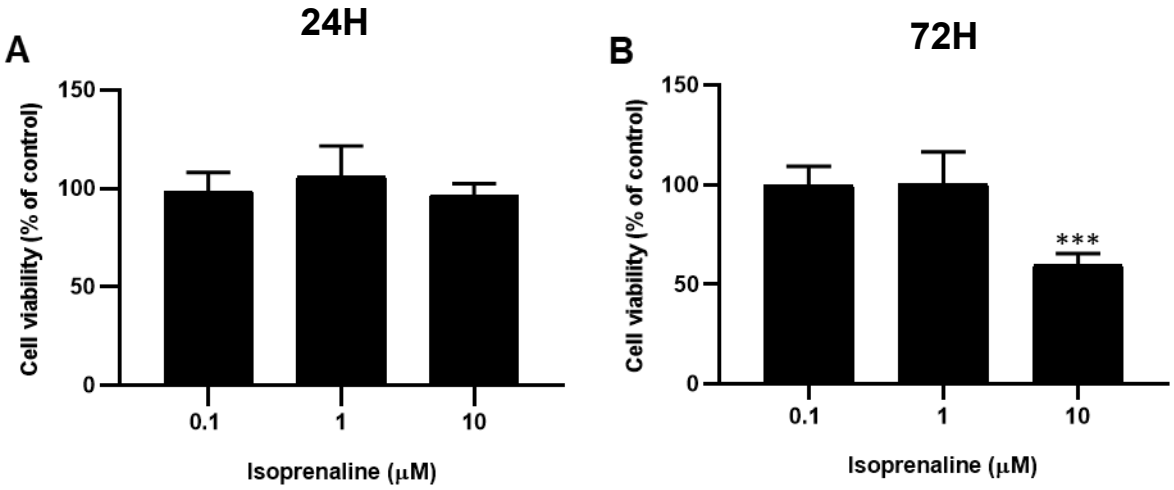



**Figure 11. Effects of the β-AR agonist isoprenaline on MCF-7 cell viability, after 24h (A) or 72h (B) incubation**. Upon seeding, MCF-7 cells were incubated for 24h for adherence, serum starved (0%) for an additional period of 24h and subsequently treated with the indicated drugs (T0) for up to 72h. The MTT assay was used to measure cell viability. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of control and presented as mean ± SEM, from 3 independent experiments. Significant differences from control: *** p<0.001; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Dunnet´s *t* test.

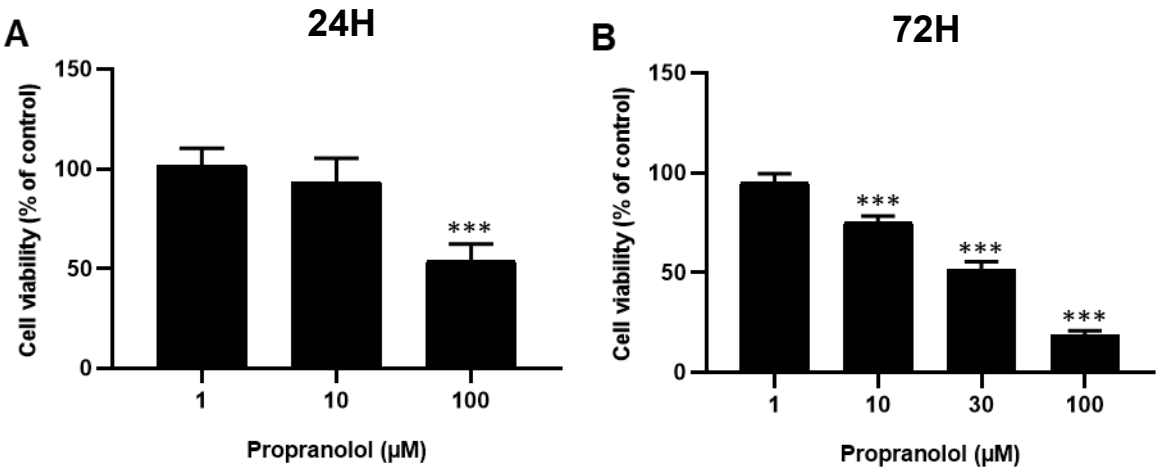



**Figure 12. Effects of the β-AR antagonist propranolol, on MCF-7 cell viability after 24h (A) or 72h (B) incubation**. Upon seeding, MCF-7 cells were incubated for 24h for adherence, serum starved (0%) for an additional period of 24h and subsequently treated with the indicated drugs (T0) for up to 72h.The MTT assay was used to measure cell viability. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of control and presented as mean ± SEM, from 3-9 independent experiments. Significant differences from control: *** p<0.001; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Dunnet´s *t* test.

A set of experiments was performed to investigate whether the effect caused by the β-AR antagonist propranolol could be attenuated by isoprenaline. These experiments were conducted using the highest concentration of propranolol and isoprenaline tested (100 µM and 10 µM, respectively). Results are shown in **Figure 13A**. Isoprenaline (10 µM), was not able to attenuate the effect of propranolol (100 µM), a competitive β-AR antagonist. These results reveal that the interaction between propranolol, a competitive β-AR antagonist, and isoprenaline, a β-AR agonist, does not follow the conventional pharmacology as the effect caused by propranolol should be attenuated by isoprenaline.

As shown in **Figure 13B**, 10 µM ICI 118551 reduced MCF-7 cell viability, as observed with propranolol. When the interaction between isoprenaline and ICI 118551 was tested, isoprenaline also did not attenuate the effect of ICI 118551, similarly to that observed with the combined treatment of isoprenaline (10 µM) and propranolol (100 µM).

The results presented in this section, demonstrate that in MCF7 cells, which have tumorigenic characteristics, the β-AR-mediated responses appears to be altered when compared with those of non-tumorigenic cells: β-agonists lose their capacity to increase cell viability whereas β-AR antagonists exhibit a capacity to reduce viability, an effect not displaced by a β-AR agonist.

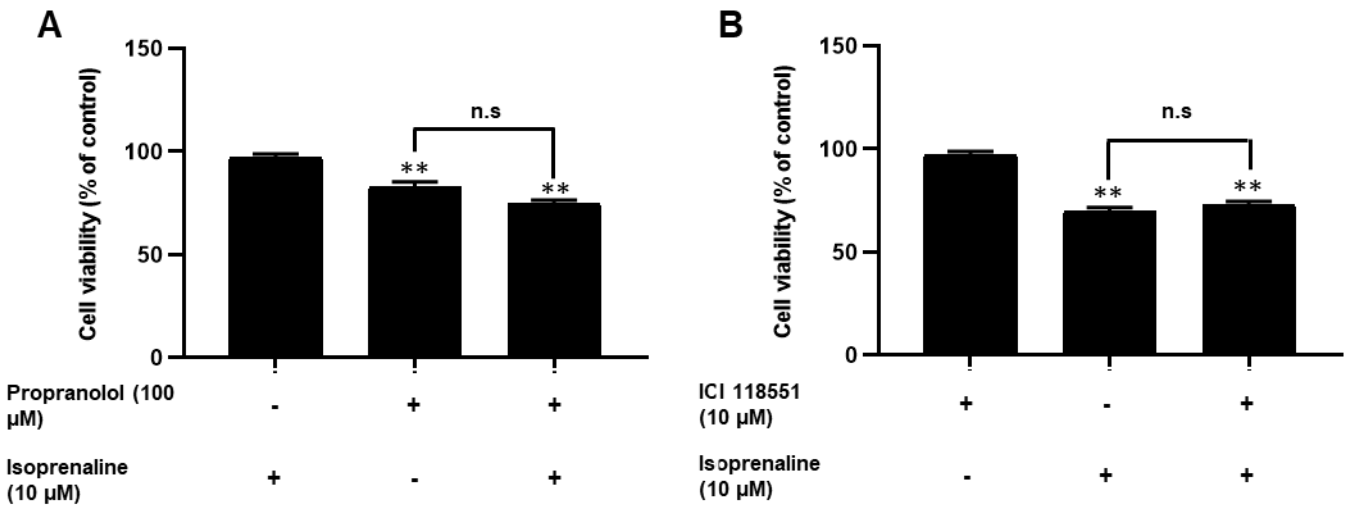



**Figure 13. Interaction between the β-AR agonist, isoprenaline, with the β-AR antagonist, propranolol (A) or with the $\beta_2$-AR inverse agonist, ICI 118551 (B) and their effects on MCF-7 cell viability after 24h of incubation**. Upon seeding, MCF-7 cells were incubated for 24h for adherence, in complete medium and subsequently treated with the indicated drugs for 24h of incubation. The MTT assay was used to measure cell viability. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of control and presented as mean ± SEM, from 8 independent experiments. Significant differences from control: ** p<0.01; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Dunnet´s *t* test. n.s – no significant differences (*Student´s t* test)

## 4.8. Influence of 17β-estradiol on the β-AR-mediated effects, in MCF-7 cell viability

The influence of 17β-estradiol in the effects of isoprenaline, in MCF-7 cell viability (MTT assay) was investigated.

After 24h of incubation, exposure to 17β-estradiol (1 - 100 nM), did not alter cell viability (**Figure 14A**). However, after a 72h exposure, 17β-estradiol was able to cause an increase in cell viability for all concentrations tested (**Figure 14B**).

Combined treatment with isoprenaline (0.1 – 10 µM) and 17β-estradiol (1 – 100 nM) in MCF-7 cell viability was also investigated (**Figure 15**). After 24h incubation, no differences in cell viability were observed for the combinations tested (**Figure 15A**). After 72h incubation, isoprenaline (0.1 and 1 µM) did not influence the increase in cell viability caused by 17β-estradiol. (**Figure 15B**). Moreover, incubation with 10 µM isoprenaline, treatment which, alone, reduced cell viability, already reduced the increase in cell viability caused by 17β-estradiol (1 – 100 nM; **Figure 15B**).

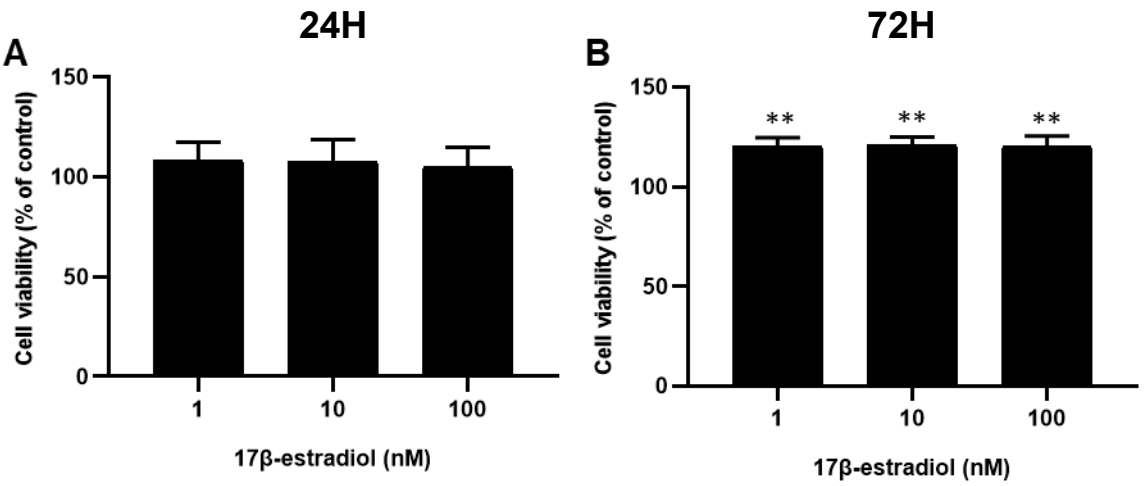



**Figure 14. Effects of the ER agonist, 17β-estradiol, on MCF-7 cell viability after 24h (A) or 72h (B) of incubation**. Upon seeding, MCF-7 cells were incubated for 24h for adherence, serum starved (0%) for an additional period of 24h and subsequently treated with the indicated drugs (T0) for up to 72h. The MTT assay was used to measure cell viability. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of control and presented as mean ± SEM, from 3-9 independent experiments. Significant differences from control: ** p<0.01, one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Dunnet´s *t* test.

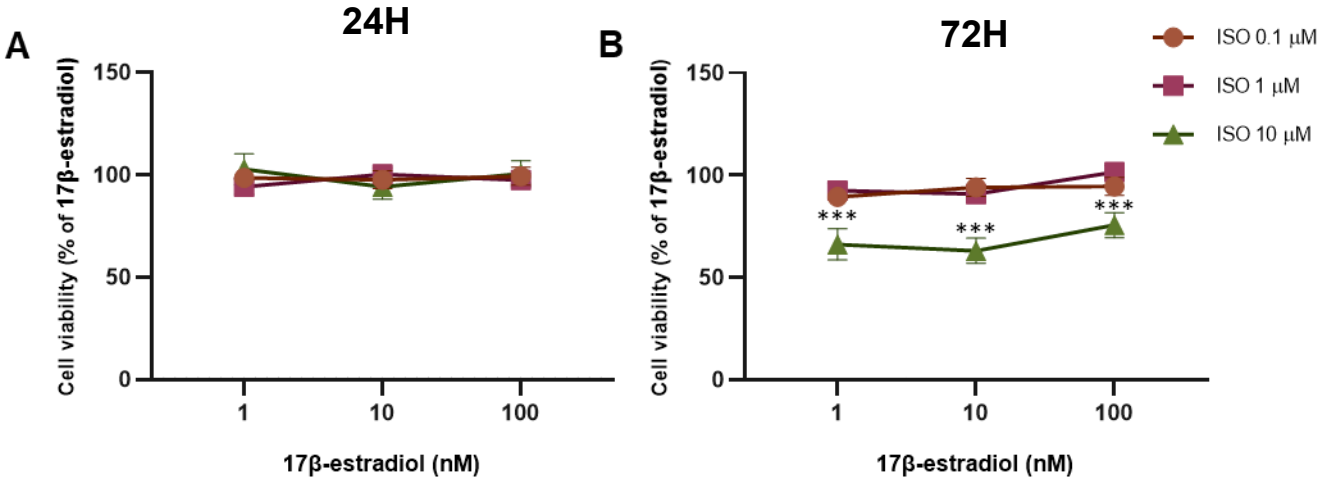



**Figure 15. Effects of the combined exposure of the β-AR agonist, isoprenaline with increasing concentrations of the ER-agonist, 17β-estradiol on MCF-7 cell viability, after 24h (A) or 72h (B) of incubation.** Upon seeding, MCF-7 cells were incubated for 24h for adherence, serum starved (0%) for an additional period of 24h and subsequently treated with the indicated drugs for up to 72h. The MTT assay was used to measure cell viability. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of the respective control (17β-estradiol) and presented as mean ± SEM, from 3-9 independent experiments. Significant differences from control (17β-estradiol alone): *** p<0.001; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Tukey *t* test.; ISO – Isoprenaline

The effect of the combination of the β-AR antagonist, propranolol, with 17β-estradiol in MCF-7 cell viability was also tested. Results are depicted in **Figure 16**. The β-AR antagonist, propranolol, reduced cell viability even in the presence of 17β-estradiol (1 – 100 nM) (**Figure 16A** for 24h of exposure and **Figure 16B** for 72h). In order to investigate if the decrease in cell viability caused by propranolol observed in the presence of 17β-estradiol is similar to that caused by propranolol alone, effects of propranolol were compared on the absence and in the presence of 17β-estradiol (1, 10 and 100 nM). As shown in **Figure 17**, 17β-estradiol failed to alter responses to 100 µM propranolol, for all concentrations tested, both after 24h (**Figure 17A**) or 72h of incubation (**Figure 17B**).

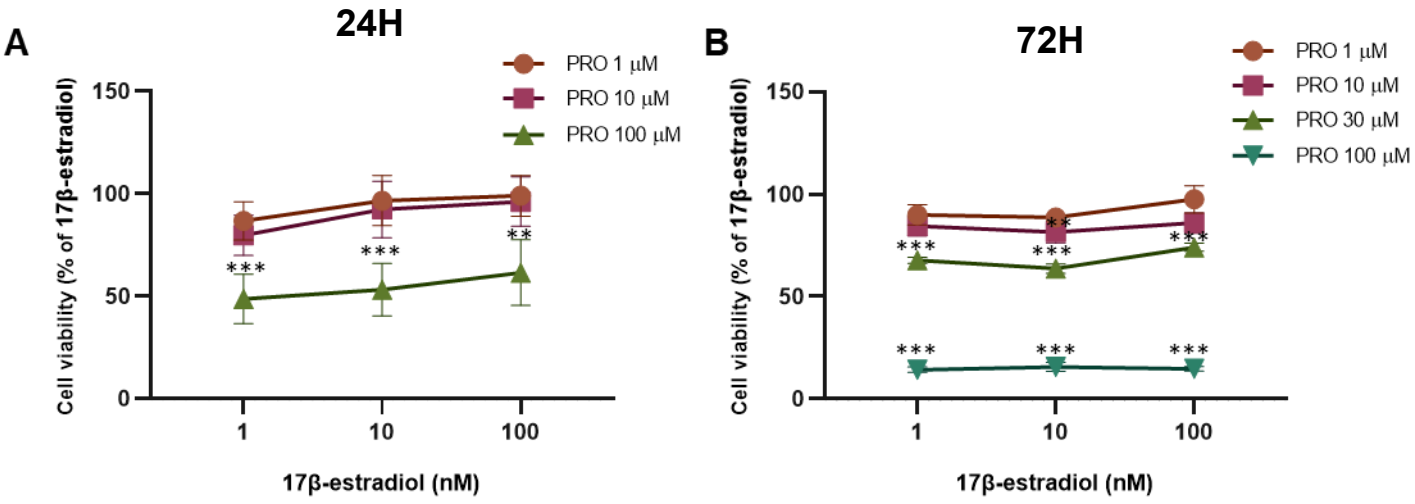



**Figure 16. Effects of the combined exposure of the β-AR antagonist, propranolol with increasing concentrations of the ER-agonist, 17β-estradiol on MCF-7 cell viability, after 24h (A) or 72h (B) of incubation.** Upon seeding, MCF-7 cells were incubated for 24h for adherence, serum starved (0%) for an additional period of 24h and subsequently treated with the indicated drugs for up to 72h. The MTT assay was used to measure cell viability. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of the respective control (17β-estradiol) and presented as mean ± SEM, from 3-9 independent experiments. Significant differences from control (17β-estradiol alone): ** p<0.01, *** p<0.001; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Tukey *t* test. PRO – Propranolol

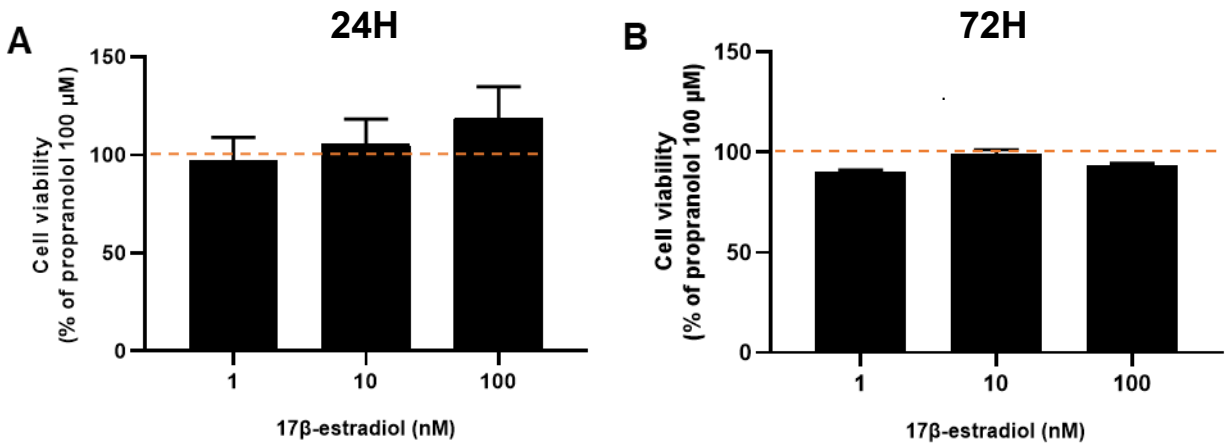



**Figure 17. Influence of 17β-estradiol in the effects caused by the β-AR, antagonist, propranolol (100 µM), on MCF-7 cell viability, after 24h (A) or 72h (B) of incubation.** Upon seeding, MCF-7 cells were incubated for 24h for adherence, serum starved (0%) for an additional period of 24h and subsequently treated with the indicated drugs for up to 72h. The MTT assay was used to measure cell viability. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of the respective control (100 µM propranolol) and presented as mean ± SEM, from 3-9 independent experiments.

## 4.9. Effects of the β-AR activation in MCF-7 cell proliferation

As the MTT assay is dependent of the rate of glycolytic NAD(P)H production in the cell, it may not entirely reflect the cell number/cell proliferation (Quent et al., 2010; Moreira et al., 2016). To further investigate the effects caused by the β-adrenergic ligands (isoprenaline and propranolol) in the MCF-7 cell line, the High Contrast Brightfield Accessory (HCB) of the real time live cell automated microscope, Lionheart FX, was used, as a method to directly monitor cell proliferation, as it counts the number of cells.

As shown in **Figure 18A**, the β-AR agonist isoprenaline (0.1 – 10 µM) did not change cell proliferation after a 24h incubation period similarly to that observed in the cell viability assay (**Figure 11A**). After a 72h incubation period, isoprenaline caused an increase in MCF-7 cell proliferation (**Figure 18B**). At the highest concentration tested (10 µM), isoprenaline failed to alter cell proliferation comparatively to the respective control (**Figure 18B**). These results are not in line with those obtained with the cell viability assay where 10 µM isoprenaline caused a significant decrease in cell viability (**Figure 11B**).

As shown in **Figure 19** the β-AR antagonist propranolol (1 – 100 µM) decreased cell proliferation in a concentration- and time-dependent manner (**Figure 19A** and **Figure 19B**, for 24h and 72h, respectively). The decrease in cell proliferation caused by

propranolol was already observed after a 24h incubation period, and increased with the incubation time: after 72h incubation, 30 µM propranolol already caused a decrease in cell proliferation whereas the effect caused by 100 µM propranolol was more marked than that observed after 24h incubation time (**Figure 19B** and **Figure 19A,** respectively)

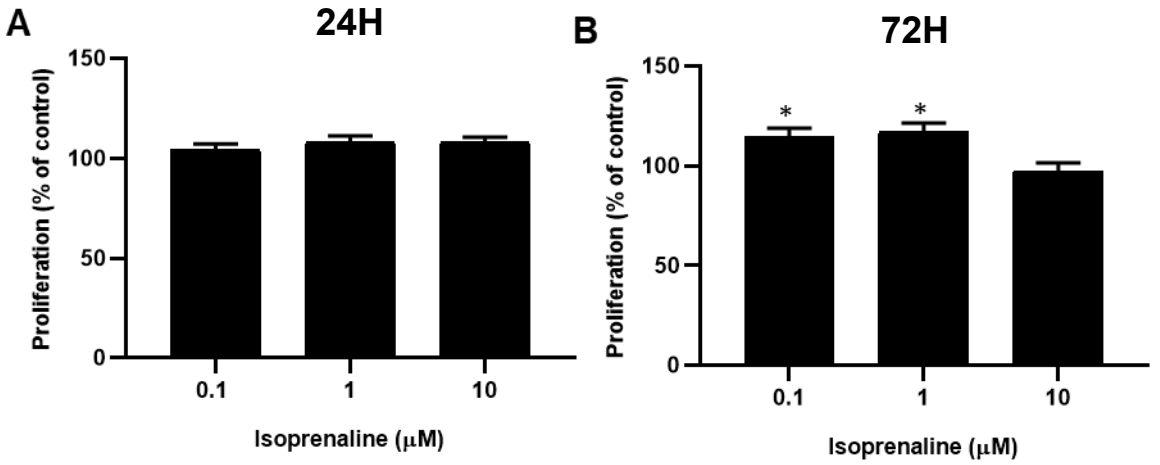



**Figure 18**. **Effects of the β-AR agonist, isoprenaline on MCF-7 cell proliferation, after 24h (A) or 72h (B) incubation.** Upon seeding, MCF-7 cells were incubated for 24h for attachment, serum starved (0%) for an additional period of 24h and subsequently treated with the indicated drugs for up to 72h. To measure cell proliferation, the number of cells was counted using high contrast brightfield images, captured using the Lionheart FX microscope, for each time point. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of control and presented as mean ± SEM, from 9 independent experiments. Significant differences from control: * p<0.05; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Dunnet´s *t* test.

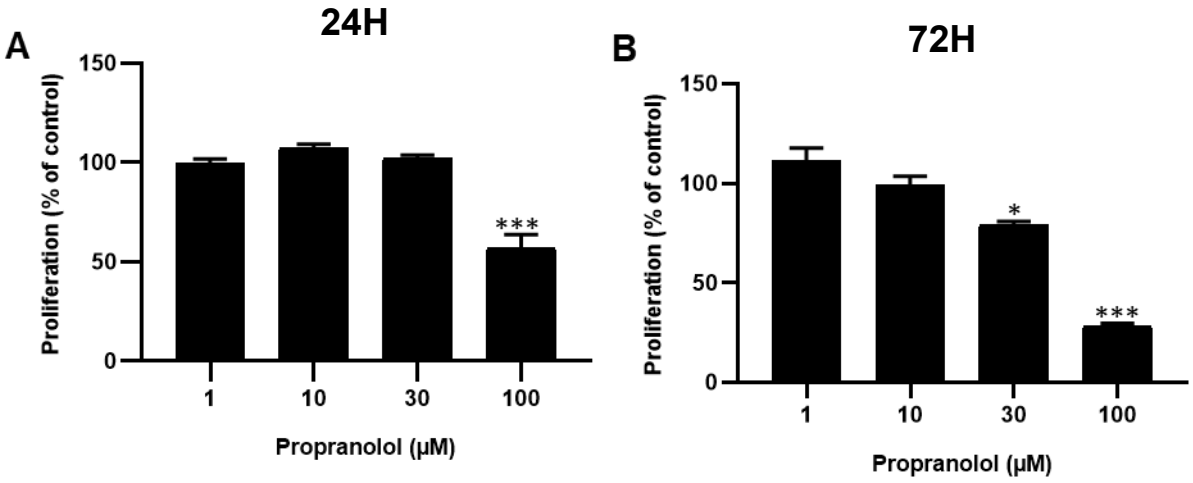



**Figure 19. Effects of the β-AR antagonist, propranolol, in MCF-7 cell proliferation, after 24h (A) or 72h (B) of incubation.** Upon seeding, MCF-7 cells were incubated for 24h for adherence, serum starved (0%) for an additional period of 24h and subsequently treated with the indicated drugs (T0). Then, cells were, incubated for up to 72h. To

measure cell proliferation, the number of cells was counted using high contrast brightfield images, captured using the Lionheart FX microscope, for each time point. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of control and presented as mean ± SEM, from 3 independent experiments. Significant differences from control: * p<0.05; *** p<0.001; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Dunnet´s *t* test.

## 4.10. Influence of 17β-estradiol on the β-AR-mediated effects, in MCF-7 cell proliferation

In order to investigate whether the influence of ER activation on the modulation of cell proliferation mediated by β-AR is identical to that caused in cell viability, the effects of isoprenaline or propranolol on cell proliferation were investigated both in the absence and in the presence of 17β-estradiol.

Treatment of MCF-7 cells with 17β-estradiol (1 – 100 nM) caused a marked increase in cell proliferation, after 72h incubation. The increase in cell proliferation induced by 17β-estradiol was observed with all concentrations of 17β-estradiol used in the present study (**Figure 20**).

The influence of 17β-estradiol on the effects of isoprenaline in cell proliferation is shown in **Figure 21.** Treatment with 17β-estradiol and isoprenaline (at 24h incubation) did not cause effects different from the effects caused by each drug alone (**Figure 21A**). However, at 72h incubation, 10 µM isoprenaline (a concentration shown to decrease cell viability, **Figure 11B**), in the presence of 17β-estradiol, reduced significantly cell proliferation (**Figure 21B**).

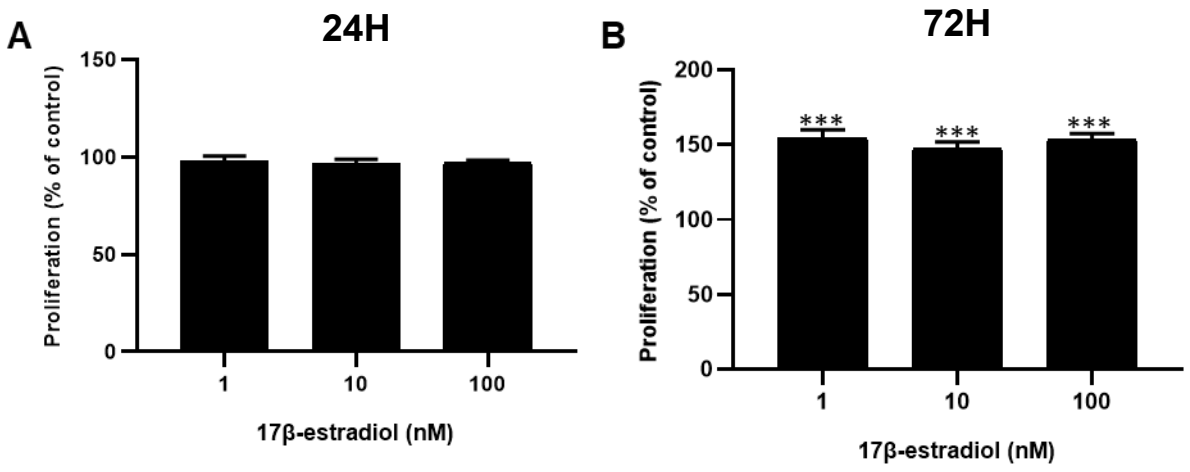



**Figure 20. Effects of the ER agonist, 17β-estradiol, on MCF-7 cell proliferation after 24h (A) or 72h (B) incubation**. Upon seeding, MCF-7 cells were incubated for 24h for adherence, serum starved (0%) for an additional period of 24h and

subsequently treated with the indicated drugs for up to 72h. To measure cell proliferation, the number of cells was counted using high contrast brightfield images, captured using the Lionheart FX microscope, for each time point. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of control and presented as mean ± SEM, from 9 independent experiments. Significant differences from control: *** p<0.001; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Dunnet´s *t* test.

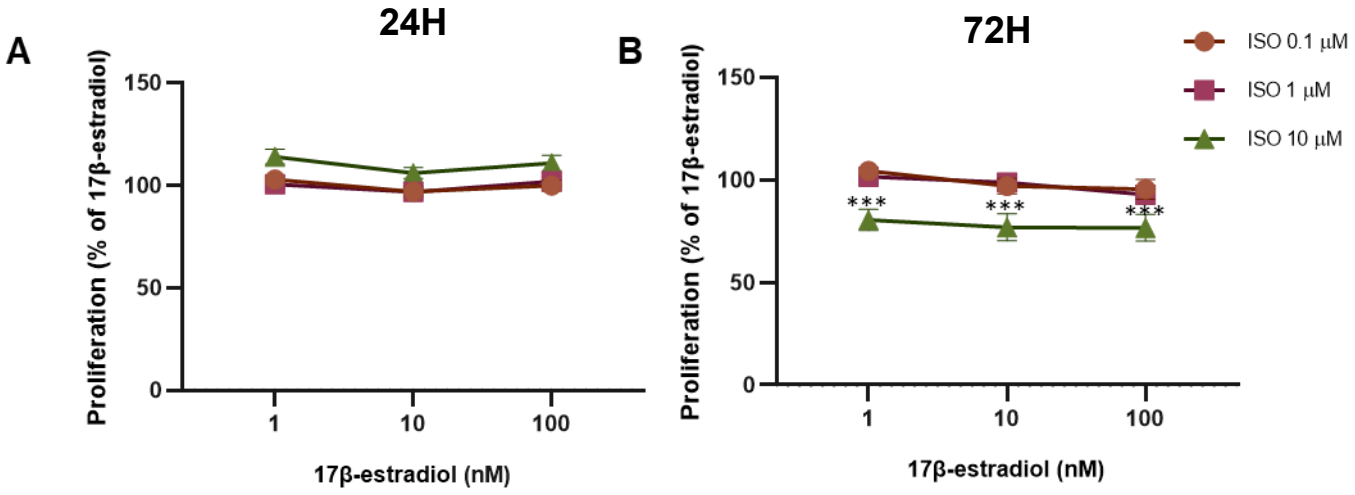



**Figure 21. Effects of the combine exposure of the β-AR agonist, isoprenaline with increasing concentrations of the ER-agonist, 17β-estradiol in MCF-7 cell proliferation, after 24h (A) or 72h (B) of incubation.** Upon seeding, MCF-7 cells were incubated for 24h for adherence, serum starved (0%) for an additional period of 24h and subsequently treated with the indicated drugs for up to 72h. To measure cell proliferation, the number of cells was counted using high contrast brightfield images, captured using the Lionheart FX microscope, for each time point. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of the respective control (17β-estradiol) and presented as mean ± SEM, from 9 independent experiments. Significant differences from control (17β-estradiol alone): *** p<0.001; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Tukey *t* test. ISO – Isoprenaline

The influence of propranolol on cell proliferation was also tested in the absence and in the presence of 17β-estradiol. The β-AR antagonist, propranolol, reduced cell proliferation even in the presence of 17β-estradiol (1 – 100 nM); (**Figure 22A** for 24h incubation and **Figure 22B** for 72h incubation). However, when cells were exposed to propranolol in the presence of 17β-estradiol (1 – 100 nM), the decrease of cell proliferation induced by propranolol was less marked than that observed in the absence of 17β-estradiol (**Figure 23A**). After 72h incubation, the decrease of cell proliferation caused by 100 μM propranolol was not influenced by 17β-estradiol (**Figure 23B**). However, when tested in a lower concentration (30 μM), 17β-estradiol already reduced the decrease in cell proliferation caused by 30 μM propranolol (**Figure 23C**).

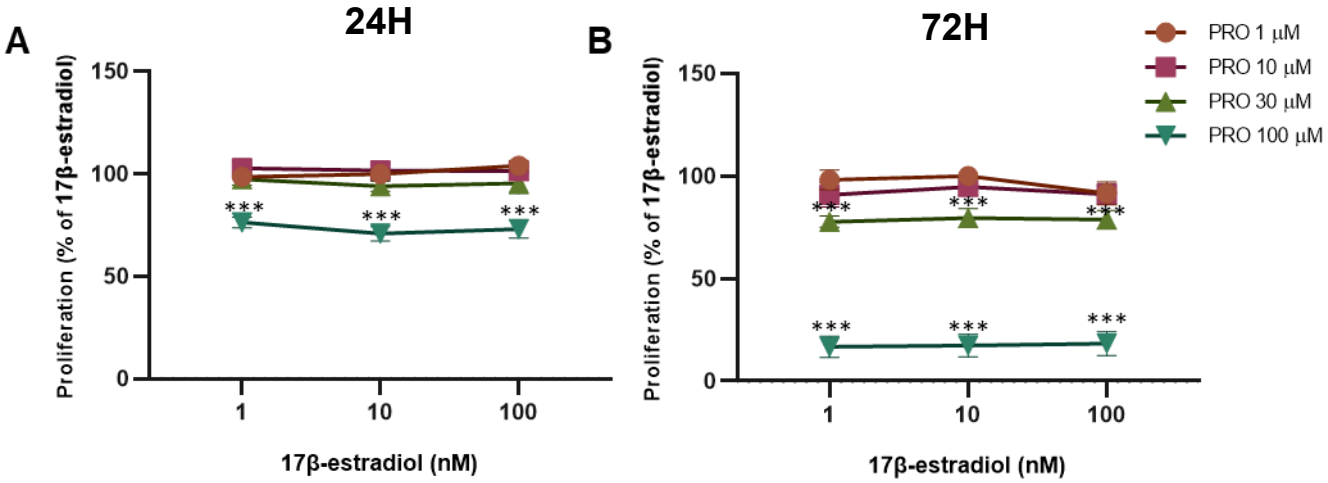



**Figure 22. Effects of the combine exposure of the β-AR antagonist, propranolol with increasing concentrations of 17β-estradiol on MCF-7 cell proliferation, after 24h (A) or 72h (B) of incubation.** Upon seeding, MCF-7 cells were incubated for 24h for adherence, serum starved (0%) for an additional period of 24h and subsequently treated with the indicated drugs for up to 72h. To measure cell proliferation, the number of cells was counted using high contrast brightfield images, captured using the Lionheart FX microscope, for each time point. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of the respective control (17β-estradiol) and presented as mean ± SEM, from 3 independent experiments. Significant differences from control (17β-estradiol): *** p<0.001; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Tukey *t* test. PRO – Propranolol

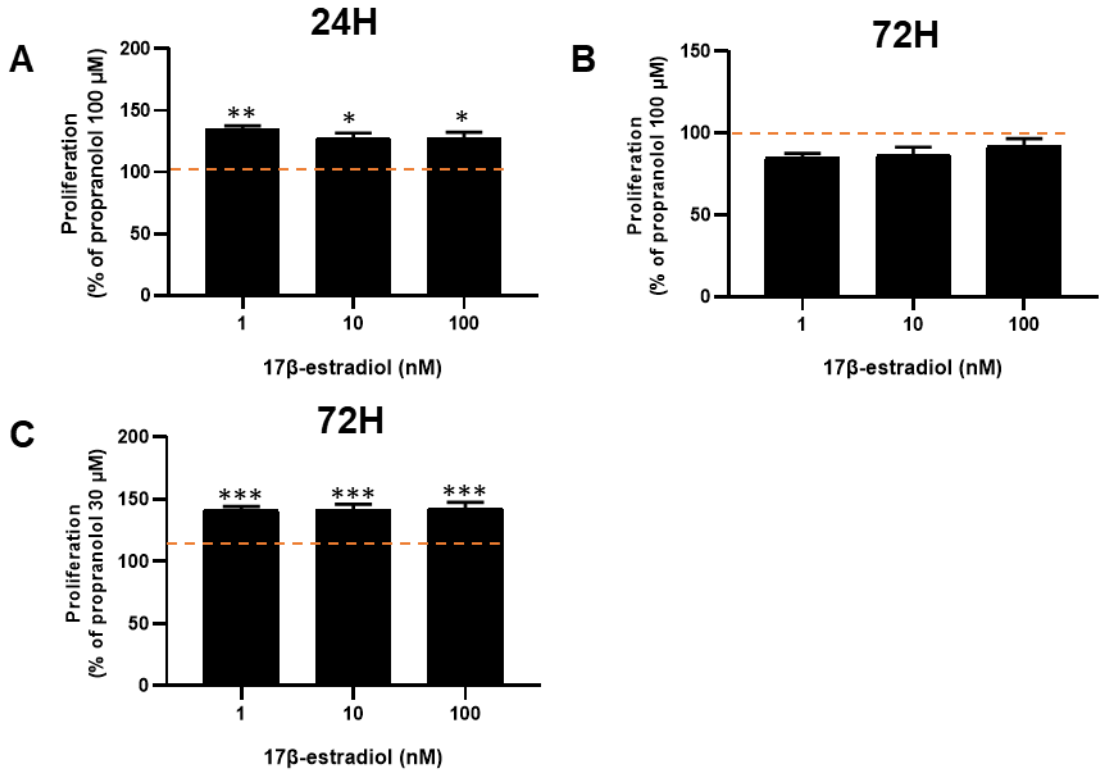



**Figure 23. Effects of the combined exposure of the ER-agonist, 17β-estradiol, and the β-AR antagonist, propranolol (30 μM – C; 100 μM – A; B), on MCF-7 cell proliferation, after 24h (A) or 72h (B and C) incubation.** Upon seeding, MCF-7 cells were incubated for 24h for adherence, serum starved (0%) for an additional period of 24h and

subsequently treated with the indicated drugs for up to 72h. To measure cell proliferation, the number of cells was counted using high contrast brightfield images, captured using the Lionheart FX microscope, for each time point. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of the respective control (100 µM propranolol in panels A and B and 30 µM propranolol in panel C) and presented as mean ± SEM, from 3 independent experiments. Significant differences from the respective control: * p<0.05; ** p<0.01, *** p<0.001; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Tukey *t* test.

## 4.11. Influence of membrane cholesterol in the β-AR effects in MCF-7 breast cancer cells

Stress as well as menopause and aging are risk factor for hypercholesterolemia (Bryant et al., 1988; Muldoon et al., 1992; Brennan et al., 1996; Davidson et al., 2002; Assadi, 2017). In order to study the role of membrane cholesterol in the modulation of the β-AR signalling, in MCF-7 breast cancer cells, the influence of methyl-β-cyclodextrin (M-βCD), a specific scavenger that extracts cholesterol from the plasma membrane, in the effects caused by the β-AR agonist, isoprenaline was investigated.

M-βCD was used at concentrations ranging from 1 to 1000 µM, the concentration-range used by others (Barman and Nayak, 2007; Guerra et al., 2016). **Figure 24** shows the influence of M-βCD (1 – 1000 µM) in the viability of MCF-7 cells after 72h incubation (the MTT assay for 24h incubation period was not performed). M-βCD, per se, resulted inhibited cell viability in all concentrations tested.

When isoprenaline (0.1 – 10 µM) was tested in the presence of M-βCD (1000 µM), a further reduction in MCF-7 cell viability was observed, compared to that caused by 1000 µM M-βCD alone (**Figure 25**).

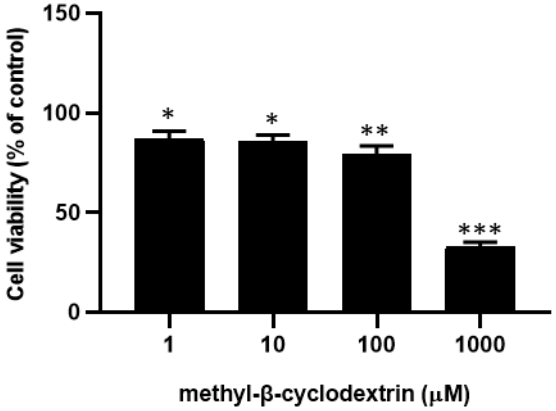


**Figure 24. Effects of methyl-β-cyclodextrin, in MCF-7 cell viability, after 72h of incubation.** Upon seeding, MCF-7 cells were incubated for 24h for adherence, serum starved (0%) for an additional period of 24h and subsequently treated with the indicated drugs for 72h. The MTT assay was used to measure cell viability. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of control and presented as mean ± SEM, from 8 independent experiments. Significant differences from control: * p<0.05; ** p<0.01, *** p<0.001; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Dunnet´s *t* test.

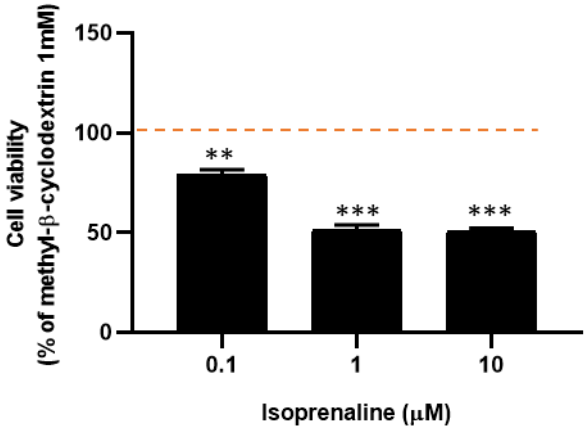



**Figure 25. Influence of methyl-β-cyclodextrin (M-βCD; 1 mM), on the effect caused by the β-AR agonist isoprenaline on MCF-7 cell viability, after 72h of incubation.** Upon seeding, MCF-7 cells were incubated for 24h for adherence, serum starved (0%) for an additional period of 24h and subsequently treated with the indicated drugs for 72h. The MTT assay was used to measure cell viability. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of methyl-β-cyclodextrin (1 mM) and presented as mean ± SEM, from 5-8 independent experiments. Significant differences from control (methyl-β-cyclodextrin; 1 mM): ** p<0.01, *** p<0.001; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Tukey *t* test.

In the presence of 1 mM M-βCD, 17β-estradiol caused an increase of cell viability after 72h incubation period, for all the concentrations tested (**Figure 26**).

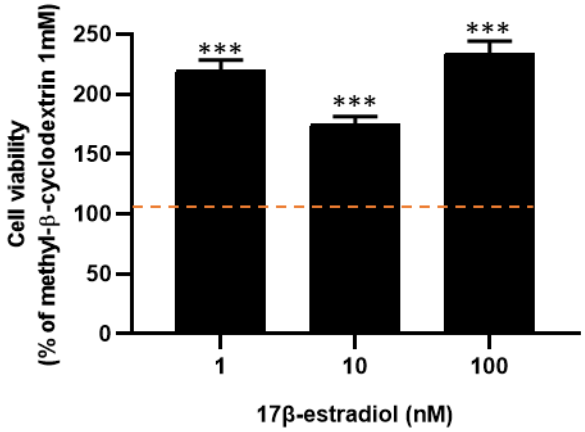



**Figure 26. Influence of methyl-β-cyclodextrin (1 mM), on the effect caused by the ER-agonist, 17β-estradiol, on MCF-7 cell viability, after 72h of incubation.** Upon seeding, MCF-7 cells were incubated for 24h for adherence, serum starved (0%) for an additional period of 24h and subsequently treated with the indicated drugs (T0). Then, cells were, incubated for up to 72h. The MTT assay was used to measure cell viability. A detailed description of the method can be

found in the Materials and Methods section. Results are expressed as percentage of methyl-β-cyclodextrin (1 mM) and presented as mean ± SEM, from 6-8 independent experiments. Significant differences from control (methyl-β-cyclodextrin; 1 mM): *** p<0.001; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Tukey *t* test.

Cell proliferation assays were also performed to further confirmed the results obtained in cell viability experiments.

After a 24h incubation period, M-βCD (1 – 1000 µM), *per se*, inhibited proliferation of MCF-7 cells, at the maximal concentration tested (1000 µM) (**Figure 27A**). After a 72h incubation period, the same pattern of effects was observed (**Figure 27B**), although the inhibition caused by 1000 µM M-βCD was more marked than that observed at 24h incubation (**Figure 27**).

In the presence of 1000 µM M-βCD, isoprenaline caused a further decrease in cell proliferation at both incubation periods (24h or 72h) (**Figure 28A** and **28B,** respectively).

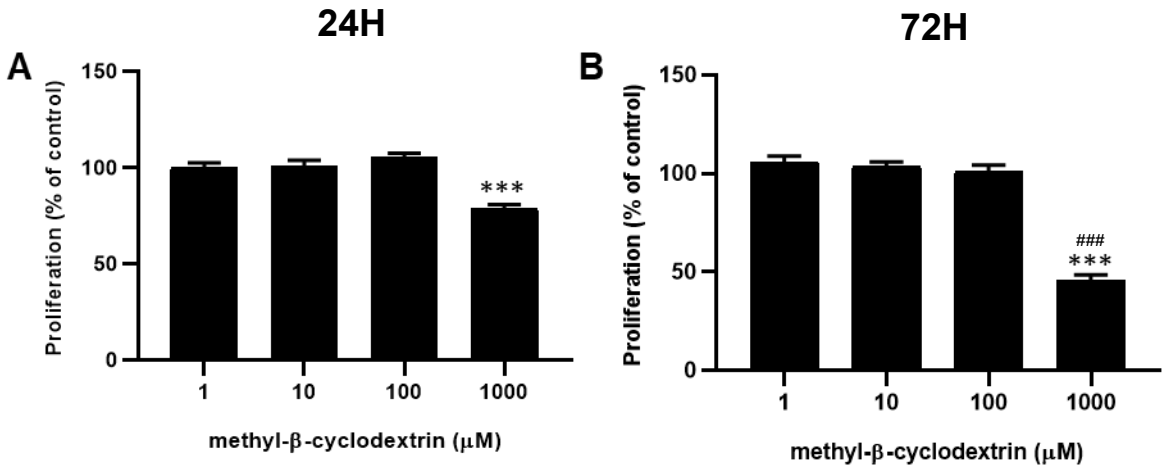



**Figure 27. Effects of methyl-β-cyclodextrin, on MCF-7 cell proliferation after 24 (A) or 72h (B) incubation.** Upon seeding, MCF-7 cells were incubated for 24h for adherence, serum starved (0%) for an additional period of 24h and subsequently treated with the indicated drugs for up to 72h. To measure cell proliferation, the number of cells was counted using high contrast brightfield images, captured using the Lionheart FX microscope, for each time point. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of control and presented as mean ± SEM, from 11 independent experiments. Significant differences from control: *** p<0.001; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Dunnet´s *t* test; Significant differences from the effect caused by 1mM methyl-β-cyclodextrin at 24h: ### p<0.001; Student´s *t* test.

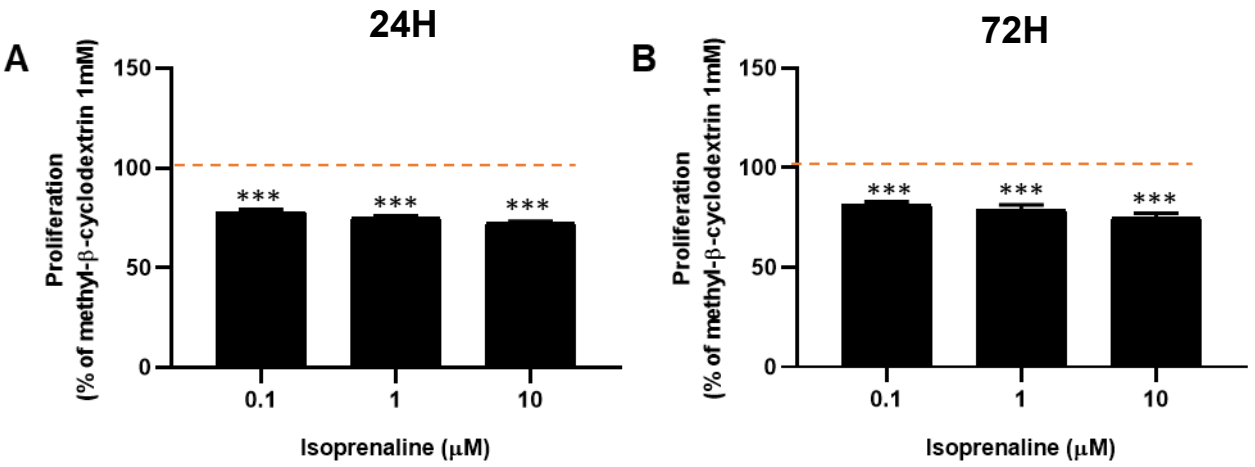



**Figure 28. Influence of methyl-β-cyclodextrin (1 mM), on the effect caused by the β-AR agonist, isoprenaline, on MCF-7 cell proliferation after 24 (A) or 72h (B) of incubation.** Upon seeding, MCF-7 cells were incubated for 24h for adherence, serum starved (0%) for an additional period of 24h and subsequently treated with the indicated drugs for up to 72h. To measure cell proliferation, the number of cells was counted using high contrast brightfield images, captured using the Lionheart FX microscope, for each time point. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of methyl-β-cyclodextrin (1 mM) and presented as mean ± SEM, from 6-8 independent experiments. Significant differences from control (methyl-β-cyclodextrin; 1 mM): *** p<0.001; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Tukey *t* test.

In the presence of 1000 μM M-βCD, 17β-estradiol caused an increase in cell proliferation but this effect was only seen after 72h of incubation (**Figure 29A** and **29B,** for 24 and 72h respectively).

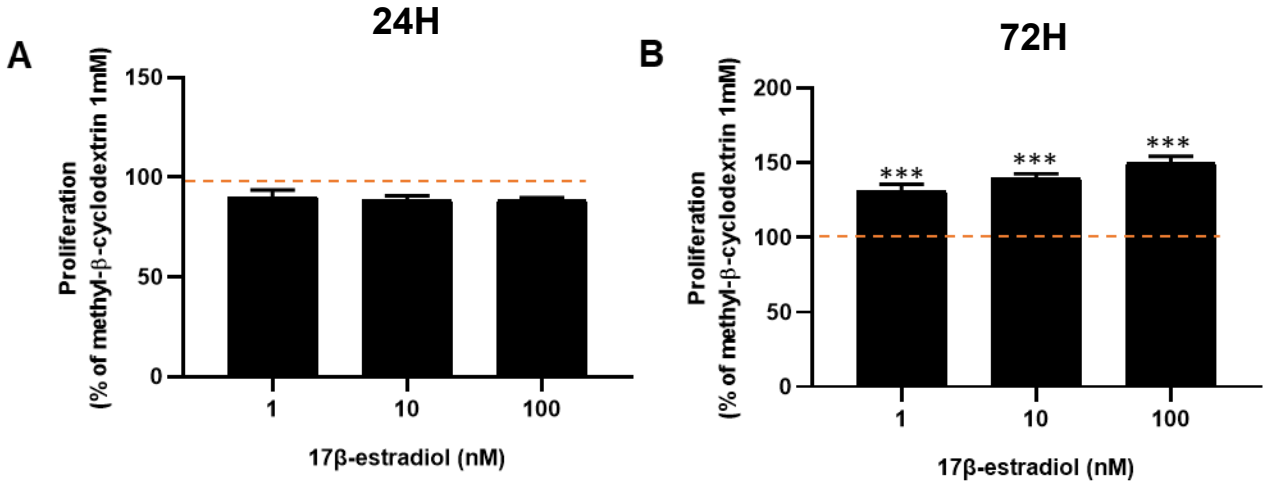



**Figure 29. Influence of methyl-β-cyclodextrin (1 mM), on the effect caused by the ER-agonist, 17β-estradiol in MCF-7 cell proliferation, after 24 (A) or 72h (B) of incubation.** Upon seeding, MCF-7 cells were incubated for 24h for adherence, serum starved (0%) for an additional period of 24h and subsequently treated with the indicated drugs for up to 72h. To measure cell proliferation, the number of cells was counted using high contrast brightfield images, captured using the Lionheart FX microscope, for each time point. A detailed description of the method can be found in the Materials

and Methods section. Results are expressed as percentage of methyl-β-cyclodextrin (1 mM) and presented as mean ± SEM, from 6-8 independent experiments. Significant differences from control (methyl-β-cyclodextrin; 1 mM): *** p<0.001; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Tukey *t* test.

## 4.12. Influence of propranolol in MCF-7 cells morphology

As 100 µM propranolol was able to decrease MCF-7 cell viability/proliferation and unable to affect MCF-10A cells (**Figures 19**; **12** and **6A**, respectively), efforts were also made in order to clarify the potential mechanisms behind the selective effects of propranolol in MCF-7 breast cancer cells.

Since it was observed by phase contrast images, a severe accumulation of vesicles in the cytoplasm of MCF-7 cells upon treatment of MCF-7 cells with propranolol (100 µM) (see **Figure 33, Annex**), the possibility that propranolol is influencing vacuole formation in MCF-7 cells was explored.

### 4.12.1. Influence of propranolol in MCF-7 vacuole formation

To investigate if propranolol influences vacuole formation, MCF-7 cells were firstly treated with MDC (monodansylcadaverine), a fluorescent marker that labels autophagic vacuoles (autophagosomes/autolysosomes).

**Figure 30A** shows fluorescence microscopy representative images obtained in cells treated with the MDC marker, in the absence (control) or in the presence of propranolol (100 µM). In control conditions, a clear staining for autophagic vacuoles was observed and when cells were treated with 100 µM propranolol, a massive increase in the number and size of autophagic vacuoles was observed in MCF-7 cells.

Acridine orange, a marker that stains acidic vesicular organelles (AVOs) such as late endosomes, lysosomes or autolysosomes (Murugan and Amaravadi; Thomé et al., 2016), was also used to further analyse the influence of propranolol in vacuole formation. Examination of acridine orange stained images from fluorescence microscopy of cells treated with propranolol (100 µM) revealed a marked increase in the number and size of AVOs (**Figure 30A**).

Quantitative analysis of the results obtained after acridine orange staining demonstrated that propranolol (10 – 100 µM) significantly increased the AVOs area occupied in each cell, in a concentration-dependent manner (**Figure 30B**). Moreover, the fluorescence

intensity of AVOs was also significantly higher suggesting that the AVOs were also more acidic, after treatment with propranolol (**Figure 30C**).

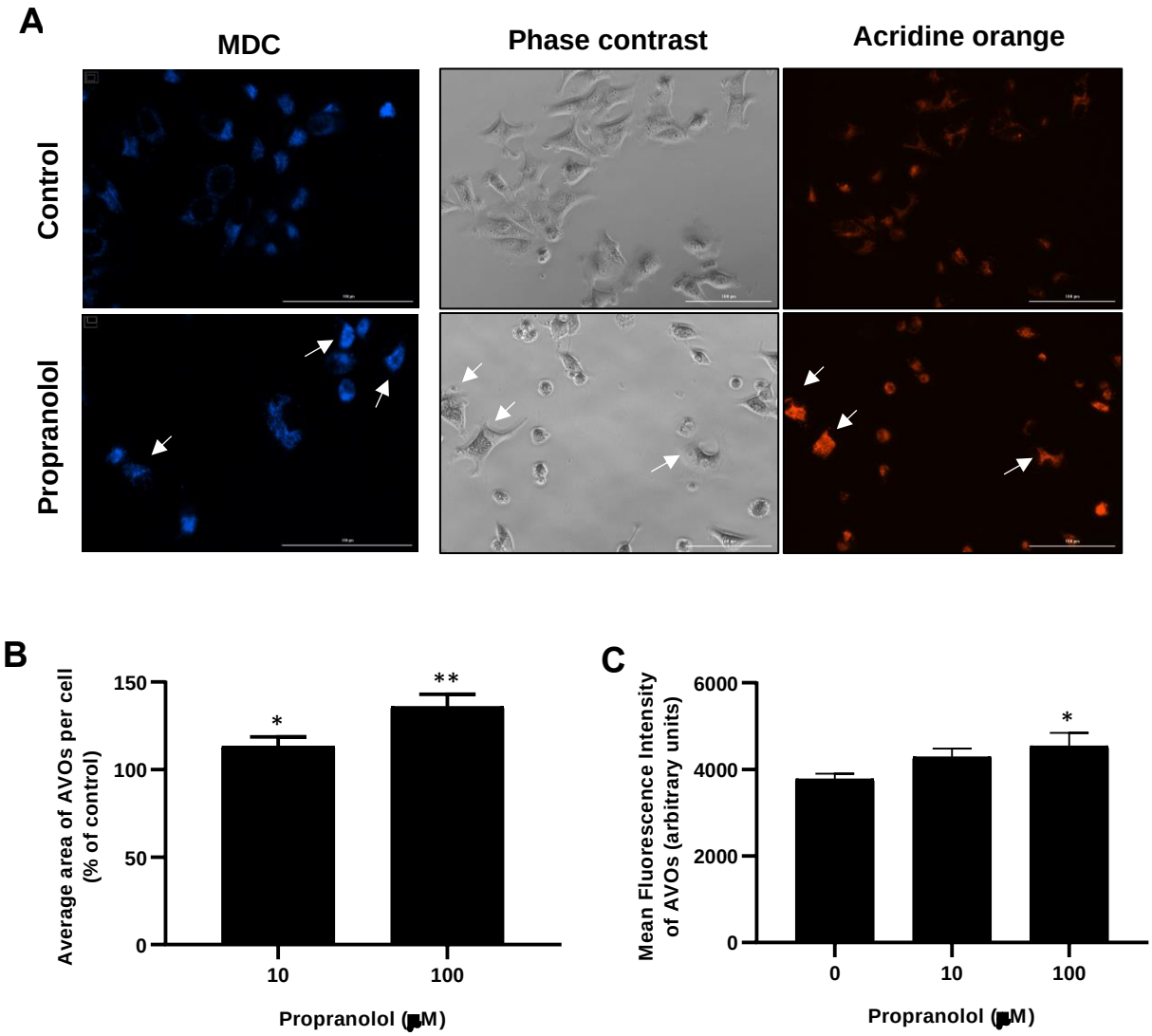



**Figure 30. Effect of propranolol (100 μM) in MCF-7 vacuole formation** A) Representative photographs of autophagic vesicles (blue fluorescence; MDC) and acidic vesicles (red/orange fluorescence; acridine orange) in non-treated and treated MCF-7 cells with propranolol 100 μM B) Average area of AVOs per cell in non-treated and treated MCF-7 cells with propranolol 100 μM (n=3) C) Mean fluorescence intensity of AVOs in non-treated and treated MCF-7 cells with propranolol 100 μM (n=3) For all the assays, MCF-7 cells were incubated for 24h, serum starved (0%) for an additional period of 24h and subsequently treated with the indicated drugs (T0). The results were obtained after 24h treatment. A detailed description of the methods used can be found in the Materials and Methods Significant differences from the respective control: * $p<0.05$; ** $p<0.01$; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Dunnet´s *t* test or Student's *t* test. Arrows indicate the presence of vacuoles (MDC) or AVOs (Acridine orange), with increased size.

### 4.12.2. Influence of autophagy inhibition on the effects caused by propranolol

The use of 3´-methyladenine (3´MA), an autophagy inhibitor, was used to investigate whether autophagy is involved in propranolol-induced decrease of viability and proliferation of MCF-7 cells (**Figure 12** and **19**, respectively).

After 24h incubation, 3´MA (5 – 10 mM) decreased cell proliferation in a concentration dependent manner. As shown in **Figure 31**, 100 µM propranolol also reduced cell proliferation which was even more pronounced in the presence of 3´MA. This result shows that the decrease of cell proliferation caused by propranolol still occurs when autophagy is inhibited. It points out that propranolol induces cell death by a mechanism that may not be entirely dependent on autophagy.

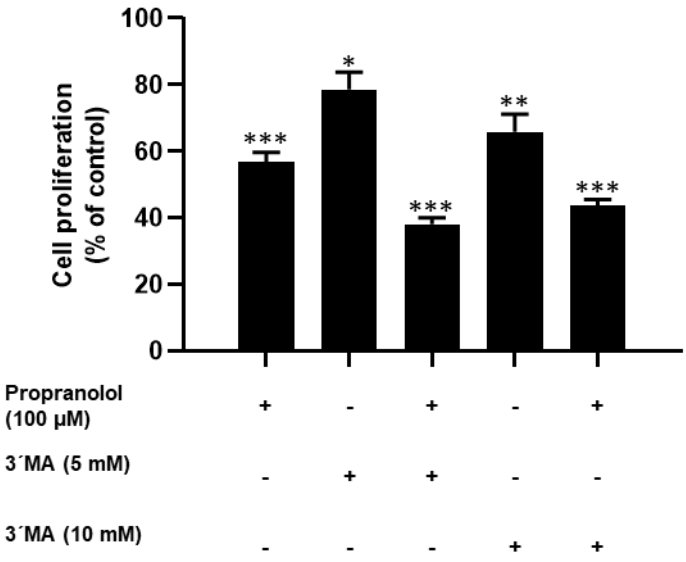



**Figure 31. Influence of 3´MA on the effect caused by propranolol (100 µM)** Upon seeding, MCF-7 cells were incubated for 24h, and subsequently treated with the indicated drugs for 24h. To measure cell proliferation, the number of cells was counted using high contrast brightfield images, captured using the Lionheart FX microscope, for each time point. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of the control and presented as mean ± SEM, from 3 independent experiments. Significant differences from respective control: * p<0.05; ** p<0.01, *** p<0.001; one-way analysis of variance (ANOVA), followed by *post-hoc* multi-comparisons Dunnet´s *t* test.

# V. Chapter: Discussion

# 5. Discussion

Stress seems to favor the increase of cancer incidence and mortality (Chida et al., 2008; Song et al., 2017). Under stress conditions, activation of the sympathetic nervous system and a long-lasting elevation of plasmatic levels of catecholamines (namely adrenaline and noradrenaline) take place (Paran et al., 1992). It is likely that these catecholamines may contribute to the increase in cancer associated with stress (Coelho et al., 2017). This putative tumorigenic effect of catecholamines is likely mediated by β-ARs, since expression of $\beta_1$- and $\beta_2$-AR subtypes has been confirmed in a very wide range of distinct cancers (Coelho et al., 2017). Moreover, epidemiological studies have shown that the clinical use of β-blockers reduce rates of progression in multiple types of cancers, due to targeting β-adrenergic signalling pathways (Schuller, 2010; Cole and Sood, 2012).

Several studies support the view that stress, through its related messengers, namely catecholamines, contributes to progression of breast cancer (Kim-Fuchs et al., 2014; Coelho et al., 2017; Cui et al., 2019). Catecholamines have been shown to drive the growth of breast cancer cells (Cakir et al., 2002), to stimulate cell migration and invasion (Cui et al., 2019), to induce an anti-apoptotic effect protecting them from chemotherapeutic agents (Sastry et al., 2007; Reeder et al., 2015), to control the angiogenesis process (Madden et al., 2011) and to promote stem-like properties in breast cancer cells (Cui et al., 2019).

The present study tests the hypothesis that catecholamines released during stress in physiological or pathophysiological conditions (particularly adrenaline which is more potent for $\beta_2$-AR than noradrenaline) may participate in the tumorigenic process in breast epithelial cells.

Isoprenaline was used as a β-AR agonist to mimic the increase in plasmatic levels of adrenaline, which is known to occur under *in vivo* conditions of stress (Yaribeygi et al., 2017). In the present study, a detailed characterization of the β-ARs subtypes involved in the effects mediated by isoprenaline was not performed. Other pharmacological ligands known to be more selective for $\beta_2$-AR (propranolol and ICI 118551) were used and provide some evidences of a role of the $\beta_2$-AR in this process.

Experiments were carried out in non-tumorigenic and tumorigenic epithelial breast cells. One of the main observations of the present study is that the β-AR mediated effects may depend on the level of "tumorigenic *status"* of the cell. This possibility is supported by the observation that the β-AR antagonist, propranolol markedly inhibits MCF-7 cell viability but not of MCF-10A cells. A different pattern of response also occurs with the β-

AR agonist, isoprenaline: in the non-tumorigenic MCF-10A cells isoprenaline increased cell viability but failed to so in the tumorigenic MFC-7 cells. Even when tested at longer incubation periods (72h), isoprenaline still did not increase MCF-7 cell viability, causing actually the opposite effect: a decrease in cell viability.

Other studies using MCF-10A cells and similar isoprenaline concentrations reported distinct results: decreased cell proliferation and increased cell adhesion (Bruzzone et al., 2014; Gargiulo et al., 2014), suggesting that isoprenaline may act by preventing the tumorigenic process. It is conceivable that these different results can be ascribed to the different experimental conditions used, since the culture conditions were shown to have a great impact in the response of MCF-10A cells (Yusuf and Frenkel, 2010). In our study, cells were routinely grown in medium with an higher concentration of hydrocortisone. There is a possibility to link the conflicting results to different concentrations of hydrocortisone in the culture medium: hydrocortisone has been reported to influence the tumorigenic potential of other stimuli (Yusuf and Frenkel, 2010). Having in mind that hydrocortisone is also a mediator of stress (Yaribeygi et al., 2017), it would be interesting to explore this possibility in future studies.

In breast tumorigenic cells, namely MCF-7 cells, controversial results have also been found with respect to the agonist action in several processes related to breast tumor progression. Numerous studies have described an inhibition of cell proliferation and cell migration by β-AR agonists (Perez Pinero et al., 2012; Gargiulo et al., 2014) while other studies described opposite results (Shi et al., 2011; Cui et al., 2019).

The increase in cell viability caused by isoprenaline in MCF-10A cells, observed in the present experimental conditions, may be a component of a cellular program that promotes the acquisition of a tumorigenic phenotype. Isoprenaline, in the same concentrations that increased viability of MCF-10A cells, also stimulated colony formation, an assay that measures the ability of cells to undergo sufficient cell proliferation to form colonies (Menyhart et al., 2016). This observation further support the hypothesis that catecholamines may promote the acquisition of a tumorigenic phenotype.

Previous studies carried out by our group, showed that MCF-7 cells acquire the capacity to produce, autocrinally, catecholamines (noradrenaline and adrenaline) (manuscript under preparation). Such autocrine catecholamines production was supported by the i) detection of adrenaline in the incubation medium, under conditions where contamination from external sources was minimized through reduction in the amount of FBS added to the incubation medium; ii) expression of key enzymes involved in the synthetic pathway

of catecholamines (by western blot, immunocytochemical detection and RT-PCR) iii) formation of methylnoradrenaline after incubation with a false substrate (methylDOPA), a reaction that requires the same machinery needed for the conversion of DOPA into noradrenaline and iv) HPLC-ECD detection of noradrenaline and adrenaline (higher levels).

An increase in the capacity of cancer cells to synthetize catecholamines was previously shown in MCF-7 cells overexpressing HER2 and in HT-29 cells (Wong et al., 2007; Shi et al., 2011), which can be associated with a development of a tumorigenic phenotype.

In the present study, we confirmed previous observations from our group that, like MCF-7 cells, MCF-10A cells express the enzymes involved in the synthesis of catecholamines, particularly the enzyme involved in the limiting step of catecholamines synthesis pathway, tyrosine hydroxylase (TH). When investigating, in more detail, the molecular characteristics of TH present in MCF-10A cells, we detected however differences on its molecular weight which suggest the involvement of a different isoform than that present in MCF-7 cells. This may indicate that the enzyme used by cancer cells to catalyse the conversion of tyrosine in DOPA may be different from that found in non-tumorigenic cells, however, this should be confirmed afterwards.

Aside from increasing cell viability and colony formation, we showed that the β-AR agonist, isoprenaline, also makes the capacity of non-tumorigenic MCF-10A cell line to produce catecholamines more like the profile observed in tumorigenic cells (increased adrenaline levels). Incubation with isoprenaline increased the amount of adrenaline present in MCF-10A cells conditioned medium, indicating that β-AR activation increases the capacity on the non-tumorigenic cells to convert noradrenaline into adrenaline. To our knowledge, this is the first observation showing that exposure of non-tumorigenic cells to exogenous β-AR agonists may contribute for the cell increased capacity to produce its own catecholamines, specially adrenaline.

In an *in vivo* system, the exposure to catecholamines released under stress conditions may mimic the exposure to exogenous β-AR agonists and offer a new possibility to understand the tumorigenic process that may be triggered by stress: a capacity to trigger an increase in the production of catecholamines (adrenaline) in predisposed non-tumorigenic cells. The ability of catecholamines to induce this type of alterations was previously described in endothelial cells after events of ischemia (Sorriento et al., 2012). However, this was never observed and/or understood as a risk factor for tumorigenesis.

We attempted to directly imply the tumorigenic potential of catecholamines using the neoplastic transformation assay. This assay has been previously used to address the

neoplastic transformation induced by several distinct carcinogens (Liu and Lin, 2004; Yusuf and Frenkel, 2010) and characterizes the ability of transformed cells to continually grow independently of contact inhibition, a marker of neoplastic transformation (Yusuf and Frenkel, 2010). In the present experimental conditions, we failed to observe any effect of β-AR activation in inducing a neoplastic transformation. Nevertheless, the carcinogen (PMA, a PKC activator), used as the positive control for neoplastic transformation (Udeani et al., 1997), caused morphologic transformation of MCF-10A cells, revealed by the occurrence of small transformed foci. These observations suggest the need, in future works, to increase the assay incubation period, in order to confirm any effect of isoprenaline (alone or in combination with other stimuli), under optimal experimental conditions.

One interesting finding of the present study was the loss of capacity of the β-AR agonist, isoprenaline, to increase cell viability of the tumorigenic MCF-7 cells. The question on the purpose of the autocrine production of catecholamines arises since, in tumorigenic cells, no increase in cell viability induced by β-AR agonist was observed. In the present study, the capacity of drugs to alter cell viability was evaluated using the MTT assay. This assay is dependent on the pool of NADPH and may not necessarily reflect cell proliferation under conditions where cell has scarce reserves of NADPH (higher NADP+/NADPH ratio) as seems to occur in tumorigenic cells (Moreira et al., 2016) caused, namely, by the increased NADPH demand for nucleotide synthesis and lipogenesis (Quent et al., 2010; Moreira et al., 2016). When an alternative assay was used to monitor cell growth (cell proliferation assay), isoprenaline evidenced a capacity to increase cell proliferation. Therefore, it is conceivable that, in tumorigenic cells, the autocrine production of catecholamines can still exert a β-AR mediated stimulatory effect of tumour progression.

In the present study we only addressed the β-AR mediated effects because all the ligands used were selective for β-ARs, particularly for the $\beta_2$-subtype. However, it is known that breast cancer cells also express other ARs subtypes, namely $\alpha_{1B}$ (Powe et al., 2011), $\alpha_{2B}$ (Vazquez et al., 2006) and $\alpha_{2C}$ (Vazquez et al., 2006; Powe et al., 2011). From these, $\alpha_{1B}$ and $\alpha_{2C}$ are positively associated with breast tumors with worse prognosis (Powe et al., 2011; Rivero et al., 2019). Thence, catecholamines can exert other effects in tumorigenic cells acting in ARs other than the β-ARs.

Tumours create an immunosuppressive microenvironment that prevents the attack from the host immune system and catecholamines, released during chronic stress stimulation, contribute for this immunosuppression (Obeid and Conzen, 2013; Eng et al., 2014; Qiao

et al., 2018). Catecholamines coming from outside the tumor, released from sympathetic nerves or from adrenal medulla (Eng et al., 2014), have been hold responsible for such immunosuppressive effects. From the results of the present and previous studies, it is reasonable to propose that catecholamines formed by non-tumorigenic cells exposed to stress conditions or by cancerous cells may have all the conditions to contribute for this AR-mediated immunosuppression, which may facilitate the tumorigenesis process.

A prolonged release of catecholamines occurs not only under chronic stress. It also occurs during menopause (Kronenberg et al., 1984; Freedman, 2014). Therefore, women would be particularly exposed to this tumorigenic pathway since menopause, *per se*, may create similar conditions to stress for an AR-mediated activation of tumorigenesis. This could explain, at least in part, the increase in incidence of breast cancer after menopause (Dall and Britt, 2017).

Ostrogen levels are also altered during menopause. In the present study a putative influence of oestrogens on the tumorigenic effects of catecholamines was explored. The results obtained further support the basis to explain the higher incidence of breast cancer observed after menopause (Dall and Britt, 2017).

Taking in account the dramatic drop in oestrogen levels during the menopause, it would be expected an exacerbation of the effects caused by catecholamines (released during the stress response and during the hot-flashes events). The results obtained showed that the loss of oestrogens can foster the tumorigenic effects of catecholamines. In non-tumorigenic cells, 17β-estradiol prevented the increase in either cell viability and in colony formation, caused by β-AR activation. Therefore, menopause has the potential to create conditions to promote tumorigenesis more efficiently than stress, not only because it promotes the formation of pro-tumorigenic messengers (adrenaline and noradrenaline), during hot flash events, but also because it loses the protective effect of oestrogens in preventing the β-AR-mediated tumorigenic effects, observed in the MCF-10A non-tumorigenic cells. These results are supported by the observation that the incidence of hot flashes is more prevalent when the levels of oestrogens are low and that women presenting persistent vasomotor symptoms (VMS), which includes hot flashes and night sweats, present higher incidence of breast cancer (Sturdee, 2008; Chlebowski et al., 2018).

Ostrogens seem to be a double-edge sword. In MCF-10A cells, they prevent the β-AR-mediated tumorigenic effect whereas in MCF-7 cells, oestrogens exert a marked tumorigenic effect. These observations support that, like what was observed with β-AR-

mediated effects, oestrogen receptor-mediated effects in MCF-10A non-tumorigenic cells are different from those observed in MCF-7 tumorigenic cells.

The dual effect of oestrogens in cell proliferation according to the "tumorigenic *status"* of the cell may explain why long term use of hormonal replacement therapy may cause an increase in the incidence of breast cancer (Mahase, 2019). It is conceivable that while no tumorigenic cells are present, oestrogens may prevent breast cancer but if any tumorigenic cellular foci are formed, oestrogens will speed up their growth, leading to tumor formation.

According to the canonic pharmacological view, agonists have affinity (ability to bind to a receptor) and intrinsic activity (ability to activate the receptor and causing a cellular response) while antagonists only have affinity but do not have (or have a very low) intrinsic activity for a given receptor. They compete for this receptor, in general, in a competitive way. Therefore, the effect of a given concentration/dose of an agonist will be reduced if applied in the presence of an appropriate concentration of antagonist while antagonists may exert biological effects by preventing the effects of agonists (the effects observed *in vivo* would be caused by inhibition of the endogenous ligands) (Kenakin and Christopoulos, 2013). The present study revealed a change of this agonist/antagonist pattern of interaction from non-tumorigenic MCF-10A to the MCF-7 tumorigenic cells. Propranolol, in the same concentration range, antagonized the tumorigenic effects of isoprenaline in non-tumorigenic MCF-10A cells whereas in tumorigenic MCF-7 cells, propranolol, *per se*, presented an anti-tumorigenic effect, that was not attenuated by isoprenaline. The lack of the competitive behavior between an agonist and an antagonist seen in MCF-7 tumorigenic cells for these β-AR ligands suggest that the mechanisms of interaction between β-AR ligands and receptors may be different from the non-tumorigenic cells. Observations about the effects of β-AR antagonists in proliferation or viability of cancer cells have been widely described (Schuller, 2010; Perez Pinero et al., 2012; Choy et al., 2016; Zhou et al., 2016a; Ashrafi et al., 2018; Brohee et al., 2018) as well as the lack of capacity of agonists to attenuate this effect (Choy et al., 2016). To our knowledge, this is the first study that directly imply that β-AR antagonists may not follow the canonical mechanism of action of being the result of a blockade of the effects of an (exogenous or endogenous) agonist.

In the last decades, evidence has emerged showing that seven-transmembrane receptors such as the β-ARs, may adopt numerous conformations, many of which favor interaction with certain cellular partners to activate a particular transduction pathway (Nygaard et al., 2013). The library of potential signaling systems in a cell by agonists

may vary according with the ligand (biased agonism) (Kenakin, 2007) and even some antagonists may activate pathways not easily observed in normal cells. Several β-AR antagonists seem to present this property and propranolol is one of the antagonists shown to have also the capacity to promote internalization of $\beta_2$-ARs and to induce activation of the ERK1/2 pathway (Azzi et al., 2003; Wisler et al., 2007). The present study shows that something is conditioning the possible conformations of β-ARs in MCF-7 cells, increasing the possibilities to see activation of pathways by β-AR antagonists.

Cholesterol is a known modulator of $\beta_2$-AR conformation, kinetics and functional characteristics (Scarpace et al., 1985; Pontier et al., 2008; Zocher et al., 2012). The same seems to occur with other relevant receptors for the control of tumorigenesis, such as the HER2 receptor (Zhang et al., 2019). Membranes of tumorigenic cells have an higher content of esterified cholesterol and saturated cholesteryl esters (Li et al., 2006; Antalis and Buhman, 2012; Chimento et al., 2018). In the present study, a first attempt was carried out in order to investigate whether a reduction in cholesterol content of tumor cells alter proliferation and viability of tumorigenic cells and, if under these conditions, the β-AR-mediated responses are altered.

MβCD was used to reduce cholesterol content. The conditions used were the same used by others which showed that, under these experimental conditions, a significant drop in cholesterol content was achieved (Rao Malla et al., 2010; Guerra et al., 2016), specially for the maximal concentration tested (1 mM). We assumed that identical degree of cholesterol depletion was achieved in the present study, although this assumption should be experimentally confirmed in future works. As reported by others (Rao Malla et al., 2010; Guerra et al., 2016), cholesterol depletion by treatment with MβCD inhibited cell viability/proliferation, suggesting that cholesterol is crucial to keep the proliferative rate of the tumorigenic cell. We also observed that cholesterol depletion altered the responses to β-AR activation and that the β-AR agonist reveals a capacity to inhibit cell viability and proliferation on top of the marked inhibition already observed after cell treatment with MβCD. In spite of the methodological limitations, this set of results support the view that cholesterol may be a factor determining the β-AR-mediated type of influence in cell proliferation and a higher cholesterol content a requirement for the cell to keep the proliferative potential.

Stress induces several metabolic disturbances, namely alterations on blood lipid profiles and hypercholesterolemia (Bryant et al., 1988; Muldoon et al., 1992; Brennan et al., 1996; Assadi, 2017). Among the metabolic alterations induced by menopause, it is also common to observe an increase in age-dependent prevalence of increased cholesterol

levels (see Kolovou and Bilianou (2008)). Therefore, the conditions that may favor tumorigenesis (stress and low oestrogens), can frequently coexist with metabolic alterations (hypercholesterolemia) and may determine alterations on cellular signaling, favoring cell proliferation and tumorigenesis through this additional mechanism.

The antitumor activity of propranolol and other β-AR antagonists has been one of the most exciting observation because it reveals the possibility of exploring the use of drugs developed for other clinical indications (repurposed drugs), in cancer (Coelho et al., 2017; Pantziarka et al., 2018). The mechanism of action proposed to explain the antitumor effect of β-AR antagonists has been the blockade of effects of endogenous adrenaline or noradrenaline coming from the blood or from the nervous system (Schuller, 2010; Cole and Sood, 2012) and through a biased agonism (Coelho et al., 2017). The present study further reinforces that β-AR antagonists like propranolol may exert an antitumor effect by acting as biased agonists.

In the present study, an attempt was made to further clarify the intracellular mechanism of action of propranolol. The results obtained suggest that the mechanism of propranolol induced cell death may involve a disturbance on the AVOs (acidic vacuolar organelles).

AVOs (which includes late endosomes, lysosomes or autolysosomes) are involved in processes that are crucial for the development of tumorigenic cells such as endosome formation (Podinovskaia and Spang, 2018) and autophagy (Huang et al., 2018; Wilde et al., 2018). The endosomal network couple endocytosis to a series of vacuolar organelles. Through this endosomal network, cells retrieve nutrients from their environment and direct endosomes to different compartment whose traffic is dependent on the metabolic needs of the cell (Podinovskaia and Spang, 2018). According to the specific cellular needs, the content may be recycled back to the cell surface or sent to the acidic lysosomes for degradation.

Endosomal membrane trafficking requires the coordination of multiple signaling events to control cargo sorting and processing and endosome maturation (Li et al., 2013). It is known that $\beta_2$-AR are also targets for endocytosis (Marullo et al., 1999) and their movement back to the cell surface (externalization) may be inhibited by propranolol (Kurz and Perkins, 1992) in the same concentration range used in the present study. Propranolol also disturbed the traffic of EGFR, a potent oncogenic protein (Shaughnessy et al., 2014) suggesting a broader action of propranolol on endosomal trafficking.

Autophagy is an important mechanism that allows cells to survive to various stresses, including starvation (Brohee et al., 2018). It is a complex intracellular clearance pathway that delivers cytoplasmic contents to the acidic lysosome for degradation, a process that

requires autophagosome-lysosome formation and an appropriate acid-dependent activation of the catabolic enzymes present inside the formed autolysosome. Propranolol was shown to block autophagy, by blocking the autophagosome-lysosome fusion and/or AVO formation (Farah et al., 2014; Brohee et al., 2018). The present study shows that propranolol turned AVOs more acidic and caused an accumulation of autophagosomes/autolysosomes, as revealed by treating cells with MDC, a selective marker for autophagic vacuoles, widely used to label these structures (Biederbick et al., 1995; Munafo and Colombo, 2001). Accumulation of autophagosomes/autolysosomes and its acidification strongly suggests that conditions that allow the acid-dependent catabolic enzymes to digest phagosomes are disturbed by propranolol and, therefore, cell cannot use efficiently, for their proliferation/survival, the products from lysosomal degradation of autophagic material. The hypothesis that propranolol is preventing the processing of the autophagic material is supported by the observation that propranolol caused an accumulation of high amounts of the autophagy markers p62 and LC3-II (Farah et al., 2014; Brohee et al., 2018), known markers of autophagy clearance.

# VI. Chapter: Conclusion

# 6. Conclusion

In synthesis, the results of the present work support the view that:

(i) catecholamines (mainly adrenaline) may play a role in promoting tumorigenesis under stress and under identical conditions where high levels of plasmatic adrenaline are attainable, such as during menopause;

(ii) the β-AR-mediated effects are different between non-tumorigenic and tumorigenic cells;

(iii) oestrogens seem to prevent the tumorigenic effects caused by β-AR activation in non-tumorigenic cells but lose this effect when cells already present tumorigenic characteristics. Taking in account the dramatic drop in oestrogen levels during the menopause, an increase of the tumorigenic risk of adrenergic activation after menopause would be expected and could explain the higher incidence of breast cancer observed after menopause;

(iv) cholesterol accumulation, which is favoured by stress and low oestrogen levels, favour the tumorigenic profile of breast cells, stimulating cell proliferation and altering the effects of β-ARs;

(v) in tumorigenic cells, β-ARs antagonists such as propranolol, influence degradation of material obtained from autophagy (or identical process) which may explain the strong inhibition of cell viability/proliferation exerted in a highly selective way in tumorigenic cells. Since propranolol pharmacodynamics and contraindications are well established, there is a high potential to repurpose this drug, used for other purposes, for the treatment or prevention of breast cancer;

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# Annex

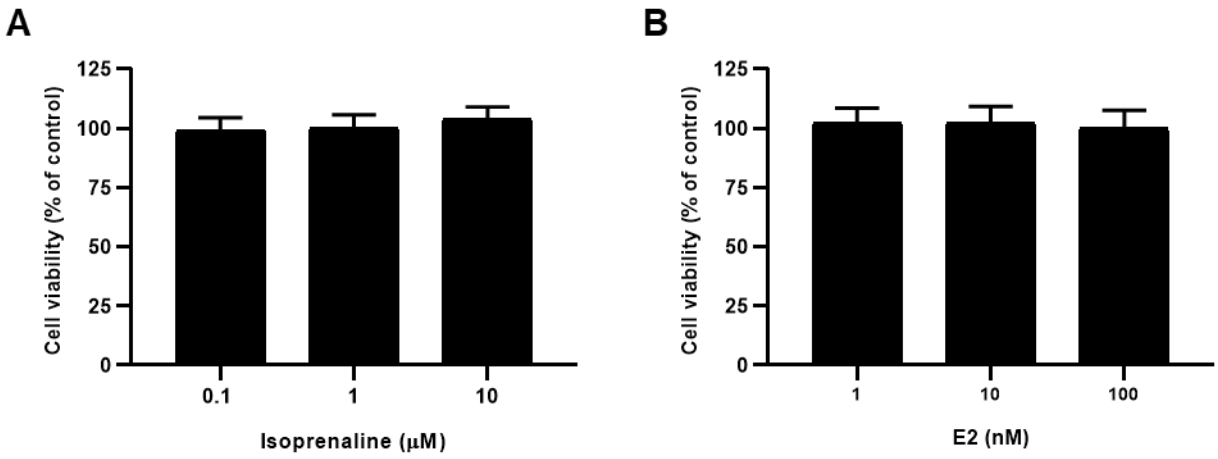



**Figure 32. Effects of the β-AR agonist, isoprenaline, or the ER agonist, 17β-estradiol, on MCF-10A cell viability (A), after 72h of incubation**. Upon seeding, MCF-10A cells were incubated for 24h for adherence and subsequently treated with the indicated drugs (T0) for additional 24h. To measure the cell viability, the MTT assay was used. A detailed description of the method can be found in the Materials and Methods section. Results are expressed as percentage of control and presented as mean ± SEM, from 6-8 independent experiments.

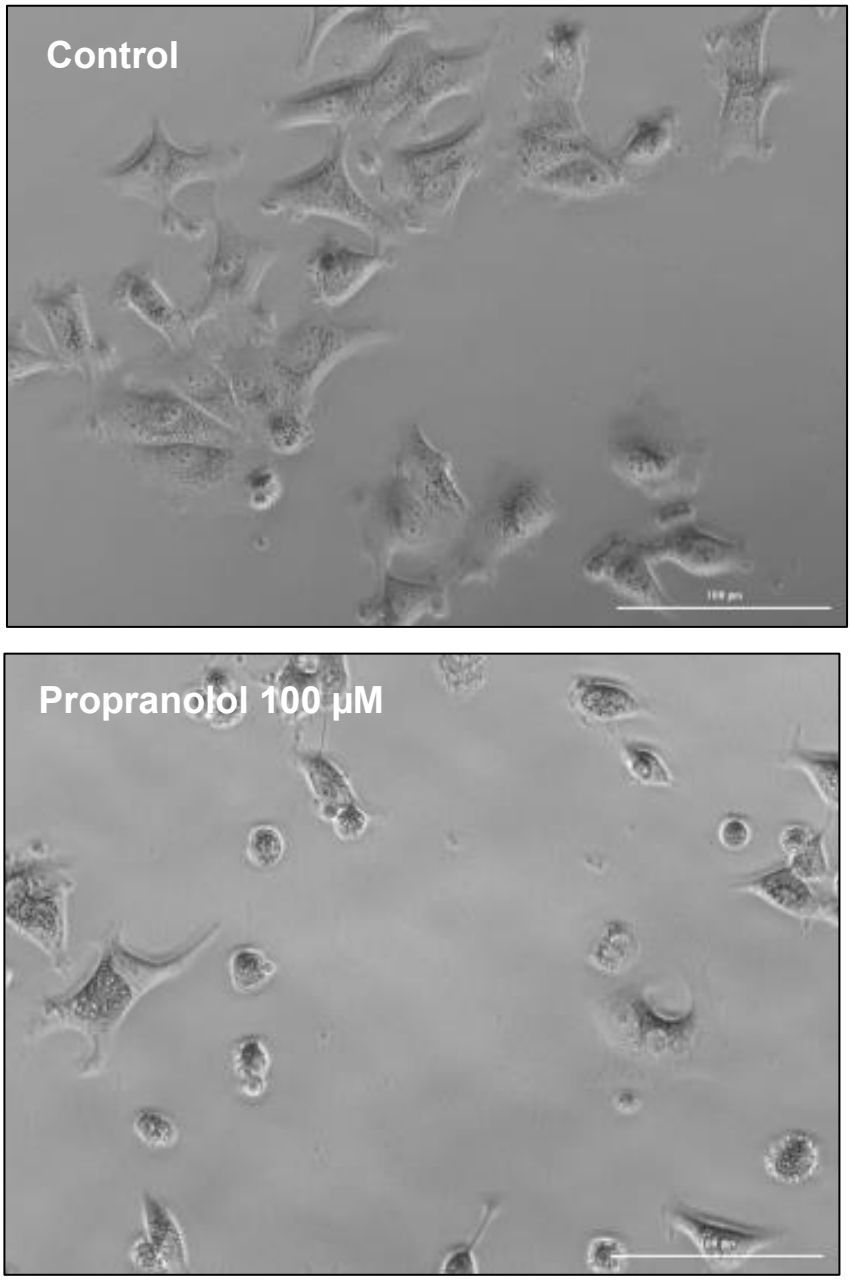



**Figure 33. Effects of propranolol (100 µM) on MCF-7 cell morphology and in the number of cells.** Images were obtained using phase contrast microscopy (Lionheart FX microscope)

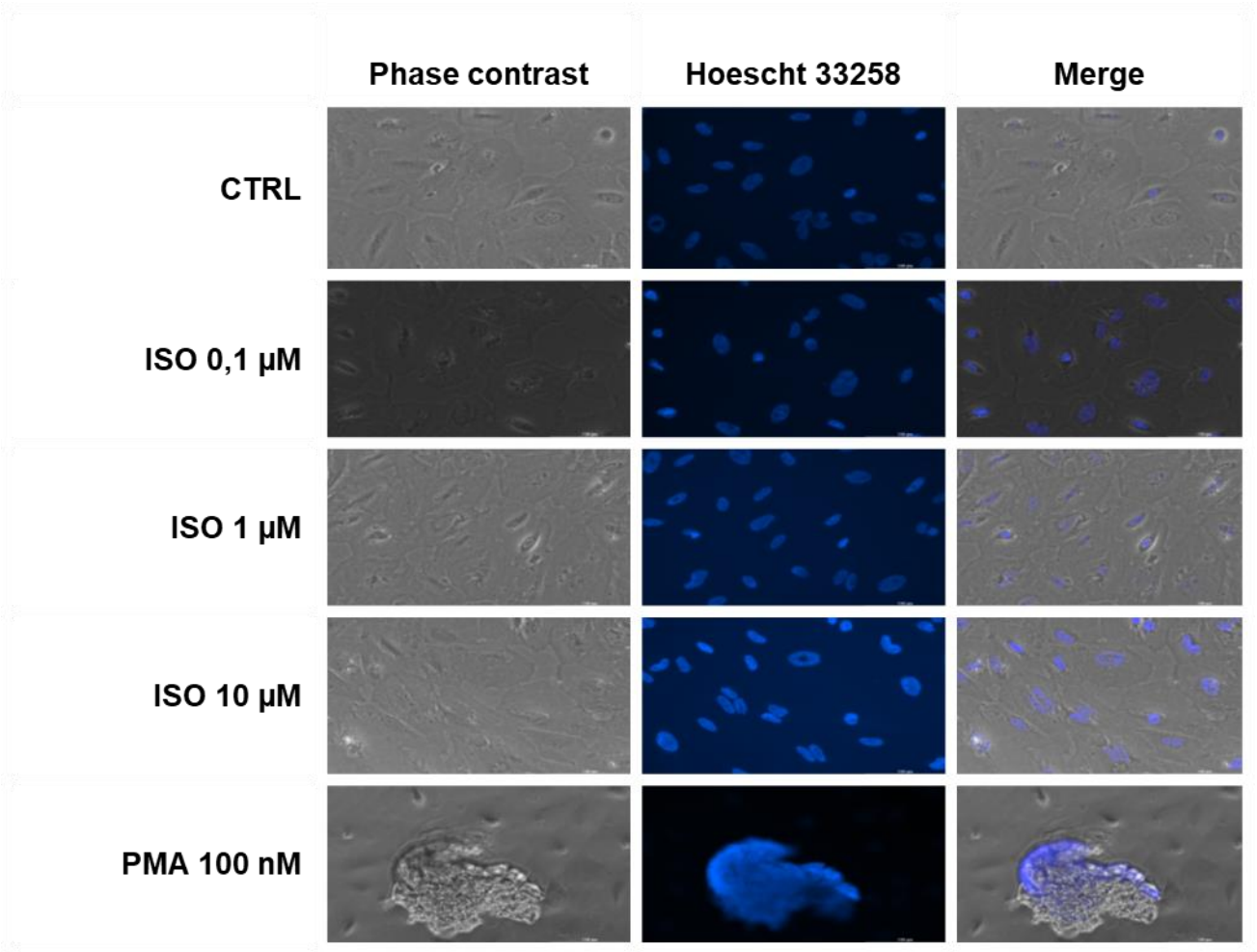



**Figure 34. Effects of the β-AR agonist, isoprenaline, on MCF-10A neoplastic transformation.** Representative images of the morphological transformation assay in MCF-10A cells, in the presence of increasing isoprenaline concentrations. PMA was also used as positive control. After reaching confluency, cells were continually grown for one month in the presence of the indicated drugs. A detailed description of the methods used can be found in the Materials and Methods. Images were obtained using the Lionheart FX microscope.