INTERNATIONAL DOCTORAL THESIS

UNIVERSIDAD DE GRANADA

Narrow leafed lupin (*Lupinus angustifolius* L.) β-Conglutin proteins as new preventive, cytotoxic and radiosensitizing agents against breast cancer cells.

Memoria presentada por **Julia Escudero Feliu** para optar a la mención de Doctor Internacional por la Universidad de Granada

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Programa de Doctorado en Medicina Clínica y Salud Pública
Granada, 2023


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Editor: Universidad de Granada. Tesis Doctorales
Autor: Julia Escudero Feliu
ISBN: 978-84-1195-461-7
URI: https://hdl.handle.net/10481/95831

Funded Grant/Project that have contributed to the development of this PhD Thesis

María Isabel Núñez Torres thanks the Excellence project PIE16/00045 (Implementation of a novel integrated platform to monitor tumour heterogeneity as a crucial determinant for individualized diagnostic and therapeutic outcome), funded by Instituto de Salud Carlos III and granted to the University of Granada and Instituto Biosanitario de Granada (ibs.GRANADA).

Jose Carlos Jiménez López thanks the EU Marie Curie Research Program FP7-PEOPLE-2011-IOF (grant ref. PIOF-GA-2011-301550); the Spanish Ministry of Economy, Industry and Competitiveness (grant ref. RYC-2011-16536 - Ramon y Cajal Research Program); the Spanish Ministry of Science and Innovation (grant ref. CPP2021-008989); and the Spanish National Research Council – CSIC (Intramural Research grant ref. 202240I002).

Josefa León López thanks the Instituto de Salud Carlos III, FEDER (PI18/01947); the MINECO grant (DPI2017-84439-R) and the Nicolás Monardes Program from the Andalusian Health Service (C-0033-2015).

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ABSTRACT

Breast cancer (BC) is the most widespread tumor in women and the second type of most common cancer worldwide. BC is characterized by an intra and inter-tumoural heterogeneity that, commonly, make this disease extremely intractable. In BC, the presence of a particular subpopulation of cancer stem cells (CSCs) is related to relapse, metastases, self-renewal capacity and radio-resistance. In this context, radiotherapy (RT) is one of the most used treatments for BC. Despite of that, it is not always effective and CSCs are partially responsible of RT resistance and failure. Ionizing radiation (IR) has also been associated with promotion of CSCs phenotype. In this context, the technical and medical advances in existing therapies in the last decade had not been able to overcome the fact that between 30 and 50% of patients with BC will develop metastasis, which contributes to the high mortality rates.

This situation urges the need to find more effective prevention and treatment strategies like the use of plant-based nutraceutical compounds. In this context, considerable interest is focused on legume seed proteins, mainly those from genus *Lupinus*, and particularly from the "Sweet lupin" group.Lupin is a legume belonging to the Fabaceae family. Interestingly, the seeds of Narrow-leafed lupin (NLL) or *Lupinus angostifolius* L. or blue lupin are attracting attention because of their potential use for inflammatory related diseases prevention and improvement. Vicilin family of proteins, and particularly β-conglutin proteins are the most abundant proteins in all lupin species including NLL. Recently, the anti-diabetic, antioxidant, and anti-inflammatory properties of β-conglutins were described, on this basis they were proposed as new potential functional foods with therapeutic and preventive properties in inflammatory-related diseases like BC.

The main goal of the present doctoral thesis is to assess whether those promising nutraceutical compounds could be natural preventive and cytotoxic agents for BC cell lines with a radiosensitizing effect on advanced malignant cells,

mediated by the implication of CSCs related genes and mechanisms. On the other hand, this thesis also studied and identified the structural domain β-conglutins having key importance in the nutraceutical properties of those NLL seed compounds.

To achieve it, we purified different isoforms of NLL β-conglutins using affinity-chromatography. We analyzed their structural modelling and studied two previously uncharacterized β-conglutin isoforms, β5 and β7, in order to assess their inflammatory amelioration properties using *in vitro* cell model, and *ex vivo* cell systems such as type 2 diabetes (T2D) cells compared with and healthy control cells. Since inhibiting the production of pro-inflammatory cytokines, iNOS expression and nitric oxide (NO) production, and regulate the antioxidant capacity of cells, β5 and β7 are newly discovered anti-inflammatory proteins. We also demonstrated, that mobile arm structural domain of β5 and β7 is involved in nutraceutical properties as compared to truncated forms, applying these features for all NLL β-conglutin protein isoforms since other functional isoform have the same behavior.

Then, we used the previously more in-deep studied β-conglutins 1, 3 and 6 to evaluate their effectiveness in terms of viability, proliferation, apoptosis, stemness properties, and mechanism of action on two different models of BC cell lines and a healthy one: an early BC model and an advanced one. NLL β-conglutins can become a preventive agent of great interest in early stages of BC, regulating the bone morphogenetic pathway (BMP) and preventing malignant transformation and epithelial to mesenchymal (EMT) enhancement in healthy cells. Those proteins can also be an interesting cytotoxic, natural and anti-stem-cell-like properties treatment for both early and advanced BC models, from very low concentrations and preserving the viability of healthy cells. These proteins could act through a regulation of BMP related genes and a dual mechanism

involving tumorigenic and stemness-related genes such as SIRT1 and FoxO1, depending on the state of p53.

NLL β-conglutin proteins have also a radiosensitizing potential in combination with conventional RT. The combined therapy of β-conglutins and IR showed lower survival fraction, higher apoptotic rates and lower DNA damage response (DDR) in all BC cell lines, especially in the most aggressive phenotype, MDA-MB-231. This combined therapy also regulated and decreased CSCs subpopulation as well as self-renewal capacity, assuring the highest effectiveness of radiation and the prevention of the CSCs phenotype acquisition, at the origin of tumor regrowth, metastasis and relapse.

In conclusion, based in this PhD Thesis results and for the best of our knowledge, this is the first time that these NLL β-conglutin proteins have been identifies as potentially anti-BC, with the capability of being used for BC management, making them a unique and novel strategy for the BC treatment at different stages and from multiple perspectives: i) prevention of the disease, ii) treatment in early and advanced cases, iii) sensitizing for radiotherapy and prevention of metastasis and relapse.

More studies must be carried out to completely understand the underlying mechanisms of action of these nutraceutical compounds in BC *in vitro* and *in vivo* systems, and their potential use for the inhibition of other cancer cell types as well.

RESUMEN

El cáncer de mama (CM) es el tumor más extendido en la mujer y el segundo tipo de cáncer más común a nivel mundial. El CM se caracteriza por una heterogeneidad intra e intertumoral que, comúnmente, hace que esta enfermedad sea extremadamente intratable. En el CM, la presencia de una subpoblación particular de células madre cancerosas (CMC) está relacionada con recidivas, metástasis, capacidad de auto-renovación y radio-resistencia. En este contexto, la radioterapia (RT) es uno de los tratamientos más utilizados para el CM. A pesar de esllo no siempre es efectiva y las CMC son parcialmente responsables de la resistencia a la RT. La radiación ionizante (RI) también se ha asociado con la promoción del fenotipo de CMC. En este contexto, los avances técnicos y médicos en las terapias existentes en la última década no han podido impedir que entre el 30 y el 50% de los pacientes con CM desarrollaran metástasis, lo que contribuye a las altas tasas de mortalidad.

Esta situación apremia la necesidad de encontrar estrategias de prevención y tratamiento más efectivas como el uso de compuestos nutracéuticos de origen vegetal. En este contexto, se centra un gran interés en las proteínas de semillas de leguminosas, principalmente las del género Lupinus, y en particular del grupo "Sweet lupin". El lupino es una leguminosa perteneciente a la familia Fabaceae. Curiosamente, las semillas de lupino de hoja estrecha (NLL) o *Lupinus angostifolius* L. o lupino azul están atrayendo la atención debido a su uso para la prevención y mejora de enfermedades relacionadas con la inflamación. La familia de proteínas "vicilinas", y particularmente las proteínas β-conglutinas, son las proteínas más abundantes en todas las especies de lupino, incluida la NLL. Recientemente, se han descrito las propiedades antidiabéticas, antioxidantes y antiinflamatorias de las β-conglutinas y, en base a esto, se han propuesto como nuevos alimentos funcionales con propiedades terapéuticas y preventivas en enfermedades relacionadas con la inflamación como el CM.

El principal objetivo de la presente tesis doctoral es evaluar si estos prometedores compuestos nutracéuticos podrían ser agentes preventivos y citotóxicos naturales para líneas celulares de CM con un efecto radiosensibilizador sobre células malignas avanzadas, mediado por la implicación de genes y mecanismos relacionados con las CMC. Por otro lado, esta tesis también estudió e identificó el dominio estructural de las β-conglutinas que tienen una importancia clave en las propiedades nutracéuticas de estos compuestos de semillas NLL.

Para lograrlo, purificamos diferentes isoformas de β-conglutinas de NLL mediante cromatografía de afinidad. Analizamos su modelado estructural y estudiamos dos isoformas de β-conglutina previamente no caracterizadas, β5 y β7, para evaluar sus propiedades de mejora inflamatoria utilizando un modelo celular *in vitro* y sistemas celulares *ex vivo* como las células de diabetes tipo 2 (T2D) en comparación con y células de control sanas. Dado que inhiben la producción de citoquinas proinflamatorias, la expresión de iNOS y la producción de óxido nítrico (NO), y regulan la capacidad antioxidante de las células, β5 y β7 son proteínas antiinflamatorias recientemente descubiertas. También demostramos que el dominio estructural del brazo móvil de β5 y β7 está involucrado en las propiedades nutracéuticas en comparación con las formas truncadas, aplicando estas características para todas las isoformas de proteína β-conglutinas NLL ya que otras isoformas funcionales tienen el mismo comportamiento.

A continuación, utilizamos las β-conglutinas 1, 3 y 6, estudiadas previamente más en profundidad, para evaluar su efectividad en términos de viabilidad, proliferación, apoptosis, propiedades relacionadas con el fenotipo de CMC y mecanismo de acción en dos modelos diferentes de líneas celulares de CM y uno saludable: un modelo de CM de estadios tempranos y uno de CM avanzado. Las

β-conglutinas de NLL pueden convertirse en un agente preventivo de gran interés en estadios tempranos de CM, regulando la vía de las proteínas morfogenéticas óseas (BMP) y previniendo la transformación maligna y el aumento de transición epitelio-mesénquima (TME) en células sanas. Esas proteínas también pueden ser un interesante tratamiento citotóxico, natural y con propiedades anti-células madre cancerosas para modelos de CM tanto tempranos como avanzados, desde concentraciones muy bajas y preservando la viabilidad de las células sanas. Estas proteínas podrían actuar a través de una regulación de genes relacionados con las BMP y un mecanismo dual que involucra genes relacionados con tumorigénesis y propiedades anti-CMC como SIRT1 y FoxO1, dependiendo del estado de p53.

Las proteínas β-conglutinas de NLL también tienen un potencial radiosensibilizador en combinación con la RT convencional. La terapia combinada de β-conglutinas y RI mostró una fracción de supervivencia más baja, tasas de apoptosis más altas y una respuesta al daño del ADN más baja en todas las líneas celulares de CM, especialmente en el fenotipo más agresivo, MDA-MB-231. Esta terapia combinada también reguló y disminuyó la subpoblación de CMC, así como la capacidad de auto-renovación, asegurando la mayor efectividad de la radiación y la prevención de la adquisición del fenotipo de CSC, en el origen del crecimiento tumoral, la metástasis y la recaída.

En conclusión, en base a los resultados de esta tesis doctoral y según nuestro mejor conocimiento, esta es la primera vez que estas proteínas β-conglutinas de NLL se identifican como potencialmente anti-CM, con la capacidad de ser utilizadas para el manejo de la enfermedad, convirtiéndose en una estrategia única y novedosa para el tratamiento del CM en diferentes etapas y desde múltiples perspectivas: i) prevención de la enfermedad, ii) tratamiento en casos

tempranos y avanzados, iii) sensibilización para radioterapia y prevención de metástasis y recidivas.

Se deben realizar más estudios para comprender completamente los mecanismos de acción subyacentes de estos compuestos nutracéuticos en los sistemas *in vitro* e *in vivo* de CM, y su uso potencial para la inhibición de otros tipos de células cancerosas también.

INTRODUCTION

1. CANCER

Cancer (also known as "malignant tumours" or "neoplasms") is a generic term that includes a group of diseases affecting any part of the body [1]. Cancer is defined by a rapid creation of abnormal cells that are able to grow beyond their usual potential and invade adjoining parts of the body, even spreading to other organs, being this last process known as metastasis[1].

According to the latest World Health Organization (WHO) report, widespread metastases are the primary cause of death from cancer in the world. In this report, different type of cancers are classified according to their incidence worldwide, being breast cancer (BC) the most common cancer with 2,26 million cases each year, followed by lung (2,21 million cases), colon and rectum (1,93 million cases), prostate (1,41 million cases), skin (non-melanoma, 1,20 million cases) and stomach (1,09 million cases) [2]. The distribution of cancer incidence can vary if we consider only male or female population, being lung cancer the most prevalent cancer for males and BC the most common among women worldwide, as show in Figure 1 [3,4]. Regarding cancer mortality, nearly 10 million deaths due to cancer happen every year, with lung cancer being the most common cause of death related to cancer, followed by colon and rectum, liver, stomach and breast [1].

Generally, cancer arises from the transformation of normal cells into tumour cells, being a multi-stage process that still remains partially unknown [1]. Generally, the development of cancer is a result of the interaction between genetic factors and three categories of external agents (physical, chemical or biological carcinogens), classified as "cancer-causing agents" by the International Agency for Research on Cancer (IARC), such as ultraviolet (UV) and ionizing

radiation (IR), components of tobacco smoke, alcohol, food and water contaminants, infections from viruses or bacteria, among others [5].

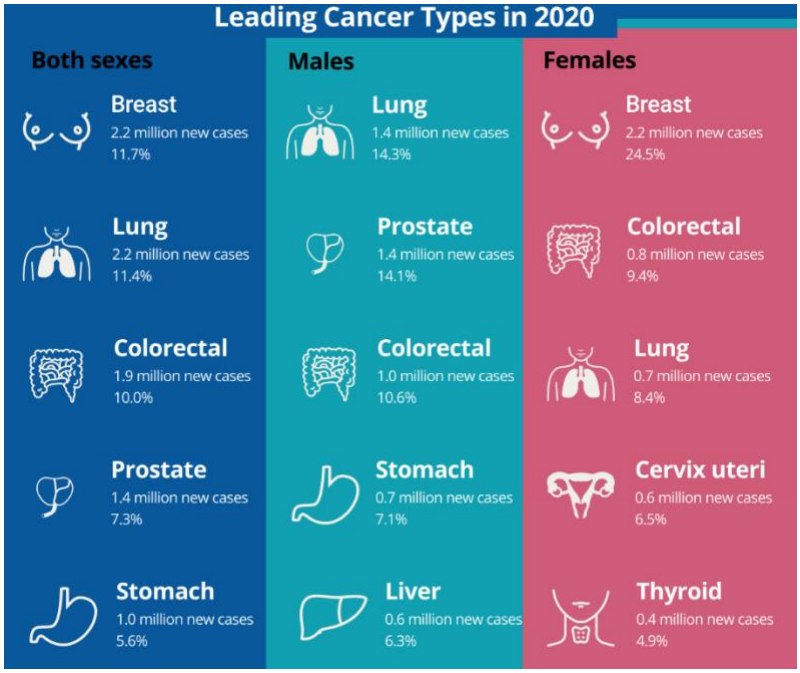


Figure 1: Leading cancer types in 2020. Source: GLOBOCAN 2020 [1]

Nearly one third of cancer deaths are due to the top 5 risk factors: behavioural and dietary risk factors: high body mass index, low fruit and vegetable intake, tobacco use and alcohol consumption. Ageing is another major factor in the development of cancer, most likely due to a combination of loss of efficiency of cellular repair mechanisms and accumulation of the aforementioned risk factors. [1]. Many cancers can be prevented by avoiding exposure to those common risk factors and, in addition, a significant proportion of cancers can be controlled and even cured by surgery, chemotherapy or radiotherapy treatments, especially If detected in early stages of the disease [6,7].

The hallmarks of cancer cells correspond to the biological functions acquired during the tumour development process, being recently updated as new mechanisms and cell types that have been added to the previously described

features [8]. Those cancer cells hallmarks include the sustaining of proliferative signaling, the evasion of growth suppressors, the resistance to cell death, the induction of angiogenesis, the activation of invasion and metastasis and genome instability, among others [8]. In addition to cancer cells and their hallmarks, the tumour microenvironment (TME), also known as tumour niche, has recently taken a huge relevance in cancer development, particularly regarding different steps of tumorigenesis [8,9]. This niche comprise a number of essential players, such as stromal cells and extracellular matrix (ECM), and the cross-talk between this components is key to tumour growth and progression [9]. Finally, the interactions of tumor and cancer cells with their microenvironment have been demonstrated to be critical for the process of development and progression of cancer [10].

2. BREAST CANCER

a. Epidemiology and risk factors

According to the data collected in 2020 by the IARC, BC is the most common tumour in women and also the most common tumour worldwide, as shown in Figure 1, and accounting for 11.7% of all new incidences [5]. However, in terms of mortality, it ranks fifth in the world with 6.9% of all cancer deaths [3].

Distribution of BC incidence and BC related deaths in 2020 for both sexes, in comparison with other types of cancers, are shown in Figure 2. The main contributor to the latter percentage is metastasis: almost 90% of BC deaths are caused by metastatic spread of primary breast tumours [11] to the lung, brain, liver and bone [12].

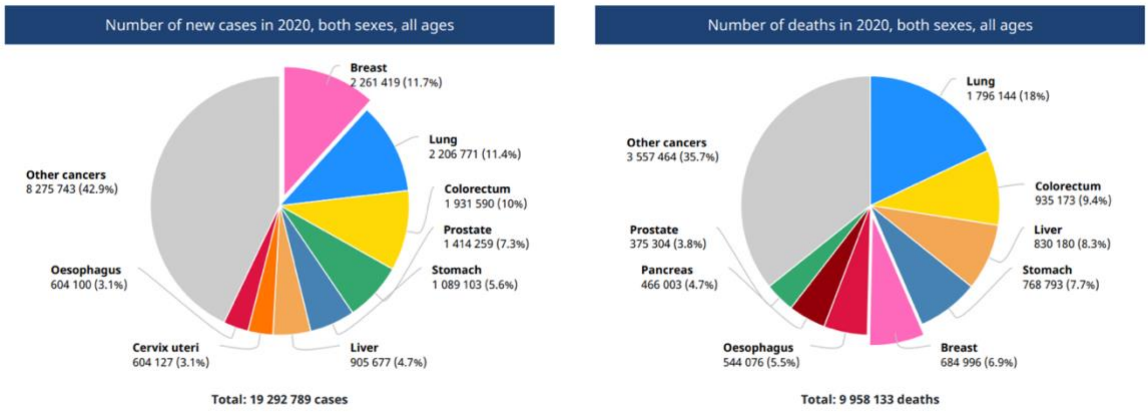


Figure 2: Number of new cases of BC and BC related deaths in 2020, in comparison with other types of cancer, for both sexes and all ages. Source: GLOBOCAN 2020 [4].

BC is the most frequently diagnosed cancer in the majority of the countries, and the leading cause of cancer death in more than 100 countries. Mortality rates have been declining in highly developed countries since the early 1900s, as a result of the improvement of detection techniques (such as mammography/population-based screening), leading to earlier diagnosis [13],

and more effective treatment regimens [3,14], that will be discussed later on this chapter. It is important to notice that most BC cases occur in women, with 100 times higher cases in women than in men [3].

As expected, a large number of general risk factors for cancer are common to BC risk factors, concretely, aging, genetic mutations, family history, unhealthy lifestyle or tobacco consumption [15]. More precisely, BC risk factors include:

- **Aging**: apart from sex, aging is one of the most important risk factors of BC, as the incidence increases with age. A large majority of BC are reported in women over the age of 40, explaining the necessity to perform mammography screening for women aged 40 or older [16]. Menopausal status is another factor to consider in the development of BC. Around the age of 40 years, the breast lobules undergo age-related lobular involution (ARLI), which accelerates after menopause and is associated with a reduced risk of breast cancer [17,18]. Hanna *et a*l. studied the association between ARLI and local inflammation of breast tissue, in order to understand the risk of developing breast cancer. This study revealed significant inverse associations between ARLI and high levels of pro-inflammatory markers, i.e. less involuted breasts are associated with an increased risk of disease and vice versa [19].

- **Family history**: A fourth part of all BC cases are related to family history [20]. The risk of developing BC becomes 2.5-fold or higher in women that have two or more first-degree relatives (women) with BC, and this inherited susceptibility to BC is attributed to genetic mutations in BRCA1 and BRCA2 tumour suppressor genes generally [20]. In fact, BRCA1 is involved in the activation of DNA damage response (DDR) and cell cycle control points and BRCA2

is related to DNA damage repair during replication [21]. Other additional genes whose mutations are related with BC are p53, ATM, PTEN and E-cadherin (CDH1), among others [14]. Particularly, p53 is associated with familial syndromes triggering a high risk of BC, and patients with those inherited mutations are estimated to be 20 times more likely to develop BC before the age of 45 [22].

- **Reproductive factors**: Early menarche, late menopause, late first pregnancy or low parity have been demonstrated to increase BC risk [23]. Recently, epidemiological reviews regarding BC have highlighted the fact that each 1-year delay in menopause can increase BC risk in 3% [21,23].

- **Estrogen**: Both endogenous (produced by the ovary) and exogenous (oral contraceptives or hormone replacement therapy, HRT) estrogens prolonged exposure are associated with the risk of BC [21].

- **Lifestyle**: Concretely, alcohol consumption, smoking, nutrition and diet play a crucial role in the development of BC. More precisely, alcohol consumption can increase the level of estrogen-related hormones and then trigger the ER pathways [22]. On top of that, the intake of >35 grams of alcohol per day can increase BC risk by 32% [24]. Tobacco and smoking have also been reported as risk factors that can affect BC mortality, and nutrition, particularly high fat intake or consumption of red meat, have been related to higher BC mortality, poor prognosis and incidence too [22,25].

- **Obesity**: A body mass index (BMI) superior or equal to 30 kg/m^2 has been associated with an increase in the risk of BC in postmenopausal women, but this remains controversial as obesity (BMI > 30) is associated with a reduced incidence in premenopausal women [22,26]. Despite of those data, obesity has been associated in general with a greater chance of developing metastatic axillary nodes in comparison with underweight or normal BMI women [26].

- **Exposure to X and gamma radiation**: Curiously, even if IR is one of the principal therapeutic approaches for BC treatment, that will be discussed later on, it remains one of the principal risk factors for cancers in general, and concretely for BC [22]. The relative risk of BC induced by radiation (X or gamma) seems to be inversely related to the age of exposure as younger breast tissue undergoes rapid cell proliferation in childhood and puberty [22,27].

b. Clinical description and classification of BC

From a clinical point of view, BC is a heterogeneous disease at the inter and intra-tumoural level, which is extremely relevant for the prognosis, treatment and perspectives of success of this pathology [28]. Other determining factors in the prognosis of BC are: type of tumour (from a histological and molecular point of view), location, degree of differentiation, presence or absence of different proteins (as already mentioned among the risks factors, E-cadherin, p53,Ki67, etc.), the involvement of the sentinel lymph node, and the age of the patient [29]. Those factors are not only related to the prognosis of the disease but also have a relation with the treatment response [29].

The location of the tumour is important regarding the diagnosis of the disease, as this characteristic may determine a good or poor prognosis of BC [1]. Depending on the location, BC can be classified as: *in situ* and invasive or infiltrative [30]. From a histological point of view, there are also different types of BC: ductal, lobular, nipple, and unspecified [31]. According to the histological and location criteria, BC is usually classified according to the tumour-node metastasis staging (TNM Staging), that subdivides BC tumours by stages [30]. This classification is essential for prognosis and treatment strategy, and is composed of:

- **Stage 0: *In situ*.** Normally, this carcinoma *in situ* is characterized by an abnormal tissue growth that does not progress but may increase the risk of subsequent invasive BC [30]
- **Stages I and II: Early invasive.** This stages are characterized by small invasive tumours contained within the breast, that may or may not have spread to the lymph nodes in the armpit . The prognosis at this point could be good depending on the treatment response [30,32,33].
- **Stage III: Locally advanced.** This stage of BC normally includes tumours > 5 cm with extensive regional lymph node involvement, non-metastatic but inoperable and inflammatory BC [33].
- **Stage IV: Metastatic**. This stage is the worst BC scenario and usually involves relapses of previous treated BC stages. The expectancy of life for this type of BC is usually five years [30]

In addition to the TNM staging, there is a molecular classification of BC, which greatly influences the prognosis, the therapy of the disease and its outcome. This classification is based on the presence or absence of oestrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor 2 receptor (HER2) [34]. Depending on the status of these receptors, BC can be:

- **Luminal A** (ER+ and PR+, HER2-). This BC subtype has low levels of Ki-67, a protein controlling fast cancer cells growth. Luminal A cancer are usually low-grade, with better clinical outcomes, slow tumour growth and less aggressive potential [35].
- **Luminal B** (ER+ and PR+, HER2+). Unlike the Luminal A tumours, Luminal B BC presents higher levels of Ki-67, entailing worst prognosis and faster growth in comparison with Luminal A BC [35]. The HER2 expression is also a major difference between the two luminal subtypes [36]
- **HER2-enriched** (ER-, PR- and HER2+): This BC subtype is hormone-receptor negative and HER2 enriched, present worse prognosis than the luminal subtype and normally tumours grow faster. Despite of that, HER2-enriched tumours are successfully treated with targeted therapies aimed at HER2 protein [35,37].
- **Basal or Triple-negative** (ER-, PR- and HER2-): The triple-negative BC (TNBC) is more common in women with BRCA1 gene mutations and corresponds to infiltrating ductal tumours with a high rate of metastases [37]. According to the literature, TNBC is the molecular subtype associated with a worst outcome, followed by the HER-2 enriched subtype, both having a strong connection with breast cancer stem cells (CSCs), a subpopulation of malignant cancer cells characterized by their main role in tumour regrowth and spread after the initial treatments, and being a cause of chemo and radio-resistance [38–40]. This particular subpopulation and its mechanisms of action will be discussed later on the introduction chapter.

Finally, another important factor regarding the clinical outcome of BC is the degree of differentiation. This factor indicates the rate of tumour growth and dissemination, and is based on the similarity of tumour cells when compared to normal breast tissue cells [39]. Tumours are differentiated into three grades: **grade I or well-differentiated** (slow growth, tumour cells similar to normal and well

organised), **grade II or moderately differentiated** (growth and appearance between grade I and grade III), and **grade III, or poorly differentiated** (rapid growth and spread, and very different appearance from normal cells) [33].

c. Treatment of BC

According to all the previously described intra and inter-tumoural heterogeneity, the staging of BC and the molecular subtypes, the different treatments for BC are combined and optimized depending on all the above mentioned factors. In general terms, the principal treatments for BC nowadays include surgery, hormonal therapy, chemotherapy, radiation therapy or radiotherapy (RT) and immunotherapies in some cases [41]. Conventional therapies include prophylaxis with tamoxifen or breast conserving surgery for *in situ* carcinoma, breast conserving surgery, RT and/or chemotherapy for stages I and II (early stage and invasive), chemotherapy, surgery, endocrine therapy and RT for stage III (locally advanced) and IV (metastatic) [30]. Depending on the subtype of BC, according to the luminal/TNBC/Her-2 classification, therapies may be more or less effective, which makes BC extremely intractable [42]. Despite of all the technical and medical advances in the existent treatments, and the recent implementation of new ones, such as immunotherapy, recent research and studies have showed that between 30 and 50% of patients will develop metastasis [43], which contributes to the failure of existing treatments and the poor cure rates worldwide. The development of drug resistance and major side effects has weakened the efficacy of the mentioned therapies, some of them becoming useless for some BC subtypes, such as TNBC: hormone therapy is not effective and resistance to chemotherapy, surgery or RT as already been described for the most aggressive tumors [44,45]. As already mentioned, this can be partially explained by CSCs subpopulations and their main role in tumor regrowth and spread after initial treatment [40].

d. Cancer Stem Cells (CSCs)

CSCs constitute a small percentage of cells within the tumour [46], which can be distinguished from other cells by the asymmetry of their cell division and by alterations in their gene expression [47]. The first evidence on the existence of CSCs date back to 1996 and was based on identical chromosomal alterations in contiguous regions of the mammary epithelium [48]. The concept of those "stem-cells" has existed for many decades, but is still evolving [28] and their origin and proportion in a given tumour have been difficult to describe [49]. There are three models that attempt to explain the origin of CSCs in tumour development [50,51]:

- **Stochastic model or clonal evolution model**. This model postulates that all cells have the same probability of being the tumour-causing cell and that all of them are involved in the maintenance of the tumour through different capacities depending on the microenvironment in which the cells are found. The existence of different subpopulations within a single tumour is explained in this model by the accumulation of mutations after each replication cycle [50,51,52].
- **Hierarchical model or CSCs model**. This model postulates that tumours are organized in a hierarchical way, in which CSCs, the cell subpopulation with stem-like properties, represent the origin [51]. According to this model, carcinogenesis starts when a healthy stem cell escapes regulation by becoming a CSC, which ends up generating the rest of the tumour [50,53]. Figure 3 compares the two described models.

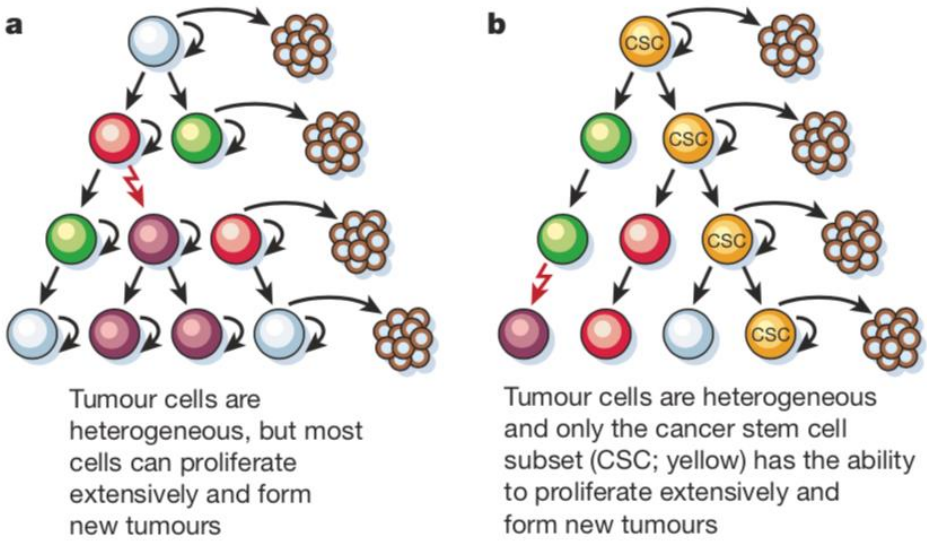


Figure 3: Comparison of stochastic (s) and hierarchical CSCs model (b). (Reya *et al*., 2001 [50]).

- **Dynamic CSCs model**. This third and most recent model is based on the hierarchical model and postulates that the phenotype of CSCs can be acquired by non-stem cancerous cells, highlighting the dynamic nature of cancer cells and CSCs heterogeneity [51]. In this model, CSCs phenotype is strongly conditioned by the tumour microenvironment (TME), non-stem cancerous cells located on the edges of the tumour mass will be exposed to the factors derived from the TME, reverting their phenotype to a more undifferentiated state, turning into CSCs [51,54]. Figure 4 summarizes the three previous described models.

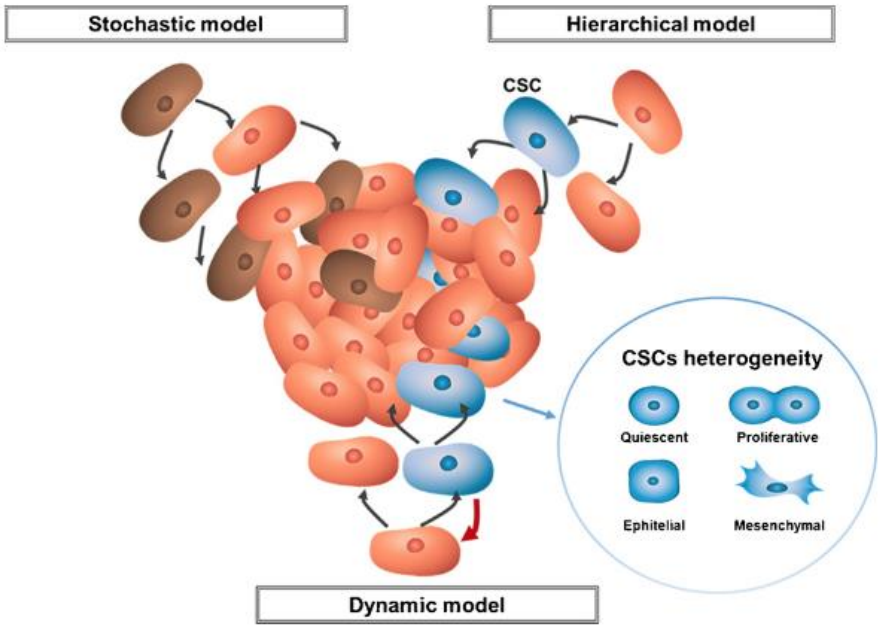


Figure 4: Comparison the three models that define tumour origin and regrowth regarding CSCs subpopulation. (P.Hernández-Camarero *et al*, 2018 [51]).

CSCs have an increased tumourigenic potential and certain characteristics of normal stem/progenitor cells [28]. These cells have been studied for their ability to originate, maintain and expand tumours [46,55]. In addition, they have the unlimited capacity for self-renewal, proliferation, differentiation to other cell lines features, survival in the bloodstream and resistance to oncological treatments such as RT and chemotherapy [55,56]. Because of this, CSCs play an important role in the processes of drug resistance, radiotherapy resistance, tumour metastasis and recurrence or relapse [57–59].

Regarding specific CSCs properties, common to breast CSCs, three characteristics provide this subpopulation with the self-renewal and malignant features that are at the origin of their maintaining, expanding and resistant phenotype. Those properties are:

- **Plasticity**: Both CSCs and non-CSCs cells are plastic and capable of undergoing phenotypic changes after the exposure to microenvironmental factors, but CSCs phenotype has a higher plasticity that also depends on the epithelial-to-mesenchymal transition (EMT) [51]. The TME play a critical role in this CSCs plasticity, from their origin to their metastatic potential [51].

- **Epithelial-to-mesenchymal transition** (EMT): EMT is an essential process during embryonic development, in which epithelial cells convert to mesenchymal ones, acquiring migratory properties [60]. This complex program has been implicated in the initiation and progression towards more invasive or metastatic phenotypes [61].

- **Quiescence**: This state correspond to a cell phase where cell does not divide, remaining in G0 cell cycle phase, allowing those quiescent cells to survive the majority of cancer treatments [61]. This feature, characteristic of CSCs subpopulation phenotype, is the key to relapse in cancer, specially BC, allowing them to reappear even decades after the initial treatment [50,61].

Metastasis is a complex process through which cells from primary solid tumours invade adjacent and distant tissues, resulting in the growth of secondary tumours. These metastases can be initiated by the formation of premetastatic niches, formed by CSCs in target organs [62]. Because of all these characteristics and properties, CSCs are becoming candidates as new therapeutic prospects in BC [63]. To this end, their characterization is essential in research. Ginestier *et al*. demonstrated that, within the tumour, these cells show increased activity for the enzyme cells show increased activity for the enzyme aldehyde dehydrogenase 1 (ALDH1) using the Aldefluor assay [64]. On the other hand, the membrane

markers established for these cells are $CD44^{high}/CD24^{low/-}$, $CD133^{+}$ or $CD29^{high}/CD61^{+}$ [28,46,55,63,65]. As a consequence of the above, targeted therapies to will be increasingly necessary for complete eradication of the tumour [55], as they are responsible for morbidity and mortality associated with the disease [63].

e. Genetic pathways of BC-related mechanisms

i. Initiation and progression of BC: relevance of Bone Morphogenetic Proteins (BMP) pathway

One of the major conserved signalling pathways involved in stem cell regulation from embryogenesis up to adult stages is the bone morphogenetic protein (BMP) signalling, involving different soluble BMP molecules, with BMP2 and BMP4 progressively emerging as the most important BMPs regarding stem cells regulation [66]. The BMP pathway is involved in numerous physiological and pathological processes, is known to regulate processes such as cell proliferation, differentiation and motility, and is actually found in the microenvironment of several tissues, contributing to tissue morphogenesis and homeostasis, such as the mammary gland [66,67]. The importance of the BMP signalling alterations in BC are emerging as to propose new therapeutic strategies for this disease [68]. In this regard, it has been reported that the microenvironment of human primary luminal breast tumours produces abnormally high amounts of soluble BMP2 compared to healthy tissue, and this higher level is associated with BMPR1B (a BMP type I receptor) overexpression by the tumour cells [66,68,69]. To test if a potential over-activation of the BMP pathway was at the origin of malignant transformation, Jung *et al* chronically exposed a model of mammary stem-cells (the MCF-10A human, non-transformed epithelial cell line) to BMP2 concentrations, leading to a transformation towards a luminal transformed

phenotype, that was further stabilized with the addition of IL-6 proinflammatory cytokine [66,68].

Regarding more advances stages of BC, BMP2 has been shown to modulate the proliferation and differentiation of BC cells [70]. In a recent study, BMP2 promoted the migration and invasion of Luminal A BC cells by regulating the reorganization of cytoskeleton and the expression of adhesion molecules on the cells [70]. Another recent study about the BMP pathway in BC showed that BMP4 signalling enhanced the expression of stem cell genes, such as CD44 (marker for CSCs in BC) and enhanced chemo-resistance and self-renewal in a TNBC model, while having an opposite effect on Luminal A and B models, indicating a context dependent role of BMP4 in BC [71]. Although more research regarding the BMP signalling in BC is required, later evidence suggests this pathway and its deregulations as crucial event regarding both the initiation of BC tumours and their progression, resistance and metastatic potential [66–71].

ii. P53/Sirt1/FoxO1 pathway

As previously mentioned, the p53 protein is involved in cancer development due to mutations of TP53 or changes in the status of p53 modulators, which prevents its activation [72]. Several studies in mice have shown that p53 mutations can result in more aggressive tumour behavior [72] and lead to metastasis, concretely though p53 gain of function, that has been related to enhanced oncogenic potential beyond the simple loss of p53 function and promotion of tumorigenesis by disruption of critical DNA damage-response (DDR) pathways [73,74]. In contrast, the wild-type protein (p53 wt) has tumour suppressor activity, allowing it to detect oncogenic events in cancer cells and eliminate them by senescence or apoptosis [74,74]. In addition to this, p53 is also involved in other processes such as the response to DNA damage, ageing, cellular metabolism, stem cell differentiation, fertility and angiogenesis [72].

Regarding the molecular mechanisms of BC progression, SIRT1, as a crucial regulator of cellular targets, is the most studied sirtuin with a promising therapeutic potential for many diseases like cancer, concretely BC [75]. The physiological functions of SIRT1, and particularly in relation with importance processes regarding cancer progression, like apoptosis or resistance to treatments, are mediated by deacetylation of histones, transcription factors, or co-activators such as p53 or forkhead box O (FOXO) [75]. The SIRT1/FoxO1 regulatory axis is an important pathway implicated in BC progression and aggressiveness, involving p53 [75–78]. Concretely, this SIRT1/FoxO1 axis is a ROS-sensitive pathway that has been described in numerous papers regarding BC progression and aggressiveness, and regarding its key role for the regulation of the stemness phenotype in BC [77]. Fukui *et al*, studied this pathway using natural compounds such as resveratrol, a dietary phenolic compound which reduced the effectiveness of paclitaxel, one of the usual chemotherapeutics agents in BC, and this reduction was mediated by up-regulation of the SIRT1/FoxO1 pathway in TNBC and Luminal B cell models [78]. Other studies showed that SIRT1-mediated FoxO1 deacetylation is a key mechanism for multidrug resistance in BC cell lines [79]. All this evidence shows the importance of this pathway of action in BC, that remains controversial nowadays, since presenting a dual effect as promoting or suppressing tumoural activity, depending on the cellular context. In this context, recent studies investigated the dual effect of the SIRT1/FoxO1 axis as tumor promotor or suppressor in different cancers [80], and p53 has emerged as a downstream effector of this axis in BC [80–82]. The controversial effect of the p53/SIRT1/FoxO1 axis can become a target for future therapies against BC resistance and progression.

iii. Autophagy

One of the processes regulated by this p53/SIRT1/FoxO1 pathway is autophagy, which has emerged as a crucial and controversial mechanism that can play a dynamic tumour-suppressive or promoting role depending on the cancer context and cellular type [83–85]. Recent studies have shown that the activation of autophagy mechanisms could supress BC metastasis [84]. The expression of LC3B (microtubule-associated protein 1 light chain 3, MAP1LC3) [86] is of high importance in the autophagy process, and sequesteome 1 (SQSTM1/p62) protein, a classic receptor of autophagy, is usually used as marker for autophagy. P62 degrades itself during the autophagy process [87] and LC3B is incorporated to the membranes of autophagosomes [88]. The interaction between p62 reduced levels and LC3B higher detection can be used to asses autophagy [88]. Recent reports have shown that promising anti-cancer agent against TNBC and HER-2 positive BC cell models induce autophagic cell-death as their mechanism of action [89]. The autophagy process, partially regulated by the p53/SIRT1/FoxO1 pathway, may be another therapeutic new approach against BC development and resistance to treatments.

3. IONIZING RADIATION (IR)

a. Description and application in BC

Ionizing radiation (IR) has been one of the main effective cancer treatments used for more than 100 years [90]. RT is used in the treatment of most tumours as IR damages cells, producing intermediate ions and free radicals that cause DNA double-stand breaks (DSBs) and prevent the cells to repair this damage, leading to cell death [91].

When implementing RT, it is very important to be aware of the concept of threshold dose, which, for a given effect, can be defined as the dose below in which there is no effect [91]. The selection of an appropriate radiotherapeutic regimen depends not only on this threshold dose but also on the characteristics of the patient, the tissues and organs where the tumour is located. In addition, RT can be combined with surgery and/or systemic therapy [92].

In BC, until recently the radiotherapeutic regimen most commonly applied in the clinical practice was conventional RT, consisting of daily fractions of 2 Gray (Gy) reaching a total dose of 45-50 Gy. Alternatively, hypo-fractionated RT is increasingly being used, which involves higher radiation doses in shorter treatment times, with the same efficacy and lower toxicity. A widely used hypofractionation regimen is associated with the use of 2.66 Gy fractions, although even 6 Gy fractions are being administered in BC [93–96]. Post-operative RT given to the breast and regional lymph nodes increases control of the disease up to 20% and improves long-term survival [97]. Despite of that, fractionated treatment regimens increase the damage to the tumour cells, and may be at the origin of the re-distribution to more sensitive cycle phases [97].

b. Limitations and non-desirable effects

Toxicity is another factor to take into account when working with radiation, as it depends mainly on the chosen radiotherapeutic regimen and the total dose administered, but also on the volume irradiated and the age of the patient. This toxicity can be manifested by two types of adverse effects [92,98]:

- **Acute**. This group of adverse effects appear in the first 3 months of treatment. They are the result of changes in cell permeability and release of inflammatory mediators.
- **Late/chronic**. These non-desirable effects usually appear after 3 months of treatment. They are the result of direct target tissue injury and severe early reactions.

In BC, skin toxicity is the most common adverse effect and usually presents as erythema, desquamation, ulceration and bleeding (acute effects), and hyperpigmentation and telangiectasia (chronic effects) [98]. De Felice *et al*. found that conventional RT and a larger volume of irradiated breast have a negative effect on acute skin toxicity [92].

The overall efficacy obtained with RT is not only dependent on all the above described parameters (threshold dose, radiotherapy regimen, tumour location, toxicity), but also on the resistance of patients to this treatment [91,92]. This resistance is due to the heterogeneity and biological complexity of certain tumours, mainly due to the CSCs subpopulation present in these tumours [90]. The resistance presented by these cells is due to a radio-adaptation mechanism, i.e. a modification of the response to radiation exposure. Two stages have been distinguished in the development of this adaptation [91]:

- **Initial adaptation**, that occurs immediately after exposure and involves physiological mechanisms.

- **Persistent adaptation**, that is gradually developed and involves mechanisms such as stimulation of DNA repair, induction of protein synthesis, activation of radioprotective systems, etc.

Radio-resistance is the major factor to be taken into account, as it can lead to recurrence after treatment and metastasis [90]. Beside the effective properties of RT for BC patients, improving survival rates and controlling the disease from early stages, one of the main limitations of this treatment could be the sided-induction of metastasis, CSCs phenotype and oncogenic metabolism by IR [97], factors that have also been associated with radioresistance. Those metabolic alterations are involved in tumour progression, including growth, invasion, metastasis and the acquisition of the CSCs phenotype. Metabolic alterations can provide lower energy levels, required by dormant CSCs, thereby contributing, as an adverse effect, to tumour recurrence and distant metastases[99]. Metabolic adaptation and reprogramming are also involved in the plasticity of CSCs acquired through EMT, being a relevant step of CSCs role during metastasis[99,100]. On top of that, IR activates the release of growth factors, including transforming growth factor-β (TGF-β) and extracellular matrix (ECM) modulators, facilitating tumour invasion and metastasis [101,102]. Even if IR has been reported to activate an antitumour immune response, this signaling can be frequently suppressed by tumour escape mechanisms, such as cell death or suppressive immune cells, and radiation *in vivo* has shown an enrichment of the fraction of cells expressing CSC markers, that also present and enhanced self-renewal capacity [97,103,104]. Figure 5 summarizes the IR-induced side effects on cancer cells, where EMT, metabolic alterations and CSCs phenotype induction play a fundamental role.

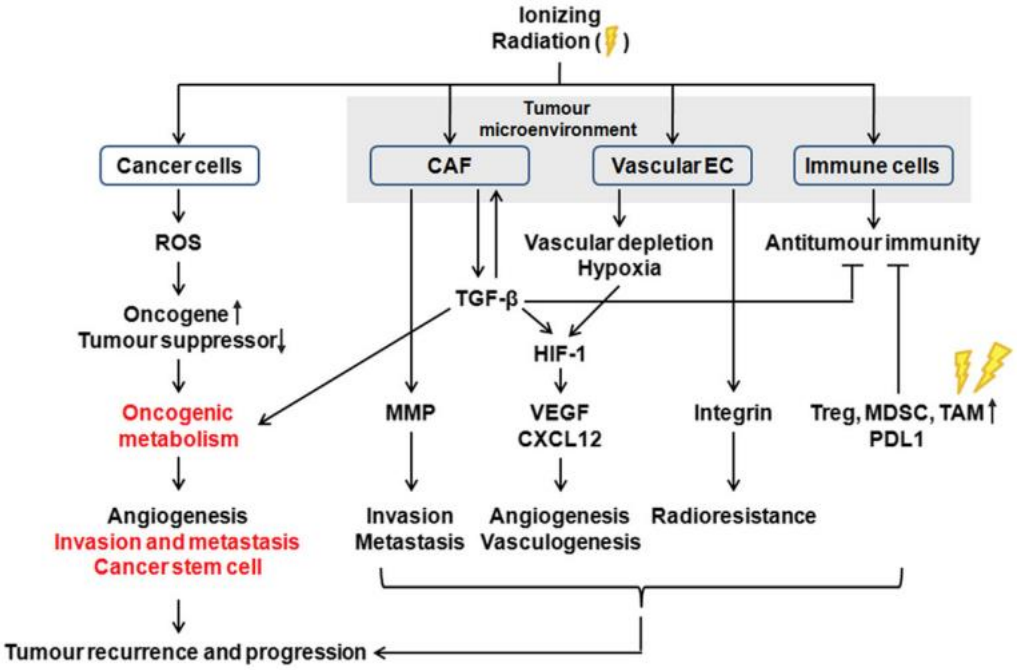


Figure 5: Non-desirable potential effects of RT (Lee *et al*, 2017 [97]).

c. CSCs resistance to IR

Within the tumour, cells undergo a number of radiation-induced effects from IR: senescence, quiescence, genomic and epigenomic instability, modification of metabolism and cell cycle, DNA damage, altered DNA repair or DNA damage response (DDR), and cell death. These effects depend on everything described so far (threshold dose, radiotherapeutic regimen, total dose, tumour volume and location), but also on the linear energy transfer (LET), which is the amount of energy deposited per unit path length in cells when interact with IR [105]. In addition, radiation can produce an effect known as the "bystander mechanism", which is a damage to non-irradiated cells adjacent to irradiated cells as a result of intercellular communication between them [106,107].

Radiation-induced effects can be repaired by tumour cells through intrinsic and extrinsic properties, which provide them with the ability to recover, inducing radioresistance. Such recovery occurs in all tumour cells, but it much faster for

the CSCs phenotype [108]. Those CSCs are proposed as directly responsible of the relapse in a tumour process, especially in BC, after having received RT. The mechanisms that may provide radioresistance to CSCs can be framed in four principal group, and are represented in Figure 6: systems repair of DNA damage, cell tumour repopulation, redistribution of the cell cycle and level of intratumour hypoxia in the TME [109].

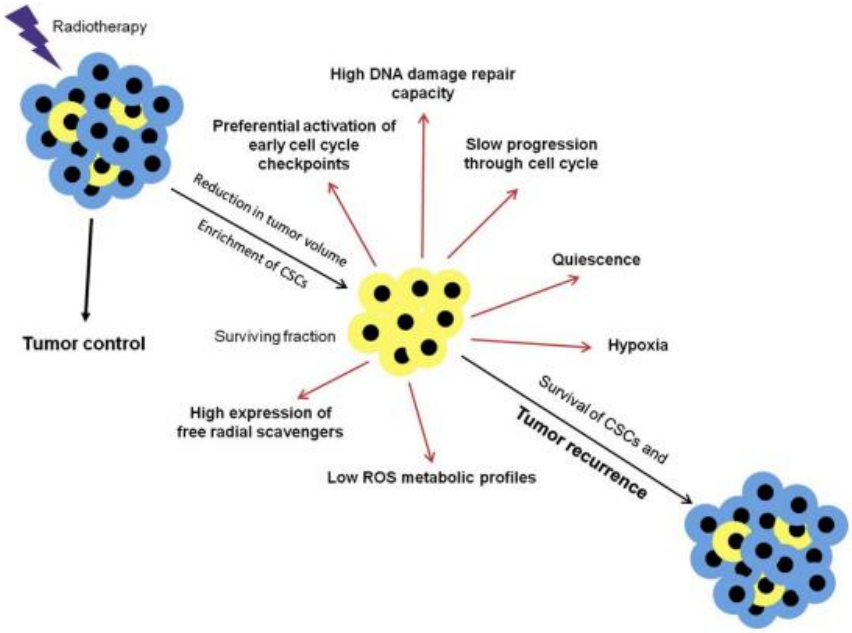


Figure 6. Activation of survival and evasion cell death mechanisms in CSCs after radiation. (Marie-Egyptienne *et al*, 2013 [109]).

In this regard, the high ability to repair DNA remains one of the principal causes for CSCs radio-resistance, that has widely been reported in several papers as an essential mechanism for cell survival that can be carried out by mismatch repair [110], base excision repair (BER) [111], nucleotide excision repair (NER) [112,113], homologous recombination (HR) repair [114] or non-homologous end joining (NHEJ) repair [115].

Regarding CSCs increased DDR capacity, the observation of γ-H2AX induction in BCSCs was fundamental. γ-H2AX is the phosphorylated form of H2AX which is the gene encoding the histone H2A variant, H2AX. Starting within a few minutes of DSB formation, H2AX becomes phosphorylated. γ-H2AX is the sensitive surrogate of DNA DSBs, which can be quantified after radiation. Several reports demonstrate that CSCs have lower γ-H2AX foci after radiation in human breast CSCs (BCSCs) [116]. As a survival mechanism used by CSCs to resist RT, Wnt/β-catenin signaling pathway is a network of proteins essential in embryogenesis, stem cell maintenance and survival. This pathway has also been shown to be important in CSCs and their responses to DNA damages. One transcriptional target of β-catenin is survivin, that has been linked to radiation resistance and its inhibition may sensitize cells to the radiation effects and induce more apoptosis according to literature [117,118]. The Wnt/β-catenin pathway is also involved in the self-renewal of CSCs through an increase in the levels of activated β-catenin following radiation. Activation of this pathway favors the proliferation of CSCs and their stability in the niches after RT, leading to radio-resistance [119,120].

Other important mechanisms for CSCs radio-resistance are, as above mentioned, the tumour niche and microenvironment, and the activation of two other developmental pathways, apart from the Wnt/β-catenin signaling one, the Notch signaling and Hedgehog pathways.

The tumour microenvironment is particularly important for CSCs, as their interactions with stromal elements constitute their niche. Within the niches, CSCs can regulate these interactions by cell-cell contact or by secretion of regulatory molecules, favoring their proliferation or inhibiting their differentiation [114]. The niches themselves can also be altered following radiation and thus affect survival of CSCs [120–122]. As a consequence, the niches produce survival cytokines

such as epidermal growth factor (EGF), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), thus inducing radio-resistance and radioprotection of CSCs [121]. Regarding the tumour niche and microenvironment, there are two other factors to be taken into account for the radio-resistance of CSCs: hypoxia and angiogenesis. Hypoxia is an oxygen deficiency, and the concentration of oxygen is a determining factor at the time of radiation due to its radiosensitizing power and its ability to generate free radicals that damage DNA [123,124]. In addition, the expression of hypoxia-inducible factor (HIF-1α) is increased, protecting CSCs from oxidative damage and contributing to their resistance to radiation [125–127]. On the other hand, angiogenesis is the formation of new blood vessels from pre-existing vessels, and it is meticulously regulated a balance of pro- and anti-angiogenic factors. Hypoxia stimulates angiogenesis via HIF-1α through the activation of proangiogenic factors such as VEGF, leading to increased radio-resistance [128].

Finally, resistance to anti-cancer treatment and accelerated repopulation of CSC after or during treatment might be also attributed to the activation of two other signaling pathways which are essential for adult tissue homeostasis and embryonic development: the Notch signaling and Hedgehog pathways. Repopulation of tumours may be one of the most common reasons for the failure of conventional fractionated courses of radiation therapy. Radiation activation of Notch pathway might be a part of the acute response to IR transcriptional activator, thereby initiating the transcription of gene products that promote progression into the S-phase of the cell cycle. In BC, IR induces the expression of Notch receptor ligands on the surface of nontumorigenic cells and activation of Notch signalling in CSCs that redistribute quiescent CSCs into the cell cycle [118]. Another developmental pathway activated in response to radiation is the TGF-β pathway, which is thought to be an antiproliferative pathway that controls tissue

homeostasis. TGF-β is produced by the mass of the nontumorigenic, radiosensitive cancer cells and activated by radiation [93,118,129].

Altogether, this evidence stands out the RT resistance of CSCs as an obstacle in cancer and BC therapy, and the role of CSCs surviving mechanisms in therapy resistance. Treatment strategies based on combination of conventional therapies targeting bulk tumor cells and CSC-specific pathway inhibition bear a promise to improve cancer cure compared to monotherapies or conventional treatment strategies [117,118].

Summarizing, BC remains a very common and highly mortal disease, especially among women, and more research is needed in order to decrease the mortality rates. The development of drug resistance and intrinsic radio-resistance, mainly exerted by CSCs, plus the major side effects of therapies like RT have weakened the efficacy of conventional treatments, some of them becoming useless for some BC subtypes, such as TNBC. This situation urges the need to find more effective prevention and treatment strategies, focusing on preventing side effects and recurrence too, as well as targeting the CSCs subpopulation [42].

4. NEW PREVENTIVE AND THERAPEUTIC APPROACHES: PLANT-DERIVED NUTRACEUTICALS

a. Nutrition, nutraceuticals and BC

As poor lifestyle, diet and health condition are some of the main risk factors for BC [6,22], nutrition has been considered a preventive strategy for cancers in the last decade, and recent studies have shown the potential of some natural products in prevention role and even treatment for different types of cancer [130,131]. Concretely, Zheng *et al.* documented the antioxidant, anti-inflammatory and immunomodulatory effect of some spices, related to potential prevention and treatment of several cancers, including BC. The main mechanism of action of the spices treatment included the induction of apoptosis, the inhibition of proliferation among others [130]. Other dietary compounds, such as cruciferous vegetables, turmeric, ginger or plum may be potential sources for prevention and treatment of liver cancer, the most common malignancy of the digestive system [131].

Various epidemiological studies have also suggested the protective effect of healthy diet (such as consumption of soy products) and reduced BC risk, and the relation between consumption of some natural products with a reduced recurrence and increased survival BC rate [132]. Concretely, Farvid *et al.* demonstrated though a prospective study that greater consumption of fruits and vegetables during adolescence is associated with lower risk of BC. Furthermore, higher intake of vegetables and fruits high in alpha-carotene during early adulthood was specifically associated with lower BC risk [132]. In this context, the European Prospective Study on Nutrition and Cancer (EPIC) is multicenter prospective cohort study that was conducted at 23 centers out of 10 European countries including more than 500.000 participants and had the objective to investigate the relationship between diet and cancer [133,134]. Salamanca-

Fernández *et al*. reported an association between high intake of saturated fat and alcohol consumption with an increased BC risk. In post-menopausal women, obesity was associated with higher BC risk[134].

With all this recent evidence, the interest in products with plant-derived nutraceutical properties has become one of the therapeutic strategies that requires resources and research nowadays, as it may be a natural source of agents for both BC prevention and treatment. In this regard, there is a strong interest focused in legumes, an economically affordable, important and alternative (from animals) source of high-quality proteins, compared to other plant foods and seeds, with highly beneficial properties for human health.

b. Legumes: a new source of functional food with therapeutic applications in cancer

Legumes are a major source of food for a significant worldwide population and relevant sources of plant rich quality proteins (20–50% of seed content) with nutraceutical and health benefit properties on human health, especially in the prevention of inflammatory-related diseases like diabetes, obesity, cardiovascular diseases or cancer [135]. Some anti-nutritional compounds have been found in legumes, which may be toxic when consumed raw, while playing a positive role when processed and treated.

Despite all of those promising qualities as potential functional foods and prevention targets, there are still a large number of underutilized potential nutraceutical food from legume seeds that remains unexplored [135,136].

Legumes, especially the seed coat, are rich in antioxidant compounds, able to terminate oxidative reaction chains, eliminating free radicals and inhibiting other oxidative reactions [135,137]. Among the antioxidant compounds present in

legumes, flavonoids should be highlighted, due to the large number of hydroxyl substitutions, which has a direct effect on their ability to donate hydrogen atoms and eliminate free radicals from the environment [138].

As a crucial part of the cancer development, progression and even metastatic invasion, proteolysis processes play important roles, as they degrade the ECM involving migration, invasion, changes in cellular adhesion, as well as the modification of the cellular microenvironment with production of growth factors [137]. In a recent review, Suárez-Martínez *et al.* studied the relevance of *Phaseolus,* the most important food legume for food consumption in the world, as a nutraceutical compound with beneficial effects regarding cancer progression. In this review, *Phaseolus* demonstrated a protective effect against cardiovascular diseases and shown favorable effects against cancer, concretely the luminal A subtype cell model, because of the antimutagenic and antiproliferative properties of their phenolics, lectins and protease inhibitors [137].

A large amount of the aforementioned diseases, and cancer in particular, are related to oxidative stress (OS) mechanisms and their deregulations, for example, when are not capable of eliminating the reactive species produced of oxygen (ROS) and nitrogen (RNS) in an efficient way [135]. These produce alterations throughout oxidative processes in different biomolecules such as proteins, lipids and nucleic acids that can either lead to cell death or produce mutations at the DNA level, increasing the possibility of developing a cancer disease [139]. In a recent report, Cid-Gallegos *et al.* demonstrated that ROS and RNS generated during carcinogenesis modify gene expression, regulate signals of transduction pathways and modulate protein function, while promoting the activation of enzymes related to angiogenesis, a crucial progress not only for cancer progression but for resistance mechanisms to existing treatments [119,139]. Phytic acid, present in legume seeds, exhibits antioxidant function that can regulate proliferation and apoptosis, according to this paper. These processes can

be identified by changes in biomarkers of colorectal cancer cells, suppressing the expression and activity of key regulatory factors [139]. Other studies have showed that the flavonoid genistein, present in *Phaseolus,* inhibits carcinogenic cells, becoming a potential strategy against BC [140].

All this recent evidence about the importance of legumes and their antioxidant compounds for cancer and BC prevention and treatment highlights the relevance of this family of functional foods as new therapeutic and preventive approaches with positive effects on human health and other inflammatory and metabolic related diseases [135–137,139,140,141]

5. NARROW LEAFED LUPIN (*Lupinus angustifolius* L.) β-CONGLUTINS

a. Description and properties

As increasing interest for prevention of diseases has been focused on legume seed proteins, as previously described, lupins, a legume of the Fabaceae family, has demonstrated the potential to be established as an alternative protein crop, capable of promoting socio-economic growth and environmental benefits in Europe [142]. Particularly, the seeds of *Lupinus angostifolius* L. or blue lupin or Narrow leafed lupin (NLL), one of the four members of the called "sweet lupin group" are attracting attention because of their nutritional attributes, presenting health benefits regarding dietary fiber, fat, available carbohydrates and polyphenolic levels, as well as antioxidant properties [143], their low alkaloid content [144] thanks to the recent domestication (genetic breeding) process of this specie, and their potential for disease improvement [145]. These properties are mainly due to the seed's high protein and dietary fiber content, which reduce the risk of a cardiovascular disease due to the impact on blood pressure and can prevent and treat type 2 diabetes [146,147].

Recent studies [148–151] have demonstrated that one of the principal compounds (β-conglutin proteins) integrated in the NLL seed mediate amelioration of these above mentioned inflammatory related diseases effects at molecular level.

β-conglutins belong to the vicilin family of proteins [152]. These NLL seed proteins were characterized by sequencing cDNA clones derived from developing seeds, identifying seven β-conglutin proteins forming the most abundant NLL conglutin family (56%) with desirable nutritional properties [153]. The 7 genes coding for individual β- conglutin proteins, named conglutin β1 to β7 have been recently identified and described, providing substantial information on the complexity of this family in terms of their gene structure,

phylogenetic relationships and relative RNA and protein abundance during seed developments [152].

b. Structure-functional aspects of NLL β-conglutin proteins

β-Conglutins show extensive structural variation [154,155]. Their sequences include by over 20 polypeptide chains, covering a broad range of molecular masses (MW 10–80 kDa) resulting from proteolytic cleavage of multiple β-conglutin precursor polypeptides [153,156].

β-Conglutins are unique proteins among cupin superfamily and among the vicilin family of proteins as they do not contain disulfide bonds in their structure. The compact structural stability is achieved thanks to electrostatic interactions and hydrogen bond establishment [154,155]. In addition, this structural stability of β-conglutin is also due to the oligomerization process yielding trimeric and tetrameric forms, with the minimal individual subunit size ranging from 20 to 75 kDa, and the putative combination of different subunits defining the multimeric structure of these conglutins in different Lupinus cultivars and species including *L. angustifolius* [157]. A heterogeneous number of up to 12 distinct frequently glycosylated polypeptides have been identified [156,158]. This post-translational modification was particularly abundant in the low MW range of polypeptides generated by proteolysis during seed germination, when seed storage proteins undergo mobilization [156,158] to contribute as C and N sources for seedling growth and plant development.

Structural features of β-conglutins are distinctive as it includes two cupin domains forming a Rossmann fold-like structure frequently found in enzymes that use molecular oxygen as a substrate [154,159]. Moreover, this is an overall similar globular structure to legume seed allergens such as peanut Ara h 1 or lentil Len c 1 sharing an average comparative superimposition value <1 Å with

NLL β-conglutins [155,159]. This structure consists of a negatively charged surface of a globular domain constituted by two conserved β-barrels, each one comprising antiparallel β-sheets. An additional mobile N-terminal arm constituted by 8–10 α-helices is a distinctive feature in NLL β-conglutin proteins compared with vicilins in most legume species [155,159]. Molecular modelling analysis has demonstrated that large structural differences between NLL β-conglutin isoforms are found mostly in this mobile arm N-terminal domain; thus, putative functional differences among NLL β-conglutins could be due to these structural differences [155,159]. Molecular modelling has also shown that one of the β-barrels in the globular domain contains a semi-conserved metal binding motif (HYX … R), typically found in oxidoreductases (Oxalate oxidase) [154,155,159]. This motif suggests that NLL β-conglutin structure is comparable with germins or germin-like proteins (oxalate oxidase activity, catalyzing the conversion of oxalates into CO2 and H2O2), and vicilin-like glucose binding proteins, associated with functional roles exerted in the cell wall, the plasma membrane, and/or plasmodesmata [160].

On the other hand, considerable structural differences have been revealed between NLL β-conglutin isoforms (β1 to β7), particularly affecting 2-D elements (loops and coils); and more noticeable differences in the N-terminal domain integrated by the mobile arm affecting number, length, amino acid composition, and polymorphism of their sequences.

c. Molecular nutraceutical properties of β-conglutin related to anti-inflammatory-related diseases amelioration, and significantly contributes to make NLL seeds as functional food

The molecular nutraceutics recently discovered of β-conglutin proteins identified as modulators of the activation of the insulin pathway and potential treatment of type 2 diabetes (T2D) [151].

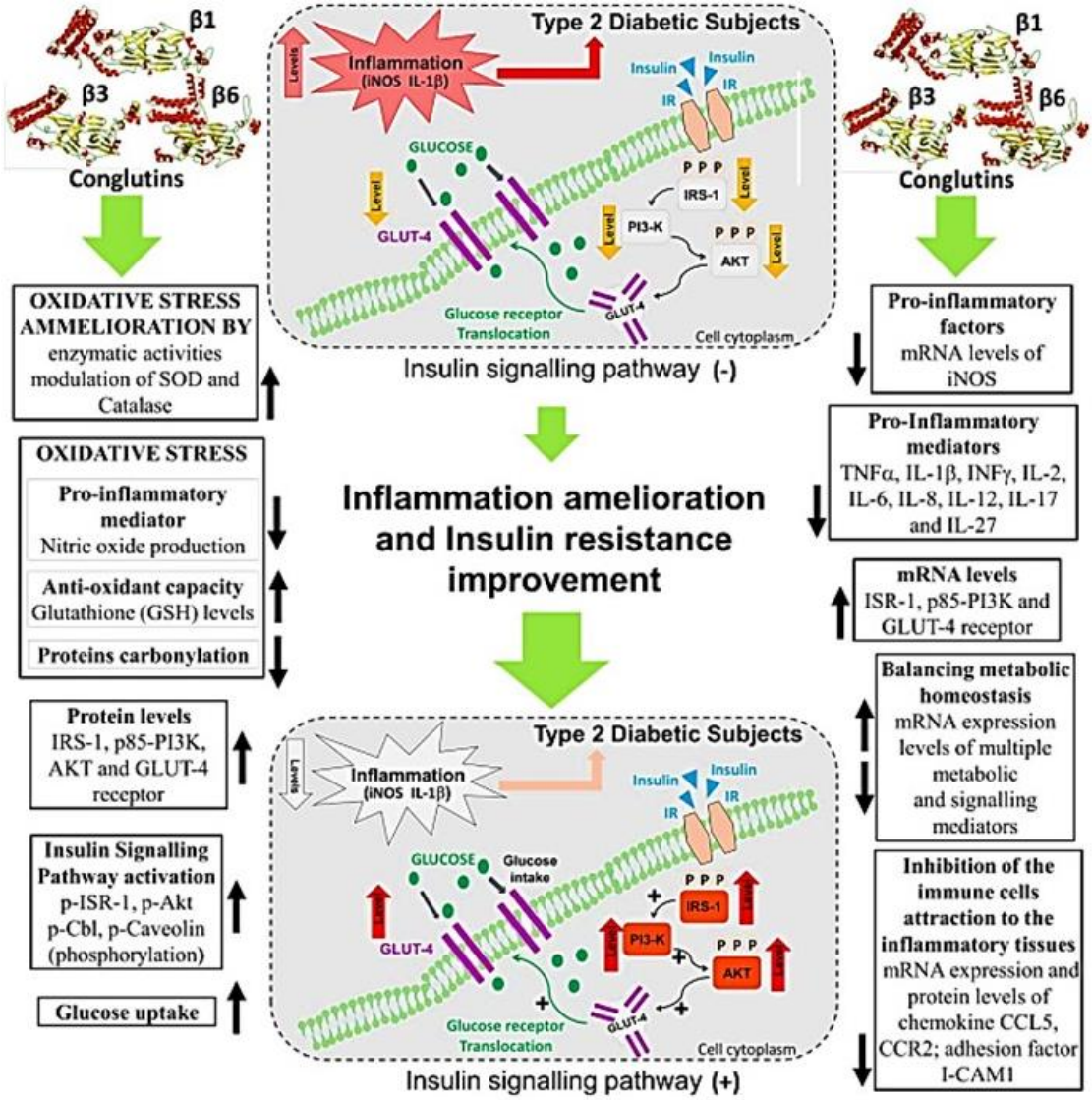


Figure 7. Molecular mechanisms of β-conglutins promoting nutraceutical properties. Insulin signalling pathway activation and type 2 diabetes state improvement are directly linked to the molecular mechanism induced by β-conglutins. Black arrows indicate increase (pointing up) or decrease (pointing down) of the action induced by β-conglutins and detailed in the boxes. (-) inhibited, (+) (Lima-Cabello *et al*, 2022 [141])

Research work using in vitro (pancreatic PANC-1 cell-line) and ex vivo (mononuclear cells from whole blood and peripheral blood from diabetic patients) cell culture approaches have shown different mechanistic actions of these proteins, making them strong new candidates to help fight T2DM. Mechanistic actions include their modulatory activity exerted on insulin

signaling pathway activation mediated by kinases. They promote the upregulation of the expression of genes such as IRS-1, p85-PI3k, AKT, and GLUT-4, as well as increasing their protein levels and activation (stimulating phosphorylation of these upstream modulators). This action was also accompanied and reinforced by decreased levels of modulatory factors such as iNOS and IL-1β, which greatly influence the development and progression of the T2DM [149–151]

Moreover, insulin resistance is a main contributor and causative problem leading to the development of the long-term T2DM complications. In this regard, NLL β-conglutin proteins are able to reverse insulin resistance through different pleiotropic effects such as (a) increasing glucose cellular uptake; (b) modulating key gene expression in the insulin-signaling pathway; (c) activation of the IRS-1/PI-3-kinase pathway through the phosphorylation of its upstream modulators, that is, IRS-1, as well as downstream mediators (Caveolin, CBL) that otherwise promote vesicular transport to the plasma membrane of glucose transporters; (d) improving insulin sensitivity; (e) regulating immune cell attraction and mobilization to the adipose tissues by controlling the expression of chemotaxis (i.e., CCL2 and CCL5) and molecular adhesion (i.e., ICAM-1 and VCAM-1) regulators; (f) decreasing cellular oxidative stress; and (g) promoting metabolic homeostasis and cell signaling [149–151] (h) in addition, it was found that among β1, β2, β3, β4 and β6-conglutin proteins were showed that β1, β3 and β6 could bind to insulin [155].

Furthermore, sustained inflammation causes and promotes development of many common diseases such as obesity linked to metabolic disease, insulin resistance leading to T2DM, dyslipidemia, and cardiovascular diseases. On the basis of the current ongoing research, NLL β-conglutins represent an outstanding alternative to tackle inflammation and inflammatory-related diseases at the molecular level. The mechanisms involved in the anti-inflammatory properties of NLL conglutin β1, β3, and β6 isoforms have been recently discovered. They

are related to their ability for (a) decreasing the production (downregulating gene expression and declining protein production) of pro-inflammatory mediators such as cytokines as TNF-α, INF-γ, IL-1β, IL-2, IL-6, IL-8, and IL-12 and inflammation modulators as NF-κB1; (b) while inhibiting chemotaxis and cell adhesion capacity of cells avoiding the enhancing inflammatory potential of immune cells after migration to pancreatic and adipose tissues [149,150]; (c) in addition to promote the reduction of the NO production and the down-regulation of the iNOS gene expression and protein production.

Therefore, oxidative stress (excessive levels of ROS generation) is also a main factor contributing to the onset and progression of T2DM under obesity or hyperglycaemic conditions, also promoting the development of insulin resistance. Hyperglycaemia damages the antioxidant defense system (glutathione production, superoxide dismutase and catalase activities), enhancing ROS production that contributes to mitochondrial dysfunction and in turn aggravates insulin resistance. NLL conglutins β1, β3, and β6 are newly discovered bioactive molecules with antioxidant activity [149,150] due to their capacity to downregulate iNOS gene expression, and to reduce NO production. They are also able to strongly reduce protein carbonylation, a covalent oxidative modification induced by ROS, leading to alterations in proteins activity. In addition, NLL β-conglutins increased the oxidative stress defense by promoting increase of glutathione levels in the cells, while regulated the activity of superoxide dismutase, and catalase [149,150].

d. Potential application of NLL β-conglutins for cancer and BC.

Due to their above explained anti-inflammatory and antioxidant related effects at the molecular level, as well as their ability to decrease insulin resistance by reverting the inflammatory effects in diabetic conditions, β-conglutin proteins

could be used in the prevention and treatment of inflammatory-based diseases, such as cardiovascular diseases, diabetes, obesity and/or cancer [135,150,151,161].

Summarizing, in the actual context of the increasing use of natural products in BC and the urgency to find new therapy strategies, leading to the implementation of new strategies improving treatments, prevention, side therapy effects and recurrence, the β-conglutin proteins from NLL and with demonstrated anti-inflammatory related effects have emerged as a potential natural and functional food alternative. In this doctoral thesis, these compounds are proposed as a potential preventive, cytotoxic and radiosensitizing treatment in BC: β1, β3 and β6 conglutin proteins from NLL could be natural modulators of different genetic pathways (as already tested in other diseases with chronic inflammation) that could regulate cell adhesion, chemotactic capacity of cells, migration, proliferation and death mechanisms in BC cell lines, also related to CSCs phenotypes, resistance to treatment and progression of BC. On top of that, this potential treatment would ensure beneficial properties for human health, such as cardiovascular, and be a very environmental healthy and inexpensive alternative for prevention and treatment of BC disease.

HYPOTHESIS

Based on all the recent evidences explained at the end of introduction section about anti-inflammatory related molecular properties of β-conglutins, and for extension their potential disease prevention and treatment for inflammation-related diseases, in addition to the current research about limitations of existing treatments like radiation and the emerging of new nutraceutical approaches in breast cancer (BC), the **hypothesis of this doctoral thesis** is:

β-conglutins from Narrow Leafed Lupin (*Lupinus angustifolius* L.) seeds could be natural preventive and cytotoxic agents for BC cell lines with a radiosensitizing effect on advanced malignant cells, mediated by the implication of Sirt1/FoxO1 and BMP's pathway in a p53 dependent manner, thus preserving the viability and properties of healthy epithelial cell lines.

OBJECTIVES

There are six main objectives of the doctoral thesis that are subdivided in multiple specific objectives.

Objectives:

1. To obtain and purify NLL β-conglutins isoforms that have not been studied until now on their inflammatory amelioration properties, and to identify the structural domain of key importance in the nutraceutical properties of NLL β-conglutins.
2. Obtain and purify β-conglutins from NLL for their use as a nutraceutical drug in *in vitro* assays for breast cancer (BC).
3. Test the efficacy of the β-conglutins treatment in an early stage BC model, the MCF-10A model of breast cancer, with a healthy epithelial cell lines as a control and three early tumoral breast cell lines derived from MCF-10A cells.
4. Assessment of the efficacy of the β-conglutins treatment in a BC model, with the same healthy epithelial line as a control and three BC cell lines.
5. Study the efficacy and role of the β-conglutins treatment as a pretreatment before cell irradiation, in order to propose those nutraceutical compounds as radiosensitizers for BC cells.
6. Elucidate the mechanism of action of these β-conglutins in different stages of BC and in single or combined treatment with irradiation.

In order to achieve the six main objectives of the doctoral thesis, **the specific objectives** are:

1. **Obtain and purify NLL β-conglutins for their use as newly discovered nutraceutical compounds in *in vitro* and *ex vivo* assays, and to unveil**

the structural domain of crucial relevance implicated in these nutraceutical properties.

a. Construction of expression plasmids for β-conglutins 5 and 7 and overexpression of those isoforms in bacteria.
b. Purification and identification of the two β-conglutins treatments.
c. Analysis of β-conglutin proteins structural modelling
d. Evaluation of their anti-inflammatory and antioxidant potential in epithelial cells, T2D patients cells and healthy controls cells.

2. **Obtain and purify NLL β-conglutins for their use as a nutraceutical drug in *in vitro* assays for BC.**
    a. Construction of expression plasmids for β-conglutins 1, 3 and 6 and overexpression of those isoforms in bacteria.
    b. Purification and identification of the three β-conglutins treatments.
    c. Purification using in order to ensure their use in cell lines *in vitro*

3. **Test the efficacy of the β-conglutins treatment in an early stage BC model, the MCF-10A model of breast cancer, with a healthy epithelial cell lines as a control and three early tumoral breast cell lines derived from MCF-10A cells.**
    a. Assess the viability and proliferation of the four cell lines after treatment with different doses of β-conglutins using MTT and Blue Trypan assays.
    b. Evaluate their clonogenical and differentiation potential after treatment.
    c. Characterize their mammosphere capacity in order to analyze stem-cell like properties and characterize CSCs by Flow Cytometry.

4. **Assessment of the efficacy of the β-conglutins treatment in a BC model, with the same healthy epithelial line as a control and three BC cell lines of advanced stages of the disease.**
    a. Test the viability and proliferation of the four cell lines after treatment with different doses of β-conglutins using MTT and Blue Trypan assays.
    b. Study apoptosis, ROS expression and DNA damage after treatment in order to evaluate the effect of β-conglutins on those cell lines.
    c. Characterize the stem-cell like properties and CSCs using three different techniques (ALDH1+ quantification, mammosphere assay and CSCs characterization by Flow Cytometry).

5. **Study the efficacy and role of the β-conglutins treatment as a pretreatment before cell irradiation, in order to propose those nutraceutical compounds as radiosensitizers for BC cells.**
    a. Analyze the colony formation fraction after combined β-conglutins and irradiation therapy *vs* standard irradiation, at different doses.
    b. Study apoptosis, ROS expression and DNA damage after combined therapy with both β-conglutins and irradiation vs standard irradiation, at 4 and 6 Gy.
    c. Characterize the stem-cell like properties and CSCs using three different techniques after combined therapy with both β-conglutins and irradiation vs standard irradiation, at 4 and 6 Gy.

6. **Elucidate the mechanism of action of these β-conglutins in different stages of BC and in single or combined treatment with irradiation.**
    a. Study the bone morphogenetic proteins (BMP) pathway in both early BC and normal BC cell models.

b. Investigate the role of the Sirt1/FoxO1 pathway and autophagy for both simple and combined treatment with irradiation for BC cell lines vs healthy control.
c. Propose a mechanism of action depending on p53 and other phenotypical characteristics of the cell lines, involving BMP genes, Sirt1/FoxO1 and caspase independent cell-death mechanisms.

MATERIAL AND METHODS

1. OVEREXPRESSION AND PURIFICATION OF β-CONGLUTIN ISOFORMS

To obtain β-conglutins proteins, specifically the β1, β3 β5, β6, and β7 isoform. This was performed by overexpression and purification, following a previously published protocol by Jiménez-Lopez *et al.* in 2016 [160] with modifications.

This protocol was performed following those six steps:

1.1 Construction of expression plasmids for each of the β-conglutin isoforms (β1, β3, β5, β6 and β7), complete and truncated (for β5 and β7) forms using a modified variant of a pET28a vector (Novagen) with a N-terminal polyhistidine (6xHis) and a pUC57 vector containing a synthetic gene encoding for each conglutin protein.

1.2 Overexpression of these β-conglutin isoforms using different expression induction methods (chemical and physical) in bacteria (*Escherichia coli*).

1.3 Purification of the β-conglutin isoforms by combination of biochemical techniques, (sonication, differential centrifugation, tandem affinity chromatography) following protocol recommendations in Jiménez-Lopez *et al.* in 2016 [160] using His-tagged proteins.

1.4 Identification of the three β-conglutin proteins previously obtained by means of SDS-PAGE and immunoblotting using a highly specific anti-conglutin β antibody (Agrisera, Sweden).

1.5 Structural assessment and analysis of NLL β-conglutins was performed by homology modeling.

2. CELL CULTURE

2.1.Cell lines

2.1.1. HepG2 model cell in vitro culture

HepG2 cells were obtained from CIC-UGR [85011430 (batch CB No 2440)]. This cell model is an immortalized cell line consisting of human liver carcinoma cells. They exhibit an epithelial-like morphology are are non-tumorigenic, with high proliferation rates [162]. They were grown in poly-L-lysine-coated 75 cm2 flasks (~2.0–2.5×106 cells/ml) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2mM glutamine and 10% heat-inactivated fetal bovine serum in the presence of 5% CO_2/95% air at 37 °C.

Cells were routinely subcultured and used in the exponential growth phase for all experiments. Cells were grown as monolayers and detached from cultures by trypsinization. Cells were washed twice with phosphate-buffered solution (PBS, Sigma) and treated with 0.25% tryp-EDTA (Lonza) for 10 min in 5% CO2/95% air at 37 °C humidified atmosphere.

The trypsinization effect was neutralized with culture medium. The cells were collected after centrifugation at 1000×g for 5 min and washed with PBS for viability assays. Cell counting and viability were checked using a Countess II FL Automated Cell Counter (Thermo Fisher) at the beginning and end of each experiment using representative wells. Viability was always > 95%. Cells were cultured to 80% confluence and then treated with LPS (1 μg/ml) for 24 h. The cells were treated with β5 or β7 purified proteins (complete or truncated forms, respectively) for 24 h alone or in combination with LPS. Aliquots of β1, β2, β3 β4, and β6 were, in this case, stored at −20 °C in PBS. The purified conglutins were thawed just before use and diluted in DMEM to desired concentrations. Ten μg of each purified conglutin (complete or truncated forms, respectively) was added to cultures, and

experiments were performed three times. After the treatments, cells were harvested for further analyses.

2.1.2. PMBC cell isolation and culture

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density-gradient centrifugation on Histopaque 1077 (Sigma, St. Louis, MO), washed three times in Hanks' balanced salt solution (Life Technologies, Grand Island, NY), and resuspended in complete medium consisting of RPMI 1640 (Sigma) supplemented with penicillin, streptomycin, and L-glutamine (100 U/ml, 100 μg/ml, and 2 mM, respectively) (Sigma).

Participants fasted for 12 h before blood collection (water only, no food intake). Venous blood was drawn into lithium–heparin tubes (BD Vacutainer System, Heidelberg, Germany) in the morning. Within 3 h, whole unseparated blood was diluted 1:3 with Dulbecco's modified Eagle's medium (DMEM) and HEPES 2.4% (Invitrogen), and agitated gently in 50 ml tubes (Greiner Bio-one); 1 ml aliquots were seeded in 24-well plates (Nunc, VWR International GmbH) and cultured for 24 h at 37 °C and 5% CO_2. From each blood draw, we performed triplicate incubations in parallel with positive and negative controls - separate cultures that included each (β5 and β7) purified β-conglutin (10 μg) including both, complete and truncated forms. The same lots of purified β-conglutin, respectively, LPS and PBS were used for all experiments. Blood cultures were removed from each well and centrifuged at 700g for 5 min at 20 °C. The supernatants were aliquoted and stored at −20 °C until further analysis [151].

Healthy control subjects and fourteen T2D patients were recruited for this study. All participants provided full written and informed consent for the procedures.

The T2DM group includes patients who were screened and diagnosed according to the American Diabetes Association guidelines. Furthermore, anthropometric data were collected:

- (A) Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters.

- (B) Blood pressure and heart rate were measured in a standardized manner. After subjects remained resting for a period of at least 5 min, blood pressure and heart rate were measured twice using a standard mercury sphygmomanometer and a heart rate monitor with a wrist receiver, respectively. In both cases, the mean of the two values was used for analysis.

- (C) We obtained samples of venous blood collected from the cubital vein in 4 mL lithium–heparin tubes.

- (D) Biochemical parameters were obtained. Fasting plasma glucose was measured by standard biochemical methods, where fasting can be defined as no caloric intake for at least 12 h.

- (E) Glycated haemoglobin (HbA1c) was measured using standard automated laboratory techniques, including high-performance liquid chromatography.

When calling patients diagnosed with T2DM for blood sample extraction, those receiving treatment for T2DM, e.g., metformin, sulfonylureas, thiazolidinedione or other medications that might affect the inflammation process, were excluded from this study.

2.1.3. Early stages of BC 2D model *in vitro*

The first BC model used for this doctoral thesis correspond to early stages of BC. This model originated from MCF-10A cells, purchased from the ATCC, that are non-tumorigenic healthy epithelial breast cells. Exposure of MCF-10A cells to BMP-2 and IL-6 led to the generation of the MC26 cell line that mimics

luminal breast tumors (*Chapellier et al., 2015*) at early stages. Since BMP2-mediated transformation was dependent on BMPR1B expression, sorted BMPR1B$^+$ MCF10A cells transformed after only a few weeks of BMP2 and IL6 chronic treatment. Three soft-agar clones from these BMP2/IL6 treated BMPR1B$^+$ MCF10A cells were selected and expanded in the presence of BMP2/IL6, giving rise to the M1B26 cell line, that mimics basal breast tumors with metastatic potential at early stages (Guyot *et al,* in submission).

A third stable BC cell line derived from MC26 cells was obtained after chronic exposure to 5-fluoracil (5-FU), a common chemotherapy agent for BC: this cell line, MC26-R correspond to an early BC luminal phenotype, resistant to chemotherapy with 5-FU.

The four cell lines (MCF-10A CT, M1B26, MC26 and MC26-R) were grown in an atmosphere of relative humidity and under standard conditions of temperature (37ºC) and CO_2 (5%). Cells were cultured using complete (DMEM)/F-12 nutrient mix medium with 2% horse serum (Life Technologies) and additional supplements: 100 ng/mL cholera enterotoxin, 10 μg/mL insulin (NovoRapid), 0.5 μg/mL hydrocortisone, 20 ng/mL epidermal growth factor EGF (all from Sigma, Saint Louis, USA), 100 units/mL penicillin and 100 mg/mL streptomycin (Life Technologies). M1B26, MC26 and MC26-R cells were cultured with the mentioned complete medium adding BMP-2 (5 ng/mL) and IL-6 (10 ng/mL). MC26-R medium was finally supplemented with 5-fluoracil (5-FU) at (2,5 μM) in order to ensure its resistance to chemotherapy with 5-FU. These three reagents were purchased by Sigma, Saint Louis, USA.

2.1.4. Normal/advanced BC 2D model *in vitro*

Three stable human breast cancer cell lines and a healthy non-tumorigenic epithelial cell lines were used for the normal BC model. The tumoral lines were: MCF-7 (ERα positive, weak for HER2), SK-BR-3 (ERα negative, HER2-positive)

and MDA-MB-231 (ERα negative, negative for HER2, triple negative). They were obtained from Horizon Discovery (Cambridge, UK). The epithelial and non-tumorigenic cell line was MCF-10A and was obtained from Horizon Discovery (Cambridge, UK) too [163].

The three tumoural cell lines were grown in an atmosphere of relative humidity and under standard conditions of temperature (37ºC) and CO_2 (5%). Those cells were cultured in a Laminar Flow Hood (TellstarBio-II-A/M), using Gibco Dulbecco's Modified Eagle Medium (DMEM) High-Glucose (Gibco, Carlsbad, CA, USA.) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 0,25 μg/mL amphotericin B and 2mM L-Glutamine. MCF-10A cells were grown in a different culture medium but under the same relative humidity, temperature and CO2 conditions. The medium used was DMEM/F-12 (Nutrient Mixture) from Gibco too, supplemented with 10% FBS, 1% penicillin-streptomycin, 0,25 μg/mL amphotericin B, 2mM L-Glutamine, 0,5 μg/mL hydrocortisone, 20 ng/ml epidermal growth factor (EGF) and 10 μg/mL Insulin. All the reagents used to supplement the culture medium were from Gibco, Carlsbad, CA, USA [163].

Cells were cultivated in monolayer until they had the optimal confluence and quantity in order to carry out the following experiments.

3. CELL IRRADIATION

BC cell lines MCF-10A, MCF-7, SK-BR-3 and MDA-MB-231 were seeded in 6 well-plates with absence or presence of the β-conglutin treatment. After 24 hours, the culture media containing (or not) this treatment was removed and replaced by normal culture medium. Then, cells were then irradiated with the X-ray equipment (Xylon Smart Maxishoot 200-E) at room temperature, under a constant current of 4.5 mA and 200kV of power, performing a range of radiation doses from 0 Gy (control) to 8Gy (0, 1, 2, 4, 6, and 8 Gy, respectively) using the field size of 15cm x 8xm with a focal distance of 15 cm.

Traceable dosimetry was performed following the TRS.398 protocol.

4. VIABILITY ASSAYS FOR BC MODELS

4.1. MTT assays

MTT viability assay was accomplished for all kind of cells, following the methodology based on the reduction of tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyl tetrazolium bromide), which gives a blue colored compound called formazan. This conversion is carried out by the mitochondrial enzyme succionate dehydrogenase, which only occurs when cells are in an active metabolic state. Additionally, the amount of formazan produced is directly proportional to the number of viable cells [163].

This assay was performed by seeding 4.000 cells per well in 96-well plates. Cells were allowed to grow overnight and treated with different concentrations of β-conglutins 1, 3 and 6 for 24, 48, and 72 hours. Then, 10μL of 5mg/ml MTT were added to each well and the cells were incubated at 37ºC and 5% of CO_2 for 4 hours. After this incubation, 100 μL of lysis buffer were added to each well. The optical density (absorbance) was measured using the Triad Multimode reader

(Dynex Technologies, Chantilly, VA, USA) at 570 nm. Non-treated cells were used as a control [163].

4.2. Trypan blue viability assays

The trypan blue viability assay is based on the principle that live cells possess intact membranes that exclude trypan blue dye, but dead cells do not, so the dye can permeate their cell membrane, allowing the counting of live cells (not-stained) and dead cells (stained) [163].

For this assay, the cells were seeded in 24 well plates (30.000 cells/well) and then were treated with the β-conglutin treatment for 24 hours. Then, cells were trypsinized and resuspended in PBS before staining with a trypan blue (BioRad Laboratories) solution containing 10 μL of cell solution and 10 μL of trypan blue dye at 0,4%. After 5 minutes of incubation, stained and non-stained cells were counted using an optical microscope, model *Olympus 1x51*, connected to a power supply *Olympus TH4-200*. Viability was calculated for each condition as (total live cells/total cells) x 100 and then, the mean percent viability for each cell line and condition was determined comparing live cells in each conditions to the live cells in the non-treated control [163].

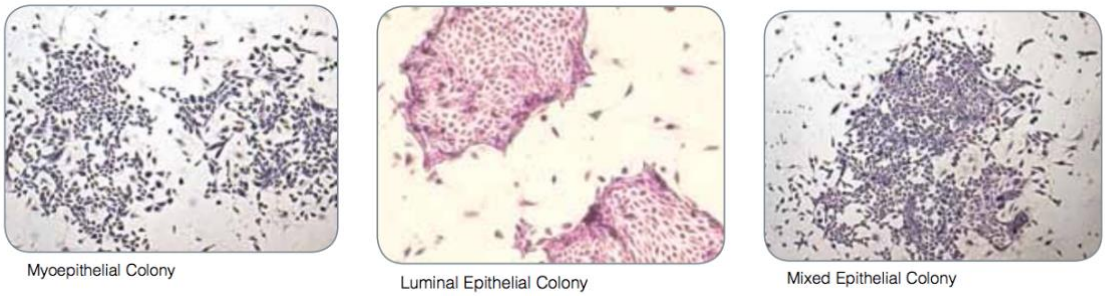
4.3.E-CFC (Epithelial Colony Formation Cell) assay for early BC cells derived from MCF-10A.

The E-CFC (mammary CFC) assay is a differentiation test that determines the frequency and immaturity of progenitor cells in a population of primary mammary cells. The cells are seeded at a limiting dilution, the progenitors will proliferate and the descendants will differentiate according to their lineage and produce colonies. Bi-lineage colonies are derived from bipotent progenitors, myoepithelial colonies from myoepithelial progenitors, and luminal

colonies from luminal progenitors. This test was firstly developed for primary cells because cell lines do not contain “true” stem and progenitor cells, but recent evidence has showed that it is possible to obtain "luminal-like" and "myoepithelial-like" colonies with MCF-10A cells.

This assay was then performed for MCF-10A cells and the three early BC cell lines derived from them. The aim was to study if the β-conglutin treatment had any impact on the number or distribution of "luminal-like", "myoepithelial-like" o mixed colonies, in order to elucidate if the treatment could have any impact on the differentiation progress of those early BC stages cell lines.

The protocol used to perform this assay started with the seeding of NIH3T3 irradiated feeder cells onto 12-well plates (32.000 cells/well). Those cells provide growth factors and matrix for our MCF-10A derived cells. NIH3T3 cells (embryonic fibroblasts) were previously cultured in 10% FBS DMEM medium and then irradiated before trypsinized and seeded. Simultaneously, our four breast cell lines were trypsinized after the β-conglutin treatment and seeded (250 cells) in the same 12-well plates. The medium used was the DMEM/F-12, normally used for the culture of our cells, but without BMP-2, IL-6 or 5-FU. After 4 or 5 days of colony growth, medium was removed and wells were double-washed with cold PBS. Then, 1 mL of 100% ice-cold methanol was added to each well and incubate for 15 minutes in agitation. After that, methanol was removed, wells were allowed to dry and wright colorant was added for 2-3 min to each well. After rinsing wells with deionized water and let dry overnight, colonies were counted and classified as "luminal-like", "myoepithelial-like" o mixed colonies depending on their characteristics, as shown in Figure 8:



(**a**) (**b**) (**c**)

Figure 8: Types of colonies after E-CFC assay in MCF-10A like cells. From: https://cdn.stemcell.com/media/files/brochure/BR29188Mammary_Epithelial_Cells_Standardized_Media_Reagents.pdf

4.4. Colony formation assay with irradiation for advanced BC cells

The colony formation assay experiments allowed the estimation of cell survival in the four cell lines (MCF-10A and the three BC cell lines, MCF-7, SK-BR-3 and MDA-MB-231) by means of dose-response curves. The irradiation was carried out following Material and Methods, *section 3*. Cells were seeded in 6-well plates with serial cell concentrations starting from a number of cells for non-irradiated control and 2 Gy irradiation dose and duplicating for each dose augmentation (4, 6 and 8 Gy). The serial concentrations used went from 500 to 4000 cells for MCF-7, 1500 to 12000 cells for SK-BR-3 and 1000 to 8000 cells for MDA-MB-231.

After the irradiation protocol, with presence or absence of the 24 hour β-conglutin treatment, cells were kept in the incubator for at least two weeks, depending on the growth for each cell lines. Then, the culture medium was removed and each well was stained adding gentian violet dye (ICN Biomedicals, 101775) for 2-3 minutes. Then, wells were washed with water the plates were allowed to dry overnight. The number of colonies was counted using the optical microscope model Olympus 1X51, connected to an Olympus TH4-200 power. A colony was defined as a grouping of cells with

at least 50 cells and with no signs of abortive colony (non-homogeneous colony, presenting holes in the center of the colony or any visual signs of cellular death). All experiments were performed in triplicate for each condition.

The cell survival fraction was calculated as follows:

FS = Number of colonies counted / (Number of seeded cells for this condition X cloning efficiency)

The survival data obtained was fitted to a linear-quadratic model and this model gave us the values for the survival fraction at 2 Gy (FS2) and the α and β parameters of the cell survival curve as indicators of intrinsic radiosensitivity. The mathematical equation of the linear quadratic (L-Q) model is as follows:

$$FS = \exp [- (\alpha D + \beta D^2)]$$

5. APOPTOSIS ASSAY

Apoptosis was analysed using the IP-Annexin V kit (BD Biosciences, Franklin Lakes, NJ, USA). 200.000 cells were seeded in 6-well plates for each cell line (MCF-10A, MCF-7, SK-BR-3 and MDA-MB-231) and treated with β-conglutins for 24 hours, in combination or not with the irradiation protocol, as previously described . Then, they were trypsinized, washed and incubated with both AnnexinV-FITC and propidium iodide for 15 minutes in the dark. Samples were immediately analyzed using the BD FACS Aria IIIu Flow Cytometer (Becton Dickinson, BD Bioscience) from the Cytometry and Microscopy Research Service of the Biosanitary Research Institute of Granada (ibs.GRANADA) [163].

For the ferroptosis assay, cells were incubated for 24 h with ferrostatin-1, an inhibitor of ferroptosis, diluted 1:1000, and the same protocol was then performed. Ferroptosis percentage was calculated with the difference between the percentage of AnnexinV negative/Propidium Iodide positive cells (necrotic cells) in the non-treated with ferrostatin-1 condition and the treated ones [163].

For both apoptosis and ferroptosis, AnnexinV-FITC was detected by a blue laser (488 nm) FSC, with a 502 LP (Long Pass) and a 530/30 filter. Propidium iodide-PI was detected by 561 YGL laser (561 nm), with a 600 LP and 610/20 filter. Apoptosis was calculated by adding both apoptotic cells (AnnexinV positive/Propidium iodide negative population) and late apoptotic cells (AnnexinV positive/Propidium iodide positive population) [163].

5.1. Caspase 3 activity

Caspase 3 activity was studied by performing Western Blot (see *Material and methods, section 9*) and incubating for 24 h with cleaved and total caspase 3 antibodies from Abcam, Cambridge, UK. Then, membranes were incubated for 1 h with anti-rabbit secondary antibody from Santa Cruz Biotechnology, Inc.,

Dallas, TX, USA. Proteins were detected and images were quantified using the *Material and methods 9* protocol. For this concrete experiment, the significant activation of caspase 3, an executor caspase, accompanied by an increased apoptotic percentage for that condition would be interpreted as an activation of caspase-dependent apoptosis [163].

6. REACTIVE OXYGEN SPECIES (ROS) QUANTIFICATION

6.1.NITRIC OXIDE DETECTION

The total amount of NO production including nitrite/nitrate contents from cultured cell supernatants of control subjects, T2D patients and HepG2 cells was measured following the recommended protocol using a commercial assay kit ab65328 (Abcam, Cambridge, UK). Briefly, supernatants were collected and centrifuged to remove cell debris. Plasma from all experimental groups was deproteinized according to the manufacturer's instructions. An equal amount (30 μl) of samples and standards were loaded into 96-well microtiter plates. A volume of nitrate reductase, enzyme cofactor, and assay buffer was added, followed by 1 h incubation at room temperature by Enhancer, Griess Reagent R1 and Greiss Reagent R2. Immediately after incubation, absorbance at 540 nm was detected with a microplate reader (iMark Biorad), and the value of the blank control (medium without cells) was subtracted. Total nitrite/nitrate concentrations were calculated with the calibrated standard curve.

6.2.ANTIOXIDANT ENZYMATIC ACTIVITY ASSAYS

The cell cultures were prepared, and after 24 h of incubation of the treated culture individually with β5 and β7 (whole protein and truncated forms, respectively), growing media was removed, and cells washed with PBS at 4C.

Cells from the different challenged with the different ◎-conglutin protein forms were collected and used for the enzymatic activity assessment of SOD and catalase, as well as the GSH measurement (Canvax, Córdoba, Spain), following manufacturer's instructions.

6.3.ROS QUANTIFICATION FOR BC SAMPLES

Intracellular reactive oxidative species (ROS) were monitored seeding 3×10^3 cells/well in a 96-well plate and performing the treatment with β-conglutins for 24 hours in combination or not with the irradiation protocol, as previously described. Then, intracellular ROS level detection protocol was performed using DCFH-DA (2′7′-Dichlorofluorescein diacetate BioReagent, suitable for fluorescence) for ROS detection (Sigma-Aldrich, St. Louis, MO, USA), performing the following protocol. Culture medium was removed after treatment time and cells were washed twice with PBS. Then, those cells were incubated with 100 μL of a 10 μM DCFH-DA concentration in serum-free medium for 30 min in the dark and inside the incubator, in order to ensure cell culture conditions. Finally, fluorescence was measured in a triad multimode reader using a wavelength of excitation of 485 nm and a wavelength of emission of 525 nm. Results of fluorescence in RFU were comparable to intracellular ROS levels in the cell lines, assuming higher levels for higher fluorescence [163].

7. DNA DAMAGE

DNA damage of the different cell lines was quantified using the γH2Ax detection kit BD Cytofix/Cytoperm from BD Biosciences, Cat. 554714 and PE-CF594 Mouse anti-H2Ax (pS139) antibody from BD Biosciences too, Cat. 564719. In this case, 2×10^5 cells were seeded in 6-well plates and then treated with the β-conglutins for 24 hours in combination or not with the irradiation protocol, as

described for the rest of experiments. After the treatments, cells were trypsinized, double washed, fixed, and permeabilized using the fixation and permeabilization buffer of the mentioned kit for 30 minutes at room temperature (RT). Finally, fixed and permeabilized cells were incubated with 100 μL of the BD Perm/Wash buffer 1X (from the same kit) for 15 minutes at RT and the anti-H2Ax antibody for 30 min in the dark at RT. Samples were immediately analyzed by flow cytometry using the BD FACS Aria IIIu Flow Cytometer (Becton Dickinson, BD Bioscience) from the Cytometry and microscopy research service of the Biosanitary Research Institute of Granada (ibs.GRANADA) [163].

For the detection of the γH2Ax population, the PE-Texas red fluorochrome was detected by a 561 yellow green laser (561 nm), with a 600 LP and 610/20 filter.

8. CANCER STEM CELLS (CSCs) QUANTIFICACTION AND CHARACTERIZATION

8.1. Quantification of Aldefluor-1 (ALDH1) positive enzyme activity

To detect aldehyde dehydrogenase 1 (ALDH1) activity, that is directly related to the number of CSCs, the aldefluor kit (Stem Cell Technologies) was used after the treatment with the β-conglutins for 24 hour in combination or not with the irradiation. After the treatments, cells were trypsinized, washed and incubated with BODIPY-amino acetaldehyde (BAAA), a fluorescent non-toxic substrate for ALDH, which was converted into BODIPY-aminoacate (BAA) and retained inside the cells. Viable ALDH1+ cells were quantified by flow cytometry on a BD FACS Aria IIIu Flow Cytometer (Becton Dickinson, BD Bioscience) from the cytometry and microscopy research service of the Biosanitary Research Institute of Granada (ibs.GRANADA). The specific inhibitor of ALDH, diethylaminobenzaldehyde (DEAB), was used to control for background fluorescence. ALDH1 positive cells were quantified with a FITC fluorochrome

that was detected by a Blue Laser (488 nm) FSC, with a 502 LP (Long Pass) and a 530/30 filter [163].

8.2. Characterization of CSCs by Flow Cytometry

The characterization of CSCs was performed according to cell surface markers using CD44-PE and CD24-FITC antibodies (Biolegend, San Diego, CA, USA) after treatment with the β-conglutins for 24 hours in combination or not with the irradiation protocol. After their trypsinization and washing with cold PBS, cells were incubated for 30 min in darkness and at 4 °C with both antibodies, having an individual non-stained control and two single CD24 or CD44 stained tubes for each cell line. All samples were then analyzed using a BD FACSAria IIIu flow cytometry (Becton Dickinson, BD Biosciences) from the cytometry and microscopy research service of the Biosanitary Research Institute of Granada (ibs.GRANADA). CD24-FITC antibody was detected by a blue laser (488 nm) FSC, with a 502 LP (Long Pass) and a 530/30 filter. CD44-PE was detected by 561 YGL laser (561 nm), with a 582/15 filter [163].

8.3. Mammospheres formation assay

To test the capacity to form spheres and its frequency, that is directly related to the number of cancer stem cells (CSC's) and the self-renewal capacity of cells, the sphere formation assay was carried out with the β-conglutins treatment (for both early stages of BC model *in vitro* and advanced BC model *in vitro*) or in combination with irradiation (for the advanced BC model).

For the early BC model *in vitro*, the sphere medium was mammary epithelial cell basal medium (MEBM) supplemented with 1X B27, 20 ng/mL bFGF2, 20 ng/mL EGF and 4U/mL heparin. For the normal or advanced BC model, the medium for sphere's growth was DMEM/F12 supplemented with 1%

P/S (penicillin/streptomycin), B27 10 μg/mL, 1 μg/mL hydrocortisone, 4 ng/mL Heparin, 10 ng/mL EGF, and 20 ng/mL FGF.

Cells were seeded with culture medium in 6 well-plate 2 days before starting the sphere formation assay and then treated for 48 hours (early BC model) or 24 hours (advanced BC model) with the β-conglutins 1, 3, and 6 as previously described for other experiments. For the combined treatment with irradiation, the normal culture medium containing the β-conglutin treatment was removed after 24 hours and cells were irradiated following *Material and Methods 3* protocol and left for another 24 hours in the incubator. Once the desired treatment was performed, cells were trypsinized and, from each condition, a 12-well triplicate with 1×10^2 cells/well was seeded in ultra-low attachment plates (Corning) in sphere culture medium prepared following the previous point. Then, spheres were allowed to grow in the incubator for 5–8 days. All reagents were purchased from Gibco (Carlsbad, CA, USA). Spheres > 50 μm diameter were counted with Leica DM500 binocular microscope from the cytometry and microscopy research service of the Biosanitary Research Institute of Granada (ibs.GRANADA) [163].

9. PROTEIN COLLECTION AND WESTERN BLOT

9.1. SDS-PAGE PROTEIN SEPARATION AND INMUNOBLOTTING FOR β-CONGLUTINS

In order to check purified β-conglutin samples, 15 μg of truncated conglutin β5 and β7 were separated by SDS-PAGE on 4–20% Mini-PROTEAN® TGX™ precast Gels (Bio-Rad) with the Mini- PROTEAN® Tetra Cell apparatus (Bio-Rad). After electrophoresis, proteins were stained with Coomassie Brilliant Blue. Proteins were electroblotted onto a PVDF membrane using a Mini-Trans-Blot Electrophoretic Transfer Cell system (Bio-Rad). The membranes were blocked for 2 h in blocking solution containing 5% (w/v) non-fat dry milk in Tris-

buffered saline (TBS) buffer, pH 7.4. Immunodetection of β-conglutin proteins was carried out by incubation with rabbit polyclonal antiserum developed in this study (see above), diluted 1:1000 in TBS buffer containing 5% (w/v) non-fat dry milk and 0.5% Tween-20. A horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Bio-Rad) served as the secondary antibody, and was diluted 1:3500 in TBS buffer and 0.5% Tween-20, and then incubated for 2 h, followed by three washing steps of 15 min each with TBS containing 0.5% Tween-20. Chemiluminescence Kit (Bio-Rad) according to the manufacturer's instructions and was visualized using the LI-COR C-DiGit Chemiluminescence Western Blot Scanner.

9.2. PROTEIN COLLECTION AND WESTERN BLOTTING FOR BC SAMPLES

Trypsinized cells which were double washed with ice-cold PBS (after treatments) and incubated with RIPA lysis and extraction buffer supplemented with protease inhibitors (Santa Cruz Biotechnology). Proteins were quantified using the Bradford assay, denatured, and subsequently separated on SDS-polyacrylamide gels. After the electrophoresis, gels were transferred to PVDF membranes using the Bio-Rad Trans Blot Turbo transfer system (Bio-Rad Laboratories, Inc., USA). Membranes were then incubated with the appropriate primary antibodies against SIRT-1, FoxO1, Cleaved and Total Caspase 3 (Material and Methods, *section 5.1*), and β-Actin (Abcam, Cambridge, UK) overnight and then secondary antibodies from Santa Cruz Biotechnology, Inc., Dallas, TX, USA, for 1 h. Finally, the proteins were detected using Amersham ECL select western blotting detection reagent (GE Healthcare, UK) on the membrane and acquiring the images with ChemiDoc MP imaging system (Bio-Rad Laboratories, Inc., USA). Western blot images were analyzed and quantified using FiJi software. Densitometry was performed for each membrane and the area and mean intensity of each condition were calculated. Finally, the ratio

between the β-actin control and the protein of interest was calculated and compared with the non-treated control ratio in each case [163].

Autophagy was studied by performing western blot (section *2.10*) and incubating for 24 h with both anti-SQSTM1/p62 antibody and anti-LC3B antibody from Abcam, Cambridge, UK. Then, membranes were incubated for 1 h with anti-rabbit and anti-mouse secondary antibodies, respectively, from Santa Cruz Biotechnology, Inc., Dallas, TX, USA. The activation of autophagy was detected if a decreased protein level of p62 was accompanied by an increased protein level of LC3B. The opposite situation describes the downregulation of the autophagy process [163].

10. ELISA ASSAYS FOR CYTOKINES, TNF-α, AND INFLAMMATORY PATHWAY QUANTIFICATION

After cell culture and β5 and β7 conglutin proteins (whole protein and truncated forms, respectively) challenges, the cells were counted and plated in six well plates (106 cells per well), with two wells per group. All experiments were made in triplicate to confirm data and reduce variation among samples.

After 24 h incubation, the treated culture media was removed and cells washed with 4 °C PBS. During protein extraction, the plates were kept on ice to avoid denaturation of cytokines. Each well was filled with 100 μL of buffer (150mM sodium chloride, 1.0% NP-40, 50mM Tris pH 8), supplemented with 1 μl of protease inhibitor (Sigma), and remained in contact with the cells for 15 s. The cells were then vigorously scraped from the bottom of the wells and transferred to a micro centrifuge tube. The tubes were centrifuged in a refrigerated centrifuge (4 °C) at 12,500×g for 15 min. After centrifugation, the supernatant was collected and 1:4 diluted for the ELISA sets for IL-1◎, IL-2, IL-6, IL-8, IL12, IL-27, TNF-α, MPO, S-TNF-R1, S-TNF-R2 and Tweak proteins (Diaclone) following the manufacturer's instructions.

11. RNA EXTRACTION and REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION (RT-qPCR)

11.1. PCR ARRAY FOR INFLAMMATORY RESPONSE

Real-time quantitative PCR technology was used to assay TNF-α, IL-1β and iNOS mRNA expression from each experimental group. Total RNA was extracted from HepG2 cells and isolated PBMC of type 2 diabetic patients and control subjects using an RNeasy Mini Kit (Qiagen). Two μg total RNA was converted to complementary DNA (cDNA) a High-Capacity cDNA Archive Kit (Applied Biosystems, Waltham, MA, USA). cDNA was prepared, diluted and subjected to real-time polymerase chain reaction (PCR), and amplified using TaqMan technology (LightCycler 480 quantitative PCR System, Roche, Basel, Switzerland) for gene expression assays. Primers and probes were used from the commercially available TaqMan Gene Expression Assays [TNFα: Hs01555410_m1, IL-1β: Hs01075529_m1, iNOS:Hs00174128_m1, respectively]. Quantitative real-time PCR (qPCR) was performed using a Light Cycler® 480 (Roche), where samples were run as duplicates for 40 cycles: 1 cycle of 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 40 cycles of 60 °C for 1 min. Cycle thresholds were measured, and the relative expression of genes was calculated by comparising Ct values. The difference in gene expression was evaluated by calculating the fold-change in expression levels based on at least a 2-fold up or down change in comparison to the gene expression levels of the control group. All gene expression levels were normalized to the housekeeping gene β beta (Assay ID: Hs99999903_m1, Applied Biosystems), which did not vary significantly between the study groups.

11.2. RNA ISOLATION AND EXTRACTION FOR BC SAMPLES

Total RNA from different conditions of the healthy MCF-10A cells and the rest of BC cell lines used was extracted from duplicate wells with a certain confluence in monolayer after 24 hours (advanced BC model) or 48 hours (early stages of BC model) treatments with β-conglutins 1, 3, and 6. For the combined treatment with irradiation, total RNA was isolated 24 hours after irradiation doses.

Total RNA was obtained using Trizol, tri-reagent following the instructions of the manufacturer (Sigma-Aldrich, St Louis, MO, USA). In our case, 350 μL of trizol tri-reagent was directly added to each duplicate well (6 well-plates) and RNA lysate was transferred to sterile and nuclease free eppendorfs in order to execute the RNA extraction with direct-zol RNA microprep kit (ZymoResearch, Ref: R2060) following the manufacturer protocol. The RNA lysate in trizol was directly diluted with 350 μL of pure ethanol and added to the zymo-spin column. After a series of consecutive centrifuges and incubations with the different buffers of the kit, RNA was binded, washed and eluted, obtaining around 15 μL of RNA for each initial trizol lysate.

11.3. RNA QUANTIFICATION FOR BC SAMPLES

For quantification of RNA, we proceed to the reading of the absorbance at 260 and 280 nm using a NanoDrop (NanoDrop™ 2000/2000c Spectrophotometers, Thermo Scientific ™). The OD260/OD280 relationship allowed us to calculate the purity of nucleic acids, whereas an optimal range of values between 1.8 and 2. RNA concentration was calculated considering an OD unit at 260 nm corresponds to a concentration of 40 mg/ml nucleic acid. The purified RNA samples were placed at 80°C for long-term storage.

11.4. REVERSE TRANSCRIPTION FOR BC SAMPLES

cDNA was synthesized by reverse transcription of total RNA using the PrimeScript RT reagent kit (Perfect Real Time) from TakaraBio, Ref: RR037A. This kit contains all the components needed for reverse transcription. The volume of each reaction was adjusted to 20 μL in each nuclease-free mini eppendorf tube. For each reaction 1 μg of the extracted RNA was used and all the buffers were added following the same order. First, 2 μL of PrimeScript buffer 5X, then 0,5 μL of oligo DT primer followed by 0,5 μL random 6-mers, then 1,5 μL of DNAse free H2O and, finally, 0,5 μL of PrimeScript RT enzyme mix I. The reaction was mixed pipetting all the reagents. The reverse transcriptase reaction was performed in a thermocycler (DOPPIO thermal cycler, VWR). The reaction tubes were heated at 37 ° C for 15 min and then the temperature rose to 85 ° C for 5 s, to come finally back to 4 ° C. Finally, cDNA were kept on ice for 5 min, and the cDNA was stored at -20 ° C.

11.5. RT-qPCR FOR BC SAMPLES

RT-qPCR assay was done using TB Green Premix Ex Taq (Tli RNase H Plus) from TakaraBio, Ref: RR420L. Each experiment was done in duplicate and reactions were performed in triplicate using the CFX384TM Real-Time PCR Systems from BioRad. The comparative threshold cycle (Ct) method was used to calculate the amplification factor. The process steps for qPCR was a holding stage (Step 1, 95℃, 30 sec) to polymerase activation/denaturation and a cycling stage (40 amplification cycles at Step1 95℃, 5 seconds and a second step of 60℃ for 30 sec). Primers used for RT-qPCR assays for both early and advanced BC models are shown in Table 1.

Table 1: Primers for RT-qPCR assays in BC models.

Primer	Forward	Reverse
BMP2	ACG CTC TTT CAA TGG ACG TG	GGA AGC AGC AAC GCT AGA AG
BMP4	CTT TAC CGG CTT CAG TCT GG	GGG ATG CTG CTG AGG TTA AA
BMPR1A	GAA AAA GTG GCG GTG AAA GT	TAG AGC TGA GTC CAG GAA CC
BMPR1B	CTG TGG TCA CTT CTG GTT GC	TTC CTT TCT GTG CAG CAT TC
BMPR2	TAG CAC CTG CTA TGG CCT TT	CTG AAT TGA GGG AGG AGT GG
ID1	GGT GCG CTG TCT GTC TGA G	TGT CGT AGA GCA GCA CGT TT
ID2	TAT TGT CAG CCT GCA TCA CC	AAT TCA GAA GCC TGC AAG GA
ID3	AAA TCC TAC AGC GCG TCA TC	AAG CTC CTT TTG TCG TTG GA
SMAD6	CTG CAA CCC CTA CCA CTT CA	AGA ATT CAC CCG GAG CAG T
CD10	CTG GAG ATT CAT AAT GGA TCT TGT AAG CAG C	CCA TCC AAG TGA GGT CAT CTA AAG TCT G
TBP	CAC GAA CCA CGG CAC TGA TT	TTT TCT TGC TGC CAG TCT GGA C

UBQ	TGG GAT GCA AAT CTT CGT GAA GAA GAC CCT GAC	ACC AAG TGC AGA GTG GAC TCT TTC TGG ATG

12. STATISTICAL ANALYSIS

The ELISA, NO determination and antioxidant enzymatic activity assays results were statistically evaluated using one-way ANOVA followed by either Games-Howell or Tukey HSD post-hoc tests (depending on whether variances were or not homogeneous, respectively). Statistical analyses were conducted at $p < 0.05$ level using the SPSS Statistics 27 software (IBM, Armonk, NY, USA). Values shown are expressed as means ± S.E.M. of the three individual experiments.

Regarding BC models, All experiments were performed at least in triplicates and the results were expressed as mean ± standard deviation, unless otherwise indicated. Statistical analyses were performed using the Shapiro-Wilk test to analyse the normality of the data set and the Two-Way Anova analysis, with Dunnett or Tukey correction using the Graphad Prism 9 software. Statistical differences between samples were considered significant when p values were $p <0.05$ (*), $p <0.01$ (**), or $p <0.001$ (***) [163].

Statistical differences between samples were considered significant when p values were $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***). Similarity matrices were performed in Microsoft Excel and XLSTAT programs, considering a dissimilarity threshold of 0.95 [163].

13. β-CONGLUTIN PROTEINS STRUCTURAL MODELLING

β5 and β7 NLL conglutin sequences Uniprot accession number F5B8W3 and F5B8W5, respectively) where used as query to search characteristic patterns, as well as functional (biologically meaningful) motifs using the PROSITE database (http://prosite.expasy.org/). Additional domain architecture analyses of these proteins was performed by using:

- Pfam v25.0 (http://pfam.sanger.ac.uk/)
- SMART v6.0 (http://smart.embl-heidelberg.de/)
- Conserved Domain Database (CDD) v3.02
- CDART (Conserved Domain Architecture Retrieval Tool) and CD-Search tools (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml/)
- InterPRO v35.0 (http://www.ebi.ac.uk/interpro/)
- ProDom release 2010.1

(http://prodom.prabi.fr/prodom/current/html/home.php/)

- CATH v3.4 (http://www.cathdb.info/)
- Superfamily v1.75 (http://supfam.cs.bris.ac.uk/SUPERFAMILY/)
- PIRSF (http://pir.georgetown.edu/pirwww/dbinfo/pirsf.shtml/)
- Functional search using PANTHER (http://www.pantherdb.org/).

Protein Data Bank (PDB) was searched for β-conglutin protein homology. The best homologous templates for β5 and β7 were selected by BLAST server (http://ncbi.nlm.nih.gov/). The BioInfoBank Metaserver (http://meta.bioinfo.pl/) and Swiss-model server for template identification (swissmodel.expasy.org) were also used for templates selection. The best templates were retrieved from PDB database and used for homology modeling. An initial structural model was analyzed for recognition of errors in 3D structure by using ProSA (prosa.services.came.sbg.ac.at/prosa.php), and for a first overall quality

estimation of the model with QMEAN (swissmodel.expasy.org/qmean/cgi/index.cgi).

Final β5 and β7 structures were subjected to energy minimization with GROMOS96 force field energy implemented in DeepView/Swiss-PDBViewer v3.7 (spdbv.vitalit.ch) to improve the van der Waals contacts and correct the stereochemistry of the model. The final quality of the models was assessed by QMEAN, the stereology with PROCHECK (www.ebi.ac.uk/thornton-srv/software/PROCHECK), and ProSA(prosa.services.came.sbg.ac.at/prosa.php) programs, and the protein energy with ANOLEA (protein.bio.puc.cl/cardex/servers/anolea). The Ramachandran plot statistics for the models were calculated to show the number of protein residues in the favoured regions.The evolutionary conservational analysis was perform using ConSurf server (consurf.tau.ac.il) through generating evolutionary related conservation scores, helping us to identify functional region in the proteins. Functional and structural key residues in the β-conglutin sequences were finally confirmed by ConSeq server.

RESULTS

CHAPTER I. METHODOLOGICAL SET UP OF THE PRODUCTION AND PURIFICATION OF NLL β-CONGLUTIN FUNCTIONAL PROTEIN ISOFORMS.

a. β-CONGLUTIN ISOFORM GENETIC CONSTRUCTS AND OVER-EXPRESSION ASSESSMENT

β-conglutins were cloned and overexpressed using the pET28b construct (Novogen) that contains an N-terminal polyhistidine (6xHis) tag. pUC57 vectors carried synthesized β conglutin sequences based on Genbank HQ670409 (β1), HQ670411 (β3), HQ670413 (β5), HQ670414 (β6), HQ670415 (β7) and truncated forms of β5 and β7 conglutin.

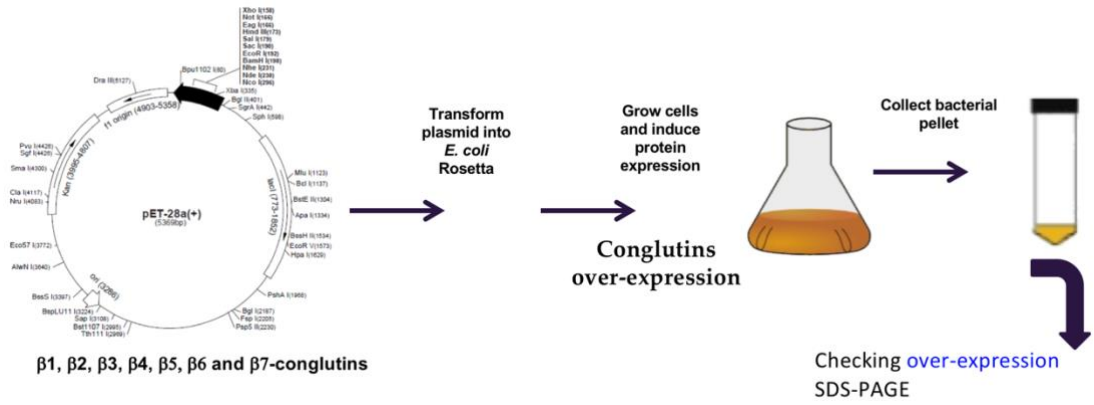


Figure 9. Cloning of the 7 β-conglutin isoforms using the pET28b construct (Novogen) that contains an N-terminal polyhistidine (6xHis) tag and bacterial overexpression.

Figure 9 shows a simplified scheme of the process of construction and over-expression of β-conglutins. In summary, sequences were altered for optimum bacterial codon usage with NcoI/XhoI restriction enzyme linkers, that were synthesized and constructed by GenScript. The bacterial expression vectors for β-conglutins were obtained via NcoI/XhoI digestion of respective pUC57-β-conglutin constructs followed by ligation of the β-conglutin fragments into the pET28b vector. We used Rosetta™ 2(DE3) pLysS Singles™ Competent Cells

(Novagen) following induction protocol previously set up by Jimenez-Lopez et al. 2016 [160] with modifications in the case of each conglutin.

b. β-CONGLUTINS ISOFORMS PURIFICATION BY AFFINITY-CHROMATOGRAPHY

Purification of β-conglutins was accomplished by protein elution performed with imidazole linear gradient (15–380 mM) on the niquel colums, where 2 mL fractions were collected. In order to know which fractions contained recombinant proteins, chromatographic fractions were analyzed using SDS-PAGE. The SDS–PAGE analyses of the eluted fractions showed a single protein band of approximately 50 kDa (Figure 10A), where recombinant β-conglutins exhibited a level of purity > 95%, and a concentration of 17–35 mg/mL.

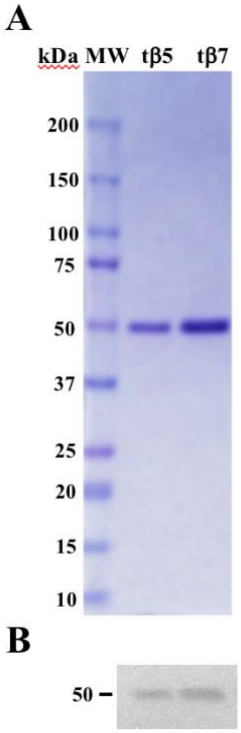


Figure 10. Purification and identification of truncated β5 and β7: truncated conglutin β5 proteins (tβ5) and truncated conglutin β7 proteins (tβ7). In A), two purified β-conglutin truncated proteins were stained with Coomassie, with a high level of purity (> 95%). In (B), immunoblotting shows the same two purified β-conglutin proteins identified by the

antiβ -conglutin antibody. MW column shows molecular weight standard in kilodalton (kDa).

To further confirm the identity of these proteins, we performed an analysis by immunoblotting using anti-β-conglutin protein antibody which confirmed the identity of the recombinant proteins (Figure 10B). Figure 10 shows an example regarding β5 and β7 proteins. All the recombinant and truncated β-conglutins showed similar results.

After the process of obtention and purification of β-conlgutins, β1, β3 and β6, already studied in several reports [148,149,151,160] and with proved anti-inflammatory and antioxidant properties, were used as a potential preventive, cytotoxic and radiosensitizing treatment in two different BC models, as described in Chapters III, IV a V. In parallel with this study, β5 and β7 proteins, as well as their truncated forms, that had never been studied before regarding an anti-inflammatory potential, were used to assess the structure-functional domain responsible of the anti-inflammatory properties of β-conglutin protein isoforms and to try to propose those two unknown isoforms as potential treatments for inflammatory-related diseases too. The following chapter (II) contains the results obtained about this sub-objective of the doctoral thesis.

CHAPTER II. ASSESMENT OF THE STRUCTURE-FUNCTIONAL DOMAIN RESPONSIBLE OF THE ANTI-INFLAMMATORY PROPERTIES OF β-CONGLUTIN PROTEINS ISOFORMS.

a. β -CONGLUTIN PROTEINS STRUCTURAL MODELLING

An extensive *in silico* analysis of seed β-conglutins described a new family of major allergen proteins in lupin (Lup a 1), and a comparison to other relevant food proteins such as Ara h 1. The surface residues are involved in changes in 2-D structural elements (loops and coils) and 3D motives, and numerous micro-heterogeneities are present in fundamental residues directly involved in domains variability. This is represented in Figure S1 (Annexes), where red lines in the alignment correspond to the globular domain, and the rest (non-marked) N-terminal is the domain of the mobile arm.

Structural analysis of the β5 and β7 conglutin isoforms and their cofactors was performed by i-Tasser (fold recognition) using proteins from the Protein Data Bank library and a validation of models and conservation through the ConSurf server. 3D structures of truncated β5 and β7 were depicted as a cartoon diagram, as shown in Figure 11, where α-helices, β-sheets and coils are depicted in red, yellow and, green respectively, integrating main proteins domains. Both studied proteins, β5 and β7, and for extension β1, β3 and β6, are built of two main domains, a globular made mainly of anti-parallel β-sheets, and a mobile-arm like domain made of a-helices. This is a distinctive feature only found in NLL β-conglutin proteins among the vicilin family of proteins in legumes [148,155,159]. In Figure 11, the 3D structure of β5 and β7-conglutins is represented, based on the obtained results.

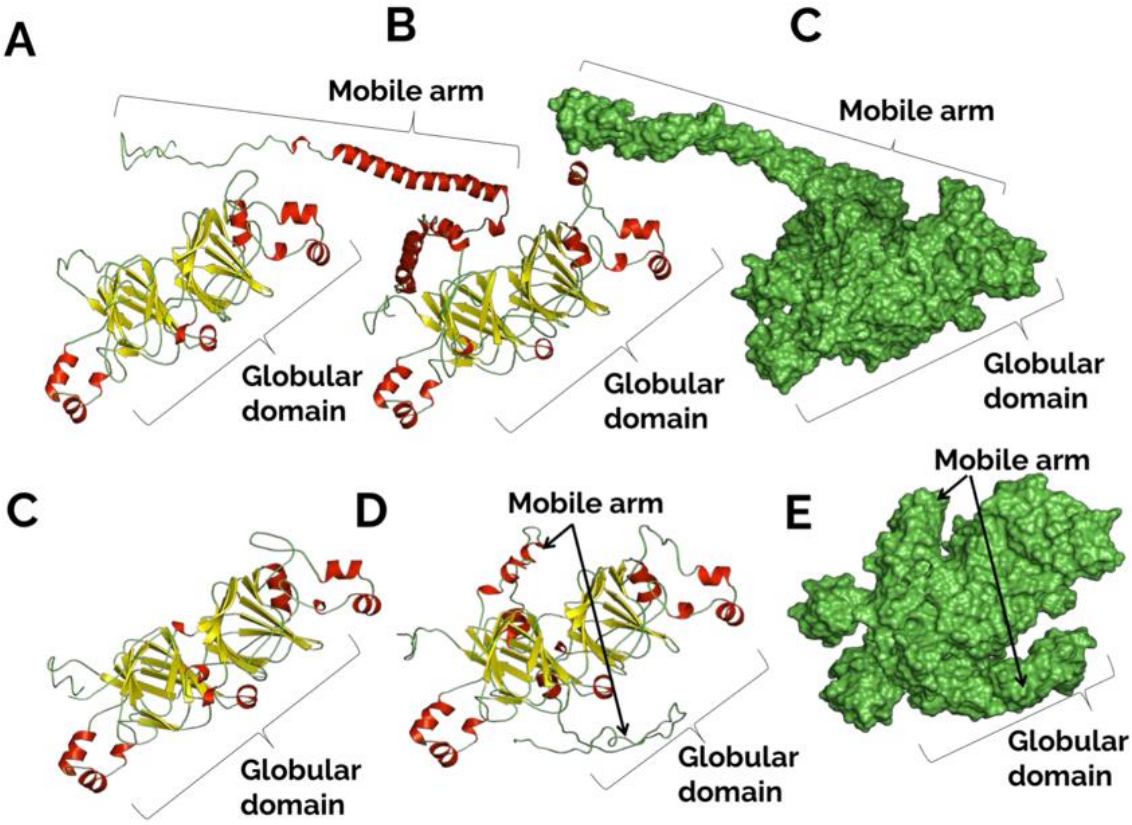


Figure 11. β5 and β7-conglutins structural analysis. 3D structure of β5 and β7-conglutins (Uniprot accession number F5B8W3 and F5B8W5, respectively) showing the mobile arm and globular domains. The structures were depicted as a cartoon diagram integrated by α-helices, β-sheets and coils (red, yellow and green colour, respectively).3D structure of (A,C) truncated 5β and 7β conglutin and 5β and 7β conglutin depicted in (B,D) cartoon and (C,E) surface.

b. β-CONGLUTIN PROTEINS INHIBITIS THE GENE EXPRESSION OF DIFFERENT CYTOKINES AND PRO-INFLAMMATORY MEDIATORS.

Real-time quantitative PCR technology was used to assay TNF-α, IL-1β and iNOS mRNA expression from each experimental group for inflammatory response analysis. Total RNA was extracted from HepG2 cells and isolated blood and peripheral blood mononuclear cell (PBMC) cultures from type 2 diabetic (T2D) of patients and control subjects using a RNeasy Mini Kit (Qiagen), as

previously described in Material and Methods. We evaluated the ability of β-conglutin protein to modulate the mRNA levels of genes of pro-inflammatory mediators as potential anti-inflammatory targets (TNFα, IL-1 β and iNOS mRNA) in HepG2 cells, cells from T2D and healthy controls cells. Results are shown in Figure 12 for HepG2 cells (Figure 12A), cells from T2D patients (Figure 12B) and healthy control cells (Figure 12C).

Regarding the HepG2 cells, the induced inflammatory state by LPS was significantly increased in HepG2 (*p <0.05 LPS vs Control), 1500, 900, and 1800–fold, for TNFα, IL-1 β and iNOS respectively. The induced inflammatory state by LPS was not inhibited significantly in HepG2 cells (**p < 0.05 LPS + truncated β-conglutins (tβ5) vs Control), 1500, 900, and 1800–fold for TNFα, IL-1 β and iNOS, respectively. In addition, the induced inflammatory state by LPS was not inhibited significantly in HepG2 cells (**p < 0.05 LPS + truncated β-conglutins (tβ7) vs Control), 1700, 900 and 1800–fold for TNFα, IL-1 β and iNOS respectively. On the other hand, the inflammatory state by LPS was significantly inhibited (•#p<0.05 β-conglutin (β5) vs LPS+ tβ5) by β-conglutin proteins at mRNA expression level in HepG2 cells -1200, -900, and -1000–fold, for TNFα, IL-1 β and iNOS respectively. Induced inflammatory state by LPS was significantly inhibited (◎#p<0.05 β-conglutin (β7) vs LPS+ tβ7) by β-conglutin proteins at mRNA expression level in HepG2 cells -1200, -900, and -1000–fold, for TNFα, IL-1 β, and iNOS respectively, versus LPS treated cells (Figure 12A).

For the T2D patients cells, In these studies, the gene expression levels of TNFα, IL-1 β and iNOS followed a similar pattern and tβ7 when compared to the control group, 500, 300, and 6000–fold for tβ5 and 500, 600, and 6000–fold for tβ7 for TNFα, IL-1 β and iNOS (**p<0.05). The results shows a significant reduction in mRNA expression levels of TNFα, IL-1 β and iNOS when challenged tβ5 and

tβ7 with β 5 and β 7 in T2D cells (◇○p<0.05 β 5 vs tβ 5) 500, 1000, and 1500–fold, and(◇○p<0.05 β 7 vs tβ 7) 500, 1000, and 1500–fold (Figure 12B).

Finally, when analyzing the same parameters in healthy control cells, the results showed that the induced inflammatory state by LPS was significantly increased in healthy control cells (*p <0.05 LPS vs Control), 900, 700, and 2500–fold, for TNFα, IL-1 β and iNOS respectively. Then, the induced inflammatory state by LPS was significantly increased in healthy control cells (**p < 0.05 LPS + truncated β-conglutins (tβ5) vs Control), 900, 200, and 1000–fold for TNFα, IL-1 β and iNOS respectively. The induced inflammatory state by LPS was also significantly increased in healthy control cells (**p < 0.05 LPS + truncated β-conglutins (tβ7) vs Control), 800, 700 and 2800–fold for TNFα, IL-1 β and iNOS respectively. Induced inflammatory state by LPS was significantly inhibited by β-conglutin β5 proteins at mRNA expression level in healthy control cells for TNFα [-400–fold, versus LPS treated cells. However, there is not a variation for IL-1 β and this state was activated for iNOS 2800 fold(•#p<0.05 β-conglutin (β5) vs LPS+ tβ5). On the other hands, induced inflammatory state by LPS was significantly inhibited (◎#p<0.05 β-conglutin (β7) vs LPS+ tβ7) by β-conglutin proteins at mRNA expression level in healthy control cells [−400, -300, -1000–fold for TNFα, IL-1 β respectively. These results indicate that β-conglutins, particularly β5 and β7, reduce the pro-inflammatory capacity in the healthy control cells by diminishing TNFα, IL-1 β and iNOS expression, thus assisting in the amelioration of the inflammatory process (Figure 12C).

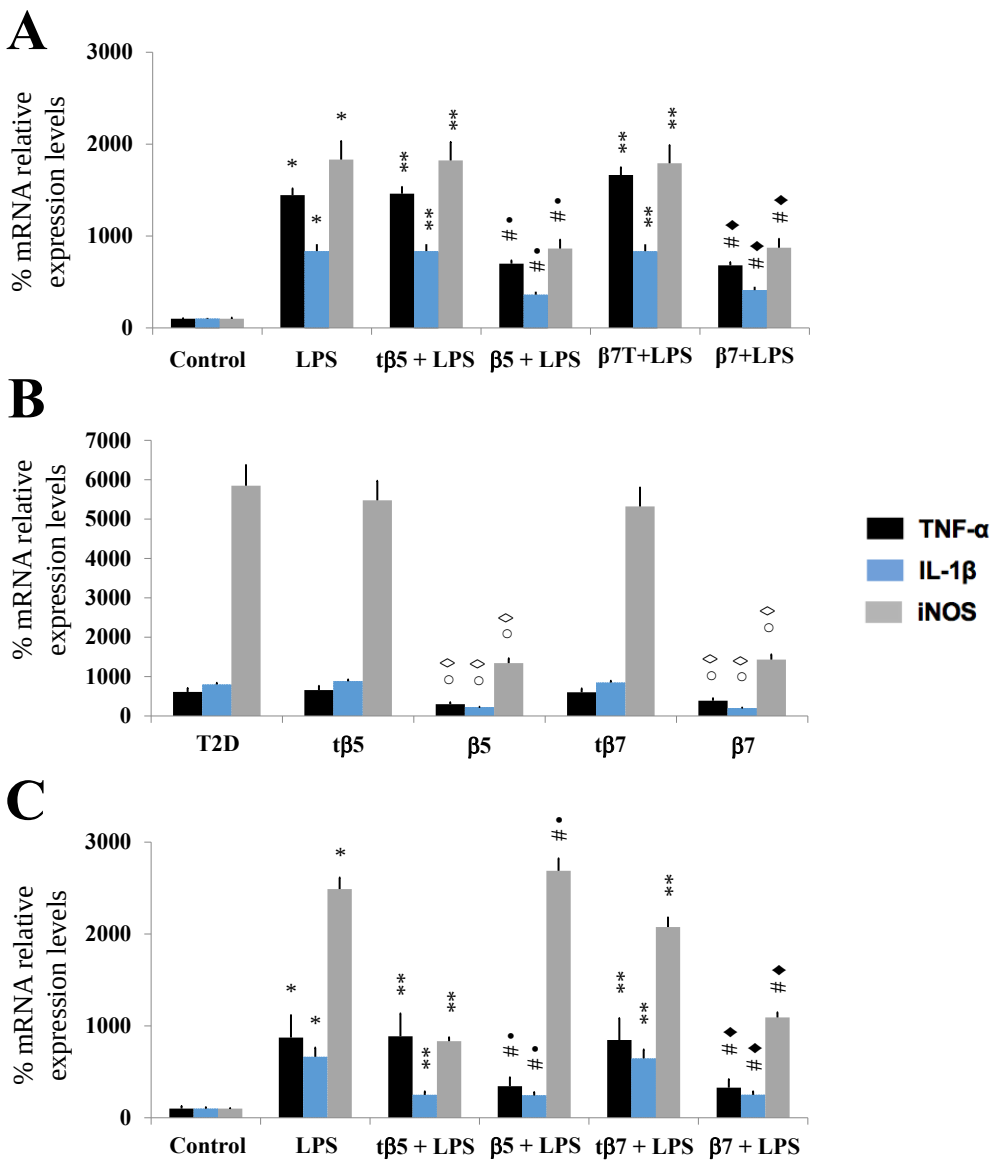


Figure 12. mRNA levels of the TNF-α, IL-1β and iNOS genes in Hep G2 cells (A), type 2 diabetes (B) and healthy control cells (C). Each group of cells were incubated for 24h in the presence of LPS, LPS + β5T, LPS + β5, LPS + β7T, LPS + β7. Bars show TNF-α, IL-1β, and iNOS in each cell type described above. *p <0.05 LPS vs Control; **p < 0.05 LPS + truncated β-conglutins (tβ5 and tβ7) vs Control; •#p<0.05 β-conglutin vs LPS+ tβ5; ◎#p<0.05 β-conglutin vs LPS+ tβ7. Data represent the mean ± SD of three independent experiments. Truncated conglutin β5 proteins (tβ5); truncated conglutin β7 proteins (tβ7); conglutin β5 proteins (β5);conglutin β5 proteins (β7).

The cell viability was assessed for the different cells treated with different isoforms and complete/truncated forms of β-conglutins and the potential

cytotoxicity of those proteins. In order to evaluate whether inflammation inductor LPS and β-conglutin produce cell cytotoxicity effects, the viability MTT assay (Material and Methods, *section 4.1*) was achieved under separate treatments with LPS adding β-conglutins, at increasing concentrations, for 24 h. The LPS plus β-conglutin treatment had no significant ($p > 0.05$) effects on cell viability . when compared with the control (non-treated) group, as represented in Tables S1 and S2 (Annexes). The cell cultures used as positive control lacked LPS and β-conglutin protein.

In order to complete the usefulness of the β-conglutin protein study, trypan blue staining was also used for assessing cells viability after treatment with LPS (1 μg/μL) and increasing concentrations (from 10 to 50 μg) of b-conglutin for 24 h, finding significant differences ($p < 0.05$) in cell viability after 24 h of incubation only at 50 μg compared to the control (Tables S1 and S2, Annexes). These results suggest that β-conglutins, in their different forms, do not affect to the cells integrity.

c. β-CONGLUTIN PROTEINS CONTROL THE ANTIOXIDANT CAPACITY OF CELLS.

In order to test the antioxidant capacity of cells and the effect of β-conglutins proteins, the antioxidant regulatory capacity of the three categories of cells (HepG2, T2D patients cells and healthy controls cells) was assessed with the measture of catalase activities, SOD and GSH production.

In this study, cell cultures were prepared and, after 24 h of incubation of β-5 and β-7 (whole protein and truncated forms, respectively) individually, growing media was removed, and cells washed with PBS at 4ºC. Cells from the different treatments with the β-conglutin protein forms were collected and used

for the enzymatic activity assessment of SOD and catalase, as well as the GSH measurement following Material and Methods, *section 6*.

Figure 13 shows the results of catalase activities, SOD and GSH production for HepG2 cells. Regarding catalase activity, In the LPS model, the induced inflammatory state in HepG2 cells was significantly observed (*p <0.05 LPS vs Control) with an increase in the catalase activities of 40 nmol/min/mL. In addition, the catalase activities were substantially increased in the induced inflammatory state by LPS in HepG2 cells, up to 50 nmol/min/mL. (**p < 0.05) with truncated β-conglutin (tβ5) and 80 nmol/min/mL with truncated β-conglutin (tβ7) in comparison to control cells. Furthermore, the levels of catalase activity were strongly reduced after the treatments with conglutin proteins (β5) in comparison to truncated β-conglutins (tβ5) in LPS-induced inflammatory statement, to -40 nmol/min/mL (●#p<0.05 β-conglutin (β5) vs LPS+ tβ5). Finally, the levels of catalase activity were strongly reduced after the treatments with conglutin proteins (β7) in comparison to truncated β-conglutins (tβ7) in LPS-induced inflammatory statement, up to -60 nmol/min/mL. (◆#p<0.05 β-conglutin (β7) vs LPS+ tβ7) (Figure 13A).

Then, SOD activity inhibition rate % in HepG2 cells was measured, as shown in Figure 13B. In the LPS model, the induced inflammatory state in HepG2 cells was significantly observed (*p <0.05 LPS vs Control) with an increase in SOD activity inhibition rate % of 50 nmol/min/mL. In addition, the SOD activity inhibition rate % was substantially increased in the induced inflammatory state by LPS in HepG2 cells up to 50 nmol/min/mL (**p < 0.05) with truncated β-conglutins (tβ5) and to 500 nmol/min/mL truncated β-conglutins (tβ7) in comparison to control cells. Furthermore, SOD activity inhibition rates % were strongly reduced after the treatments with conglutin proteins (β5) in comparison to truncated β-conglutins (tβ5) in LPS-induced inflammatory

statement, with a reduction of -400 nmol/min/mL. (•#p<0.05 β-conglutin (β5) vs LPS+ tβ5). Finally, SOD activity inhibition rates % were strongly reduced after the treatments with conglutin protein (β7) in comparison to truncated β-conglutin (tβ7) in LPS-induced inflammatory statement, with -400 nmol/min/mL. (◆#p<0.05 β-conglutin (β7) vs LPS+ tβ7) (Figure 13B).

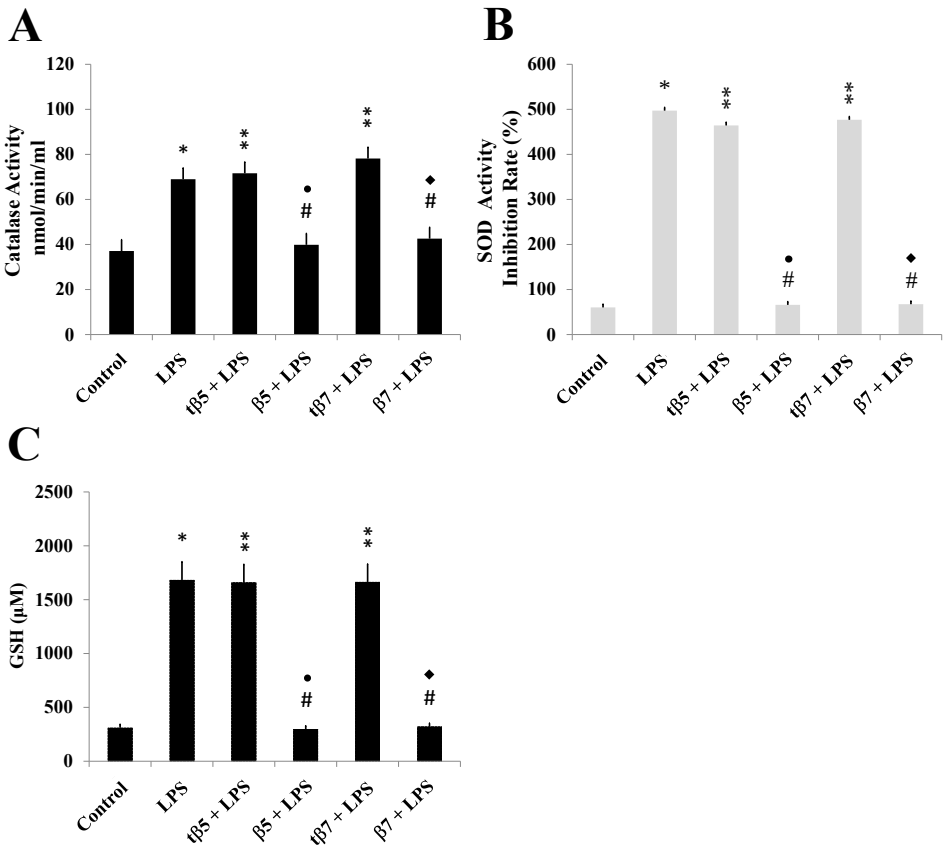


Figure 13. Effect of β-conglutins on antioxidant enzymatic activities and production of glutathione (GSH) in HepG2 cells. Cells were incubated for 24 h with β-conglutin proteins. Catalase activities (A), SOD (B) as well as GSH production (C) were measured. *p <0.05 LPS vs Control; **p < 0.05 LPS + truncated β-conglutins (tβ5 and tβ7) vs Control; •#p<0.05 β-conglutin vs LPS+ tβ5; ◆#p<0.05 β-conglutin vs LPS+ tβ7. Data represent mean ± SD from three independent experiments.

Finally, the GSH production in HepG2 cells was studied. In the LPS model the induced inflammatory state in HepG2 cells was significantly observed (*p <0.05 LPS vs Control) an increase in GSH (μM) production of 2000 nmol/min/mL. In addition the GSH (μM) production was substantially increased in the induced inflammatory state by LPS in HepG2 cells 1800 nmol/min/mL. (**p < 0.05) with truncated β-conglutins (tβ5) and 2100 nmol/min/mL truncated β-conglutins (tβ7) in comparison to control cells. Furthermore, GSH (μM) production were strongly reduced after the treatments with conglutin proteins (β5) in comparison to truncated β-conglutins (tβ5) in LPS-induced inflammatory statement -400X nmol/min/mL. (•#p<0.05 β-conglutin (β5) vs LPS+ tβ5). In addition GSH (μM) production were strongly reduced after the treatments with conglutin proteins (β7) in comparison to truncated β-conglutins (tβ7) in LPS-induced inflammatory statement -400XXX nmol/min/mL. (◆#p<0.05 β-conglutin (β7) vs LPS+ tβ7) (Figure 13C).

Following the same reasoning, Figure 14 shows the results of antioxidant capacity for TD2 cells. Regarding catalase activities in a TD2 model, the levels of catalase activity were similar to tβ5 and tβ7 when compared to the control group. Those levels were strongly reduced after the treatments with conglutin proteins (β5) in comparison to truncated β-conglutins (tβ5), up to -40 nmol/min/mL. (✶✶p<0.05 β-conglutin (β5) vs LPS+ tβ5). On top of that, the levels of catalase activity were strongly reduced after the treatments with conglutin proteins (β7) too, in comparison to truncated β-conglutins (tβ7) in -60 nmol/min/mL. (++<0.05 β-conglutin (β7) vs LPS+ tβ7) (Figure 14A).

Then, the SOD activity inhibition rate % in TD2 cells was assessed.
The percentages of SOD activity inhibition rate were similar for tβ5 and tβ7 when compared to the control group. Interestingly, those inhibition rates % were strongly reduced after the treatments with conglutin proteins (β5) in comparison

to truncated β-conglutins (tβ5) in -700 nmol/min/mL. (✶✶p<0.05 β-conglutin (β5) vs LPS+ tβ5). In addition, they were also strongly reduced after the treatments with conglutin proteins (β7) in comparison to truncated β-conglutins (tβ7) in -700 nmol/min/mL. (++<0.05 β-conglutin (β7) vs LPS+ tβ7) (Figure 14B).

Finally, when studying GSH production in TD2 cells, results were also similar for tβ5 and tβ7 when compared to the control group. Then, GSH levels were strongly reduced after the treatments with conglutin proteins (β5) in comparison to truncated β-conglutins (tβ5) -1000XXX nmol/min/mL. (✶✶p<0.05 β-conglutin (β5) vs LPS+ tβ5). In addition GSH were strongly reduced after the treatments with conglutin proteins (β7) in comparison to truncated β-conglutins (tβ7) in -1000XXX nmol/min/mL. (++<0.05 β-conglutin (β7) vs LPS+ tβ7) (Figure 14B).

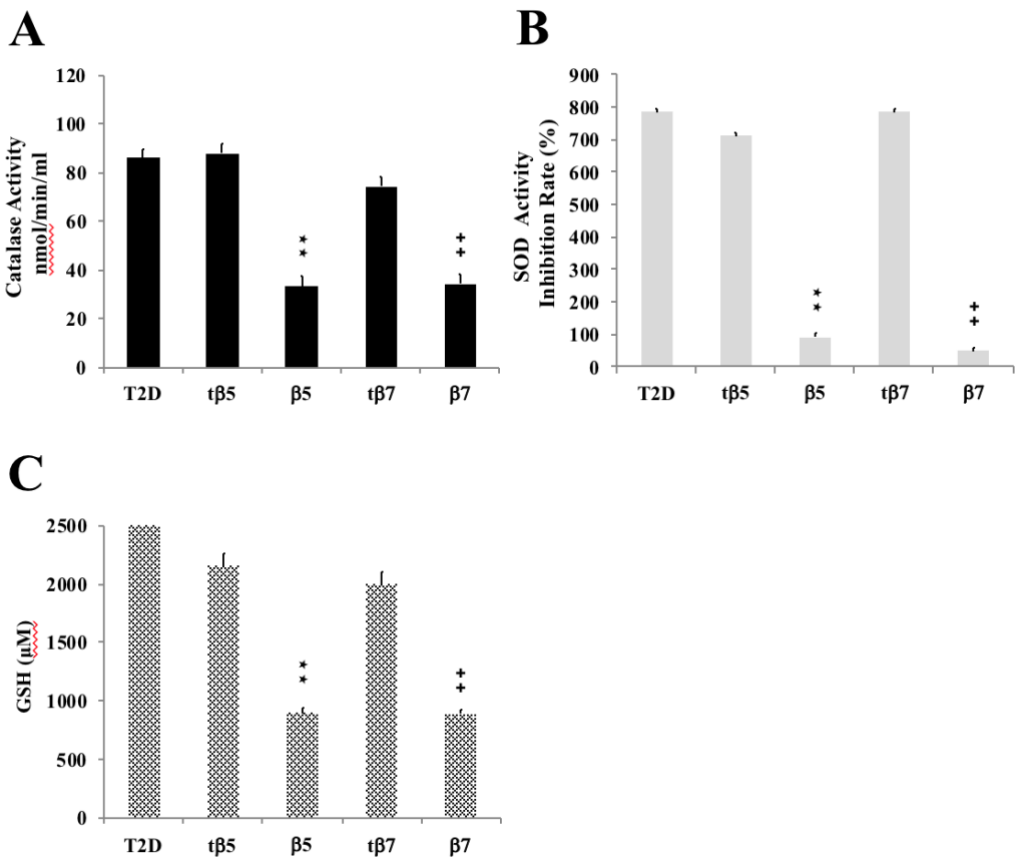


Figure 14. Effect of β-conglutins on antioxidant enzymatic activities and production of glutathione (GSH) in TD2 cells were incubated for 24 h with β-conglutins protein.

Catalase activities (A) and SOD (B)as well as GSH (C) production were measured. *p <0.05 LPS vs Control; **p < 0.05 LPS + truncated β-conglutins (tβ5 and tβ7) vs Control; •#p<0.05 β-conglutin vs LPS+ tβ5; ◆#p<0.05 β-conglutin vs LPS+ tβ7. Data represent mean ± SD from three independent experiments.

Finally, the same experiments were performed on healthy subject cells cells and results are shown in Figure 15. First, catalase activities were measured in healthy subject cells (Figure 15A). In the LPS model, the induced inflammatory state was significantly observed (*p <0.05 LPS vs Control), with an increase in the catalase activities of 40 nmol/min/mL. In addition, the catalase activity was substantially increased in the induced inflammatory state by LPS in 50 nmol/min/mL (**p < 0.05) with truncated β-conglutin (tβ5) and 80 nmol/min/mL with truncated β-conglutin (tβ7) in comparison to control cells. Furthermore, the levels of catalase activity were strongly reduced after the treatments with conglutin protein β5 in comparison to truncated β-conglutin (tβ5) in LPS-induced inflammatory statement -40 nmol/min/mL. (•#p<0.05 β-conglutin (β5) vs LPS+ tβ5). Finally, the levels of catalase activity were also strongly reduced after the treatments with conglutin protein β7 in comparison to truncated β-conglutin (tβ7) in LPS-induced inflammatory statement in -60 nmol/min/mL. (◆#p<0.05 β-conglutin (β7) vs LPS+ tβ7) healthy subject cells (Figure 15A).

Regarding SOD activity inhibition rate (%) in healthy subject cells, in the LPS model, the induced inflammatory state was significantly observed (*p <0.05 LPS vs Control), with an increase in SOD activity inhibition rate (%) of 500 nmol/min/mL. In addition, the SOD activity inhibition rate (%) was substantially increased in the induced inflammatory state by LPS in healthy subject cells 50 nmol/min/mL. (**p<0.05) with truncated β-conglutins (tβ5) and 500 nmol/min/mL truncated β-conglutins (tβ7) in comparison to control cells. Furthermore, SOD activity inhibition rate % were strongly reduced after the

treatments with conglutin proteins (β5) in comparison to truncated β-conglutins (tβ5) in LPS-induced inflammatory statement -400 nmol/min/mL. (•#p<0.05 β-conglutin (β5) vs LPS+ tβ5). In addition SOD activity inhibition rate % were strongly reduced after the treatments with conglutin proteins (β7) in comparison to truncated β-conglutins (tβ7) in LPS-induced inflammatory statement -400 nmol/min/mL. (◆#p<0.05 β-conglutin (β7) vs LPS+ tβ7) in healthy subject cells (Figure 15B).

Finally, GSH production was assesed in healthy subject cells. In the LPS model, the induced inflammatory state was significantly observed (*p <0.05 LPS vs Control), with an increase in GSH (μM) production of 2000 nmol/min/mL. In addition, the GSH (μM) production was substantially increased in the induced inflammatory state by LPS in 1800 nmol/min/mL. (**p < 0.05) with truncated β-conglutin (tβ5) and with 2100 nmol/min/mL in truncated β-conglutin (tβ7), in comparison to control cells. Furthermore, GSH (μM) production was strongly reduced after the treatments with conglutin protein β5 in comparison to truncated β-conglutin (tβ5) in LPS-induced inflammatory statement, with a reduction of 400 nmol/min/mL (•#p<0.05 β-conglutin (β5) vs LPS+ tβ5). Finally, GSH (μM) production was also strongly reduced after the treatments with conglutin protein β7 in comparison to truncated β-conglutin (tβ7) in LPS-induced inflammatory statement, with a reduction of -400 nmol/min/mL. (◆#p<0.05 β-conglutin (β7) vs LPS+ tβ7) (Figure 15C).

Summarizing, the β-conglutin proteins β5 and β7 improved glutathione (GSH) levels, lower SOD and catalase antioxidant enzymatic activities in all cells studied.

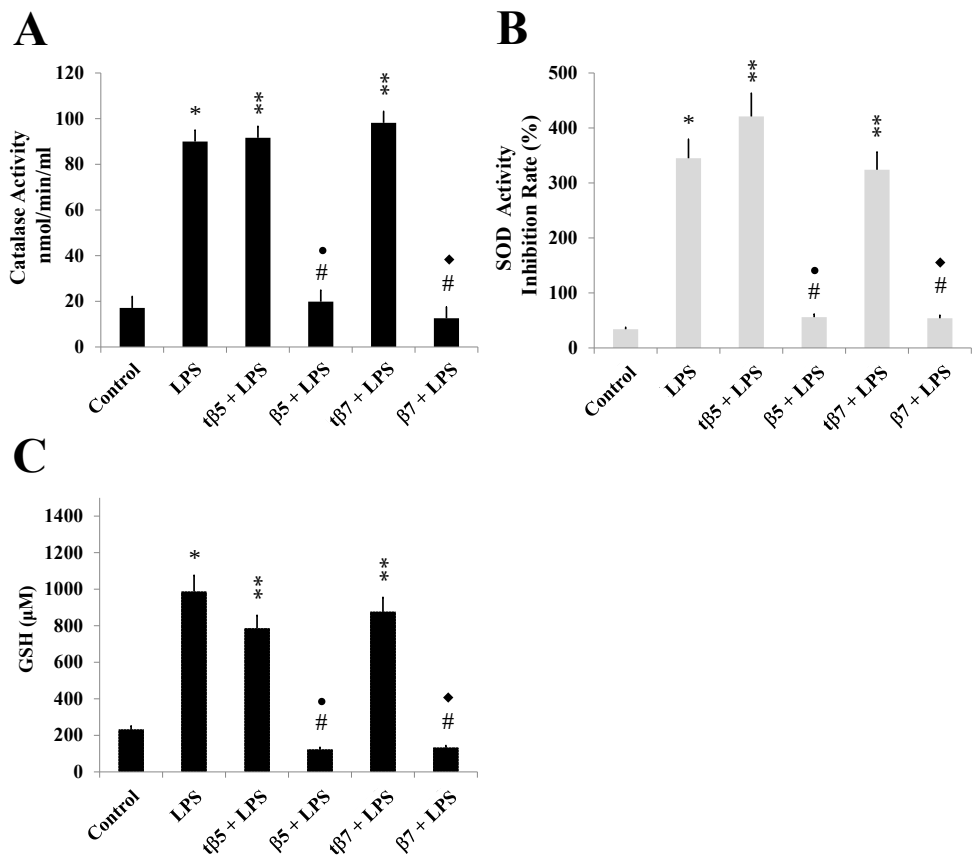


Figure 15. Effect of β-conglutins on antioxidant enzymatic activities and production of glutathione (GSH) in cells of healthy subject were incubated for 24 h with β-conglutins protein. Catalase activities (A) and SOD (B)as well as GSH (C) production were measured. *p <0.05 LPS vs Control; **p < 0.05 LPS + truncated β-conglutins (tβ5 and tβ7) vs Control; •#p<0.05 β-conglutin vs LPS+ tβ5; ◆#p<0.05 β-conglutin vs LPS+ tβ7. Data represent mean ± SD from three independent experiments.

β-conglutin protein β5 and β7 achieved a functional effect as the strong reduction of cell oxidative stress is induced by inflammation, through raising glutathione (GSH) levels, modulation of superoxide dismutase (SOD) and catalase enzymes activities. However, truncated conglutin β proteins tβ5 and tβ7 are not able to perform the same effects on cells.

As the final part of the antioxidant and anti-inflammatory study regarding β conglutins, the nitric oxide (NO) detection was performed in the three cell

models used for the previous experiments. The total amount of NO production including nitrite/nitrate contents from cultured cell supernatants of control subjects, T2D patients and HepG2 cells was measured following Material and Methods, *section 6*. Results are shown in Figure 16.

First of all, in the healthy subjects' cells, total NO in the LPS model, with induced inflammatory state, was significantly increased in 450 nmol/min/mL (*p <0.05 LPS vs control). In addition, total NO was substantially increased in 480 nmol/min/mL in the induced inflammatory state by LPS with truncated β-conglutin (tβ5) (**p < 0.05 tβ5 vs control). In concordance with the previous results, the same effect was obtained for the total NO in the induced inflammatory state by LPS with truncated β-conglutins (tβ7), with an increase of 650 nmol/min/mL (**p < 0.05 tβ7 vs control). On the other hand, the levels of total NO were strongly reduced after the treatments with conglutin protein β5 in comparison to truncated β-conglutin (tβ5) in LPS-induced inflammatory statement, with a 100 nmol/min/mL reduction (•#p<0.05 β-conglutin (β5) vs LPS+ tβ5). Furthermore, the total NO was strongly reduced after the treatment with conglutin protein β7 in comparison to truncated β-conglutins (tβ7), in LPS-induced inflammatory statement, with a difference in 60 nmol/min/mL (◆#p<0.05 β-conglutin (β7) vs LPS+ tβ7). Those results are shown in Figure 16A.

In the TD2 model, the total NO levels were similar to tβ5 (750 nmol/min/mL) and tβ7 (700 nmol/min/mL) when we compared to the control group 800 nmol/min/mL. Furthermore, NO levels were strongly reduced after the treatments with conglutin protein β5 in comparison to truncated β-conglutin (tβ5), with a -700 nmol/min/mL reduction (•#p<0.05 β-conglutin (β5) vs LPS+ tβ5). Finally, NO levels were also strongly reduced after the treatment with conglutin protein β7 in comparison to truncated β-conglutin (tβ7), in -700 nmol/min/mL (•#<0.05 β-conglutin (β7) vs LPS+ tβ7) (Figure 16B).

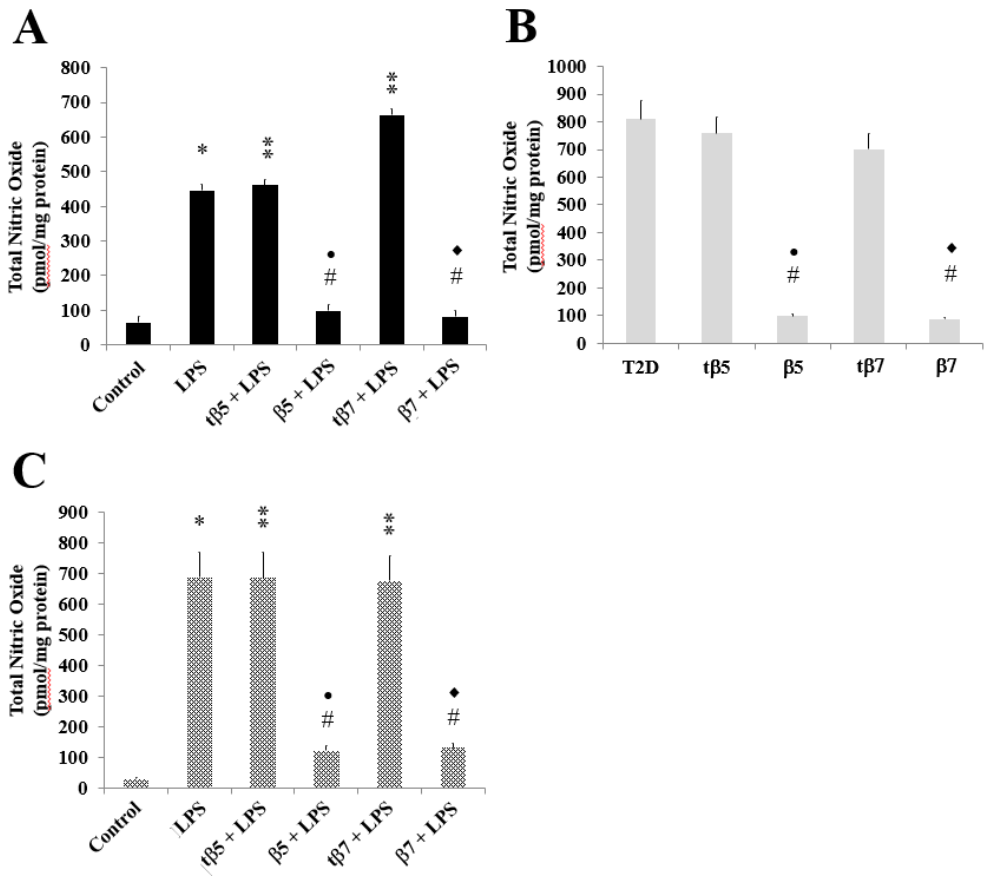


Figure 16. Effect of β-conglutin (complete and truncated) on the production of nitric oxide (NO) in culture supernatant from control groups, healthy subjects (A), type 2 diabetes (B) and HEPG2s cells. Each group of blood cells were incubated for 24h in the presence of LPS, LPS + β5T, LPS + β5, LPS + β7T, LPS + β7. *p <0.05 LPS vs Control; **p < 0.05 LPS + truncated β-conglutins (tβ5 and tβ7) vs Control; •#p<0.05 β-conglutin vs LPS+ tβ5; ◆#p<0.05 β-conglutin vs LPS+ tβ7. Data represent the mean ± SD of three independent experiments.

Finally, total NO levels in the LPS model the induced inflammatory state in HepG2 cells was significantly observed (*p <0.05 LPS vs control) an increase of 700 nmol/min/mL. In addition, total NO was substantially increased in the induced inflammatory state by LPS with truncated β-conglutin (tβ5), 700 nmol/min/mL (**p < 0.05 tβ5 vs control). The total NO was also significantly increased in the induced inflammatory state by LPS with truncated β-conglutin

(tβ7), also in 700 nmol/min/mL (**p < 0.05 tβ7 vs control). On the other hand, the levels of total NO were strongly reduced after the treatments with β-conglutin protein β5 in comparison to truncated β-conglutin (tβ5) in LPS-induced inflammatory statement, by 100 nmol/min/mL (•#p<0.05 β-conglutin (β5) vs LPS+ tβ5). Furthermore, the total NO levels were also significantly reduced after the treatments with conglutin protein β7 in comparison to truncated β-conglutins (tβ7) in LPS-induced inflammatory state, in 150 nmol/min/mL (◆#p<0.05 β-conglutin (β7) vs LPS+ tβ7) (Figure 16C).

CHAPTER III: SIMPLE β-CONGLUTIN TREATMENT IN EARLY BC MODEL

a. β-CONGLUTINS INHIBIT EARLY BC STAGES CELL LINES GROWTH *IN VITRO*

In order to test the effect of β-conglutin proteins in terms of cell growth, proliferation and viability in different types of early BC stages cell lines, the MTT and Trypan Blue assays were performed in this cell line model, where epithelial and non-tumorigenic cell line, MCF-10A, was used as a control. The three tumoural cell lines studied are described in *Material and Methods (section 2.1.3)* and correspond to: MC26 cell line, that mimics luminal breast tumors at early stages, MC26-R, derived from MC26 cells, that mimics luminal breast tumors with resistance to conventional therapy with 5-FU and M1B26, that correspond to the first stages of basal breast tumor, with more aggressive phenotype and metastatic potential.

The MTT assay was performed with serial concentrations of each β-conglutin treatment (0–10 ng/mL) during 24 hours (Figure 17 a, b and c) and 48 hours (Figure 17 d, e and f). The β-conglutin treatments used were β1, β3 and β6, as those were the β-conglutins that had already demonstrated in previous studies the anti-inflammatory and antioxidant properties of interest regarding potential BC prevention and therapeutic treatment.

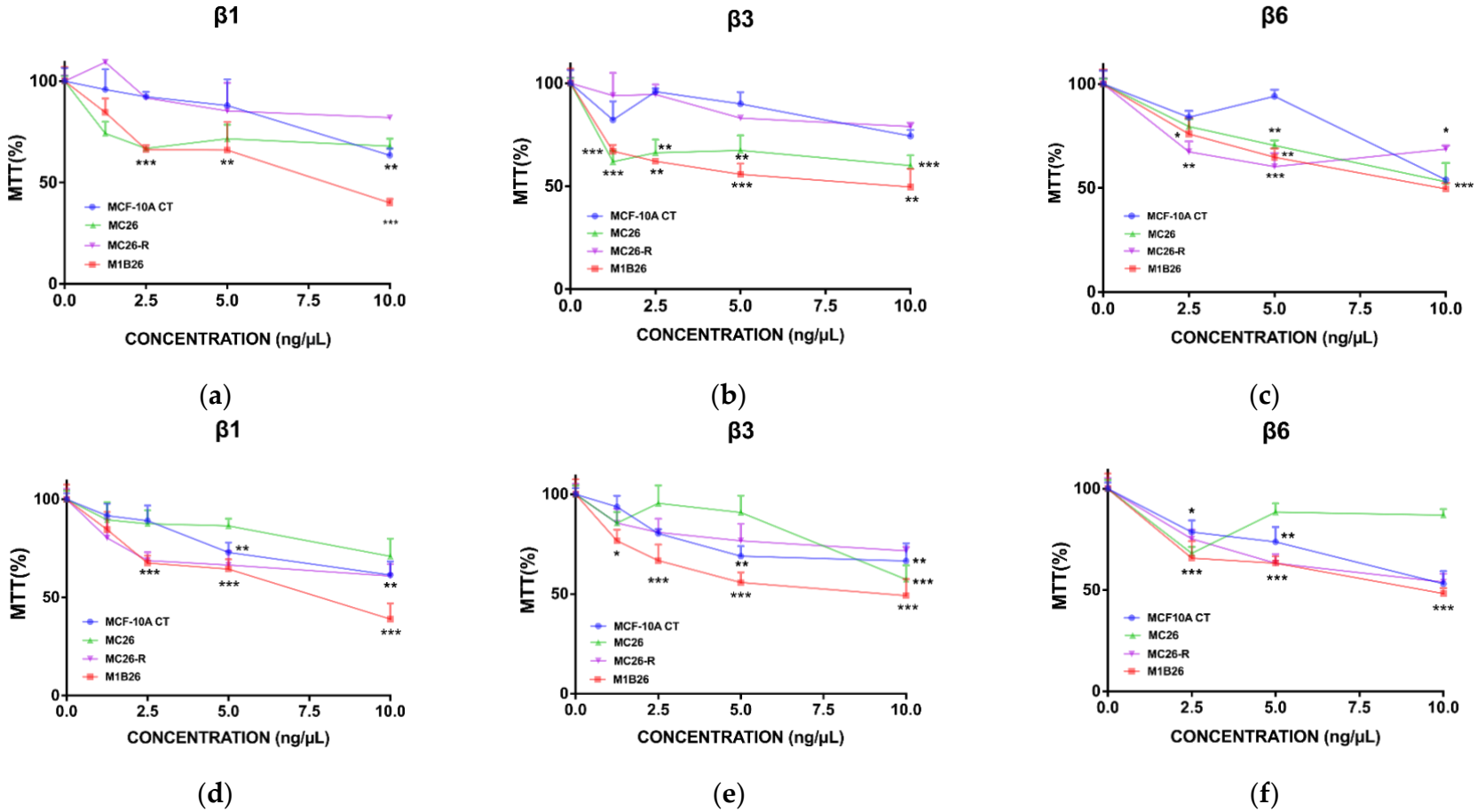


Figure 17: Inhibition of cell growth in an early BC model *in vitro* after 24 and 48 hours treatment. MTT assay after 24 hours was performed for (a) β1, (b) β3 and (c) β6 treatments and absorbance was measured. MTT assay after 48 hours was performed for (d) β1, (e) β3 and (f) β6 treatments and absorbance was measured. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells.

Figure 17 shows how β-conglutins significantly decreased the proliferation rates of M1B26 cell line from very low doses and for both treatment times, showing a greater effect for β1 and β3 at the mentioned low doses [1,25-2,5 ng/μL] (Figure 17 a b,d and e). Regarding luminal-like malignant cells, MC26 shows a similar effect for the 24-hour treatment, and seems to lose this proliferation inhibition effect after 48 hours (Figure 17 d,e). Interestingly, MC26-R showed resistance to β-conglutin treatment at low doses, except for β6 treatment at 24 hours. Finally, the main finding of this first MTT assay is the resistance of the healthy epithelial cell line, MCF-10A, to β-conglutin treatment, especially at low doses, where a significant inhibition of growth is found for M1B26 and MC26 at 24-hour treatment.

In order to ensure that the inhibition of proliferation is accompanied by a cytotoxic effect, the second experiment performed was the vital dye Trypan Blue assay, following the protocol in *Material and Methods (section 4.2)* for 24 hours (Figure 18 a, b and c) and 48 hours (Figure 18 d, e and f).

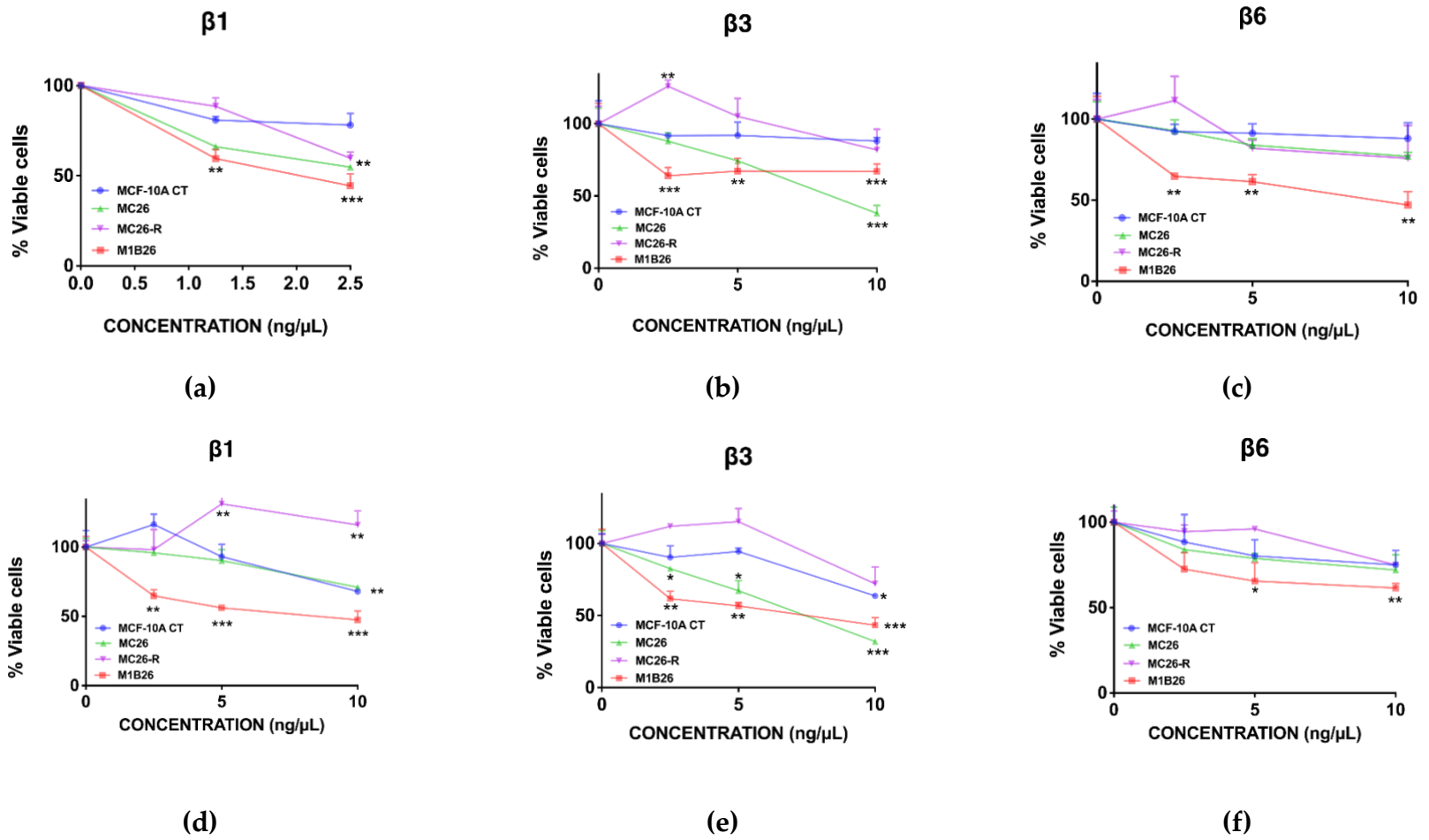


Figure 18: Cytotoxic effect of β-conlgutins in an early BC model *in vitro* after 24 and 48 hours treatment. Trypan blue dye assay was performed for (a) β1, (b) β3 and (c) β6 treatments after 24 hours and for (d) β1, (e) β3 and (f) β6 treatments after 48h in order to determine the cytotoxic effect of β-conglutins versus control. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells.

Interestingly, the trypan blue viability assay gave some contradictory data regarding Figure 17. For the M1B26 cell line, the growth-inhibition effect of β1 and β3 treatments from very low doses was maintained, showing a greater cytotoxic effect after 48 hours of treatment (Figure 18d and e). For the MC26 cell line, the effect of the treatment of β1 and β at 24 hours is *grosso-modo* the same, but we do not appreciate any loss of effect after 48 hours of treatment. Contrarily, the viability of MC26 cells seems to be highly affected at 48-hour treatment, especially for β3 (Figure 18, e). Finally, the resistance of MC26-R and MCF-10A cell lines is confirmed with the vital trypan blue dye assay at low doses for β1 and β3 at both treatment times. This viability assay showed no significant effect of β6 conglutin except for M1B26, contrarily to the MTT results that suggested an inhibition of growth for the resistant to chemotherapy cell-line MC26-R.

The differences between those two assays reside in the technique used to asses viability (see *Material and Methods 4.2*) and, normally, trypan's blue assay gives more precise information regarding the cytotoxicity of treatments. Due to the controversial data and the lack of effect of β6 conglutin in the viability assays at low doses, only β1 and β3 treatments were selected as potential treatments for the rest of experiments. Concentrations chosen with the aim of assuming the higher inhibition of viability with the higher preservation of healthy MCF-10A cells, so, based on Figure 11, the doses selected were 1,25 and 2,5 ng/μL for both β1 and β3 and treatment time was fixed to 48h as the effect was higher for BC cells and MCF-10A viability was assured at those low doses. With those experimental conditions fixed, both MTT and trypan blue's viability assays were performed after 72 hours, and results are shown in Figure 19.

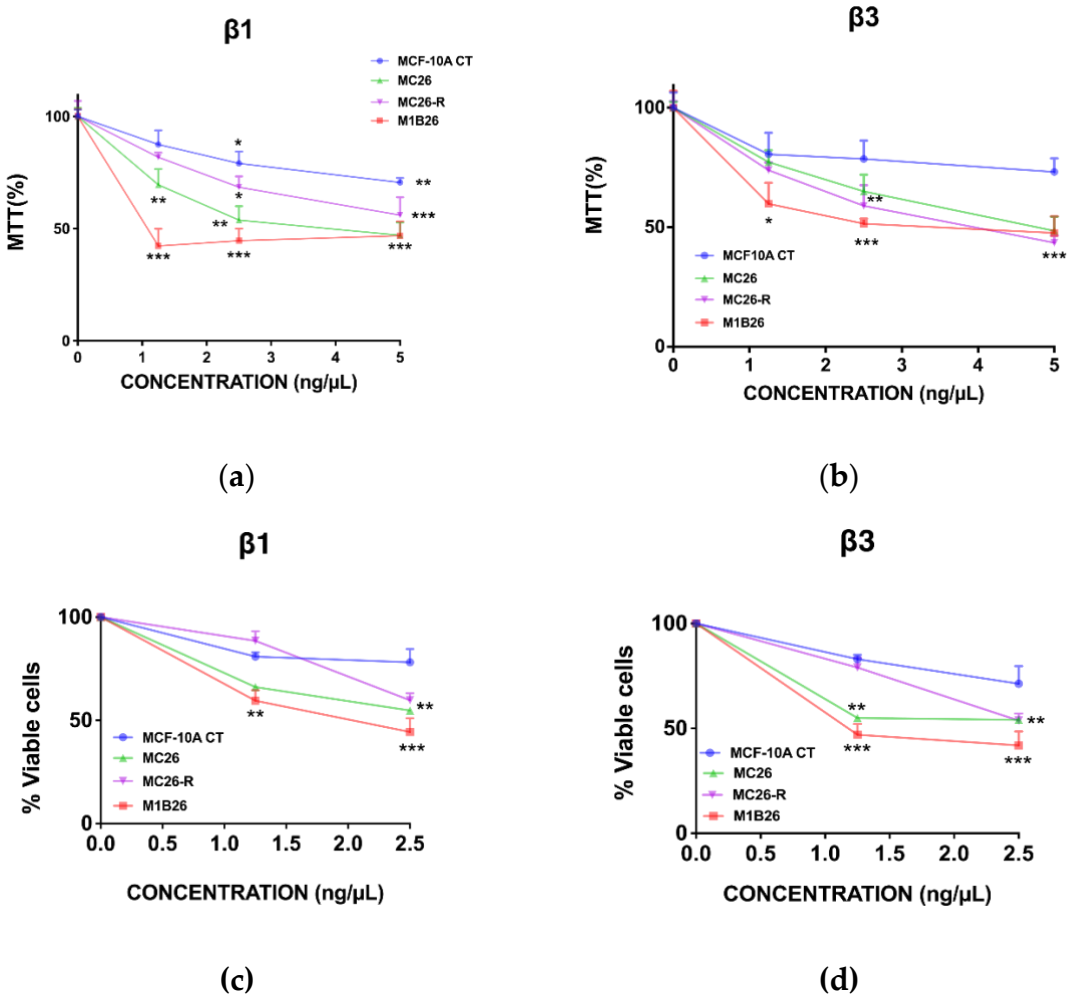


Figure 19: Inhibition of cell growth in an early stage BC model *in vitro* after 72 hours. MTT assay was performed for (a) β1 and (b) β3 treatments and absorbance was measured. Trypan blue dye assay was performed for (c) β1 and (d) β3 treatments in order to determine the cytotoxic effect of β-conglutins versus control. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells.

Results for both MTT and trypan blue assays confirmed the right choice of doses and treatments for this BC model. In Figure 19a and c, β1 treatment was able to inhibit both cell growth and cytotoxicity of all BC cell lines significantly, with the maintain of +/-80% of the viability of MCF-10A cells. MC26-R shows less resistance to treatment than for the previous treatment times. β3 treatment at 72 hour shows the most interesting result for this early BC stages model: β3 seems

to affect the viability of the three BC cell lines very significantly, without any significant differences in the MCF-10A cells, and at very low-doses.

b. β-CONGLUTINS REDUCES TOTAL NUMBER OF COLONY AND REDUCE BASAL AND LUMINAL-LIKE PHENOTYPE LIKE IN EARLY STAGES OF BC GROWTH.

In order to assay the effect of the selected β-conglutin treatments for this BC model, the E-CFC (mammary CFC) assay was performed. It is a differentiation test that determines the frequency and immaturity of progenitor cells in a population of primary mammary cells. Even if this test was firstly developed for primary cells, recent evidence has showed that it is possible to obtain "luminal-like" and "myoepithelial-like" colonies with MCF-10A cells. Cells were treated during 48 hours with β1 and β3 at the selected study doses: 1,25 and 2.5 ng/uL respectively. Results are shown in Figure 20.

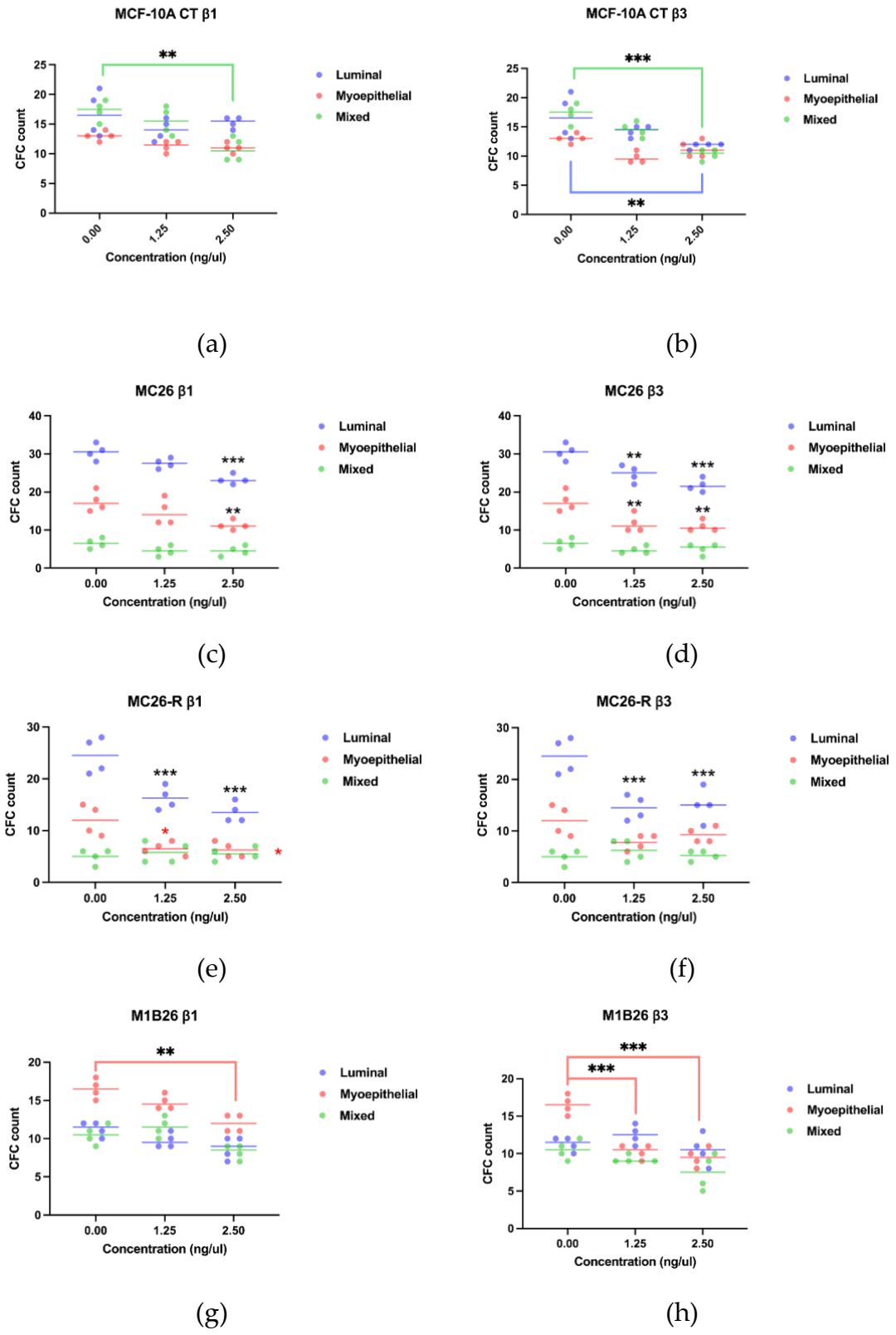


Figure 20: E-CFC count after 48h of β1 and β3 treatments in the early BC stages cell lines MCF-10A (a and b), MC26 (c and d), MC26-R (e and f) and M1B26 (g and h). Cells were seeded after 48 hours of each treatment and different types of "luminal-like",

"myoepithelial-like" and "mixed" colonies were stained and counted. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells.

MCF-10A cells showed a significant decrease in the number of mixed colonies for β1 treatment, and for both "luminal-like" and mixed colonies with β3 treatment (Figure 20a and b). MC26 cells, a luminal early BC cell line, only showed significant decreasing in the number of "luminal-like" colonies for both treatments (Figure 20c and d). Interestingly, the MC26-R cell line, derived from MC26 but with acquisition of chemotherapy resistance to 5-FU, presents a significant lower number of both "luminal-like" and "myoepithelial-like" (or basal-like) colonies. Finally, the basal-like early BC subtype cell line, M1B26, only presents significant decrease in the number of "myoepithelial-like" colonies (Figure 20g and h). Regarding total E-CFC count, all treatment decreased the colony-forming capacity, especially in the BC cell lines.

c. β-CONGLUTINS EFFECT IS MEDIATED BY BONE MORPHOGENETIC PROTEINS (BMPs) PATHWAY

As reported in the introduction chapter, the over-activation of the BMP pathway has been established at the origin of malignant transformation for MCF-10A cells and involved in the progression and evolution of the subsequent malignant phenotypes derived from healthy cells. In order to study it the proposed β-conglutin treatment could have any impact on this pathways of action, qPCR of interest BMP pathway related genes has been performed for the four cell lines, with β1 and β3 treatments. Figures 21, 22 and 23 showed the results of the genetic expression of BMP receptors (BMPR1A, BMPR1B and BMPR2), BMP2 and BMP4 (main BMP genes related with BC initiation and progression), Inhibitors of DNA-binding (ID), known as important modulators in the regulation of cell proliferation and differentiation, SMAD6, a BMP-specific

inhibitory Smad and CD10, a marker for basal-type cells and associated with BC progression, dissemination and CSCs.

For the healthy epithelial cells, β1 conglutin significantly decreased all the BMPR genetic levels, accompanied with an augmentation for ID2. β3 conglutin treatment decreased receptors 1A and 2 levels, as well as the expression of BMP4 (Figure 21a,c and Figure 22a). Both β-conglutins severally decrease BMPR1B levels for MC26 cells, with a lower expression of both BMP2 and BMP4, especially with β3. ID1 and 2 levels were also significantly lower in comparison with the non-treated control for β3 treatment in MC26 cells (Figure 21b,f and Figure 22b). Regarding the chemotherapy-resistant cell line, MC26-R, the BMP-receptors, BMP2, BMP4 and the three ID were drastically lower for both treatments, especially for β3. Finally, for the basal-like BC cell line, M1B26, no significant changes were found for the BMP receptors, but both treatments induced lower levels of BMP2 and 4 and the three IDs (Figure 21d,h and Figure 22d). SMAD6 genetic expression was only reduced for M1B26 and MC26-R treated cells (Figure 23a), and CD10 expression presented significant lower levels for the healthy cells, MCF-10A, treated with both β -conglutins, M1B26 with β1 and MC26-R with β3 (Figure 23b).

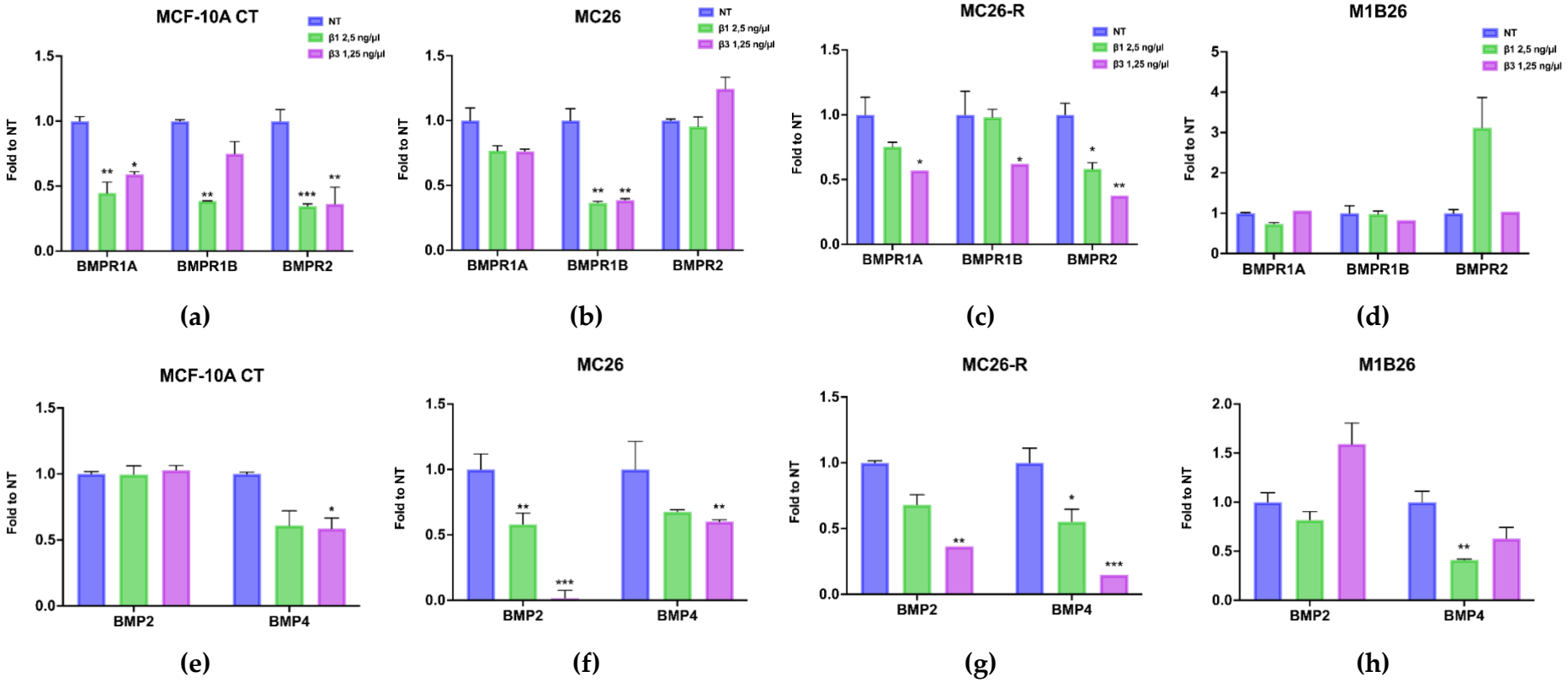


Figure 21: Results of genetic expression of BMP receptors (BMPR1A, 1B and 2), of BMP-2 and BMP-4 and BMP's related genes IDs (inhibitors of DNA-binding) after 48h of β1 and β3 treatments in the early BC stages cell lines. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells.

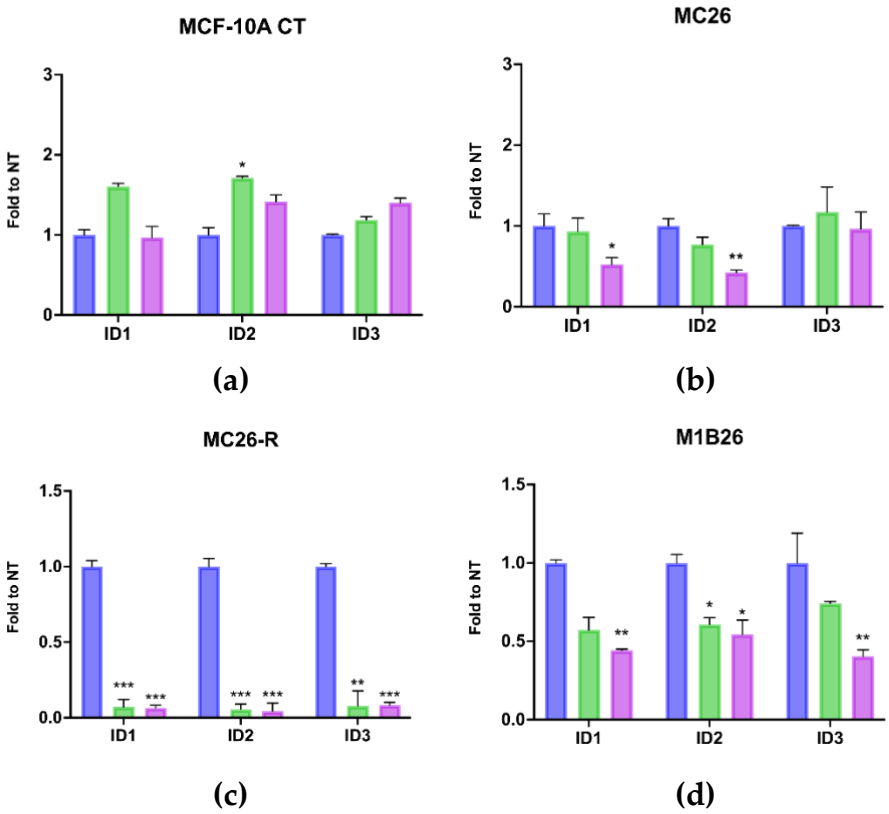


Figure 22: Results of genetic expression BMP's related genes IDs (inhibitors of DNA-binding) after 48h of β1 and β3 treatments in the early BC stages cell lines. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells.

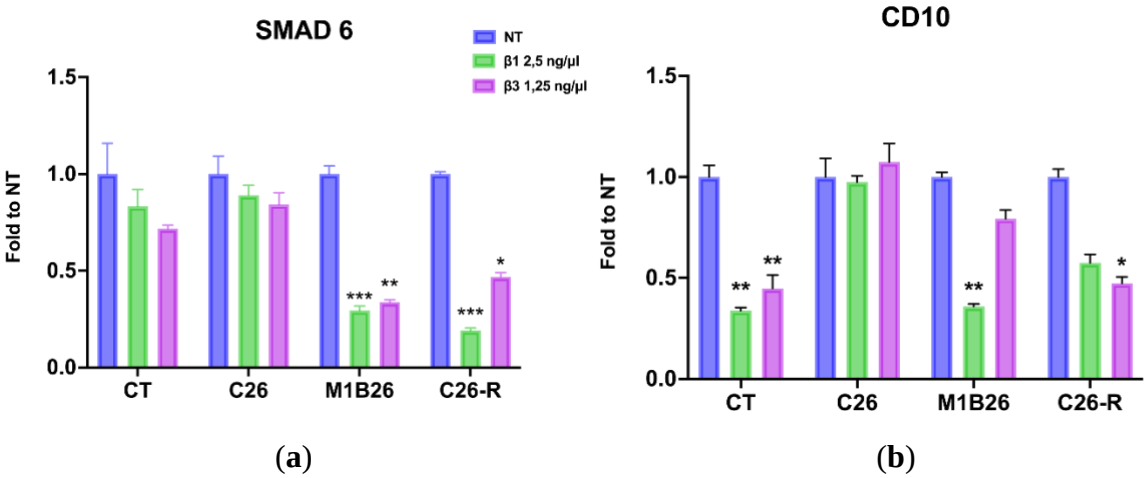
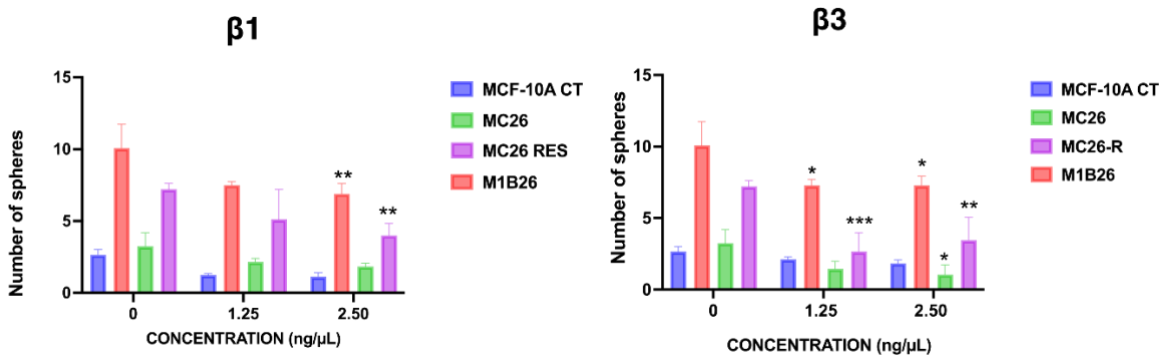


Figure 23: Results of genetic expression of BMP's related genes SMAD 6 (a) and CD10 (b) after 48h of β1 and β3 treatments in the early BC stages cell lines. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells.

d. β-CONGLUTINS REDUCE STEM-LIKE AND PROMOTES DIFFERENTIATION IN EARLY STAGES OF BC CELLS

As β-conglutin treatment seem to have a crucial role in basal/luminal progenitors and BMP pathway, we finally analyzed the self-renewal capacity of cells after β-conglutin treatments through the mammosphere assay, in order to fully characterize CSCs and compare those results with the genetic expression of stem-cell related genes. As shown in Figure 24, β1 and β3-conglutins reduced the number of mammospheres in M1B26 and MC26-R cells, the most aggressive phenotypes within the early BC stage model. Interestingly, none of the β-conglutins were able to reduce the capacity to form spheres significantly in MCF-10A or MC26 cell line.



(a) **(b)**

Figure 24: Quantification of mammospheres after treatment with β1-conglutin and β3-conglutin (2.5 ng/μL and 1.25 ng/μL). Spheres were counted in (**a**) for β1-conglutin treatment and (**b**) for β3-conglutin. Spheres > 50 μm were counted and captured using Leica DM500 binocular microscope. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells.

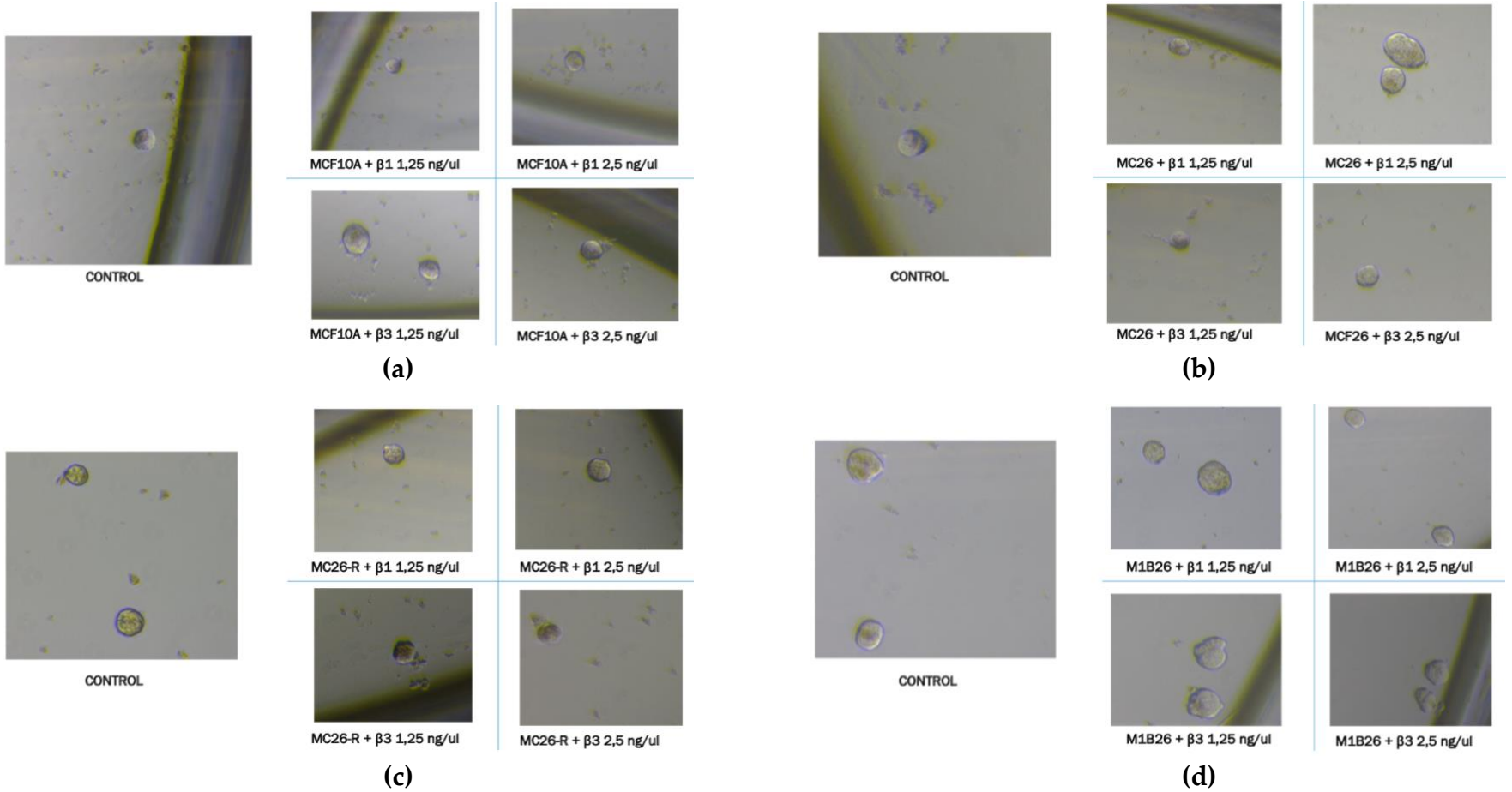


Figure 25: Characterization of mammospheres after treatment with β1-conglutin and β3-conglutin (2.5 ng/μL and 1.25 ng/μL). Treatment was performed 48 h before seeding mammospheres from (**a**) MCF-10A, (**b**) MC26, (**c**) MC26-R, and (**d**) M1B26. Spheres > 50 μm were counted and captured using Leica DM500 binocular microscope at 10x magnification.

Regarding the aspect of mammospheres, β-conglutins treated cells presented signs of differentiation in some of the spheres formed, as shown in the MCF-10A assay (Figure 25a) and in the MC26-R (Figure 25c). Those small cells "scaping" the sphere could be a sign of differentiation and non-survival potential (self-renewal capacity) of the spheres formed for those two cell-lines.

CHAPTER IV: SIMPLE β-CONGLUTIN TREATMENT IN NORMAL/ADVANCED BC MODEL

a. β-CONGLUTINS INHIBIT BC CELL LINES GROWTH *IN VITRO*

In order to test the effect of β-conglutin proteins in terms of cell growth, proliferation and viability in more advanced BC cell lines, the MTT and trypan blue assays were performed in a normal BC cell line model, where the same epithelial and non-tumorigenic cell line, MCF-10A, was used as a control. The three tumoural cell lines studied are described in *Material and Methods (section 2.1.2)* and correspond to more advanced BC stages: MCF-7 cell line has a luminal A phenotype, SK-BR-3 is a luminal B phenotype like cell line and MDA-MB-231 has the most aggressive BC phenotype, the triple negative (TNBC).

The MTT assay was performed with serial concentrations of each β-conglutin treatment (0–10 ng/mL) during 24 hours (Figure 26a, b and c) and 72 hours (Figure 27a, b and c**).** After 72 hours of treatment, the three β-conglutins had a dose-dependent effect on the BC cell lines treated, with very significant differences in comparison with the healthy cell line MCF-10A, that, as well as in the early BC model, seems to be very resistant to this treatment, especially at very low doses. β1 induced a very significant effect in SK-BR-3 and MDA-MD-231 from very low doses thus maintaining the viability of MCF-10A cells around 90%. On the other hand, β3 was less effective than β1-conglutin in all cell lines, including the non-tumoural one. Finally, β6 was the least effective in terms of MTT viability although it also showed a significant effect in comparison with the non-treated control for all cell lines, especially at higher doses for MCF-7. All β-conglutins studied in this first experiment induced cell growth inhibition more efficiently in the tumor cell lines compared to the non-tumoural one.

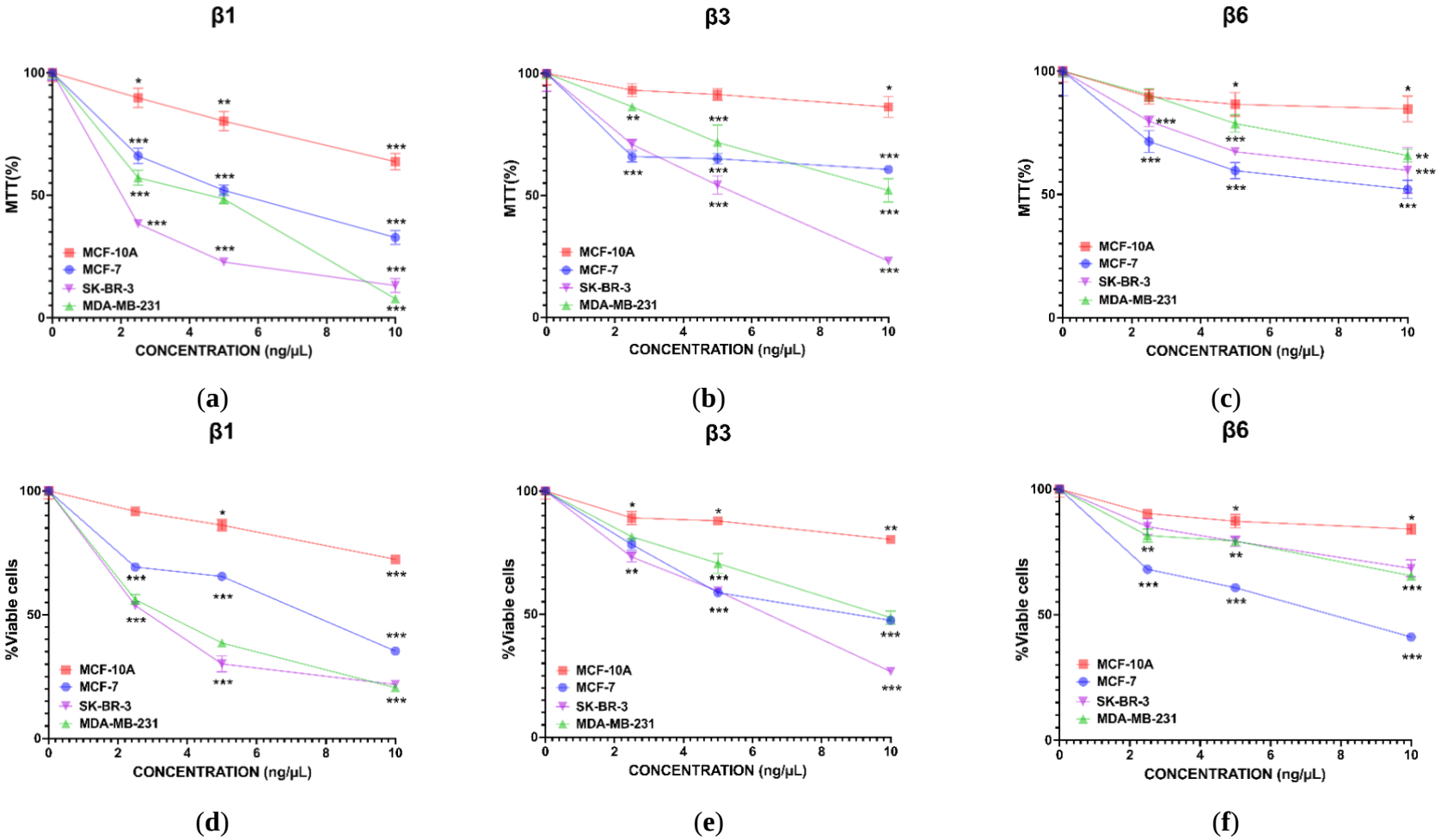


Figure 26: Inhibition of cell growth in an advanced BC model *in vitro* after 24 hours. MTT assay was performed for (a) β1, (b) β3 and (c) β6 treatments and absorbance was measured. Trypan blue dye assay was performed for (d) β1, (e) β3 and (f) β6 treatments in order to determine the cytotoxic effect of β-conglutins versus control. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells.

In order to ensure that the inhibition of proliferation is accompanied by a cytotoxic effect, the second assay was performed with the vital dye trypan blue following the protocol in *Material and Methods 4.2* (Figure 27d,e and f). The effect of these β-conglutins after 24 h of treatment was similar, as shown in Figure 26. The data obtained for the viability of our normal BC cell line model were used to construct a proximity matrix. We calculated the Pearson correlation between each pair of β-conglutins. As shown in Table 2, no similarities were found between the three treatments at 72 hours. This result confirms that the use of three different treatments is justified as each one has a different effect on the viability of our BC cell lines. Similarly, we calculated the Pearson correlation coefficients between each pair of cell line. In this case, we found similarity in the behavior of the MCF-10A and MCF-7 cell lines after treatments (Table 3).

Table 2: Similarity matrix for paired β-conglutins treatments. Data represent Pearson's correlation coefficients. Bold numbers indicate similarity. Similarity threshold were 0.95.

	β1	β3	β6
β1	1.000	0.400	0.200
β3	0.400	1.000	0.800
β6	0.200	0.800	1.000

Table 3: Similarity matrix for paired cell lines treated. Data represent Pearson's correlation coefficients. Bold numbers indicate similarity. Similarity threshold were 0.95.

	MCF-10A	MCF-7	SK-BR-3	MDA-MB-231
MCF-10A	1.000	1.000	-0.500	-1.000
MCF-7	1.000	1.000	-0.500	-1.000
SK-BR-3	-0.500	-0.500	1.000	0.500
MDA-MB-231	-1.000	-1.000	0.500	1.000

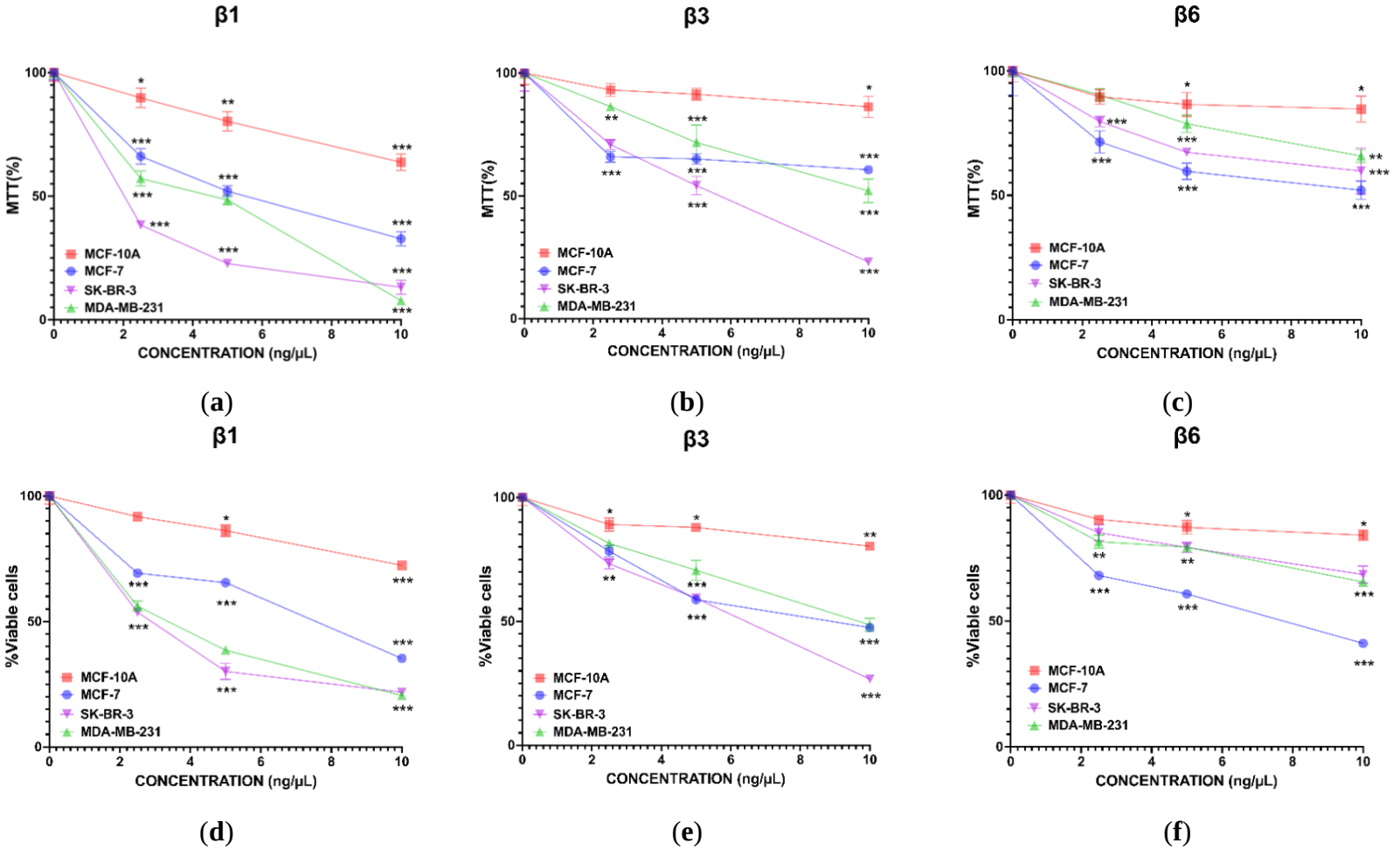


Figure 27: Inhibition of cell growth in an advanced BC model *in vitro* after 72 hours. MTT assay was performed for (a) β1, (b) β3 and (c) β6 treatments and absorbance was measured. Trypan blue dye assay was performed for (d) β1, (e) β3 and (f) β6 treatments in order to determine the cytotoxic effect of β-conglutins versus control. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells

b. β-CONGLUTINS INDUCE CASPASE-INDEPENDENT APOPTOSIS IN BC CELL LINES

Results of viability assays and similarity analysis confirmed that β-conglutins affect the viability of BC cell lines in comparison with the healthy epithelial one, with and individual effect for each single treatment and some similarity, in terms of evolution or tendency of the viability curve. In order to study deeply this cytotoxic effect, an apoptosis assay was performed for all the cell lines 24 hours after treatments with these proteins. Concentrations were chosen based on the cell growth and viability results shown in Figure 27, so that the viability of MCF-10A cells was preserved to the maximum and a significant effect in reducing the viability in at least one of the other three BC cell lines was found. The apoptosis assays were performed according to the protocol described in *Material and Methods (section 5).*

While non-statistically significant changes were found in MCF-10A cells (Figure 28a), β1 and β3 induced a significant peak of apoptosis in comparison with the non-treated control in SK-BR-3 cell line (Figure 28c). For MCF-7 and MDA-MB-231, the three treatments (β1, β3, and β6) induce apoptosis, especially in MCF-7 where a 40% of apoptotic cells is reached with β1, β3, and β6 treatments (Figure 28b and d). For the MDA-MB-231cell line, β1 induced the higher percentage of apoptosis in comparison with the other two β-conglutins.

To elucidate if this apoptosis activation is mediated through activation of caspases (caspase-dependent apoptosis), protein levels of Caspase 3 (total and cleaved) were studied by Western Blotting following *Material and Methods (section 5.1*). Interestingly, results shown in Figure 29 showed that neither of the three β-conglutins induced a significant activation of caspase 3 in any of the cell lines tested. The induced apoptosis is caspase-independent.

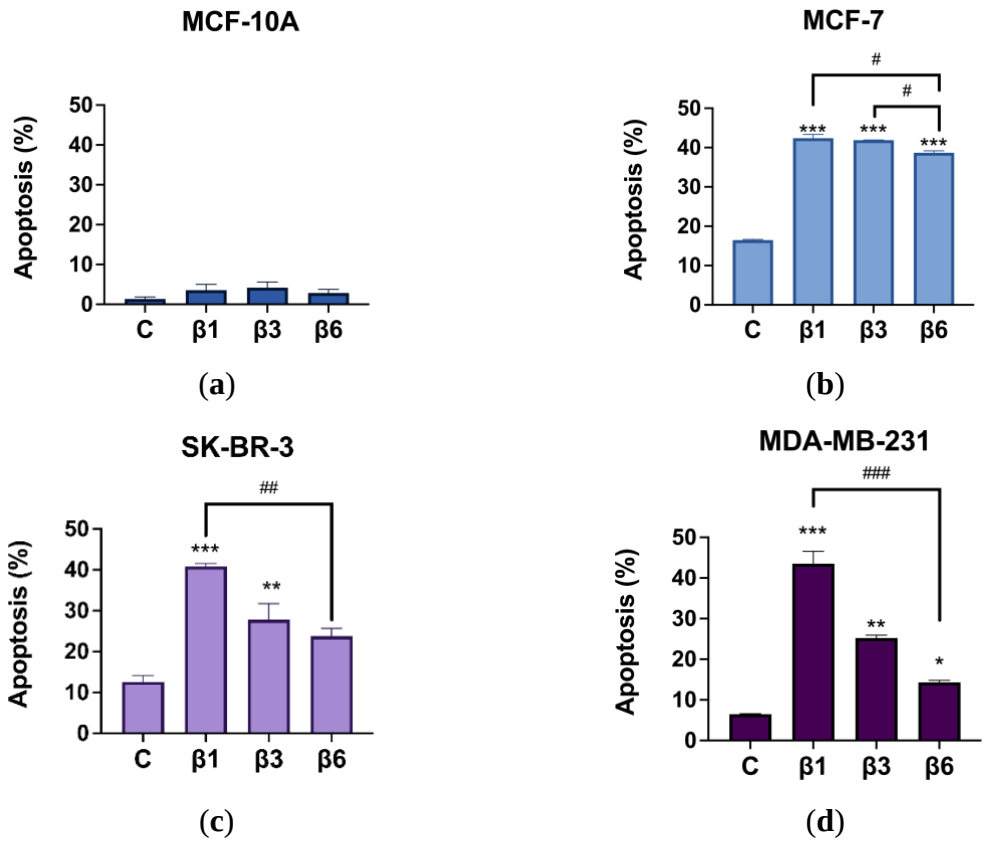


Figure 28: Apoptotic cells in an advanced BC model *in vitro* after 24 hours treatment of β1 (2.5 ng/μL), β3 (5 ng/μL) or β6-conglutins (10 ng/μL). Cells were incubated during 15 min with both annexinV-FITC and propidium iodide-PI (IP-AnnexinV kit from BD, Biosciences, UK). Apoptosis percentage was calculated with both early and late apoptotic cells. Samples were analyzed using BD FACS aria IIIu flow cytometer (Becton Dickinson, BD Bioscience). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells and # $p < 0.05$, ## $p < 0.01$ vs other treatments.

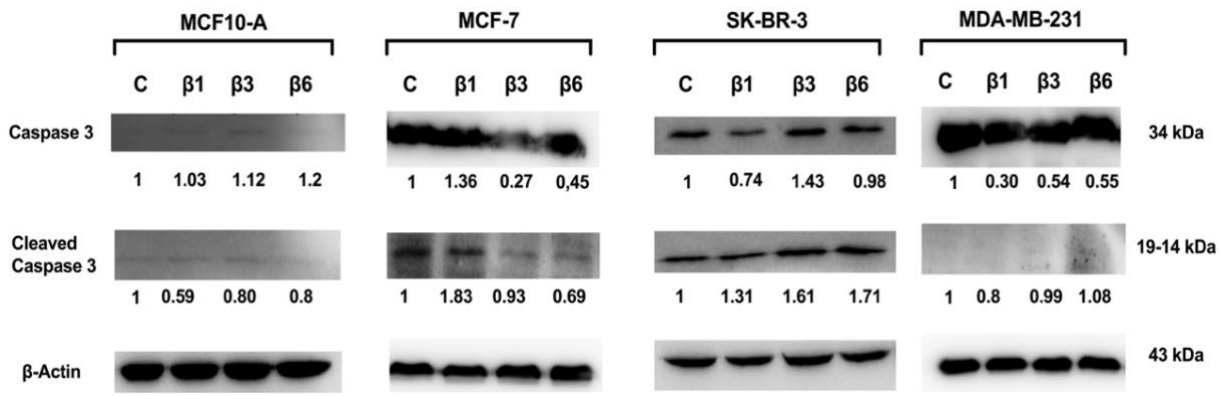


Figure 29: Western blot analysis of cleaved caspase 3 and total caspase 3. β-actin was used as a control**.** Cells were treated with β1-conglutin (2.5 ng/µl), β3-conglutin (5 ng/µl) or β6-conglutin (10 ng/µl) for 24h in MCF10-A, MCF-7, SK-BR-3 and MDA-MB-321.

To elucidate if other caspase-independent death mechanisms activated by β-conglutins could be ferroptosis, the same Apoptosis experiment was performed by adding ferrostatin-1 (1 µM), an inhibitor of ferroptosis, to each condition during 24 h (Figure 30). MCF-10A cells were not included for this experiment as no activation of apoptosis was observed. β-conglutins did not exert any effect on the percentage of ferroptosis, except for β1-conglutin which inhibits this process of cell death in MCF-7 cells (Figure 30a). These results are in concordance with the level of ROS and the DNA damage measured after the treatment of cells with these proteins, that are explained and represented in *Results (chapter II, c)* and Figure 31, respectively.

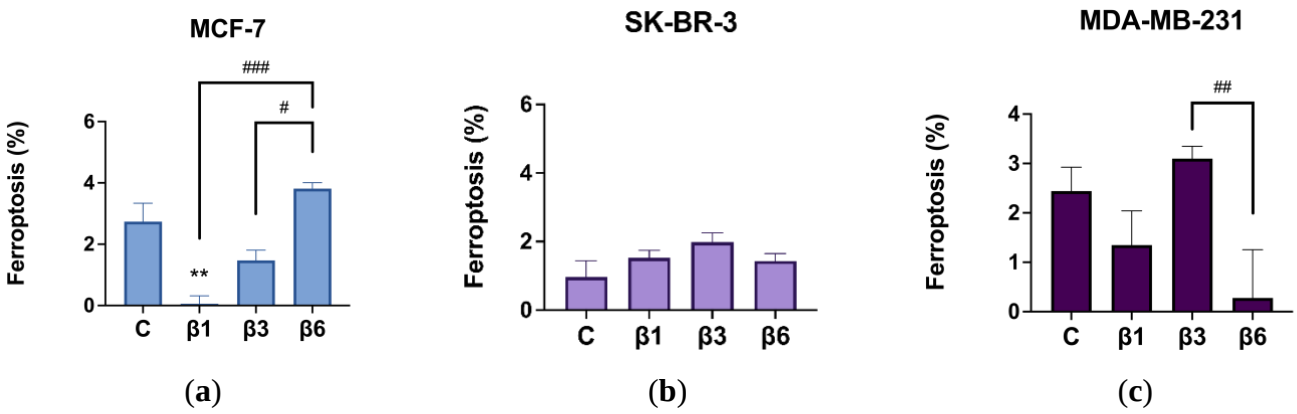


Figure 30: Ferroptotic cells in an advanced BC model *in vitro* after 24 hours treatment of β1 (2.5 ng/μL), β3 (5 ng/μL) or β6-conglutins (10 ng/μL). Cells were incubated during 15 min with both annexinV-FITC and propidium oodide-PI (IP-AnnexinV kit from BD, Biosciences, UK). Ferroptosis percentage was calculated with both early and late apoptotic cells. Samples were analyzed using BD FACS aria IIIu flow cytometer (Becton Dickinson, BD Bioscience). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells, and # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ vs. other treatments.

c. β-CONGLUTINS INHIBIT ROS AND DNA DAMAGE EXPRESSION IN BC CELL LINES

Treatment of all BC cell lines for 24 hours induces an inhibition of ROS levels accompanied by an inhibition of DNA damage. β1 and β3-conlgutins are the most effective treatments for both MCF-7 and SK-BR-3, while β6 has no significant effect on SK-BR-3. Interestingly, no significant changes in ROS levels or DNA damage were found in MCF-10A non-tumorigenic cells.

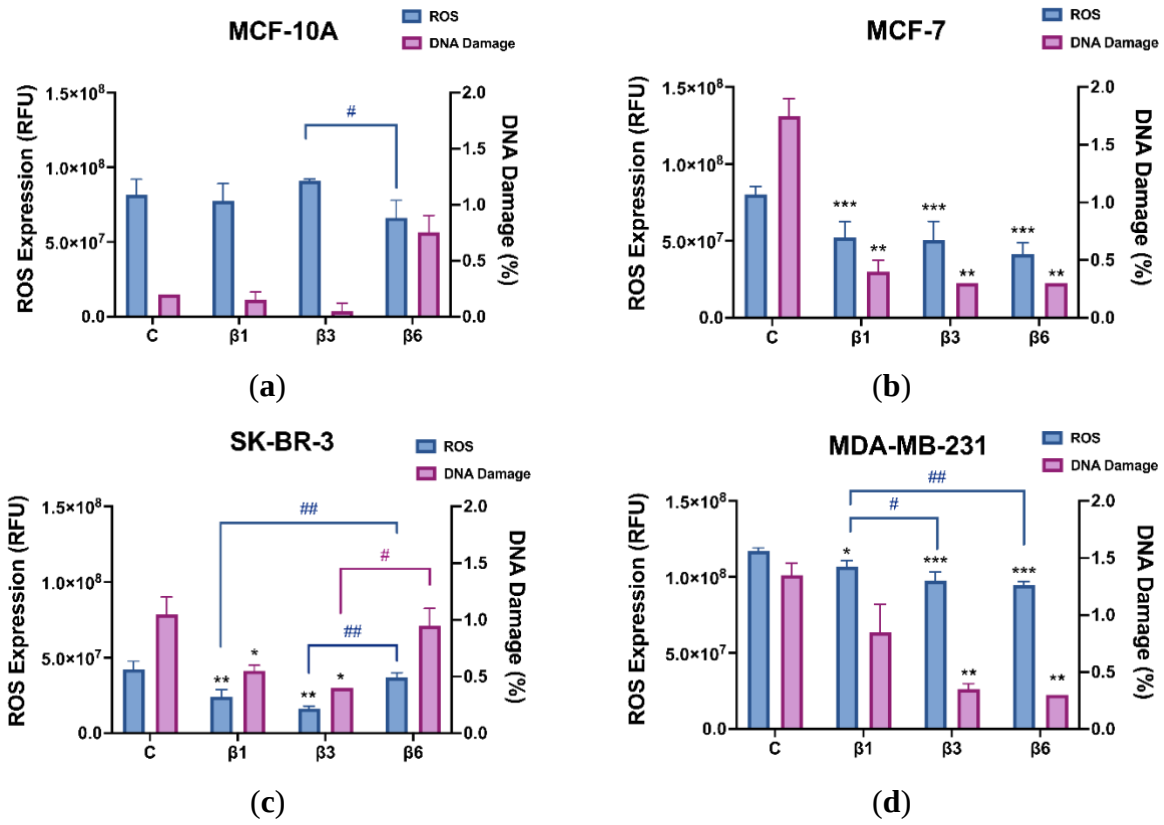


Figure 31: ROS levels and DNA damage percentage after treatment with β1-conglutin (2.5 ng/μL), β3-conglutin (5 ng/μL), or β6-conglutin (10 ng/μL) for 24 h in (**a**) MCF-10A, (**b**) MCF-7, (**c**) SK-BR-3, and (**d**) MDA-MB-321. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells, and # $p < 0.05$, ## $p < 0.01$ vs. other treatments.

d. IMPLICATION OF SIRT1/FOXO1 PATHWAY IN β-CONGLUTINS EFFECT ON BC CELLS *IN VITRO*

In order to elucidate the potential mechanism of action of the β-conglutins, we analyzed the changes in SIRT1 expression, implicated in BC growth and progression [77] that are sensible to cellular stress induced by ROS [164–166] acting through the activation of FoxO1 transcription factor, among others [167,168]. As shown in Figure 32, treatments with β-conglutins induce a decrease in the expression of SIRT1 in MCF-7 and SK-BR-3 cells. On the contrary, these proteins induce an increase in SIRT1 expression in MDA-MB-231 cells. These changes in SIRT1 expression are accompanied by similar changes in FoxO1 expression.

Since one of the processes regulated by the SIRT1/FoxO1 pathway is autophagy, LC3B and p62 protein expression after treatment was studied using the same technique (Figure 32). We found an increase of LC3B levels after β1 and β3-conglutin treatment with a decrease of p62 levels in the MDA-MB-231 cell line, which may suggest that autophagy processes are activated under these conditions. As expected, we found decreased expression in LC3B and increased expression of p62 in SK-BR3 cells, indicating inactivation of autophagy. Interestingly, an increase of LC3B levels after β1 and β3 and β6-conglutin treatment with an increase of p62 levels was found in the MCF-7 cell line, which may suggest that autophagy processes are not triggered (up nor down regulation) in this cell line after treatments. Results for MCF-10A showed no significant changes for SIRT1, FoxO1, or LC3B, but a subtle increase in p62, especially after treatment with β1, that is not related to autophagy in this case as it is not accompanied by any significant changes in LC3B expression. Autophagy is not activated for MCF-10A cells.

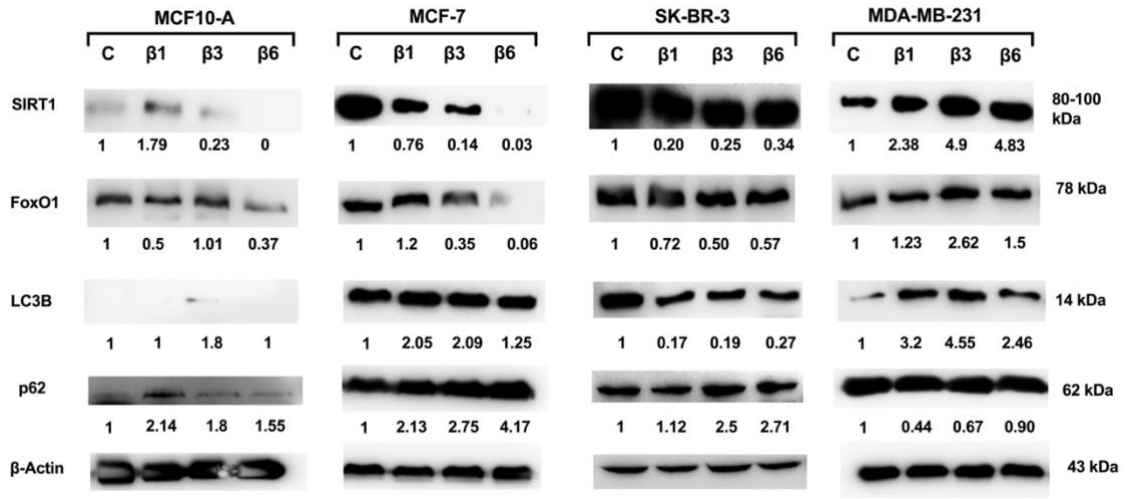


Figure 32: Western blot analysis of SIRT1, FoxO1, LC3B and p62. β-actin was used as a control**.** Cells were treated with β1-conglutin (2.5 ng/μl), β3-conglutin (5 ng/μl) or β6-conglutin (10 ng/μl) for 24h in MCF10-A, MCF-7, SK-BR-3 and MDA-MB-321.

e. IMPLICATION OF BMPs PATHWAY IN β-CONGLUTINS EFFECT ON BC CELLS *IN VITRO*

As previously reported in the introduction chapter, the BMP signalling in BC and its deregulations are crucial events regarding both the initiation of BC tumours and their progression, resistance and metastatic potential. In order to study it the proposed β-conglutin treatment could have any impact on this pathways of action, qPCR of interest BMP pathway related genes has been performed for the four cell lines, with the three β-conglutin treatments. Figure 33 shows the results of the genetic expression of BMP receptors (BMPR1A and BMPR2) and BMP2 and BMP4 (main BMP genes related with BC progression and CSCs regulation).

For MCF-10A cells, and according to the previous results obtained for the same cell line in the early BC model, β1 and β3 significantly decrease the levels of BMP receptors 1A and 2, and β1 decreases both BMP2 and BMP4 genetic expression in a significant manner too (Figure 33a,b). For MCF-7, only β1 had an effect on the BMP2 and BMP4 genetic expression, with a significantly lower level in comparison to the non-treated control (Figure 33c,d). For SK-BR-3, the three β-conlgutins caused the inhibition of the BMPR1A and BMPR2 levels. Interestingly, no BMP2 detection was obtained for this cell-line. Apart from that, β1, 3 and 6 significantly decreased the levels of BMP4 in SK-BR-3 treated cells. Finally, for MDA-MB-231 cells, β3 and β6 cause lower levels of both receptors and, in addition, both β1 and β3, specially the last one, caused a very low rate of BMP2 levels (Figure 33g, h).

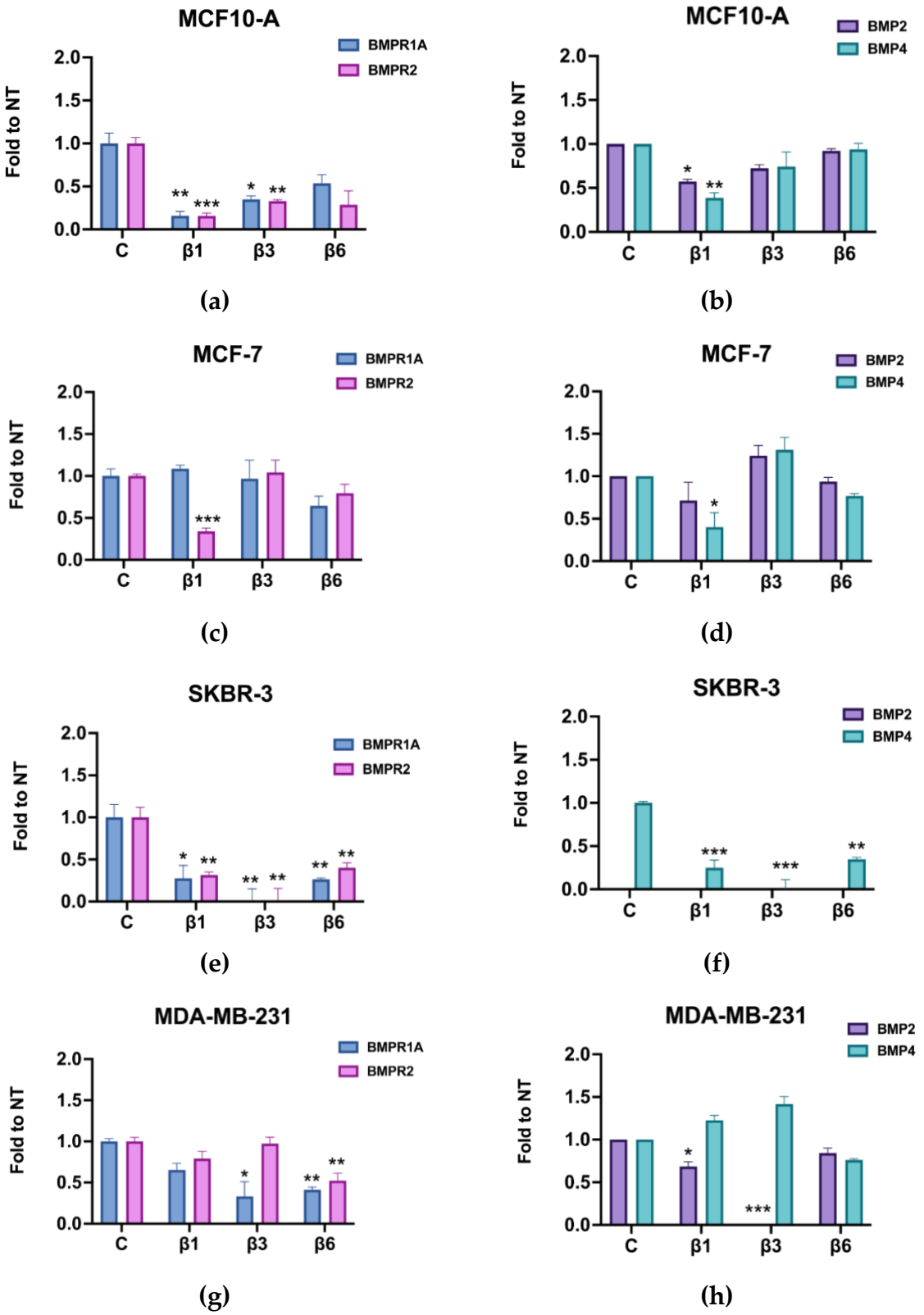


Figure 33: Results of genetic expression of BMP receptors (BMPR1A and 2) and of BMP-2 and BMP-4 after 24h of β1, β3 and β6 treatments in the normal BC cell lines. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells.

f. β-CONGLUTINS REGULATE STEMNESS PHENOTYPE IN BC CELLS *IN VITRO*

Next, we studied whether β-conglutins regulated stemness in our *in vitro* model of BC since SIRT1 was identified as a central regulator of progression and metastasis in BC through cancer stem cells (CSCs) and the therapeutic potential of this subpopulation was also described recently. Breast CSCs were characterized after treatment with β-conglutins using specific characteristics such as ALDH1 activity and CD44 high/CD24 low expression. The results were compared with a control (non-treated cells). ALDH1 activity (Figure 34) decreased significantly in all cases, except for MCF-7, in which β1 and β3 had no effect (Figure 34b). In addition, β6 was the most effective conglutin in reducing ALDH1 activity in all cell lines tested.

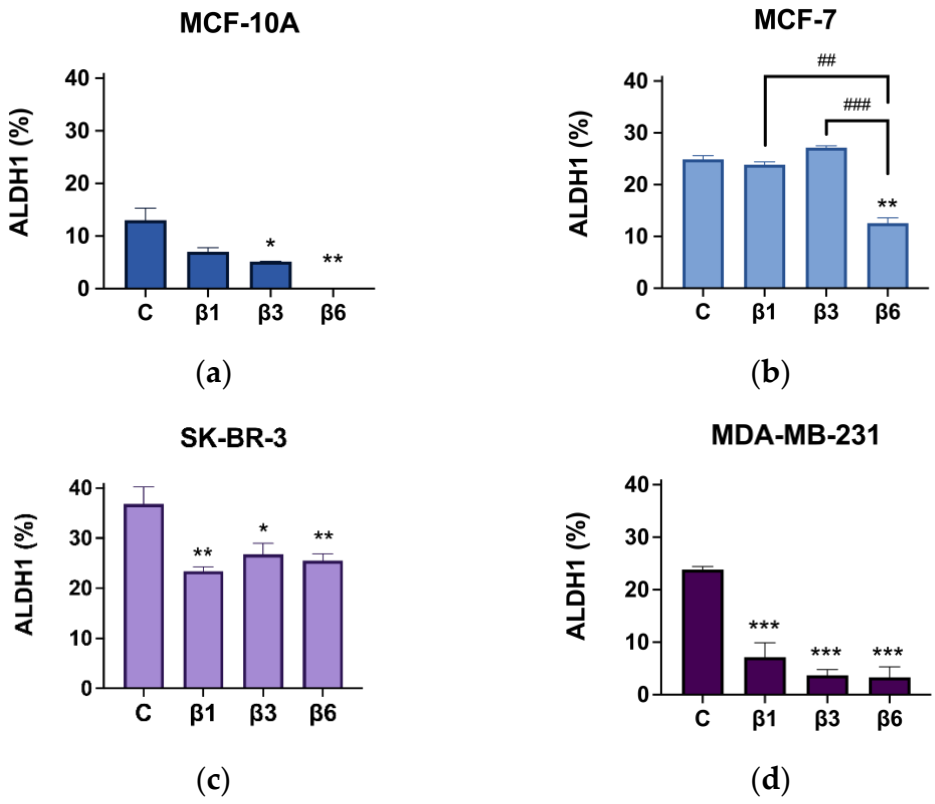


Figure 34. ALDH1 activity after treatment with β1-conglutin (2.5 ng/μL), β3-conglutin (5 ng/μL), or β6-conglutin (10 ng/μL) for 24 h in (**a**) MCF10-A, (**b**) MCF-7, (**c**) SK-BR-3,

and (**d**) MDA-MB-321. Viable ALDH1+ cells were quantified by flow cytometry on a BD FACS aria IIIu flow cytometer (Becton Dickinson, BD Bioscience). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells and ## $p < 0.01$, ### $p < 0.001$ vs. other treatments.

Similarly to the results obtained for ALDH1 activity, treatments with β-conglutins decreased the expression of the surface markers CD44high/CD24low in the four cell lines tested with comparable efficacy, except for MDA-MB-231 cells. In this case, only β3-conglutin showed a significant decrease in the surface markers (Figure 35).

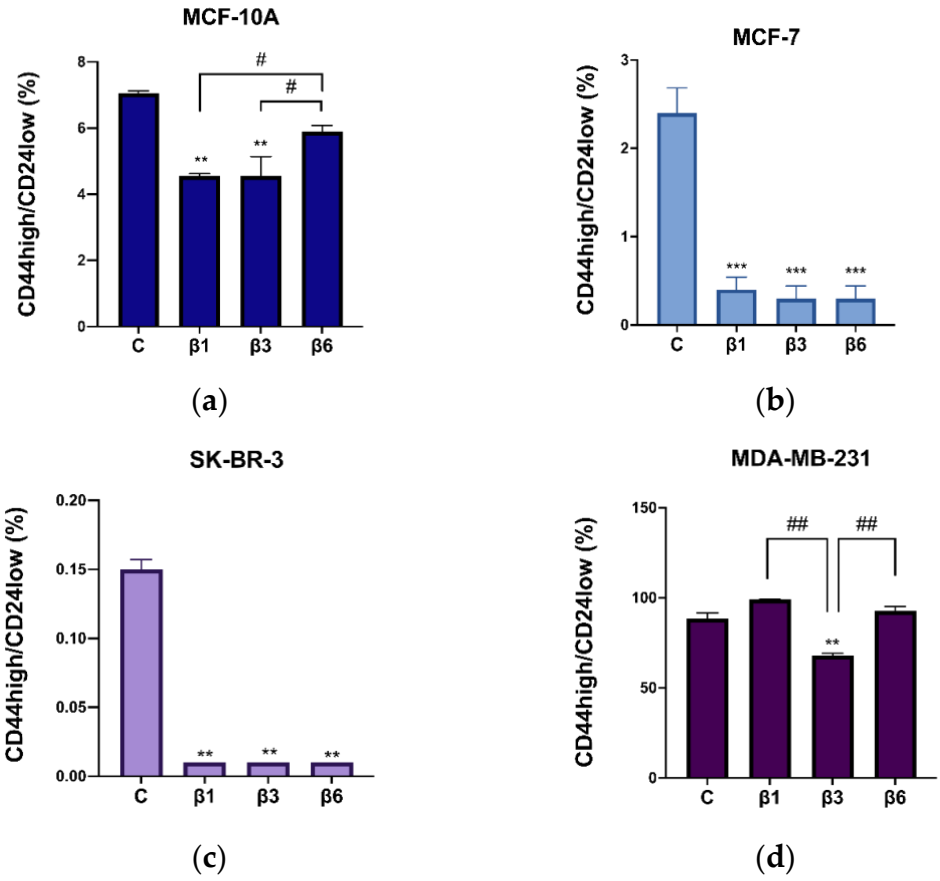


Figure 35. Expression of CD44high/CD24low with β1-conglutin (2.5 ng/μL), β3-conglutin (5 ng/μL), or β6-conglutin (10 ng/μL) for 24 h in (**a**) MCF10-A, (**b**) MCF-7, (**c**) SK-BR-3, and (**d**) MDA-MB-321. Samples were analyzed by flow cytometry on a BD FACS aria IIIu flow cytometer (Becton Dickinson, BD Bioscience). ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells and # $p < 0.05$, ## $p < 0.01$ vs. other treatments.

We finally analyzed the self-renewal capacity of cells after β-conglutin treatments through the mammosphere assay. As shown in Figure 36, β1, β3, and β6-conglutins reduced the number of mammospheres in MCF-10A, SKBR-3, and MDA-MB-231 cells. Interestingly, none of the β-conglutins were able to reduce the capacity to form spheres in MCF-7.

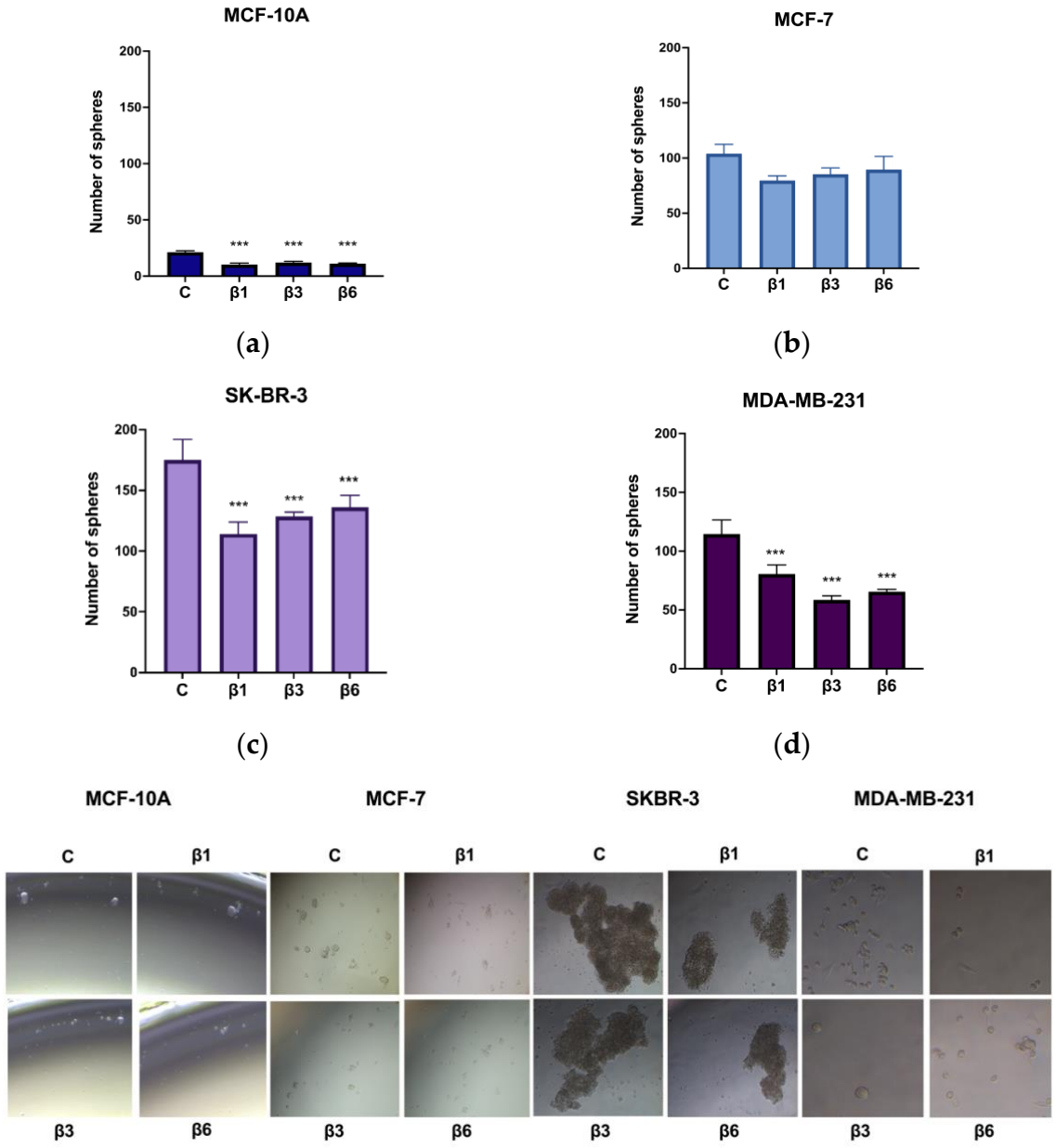


Figure 36. Quantification of mammospheres after treatment with β1-conglutin (2.5 ng/μL), β3-conglutin (5 ng/μL), and β6-conglutin (10 ng/μL). Treatment was performed 24 h before seeding mammospheres from (**a**) MCF-10A, (**b**) MCF-7, (**c**) SK-BR-3, and (**d**)

MDA-MB-231 and spheres were characterized in (**e**). Spheres > 50 μm were counted and captured using Leica DM500 binocular microscope at 10x magnification. *** $p < 0.001$ vs. non-treated cells.

CHAPTER V: COMBINED β-CONGLUTIN PRE-TREATMENT + IR TREATMENT IN A NORMAL BC MODEL

a. β-CONGLUTINS POTENTIATE THE EFFECT OF CONVENTIONAL IR *IN VITRO*.

The final chapter of results of this doctoral thesis is focused on the potential radiosentizing effect of β-conglutin treatment in combination with the standard ionizing radiation (IR) therapy. In order to study this potential effect and its mechanisms of action, the normal BC model, used in chapter II, was chosen, and the simple β-conglutin treatment results were used to optimize experiments.

A colony formation assay with the proposed combined therapy (β-conglutin treatment + ionizing radiation) (*Material and Methods section 4.4*) was performed and the dose-response curve for each cell line, using different concentrations of each β-conglutin and different radiation doses from 0 to 8 Gy, were obtained. Results are shown in Figure 37, where only the concentration of each β-conglutin corresponds to the simple β-conglutin treatment doses used in chapter II, as lower doses showed no significant effect for this combined therapy assay and higher doses could potentially cause damage to the healthy epithelial cells, as discussed in the previous chapter. Survival fraction was calculated following the protocol at *Materials and Methods (section 4.4)* and represented with logarithmic scale.

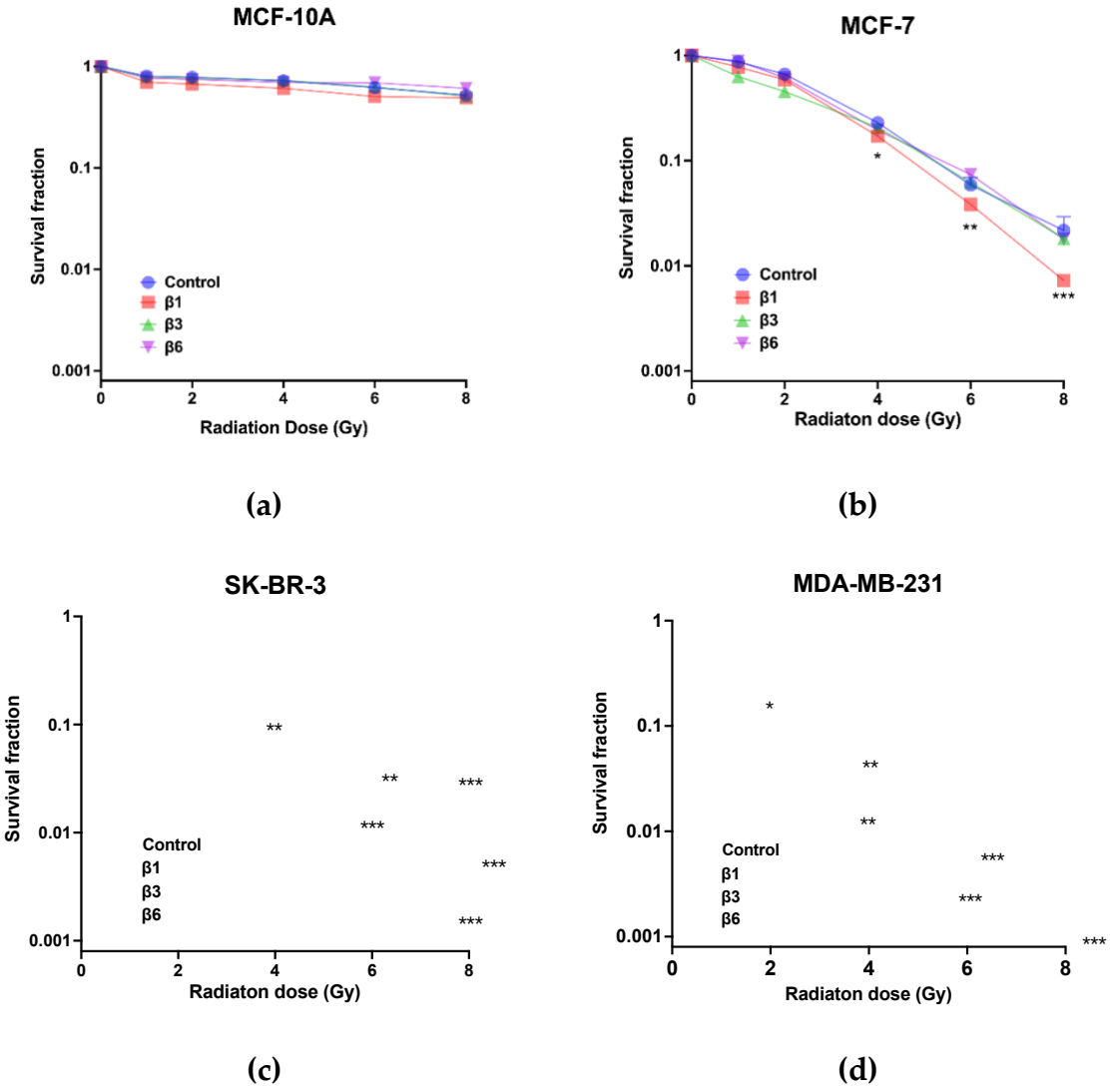


Figure 37. Dose-response curves (survival fraction) for the four cell lines: (**a**) MCF10-A, (**b**) MCF-7, (**c**) SKBR-3 and (**d**) MDA-MB-231 with a 24h treatment with selected β-C1; β-C3 and β-C6 concentrations and a combined added radiation dose: 0, 1, 2, 4, 6 and 8 Gy. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells

The results of the traceable dosimetry in Figure 37 showed that the β-conglutin treatment potentiates the effect of the IR treatment for the three tumor cell lines, especially for MDA-MB-231, the most aggressive BC cell line, but doesn't cause any significative changes regarding the resistance of the conventional IR treatment in the healthy non tumorigenic cells, MCF-10A. This first result is very promising as the β-conglutin proteins may be natural radiosensitizers for malignant cells without changing the intrinsic radio

resistance of epithelial healthy cells. For that reason, MCF-10A cell line will not be studied for the proposed combined therapy as it doesn't seem to show any significant differences.

On top of that, the radiosensitizing effect of the β-conglutins is different for each treatment and each tumor cell line: more precisely, β1 decreases significantly the survival fraction of MCF-7 cells for doses > 4 Gy, while β3 and β6 showed no significant effect compared to the conventional IR treatment in terms of long-term survival. On the other hand, β1 significantly reduced the survival fraction for SK-BR-3 cells at 4 Gy and β1, β3 and β6 reduced it even more for 6 and 8 Gy. Finally, for MDA-MB-231, the TNBC phenotype cell line, the radiosensitizing effect is the highest, with significant reduction of the survival fraction from 2 Gy with β1 and with the three β-conlgutins for higher doses. In this case, for example, the same survival fraction is obtained at 2 Gy with combined therapy (β-conlgutins + IR) than 4 Gy with the conventional IR, which could be a promising effect to reduce IR doses, preventing non-desirable side effects, but obtaining the same effect in terms of cell survival.

In order to understand the mechanism of action of these potential natural radiosensitizers, it is important to study how cell death mechanisms are triggered and what pathway these proteins use to achieve their desirable effect. Radiation doses were fixed at 4 Gy and 6 Gy in order to evaluate the radiosensitizing effect in all BC cell lines, as 2 Gy only showed a potential radiosensitazion. β-conglutins concentrations were the same as those used for the simple treatment in chapter II and were represented in Figure 30 (β1: 2.5 ng/uL, β3: 5 ng/uL, β6: 10 ng/uL).

b. β-CONGLUTINS IN COMBINATION WITH IR INDUCE GREATER CASPASE DEPENDENT AND INDEPENDENT APOPTOTIC LEVELS THAN CONVENTIONAL TREATMENT IN BC CELL LINES

Results of dose-response curves and survival fraction for the combined β-conglutin + IR treatment confirmed that β-conglutins could be natural radiosensitizers for BC cells, especially for MDA-MB-231. In order to study deeply this effect and how death mechanisms are triggered to obtain the lower survival rates, an apoptosis assay was performed for all the cell lines 24 hours after treatments with these proteins and 1 hour after IR therapy. The apoptosis assays were performed according to the protocol described in *Material and Methods section 5.*

While non-statistically significant changes were found in SK-BR-3 cells at 4 Gy (Figure 38b), as expected and in concordance with Figure 37, β1 induced a significant peak of apoptosis in comparison with the IR control at 6 Gy. For MCF-7, only β3 induce a higher rate of apoptosis than conventional IR at 4 Gy (Figure 38a) and no significant differences were found at 6 Gy, where all the conditions showed extremely high apoptotic rates (>50%). Finally, and as expected, for MDA-MB-231 cells, β1 and β3 induced 4 times more apoptosis in combination with a 4 Gy dose than the only irradiated control. For 6 Gy, only β1 showed a significant augmentation of the apoptosis percentage in comparison with the irradiated control.

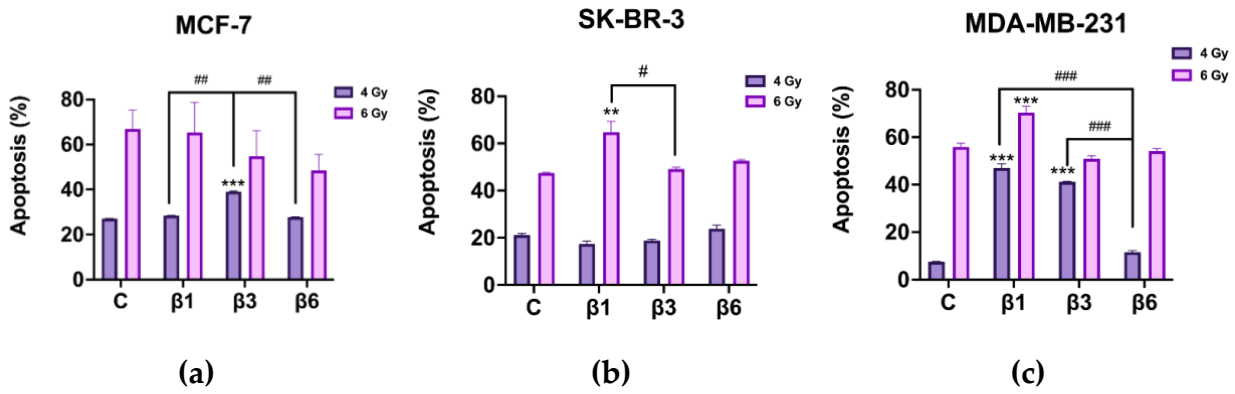


Figure 38: Apoptotic cells in an advanced BC model *in vitro* after 24 hours pre-treatment of β1 (2.5 ng/μL), β3 (5 ng/μL) or β6-conglutins (10 ng/μL) and posterior IR treatment at 4 and 6 Gy in (a) MCF-7, (b) SK-BR-3 and (c) MDA-MB-231. Cells were trypsinized 1 h post IR and incubated during 15 min with both annexinV-FITC and propidium iodide-PI (IP-AnnexinV kit from BD, Biosciences, UK). Apoptosis percentage was calculated with both early and late apoptotic cells. Samples were analyzed using BD FACS aria IIIu flow cytometer (Becton Dickinson, BD Bioscience). ** $p < 0.01$ and *** $p < 0.001$ vs. non-treated cells, and # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ vs. other treatments.

To elucidate if this apoptosis increase is mediated through activation of caspases (caspase-dependent apoptosis), protein levels of caspase 3, an executioner caspase, were studied by western blotting following *Material and Methods 5.1* at 4 Gy (Figure 39) and 6 Gy (Figure 40). Interestingly, results shown in Figure 39 showed that neither of the three β-conglutins induced a significant activation of caspase 3 in any of the cell lines tested at 4 Gy. In fact, β-conglutins seem to reduce caspase 3 levels, and the induction of apoptosis by β3 for MCF-7 and β1 and β3 for MDA-MB-231 at 4 Gy are caspase-independent Interestingly, as shown in Figure 40, when the IR dose was increased to 6 Gy, β3 and β6 produce an augmentation of caspase 3 protein level in all the cell lines that was not accompanied by an apoptosis higher rate (Figure 31a, b and c). The higher levels of apoptosis at 6 Gy (induced by β1 for both SK-BR-3 and MDA-MB-231 cells) are also caspase-independent, as β1 showed no augmentation of caspase 3 protein levels. Although the apoptosis induced after β-conglutins + IR is caspase-

independent, the elevation of caspase 3 levels at 6 Gy must be considered and studied in the discussion chapter.

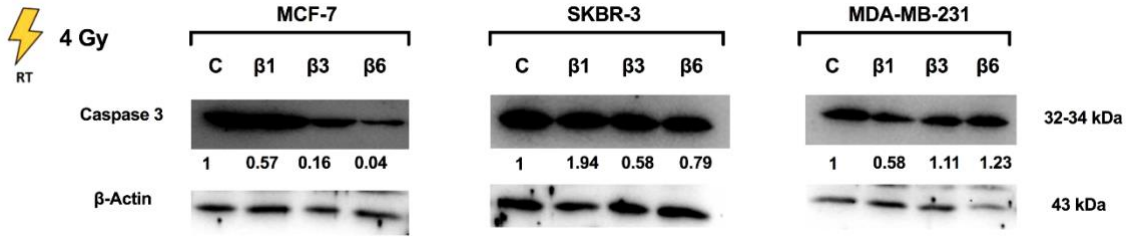


Figure 39: Western Blot analysis of total Caspase 3. β-actin was used as a control**.** Cells were treated with β1-conglutin (2.5 ng/μl), β3-conglutin (5 ng/μl) or β6-conglutin (10 ng/μl) for 24 h in MCF-7, SK-BR-3 and MDA-MB-321 and then irradiated at 4 Gy. Protein was collected 24 h post IR.

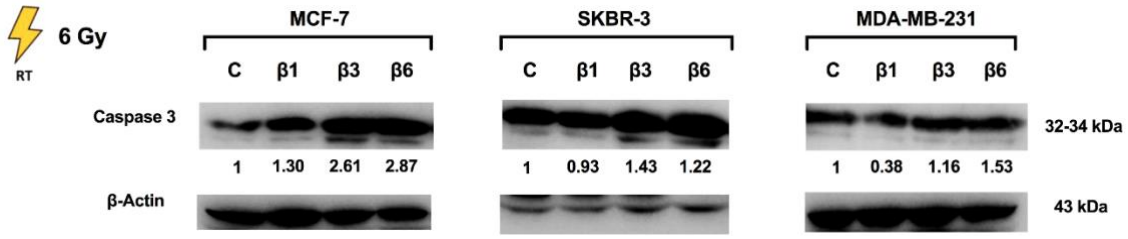
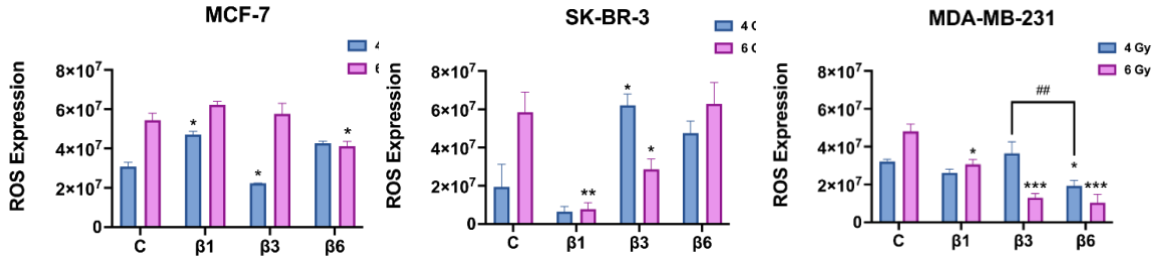


Figure 40: Western blot analysis of total caspase 3. β-actin was used as a control**.** Cells were treated with β1-conglutin (2.5 ng/μl), β3-conglutin (5 ng/μl) or β6-conglutin (10 ng/μl) for 24 h in MCF-7, SK-BR-3 and MDA-MB-321 and then irradiated at 6 Gy. Protein was collected 24 h post IR.

c. β-CONGLUTINS IN COMBINATION WITH IR DEREGULATE ROS LEVELS AND REDUCE DNA DAMAGE RESPONSE IN BC CELL LINES

In order to elucidate the effect post combined treatment in the studied cell lines, ROS and DNA damage levels were measured following *Material and Methods sections 6 and 7*. Treatment of all BC cell lines for 24 hours followed by IR

at 4 and 6 Gy for 1 h induced an de-regulation of ROS levels depending on the β-conglutin pre-treatment performed and the cell line, as observed in Figure 41. For MCF-7, β1 treatment induced a peak of ROS levels at 4 Gy while asignificant inhibition of ROS levels was observed at the same dose for β3 treatment. At 6 Gy, combined therapy with β6 treatment decreased ROS levels, but neither β1 or β3 showed any significant effect (Figure 41a). For SK-BR-3, an augmentation of ROS with β3 treatment and 4 Gy IR dose was observed, while β1 and β3 induce lower ROS levels with a 6 Gy dose (Figure 41b). Finally, for MDA-MB-231, no significant changes were found in ROS levels at 4 Gy dose, in comparison with the IR control, but a significant inhibition was found for β1 ($p < 0.05$) and β3 and β6 ($p < 0.001$) when the IR dose was increased to 6 Gy (Figure 41c).



(**a**) (**b**) (**c**)

Figure 41: ROS levels after treatment with β1-conglutin (2.5 ng/μL), β3-conglutin (5 ng/μL), or β6-conglutin (10 ng/μL) for 24 h and a posterior IR at 4 and 6 Gy in (**a**) MCF-7, (**b**) SK-BR-3, and (**c**) MDA-MB-321. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells, and # $p < 0.05$, ## $p < 0.01$ vs. other treatments.

Regarding DNA damage levels, after 24 h of β-conglutins and IR, the quantification of H2AX gave information about the DDR after treatments (Figure 42). The de-regulation of ROS levels was accompanied by an inhibition of DDR in general terms. For MCF-7, the three β-conglutins induced significant lower DDR percentage than the IR control at 4 Gy, while only β3 induced lower significant DDR leves for the higher dose, 6 Gy. In SK-BR-3, no significant

changes were found for 4 Gy dose, but all the treatments induced lower DDR rates for 6 Gy ($p < 0.05$ for β1 and β3, $p < 0.01$ for β6). Finally, β1 and β3 induce significant lower DDR at 4 Gy while only β1 combined with a 6 Gy IR dose induced a decreased DDR in MDA-MB-231 (Figure 42c).

Those results suggest that the apoptosis higher rates, found for β3 at 4 Gy in MCF-7, β1 at 6 Gy for SK-BR-3, β1 and β3 at 4 Gy and β1 at 6 Gy in MDA-MB-231 (Figure 31), are accompanied by lower ROS levels and reduced DDR simultaneously.

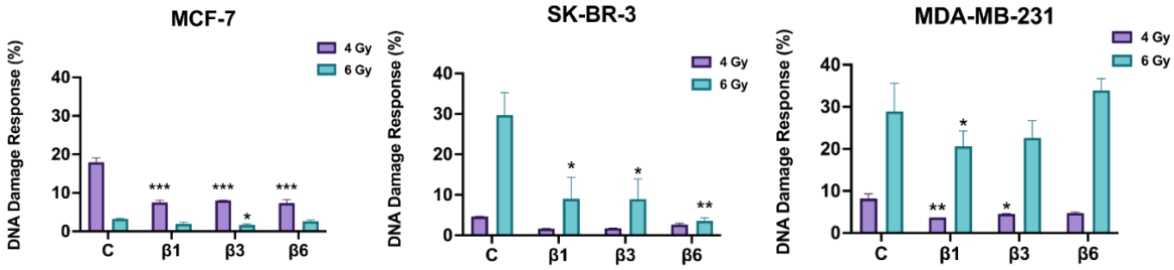


Figure 42: DNA damage response (%) after treatment with β1-conglutin (2.5 ng/μL), β3-conglutin (5 ng/μL), or β6-conglutin (10 ng/μL) for 24 h and a posterior IR at 4 and 6 Gy in (**a**) MCF-7, (**b**) SK-BR-3, and (**c**) MDA-MB-321. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells, and # $p < 0.05$, ## $p < 0.01$ vs. other treatments.

d. IMPLICATION OF SIRT1/FOXO1 PATHWAY AND AUTOPHAGY IN COMBINED β-CONGLUTINS AND IR EFFECT ON BC CELLS *IN VITRO*

In order to elucidate the potential mechanism of action of the β-conglutins as a radiosensitizer in combination with IR, following the same protocol as in chapter II, we analyzed changes in SIRT1 and FoxO1 expressions at both 4 Gy and 6 Gy. As shown in Figure 43, regarding the combined treatment at 4 Gy, β-

conglutins induce a decrease in the expression of SIRT1 in the three cell lines studied, that is accompanied with a lower expression of FoxO1 levels, except for MDA-MB-231 cells, where FoxO1 is increased. On the contrary, regarding the combined treatment at 6 Gy (Figure 44), these proteins induce an increase in SIRT1 expression for β3 and β6 in MCF-7 and for β1 in MDA-MB-231 cells. The rest of treatments continue to induce lower levels of SIRT1. These changes in SIRT1 expression at 6 Gy are accompanied by similar changes in FoxO1 expression.

Since one of the processes regulated by the SIRT1/FoxO1 pathway is autophagy, which is closely related to IR resistance, LC3B and p62 protein expression after treatment was studied using the same technique and protocol for autophagy assessment than in chapter two. Results at 4 Gy and 6 Gy are showed in Figure 43 and 44, respectively. We found an increase of LC3B levels after β1, β3 and β6-conglutin treatment with a decrease of p62 levels in the MDA-MB-231 cell line at 4 Gy and the same result after β6 treatment and 6 Gy IR, which suggests that autophagy processes are activated under these conditions. As expected, we found decreased expression in LC3B and increased expression of p62 in SK-BR3 cells at 4 Gy (β 1 and β 3) and 6 Gy (all β-conglutins), indicating inactivation of autophagy. Interestingly, a decrease of LC3B levels after β1 and β3 and β6-conglutin treatment with a decrease of p62 levels was found in the MCF-7 cell line in all cases, which may suggest that autophagy processes are not triggered (up nor down regulation) in this cell line after treatments.

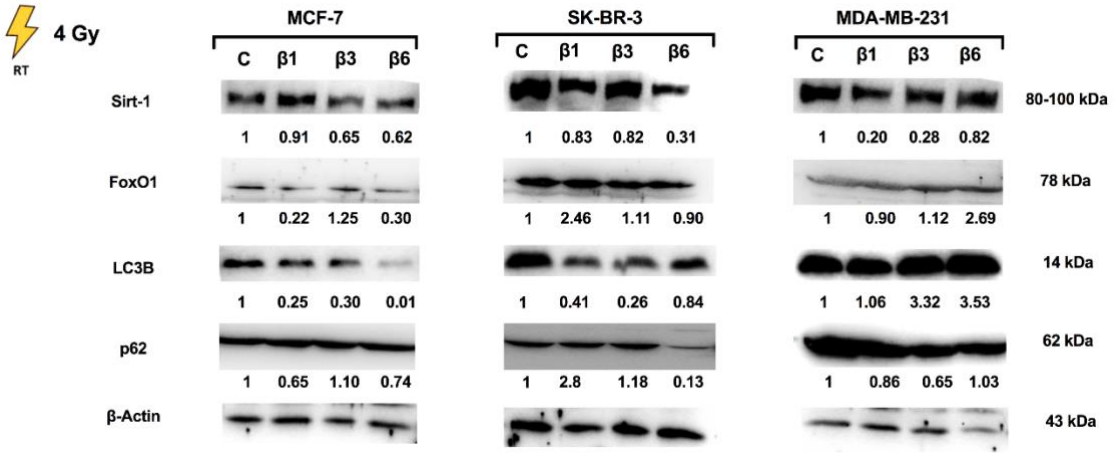


Figure 43: Western Blot analysis of SIRT1, FoxO1, LC3B and p62. β-actin was used as a control**.** Cells were treated with β1-conglutin (2.5 ng/µl), β3-conglutin (5 ng/µl) or β6-conglutin (10 ng/µl) for 24h in MCF10-A, MCF-7, SK-BR-3 and MDA-MB-321.

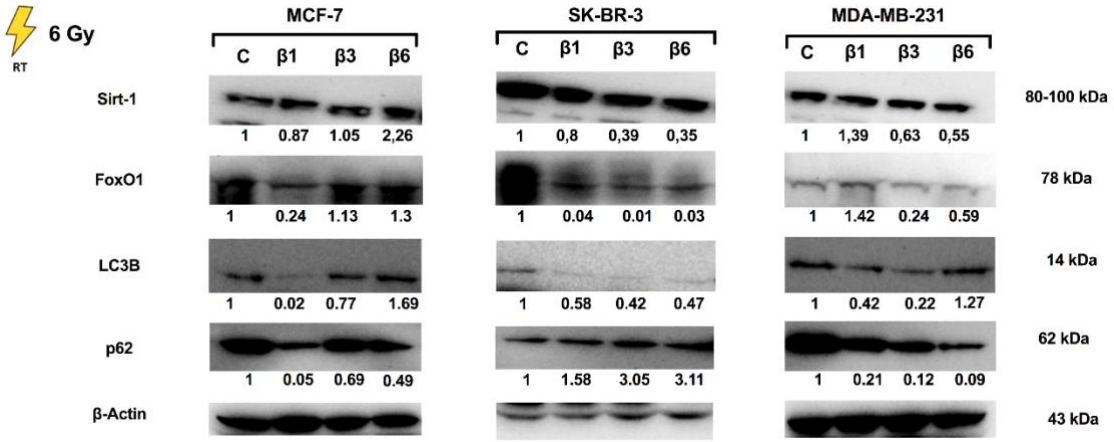


Figure 44: Western blot analysis of SIRT1, FoxO1, LC3B and p62. β-actin was used as a control**.** Cells were treated with β1-conglutin (2.5 ng/µl), β3-conglutin (5 ng/µl) or β6-conglutin (10 ng/µl) for 24h in MCF10-A, MCF-7, SK-BR-3 and MDA-MB-321.

e. β-CONGLUTINS AND IR COMBINED TREATMENT REGULATE *IN VITRO* STEMNESS PHENOTYPE MORE EFFICIENTLY THAN CONVENTIONAL IR IN BC CELLS

Finally, we studied whether β-conglutins pre-treatment, in combination with IR therapy, regulated stemness in our *in vitro* model of BC, since conventional IR has showed a potential enhancement of CSCs phenotype as a non-desirable effect, despite its therapeutic properties in BC. Breast CSCs were characterized after treatment with β-conglutins and IR using specific characteristics such as ALDH1 activity and CD44 high/CD24 low expression, as already performed in chapter II. The results were compared with an irradiated control for each dose (non-pretreated with β-conglutins cells). ALDH1 activity (Figure 45) decreased significantly for MCF-7 at 4 Gy with the three β-conglutins, while no significant differences were found at 6 Gy. Interestingly, for SK-BR-3, only β1 at 4 Gy showed a significant augmentation of ALDH1 activity, that can be in concordance with survival (Figure 37b) and apoptosis (Figure 38b) results at this dose. Finally, β3 and 6 treatments induce lower ALDH1 levels in MDA-MB-231 at 4 Gy, while only β1 induced those lower levels in 6 Gy. It is important to mention that, for the three cell lines IR controls, the ALDH1 activity was dramatically decreased with 6 Gy in comparison with 4 Gy non-pretreated cells. As well as in the traceable dosimetry experiment (Figure 37), some β-conglutins treatments at 4 Gy reduced the ALDH1 levels as the 6 Gy IR single therapy, for example, β3 and 6 at 4 Gy in MDA-MB-231 showed similar ALDH1 percentages than the 6 Gy control, and β6 treatment in MCF-7 at 4 Gy induced lower ALDH1 activity than the 6 Gy control.

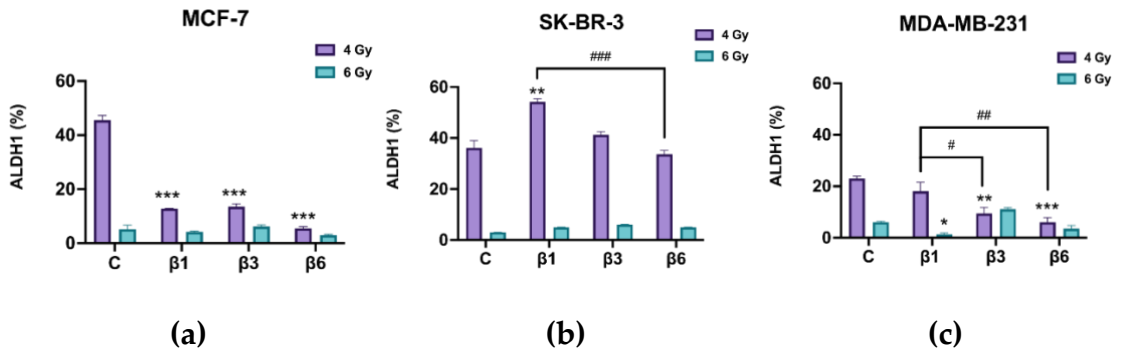


Figure 45. ALDH1 activity after a 24 h pre-treatment with β1-conglutin (2.5 ng/μL), β3-conglutin (5 ng/μL), or β6-conglutin (10 ng/μL) and a posterior IR treatment at 4 and 6 Gy in (**a**) MCF-7, (**b**) SK-BR-3, and (**c**) MDA-MB-321. Viable ALDH1+ cells were quantified by flow cytometry 1 h post IR treatment on a BD FACS aria IIIu flow cytometer (Becton Dickinson, BD Bioscience). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells and ## $p < 0.01$, ### $p < 0.001$ vs. other treatments.

Similarly, to the results obtained for ALDH1 activity, treatments with β-conglutins generally decreased the expression of the surface markers CD44high/CD24low in the three cell lines tested in comparison with the irradiated control. Concretely, all treatments induced lower expression of CD44high/CD24low in MCF-7 and SK-BR-3 at 4 Gy, while only β3-conglutin showed a significant decrease in the surface markers for MDA-MB-231 at 4 Gy. At 6 Gy, all β-conglutins induce lower expression of CD44high/CD24low in MDA-MB-231 cells, but only β3-conglutin showed a significant decrease for MCF-7 as well as β1 in SK-BR-3 (Figure 46).

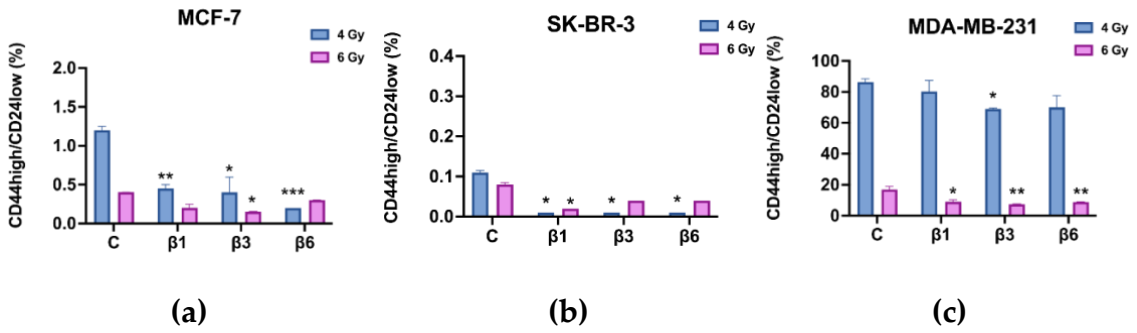


Figure 46. Expression of CD44hih/CD24low with a 24h pre-treatment of β1-conglutin (2.5 ng/μL), β3-conglutin (5 ng/μL), or β6-conglutin (10 ng/μL) and a posterior IR treatment at 4 Gy and 6 Gy in (**a**) MCF-7, (**b**) SK-BR-3, and (**c**) MDA-MB-321. Samples were analyzed by flow cytometry 1h post IR on a BD FACS aria IIIu flow cytometer (Becton Dickinson, BD Bioscience). ** $p < 0.01$, and *** $p < 0.001$ vs. non-treated cells and # $p < 0.05$, ## $p < 0.01$ vs. other treatments.

We finally analyzed the self-renewal capacity of cells after β-conglutin pre-treatments followed by IR through the mammosphere assay. As shown in Figure 47a, b and c, β1, β3, and β6-conglutins significantly reduced the number of mammospheres in MCF-7. For MDA-MB-231 cells, β1 and β3 reduced the number of mammospheres at both doses too. Interestingly, only β-conglutin 1 in combination with 6 Gy was able to reduce the capacity to form spheres in SKBR-3. The characterization of those spheres by microscopy is showed in Figure 38 d where, in concordance with ALDH1 and CD24low/CD44high results, β-conlgutins combined treatment with 4 Gy IR can show a reduced self-renewal capacity of cells in a greater way than the 6 Gy IR single dose, concretely for β1 in MCF-7 and MDA-MB-231.

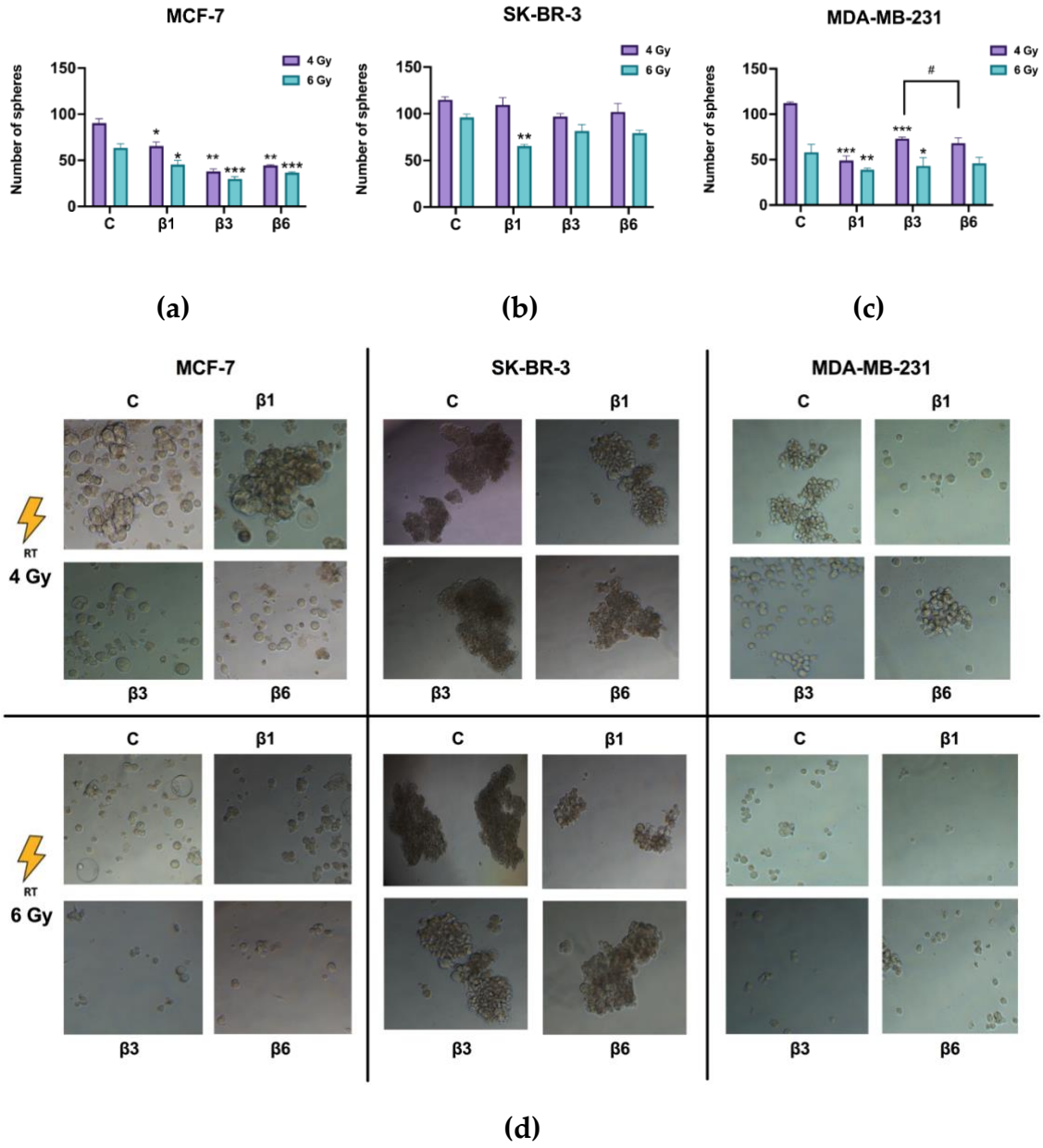


Figure 47. Quantification of mammospheres after a 24h pre-treatment with β1-conglutin (2.5 ng/μL), β3-conglutin (5 ng/μL), and β6-conglutin (10 ng/μL) and posterior IR treatment at 4 and 6 Gy. IR Treatment was performed 24 h before seeding mammospheres from (**a**) MCF-7, (**b**) SK-BR-3, and (**c**) MDA-MB-231 and spheres were characterized in (**e**). Spheres > 50 μm were counted and captured using Leica DM500 binocular microscope at 10x magnification. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. non-treated cells.

DISCUSSION

The main objective of this doctoral thesis is the application of the recently discovered NLL β-conglutin proteins in BC *in vitro*. Of course, the first step in order to achieve this objective was to obtain and purify the β-conglutin protein isoforms following an already published protocol by Jimenez-Lopez et al [160], with modifications, prior to the use of those nutraceutical compounds in BC in vitro. However, not all β-conglutin proteins had been characterized in depth in the previous published studies, and the part of their structure responsible for the interesting anti-inflammatory and antioxidant properties exerted by the β-conglutin proteins and described in previous reports had not been identified yet [148,149,151,160]. For all of those reasons, a functional study, carried out in parallel with the application of already studied β-conglutin proteins in BC in vitro, was performed, with the objective of assessing the structure-functional domain responsible for the properties of the β-conglutin proteins isoforms, using β5 and β7 isoforms, that had not been studied before.

In this context, we performed the study of the effect of β-conglutin proteins β5 and β7 and their respective truncated forms (tβ5 and tβ7) in Hep2G cells, T2D patients' cells and healthy control cells, with the aim of analyzing the expression of pro-inflammatory mediators (TNF-α, IL-1β and iNOS) as potential pro-inflammatory targets. Regarding this, other studies of nutraceutical compounds, like soybean, have demonstrated that germination of soybean produce bioactive peptides capable of inhibiting the synthesis of NO, TNFα, and PGE2 and the expression of iNOS and COX-2 in LPS-stimulated RAW 264.7 macrophages, directly related with the inflammation process[169]. On top of that, recently, the anti-inflammatory properties of narrow-leafed lupin (NLL) γ-conglutin protein from mature seeds using in vitro human PANC-1 pancreatic cell-line have been studied in both, an induced inflammation model using bacteria lipopolysaccharide (LPS), and an induced insulin resistance cell model, with the aim of assessing the capability of NLL γ -conglutin to improve the

oxidative stress homeostasis of cells, the inflammatory induced state and the IR improvement at molecular level by decreasing several pro-inflammatory mediators genes expression and proteins levels, as well as up-regulating of insulin signaling pathway gene expression. These studies have shown that NLL γ -conglutin achieved a plethora of functional effects as the strong reduction of cell oxidative stress induced by inflammation through decreasing proteins carbonylation, nitric oxide synthesis and inducible nitric oxide synthase (iNOS) transcriptional levels, and raising glutathione (GSH) levels and modulation of superoxide dismutase (SOD) and catalase enzymes activities. γ -conglutin induced up-regulated transcriptomic and protein levels of insulin signalling pathway IRS-1, Glut-4, and PI3K, improving glucose uptake, while decreasing pro-inflammatory mediators as iNOs, TNFα, IL-1 β , INF γ , IL-6, IL-12, IL-17, and IL-27. These results suggest a promising use of NLL γ -conglutin protein in functional foods, which could also be implemented in alternative diagnosis and therapeutic molecular tools helping to prevent and treat inflammatory-related diseases [170].

In fact, different diseases, such as T2D, obesity, and metabolic syndrome, are associated with and chronically sustained by inflammation. Multiple mechanisms could be underlying inflammatory response diseases, where multiple stressor factors affect pancreatic islets function, and contributing to the pathology depending on aspects such as genetic background and environment [171]. Diabetes is a chronic disease that occurs when pancreatic islets fail to produce sufficient insulin and/or the sensitivity of glucose-metabolizing tissues to insulin decreases. During the establishment of T2D, chronic hyperglycemia may lead to organ damage. Pancreatic beta cells mediate tissue damage and promote inflammatory responses together with immune system cells by releasing pro-inflammatory mediators and other cell attractants as chemokines, establishing a feed-forward process that further increases immune system cell

content and promotes a chronic inflammatory state [172]. Increased levels of cytokine IL-1 β or iNOS as a consequence of stressor factors are among the main contributors to inflammatory development since IL-1 β -mediated pancreatic β -cell dysfunction is involved in the pathogenesis of pancreatic β -cell dysfunction and establishment of T2D. For instance, sustained exposure of the pancreatic β cell to IL-1 β activates the expression of iNOS resulting in excessive production of NO, which induces the expression of pro-inflammatory genes [150,173].

T2D is characterized by impaired insulin secretion and/or insulin sensitivity [174], and sustained by chronic subclinical inflammation. Recently results raise the possibility of using these particular β -conglutin proteins in the prevention and treatment of diabetes, as well as their potential as anti-inflammatory molecules. Our results showed that iNOS mRNA and protein levels were substantially increased in T2D in all experimental groups when compared to control [151].

Regarding the antioxidant regulatory capacity of cells, removal of free radicals is strongly dependent of enzymatic activities as superoxide dismutase (Cu/Zn-SOD), catalase (CAT) and glutathione (GSH) levels, representing crucial indicators of the cellular anti-oxidant capacity, and the oxidative stress cell state [175], and, for those reasons, we studied CAT, SOD and GSH parameters after with the β-conglutin treatment. Our results highlight the potential implications of β-conglutin (β5 and β7) to decrease the pro-inflammatory capacity in HepG2, T2D cells and healthy control cells by decreasing gene expression levels of TNFα, IL-1 β and iNOS, thus supporting the inflammatory process amelioration at molecular level. The body encloses a complex antioxidant defense grid that relies on endogenous enzymatic and non-enzymatic antioxidants. These molecules collectively act against free radicals to resist their damaging effects to vital biomolecules and ultimately body tissues. Based on their response to general free

radical invasion, they can be categorized into first, second, third and even fourth line defense antioxidants. The role and effectiveness of the first line defense antioxidants which basically include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) is important and indispensable in the entire defense strategy of antioxidants [176].

As already commented earlier on, recent studies have revealed that NLL γ-conglutin achieved a plethora of functional effects as the strong reduction of cell oxidative stress induced by inflammation through decreasing proteins carbonylation, nitric oxide synthesis and inducible nitric oxide synthase (iNOS) transcriptional levels, and raising glutathione (GSH) levels and modulation of superoxide dismutase (SOD) and catalase enzymes activities [170]. In our study, this lowering in the cellular pro-inflammatory capacity could be the result of the antioxidant capacity of β-conglutin since changes in GSH levels, SOD and catalase activities was shown, helping to keep redox homeostasis in T2DM and other inflammatory-dependent diseases also affected by the oxidative stress. [177] Interestingly, recent reports showed that the modulation of these antioxidant factors by γ-conglutin in the inflammatory LPS-induced PANC-1 cells, as well as in insulin resistant-C cell model, by measuring SOD and catalase activities, GSH levels and NO production, before and after the treatment with γ -conglutin. These studies indicate a statistically significant ($p < 0.05$) decreased levels of GSH. Furthermore, the levels of SOD and catalase activity were strongly reduced after the same treatments with γ -conglutin protein in LPS-induced inflammatory statement. These data showed that high GSH and low SOD levels and catalase activities might be regulated by γ -conglutin protein through direct or indirect marked effects in avoiding lipids and protein oxidative modifications, which is also supported by the concomitant large reduction of oxidative carbonylation and an overall oxidative stress balance improvement, translated also to an inflammation molecular cellular statement amelioration by γ-conglutin protein as an anti-oxidant protein [170].

Finally, regarding the effect of β -conglutins on NO production in HepG2, T2D cells and healthy subject cells, it is important to mention that NO is a signaling molecule that plays a key role in the pathogenesis of inflammation. NO is considered a pro-inflammatory mediator that induces inflammation when over-produced in abnormal situations. The link between pancreatic β -cell stress and inflammation in T2D activates the expression of a variety of pro-inflammatory genes of cytokines and chemokines (e.g., TNF-α, IL-6, IL-12, CCL2, CCL5, and other pro-inflammatory mediators such as NO), in addition to the excessive production of ROS. This pro-inflammatory cytokines expression would activate inflammatory signaling pathways and the attraction of immune cells that further aggravate local inflammation, causing β -cell apoptosis and T2D [178]. NO production by inducible NO synthase (iNOS) is a sign of inflammation progression. The over-production of NO is detrimental for the host, leading to the development of inflammatory-related diseases [179].

In recent reports, the effect on the NO production was determined in T2D blood cell culture challenged with individual β1, β2, β3, β4, β6 isoforms, or β-conglutins mix after LPS treatment for 24 h, and also adding at the same time LPS+ β -conglutin isoforms or LPS + mix. LPS added to blood cell culture significantly increased NO production, compared to the control group. A range of β -conglutin treatments was tested to see if they could reverse the effects of LPS on NO production. The blood cultures of patients with T2D had significantly elevated levels of NO compared to blood cultures from normal donors, adding β1, β3, or β6 conglutins to the T2D blood cultures able to reduce NO production, but adding β2 or β4 conglutins had no effect. Interestingly, adding the β-conglutin mix (β1+ β2+ β3+ β4+ β6) to T2D blood cultures also strongly suppressed NO production.

Soybean lunasin, a short 2S-albumin-derived peptide, has anti-inflammatory effects by inhibiting NO production in macrophages [180]. However, this is the

first time that a legume protein belonging to *L. angustifolius* has suppressed NO production in blood culture cells from T2D patients, with potential anti-inflammatory effects [150]. Recently, the NO production in induced inflammation cell models treated with γ -conglutin protein for 24 h were analyzed. Statistically significant decreased levels of NO were found ($p < 0.05$) in the LPS-induced cells and insulin resistant-C cells in comparison to inflammation induced cells without γ-conglutin protein treatment, showing again how γ-conglutin is able to ameliorate the inflammatory state of cells promoting lowering NO [181].

Our results, regarding all the discussed information, showed that β-conglutin proteins β5 and β7 achieved functional effects with a strong reduction of cell oxidative stress induced by inflammation through raising glutathione (GSH) levels, modulation of superoxide dismutase (SOD) and catalase enzymes activities. However, truncated conglutin β proteins tβ5 and tβ7 are not able to do the same effects.

Regarding all the discussed results, we have shown that β-conglutin proteins 5 and 7 also possess the anti-inflammatory and antioxidant properties that had already been described in recent reports about β-conglutin proteins 1, 3 and 6 and inflammatory-related diseases. In addition, as a novel discovery, we have demonstrated that the responsible domain of functional properties of those β-conglutins 5 and 7, and in extension, β-conglutin proteins 1, 3 and 6, is the mobile-arm structural-functional domain, as, when truncated conglutin β proteins tβ5 and tβ7 were used as treatments, none of the anti-inflammatory nor antioxidant properties were exhibited. This promising result not only shows the importance of the mobile-arm structural-functional domain to achieve this potential nutraceutical effect, but also confirms β5 and β7 conglutins as potential preventive and therapeutic agents against inflammatory-diseases like cancer, particularly BC, as discussed below.

To the best of our knowledge, this study is the first attempt to evaluate the molecular effects of NLL β-conglutin proteins 1, 3 and 6 in cancer cell lines, concretely in BC ones, from early to advanced stages and even metastatic BC cell lines. To this aim, we studied the effect of NLL β-conglutin proteins, obtained by overexpression and purification, in two different BC models, and in combination or not with IR[163]. The first model used mimics the early stages of luminal and basal BC tumors, derived from MCF-10A cell, a healthy non-tumorigenic cell line used as a healthy control. Our results showed that β1 and β3 conglutins inhibited cell growth, had a cytotoxic effect, reduced the number of both luminal and basal progenitors depending on the cell line and regulated CSCs reducing stemness properties, especially in cell lines corresponding to the most aggressive BC subtypes for this model, M1B26 (basal cell with metastatic potential) and MC26-R (luminal cell resistant to conventional 5-FU chemotherapy) preserving the viability of healthy cells, MCF-10A. We also studied the regulation of the BMP pathway under the treatment conditions, obtaining a potential mechanism of action for this proteins in early stages of BC and in prevention of MCF-10A malignant transformation.

β1 and β3 conglutin treatments induce lower growth rates and present a cytotoxic effect for early luminal and basal BC cell lines (MC26 and M1B26 respectively) at 24 h and 48 h, preserving the viability of MCF-10A healthy cells and MC26-R, luminal and resistant to 5-FU cells. This resistance disappears at 72 htreatment and very low doses, where the viability of MCF-10A cells is still preserved. In fact, drug resistance is one of the major obstacles for BC treatment improvement nowadays, as it limits the effectiveness of chemotherapy drugs [182]. Determining mechanisms to reverse this chemoresistance would help to establish new strategies for BC and improve survival rates [183]. Recent reports have tried to overcome this resistance using several anticancer drugs or novel agents [183,184] and obtaining promising results in terms of reversing chemotherapy resistance

to improve BC treatment. Our results suggest that β-conglutins could potentially be an alternative natural chemotherapy for early BC cell lines, overcoming the resistance to 5-FU with an alternative natural treatment.

β-conglutins also showed a significant effect in terms of reducing the number of "luminal-like", "myoepithelial-like" and mixed or "bi-potent" colonies depending on the phenotype of the cell line, as assayed in the E-CFC test. This assay was used to identify differentiated epithelial progenitor that can form *in vitro* colonies [185,186] with MCF-10A derived early BC cells. Our results showed a lower number of luminal and bi-potent colonies in MCF-10A cells, a decreased luminal colony population for MC26 cells, a lower number of myoepithelial or basal colonies in M1B26 and a lower number of both basal and luminal colonies for the resistant cell line, MC26-R. In fact, the luminal progenitors compartment has been reported to have enhanced susceptibility to oncogenic events that might confer more genetically unstable features leading to the generation of tumors [187,188]. Other reports have demonstrated that an increased frequency of bi-potent progenitors, that can give rise to both luminal and myoepithelial compartments, is related to the stem-cell phenotype (spheres) compared to the normal tissue [189]. Finally, a recent report has revealed that a large amount of BRCA1-associated and basal-like breast tumors are derivated/originated from luminal progenitors rather than from myoepithelial/basal stem-cells [187]. According to this evidence, the lower number of luminal and bi-potent colonies observed in MCF-10A cells after the E-CFC assay could be considered as a preventive event for luminal differentiation to tumoural and CSC phenotype. As well, the lower number of luminal-like colonies in both MC26 and MC26-R cells could also be related to a lower luminal differentiation capacity for those BC cell lines.

Regarding the transformation of healthy cells into luminal or basal BC cells, increasing evidence indicates that the microenvironment plays an active role in cancer, but the role of microenvironment in initial steps of transformation remains unexplored [68,190]. BMP proteins and its pathway have been studied and

proposed as main actors in this process, identifying BMP2 as an important factor of the stem-cell niche that regulates the luminal differentiation of mammary progenitors, leading to tumor development [68]. Jung *et al.* unveiled that the human primary luminal breast tumors produces abnormally high amounts of BMP2, associated with BMPR1B, a receptor of the BMP pathway, overexpression by the tumor cells [66]. Chapellier *et al.* also discovered that BMP2 and IL-6 chronical exposure enabled MCF-10A transformation in a BMPR1B dependent manner and BMP2 sustained and expanded luminal progenitors [68]. Finally, in BC, it has been demonstrated that the niche-secreted BMP2/BMP4 promoted stem-cell transformation, and the up-regulation of BMP4 could be involved in transformation initiation in an inflammatory context [68,191,192]. All this evidence leads us to study the effect of the β-conglutins treatment in the BMP pathway related genes. Our results showed a decreased level of BMP receptors, particularly BMPR1B, and BMP4 in MCF-10A cells. In the tumoural cell lines, β-conglutins induced lower levels of BMPR1B and BMP2 in MC26 cells and MC26-R cells. BMP4 was significantly decreased after β-conglutin treatment for MC26-R and M1B26 cell lines too. According to the previous reports, β-conglutins could inhibit luminal transformation in MCF-10A cells by decreasing BMPR1B expression and prevent a transformation in an inflammatory context by decreasing BMP4 levels [68,192]. Regarding more advances stages of BC, BMP2 has been shown to modulate the proliferation and differentiation of BC cells [70]. In a recent study, BMP2 promoted the migration and invasion of luminal A BC cells by regulating the reorganization of cytoskeleton and the expression of adhesion molecules on the cells [70]. Another recent study about the BMP pathway in BC showed that BMP4 signalling enhanced the expression of stem cell genes and enhanced chemo-resistance and self-renewal in a TNBC model [71]. According to those evidences, the lower BMP2 levels in MC26 (luminal) and the lower BMP4 levels in M1B26 (basal) found after β-conglutins treatment could inhibit tumor progression and invasion and reduce chemo or radio resistance on BC cells.

Interestingly, the resistant cell line, MC26-R, also showed lower BMP2 and BMP4 levels, that could be at the origin of their sensibility to this treatment.

Other important genes related to the BMP pathway have been studied after treatment with β-conglutins 1 and 3 in this early BC cell line model. Concretely, the three tumoural cell lines showed lower expression of IDs (Inhibitors of DNA-binding), that are important modulators in the regulation of cell proliferation and differentiation [193]. Overexpression of ID2 can be used as a prognostic marker in BC patients and aberrant expression levels of the ID proteins have been reported in BC [193,194]. Recent studies have suggested that ID proteins act as crucial factors that regulate tumour progression and have shown a principal role in tissue invasion, tumor angiogenesis and metastasis [195]. Our results showed that the three BC cell lines studied presented lower ID expression, especially for ID2, after the β-conglutin treatment. Regarding other BMP related genes, the overexpression of Smad6, a BMP-specific inhibitory Smad, has been related to the potentiation of MCF-10A cells invasions as well as enhancing the aggressiveness of TNBC cells [196]. In this context, we demonstrated that β-conglutin treatment reduced Smad6 expression in M1B26 and MC26-R cells, which present a more aggressive phenotype than MC26. The inhibition of Smad6 in this basal and luminal-resistant phenotypes could prevent cell invasion and reduce aggressiveness. In fact, knockdown of Smad6 has already been reported as an inhibitor of TNBC cell invasion [196]. Finally, our results showed a decreased expression for CD10 after treatment in MCF-10A, M1B26 and MC26-R cell lines. CD10, a remarkable member of the major class of widely expressed cell surface proteins, endopeptidases, has become largely used in cancer diagnosis over the years, although its function in oncogenesis remains unclear [197]. Recent papers showed that CD10 expression is associated with tumor invasion and cancer stem cell phenotype in BC [198] and that CD10 is associated with the sphere-forming ability of BC cells, linked with higher CSCs phenotype and worst prognosis [199].

Our results regarding the lower CD10 expression after β-conglutin treatment could be linked to a lower probability of CSCs phenotype and tumor invasion, as well as with a reduced sphere-forming capacity.

In this context, as already described, the BMP pathway is closely related to stemness phenotype. In fact, BMP2 has been reported to facilitate epithelial-to-mesenchymal (EMT) transition and bone metastasis in BC, contributing to stem cell transformation and BC initiation [200]. ID proteins have also recently been associated with EMT process [193]. In order to elucidate the role of β-conglutins in the stemness phenotype and CSCs subpopulation, the mammosphere assay was performed, showing a decreased number of spheres for both MC26-R and M1B26 after treatments. This reduction of self-renewal capacity could be linked to the regulation of BMP pathway, concretely the inhibition of IDs and CD10, the reduced levels of BMPR1B and BMP2/4 and the down-regulation of Smad6, genes that have all been linked to EMT and CSCs phenotype. In this regard, β-conglutins could be a potential treatment targeting CSCs phenotype. According to all of our results *in vitro* for the early BC model, those β-conlgutins could be potential cytotoxic/natural treatments, alternative to conventional chemotherapy, to effectively treat early BC stages, fight chemotherapy resistance and preserve healthy cells. They could also offer a preventive strategy for MCF-10A cells, reducing the probabilities of a malignant transformation.

Once the β-conglutin treatment was tested in an early BC model, the aim was to study its effect in a normal or more advanced BC model in comparison with the same healthy epithelial cell line, MCF-10A. Our results showed that β1, β3, and β6-conglutins inhibited cell growth, had a cytotoxic effect, and regulated CSCs reducing stemness properties, especially in cell lines corresponding to the most aggressive BC subtypes (luminal B and TNBC), preserving the viability of healthy cells, MCF-10A [163]. We proposed a mechanism of action focused on the SIRT1/FoxO1 axis in a p53-dependent manner and also studied the impact of the

treatment in the BMP pathway. In fact, the four cell lines studied in the BC normal model in chapter II, MCF-10A, MCF-7, SK-BR-3 and MDA-MB-231, present different status of p53, as reported in several scientific papers [201-179]. First, MCF-10A, the epithelial healthy BC cell line is p53 wild-type [201], as well as BC cell line MCF-7 [202]. On the other hand, MDA-MB-231 cells, presenting the most aggressive phenotype type, is a mutant p53-expressing cell line, concretely, the p53-R280K, a gaining function mutation [201,202]. SK-BR-3 cells also harbored a mutated p53 without gaining functionality, as the mutation is in this case functional p53-E280K [203,204]. Recent evidence has already described different pathways of action and progression of BC cell lines treatments and characteristics depending on p53 status [201,202,205]. Considering the described differences, the recent evidence about the importance of this genetic mutation, and our results for each cell line, it seems that the final effect of the β-conglutins is strongly linked to the state of p53[163]. In fact, a similar effect was found in MCF-7 and MCF-10A cells after β-conglutin treatment, both harboring a wild-type p53, as previously reported [206,207]. Interestingly, the three β-conglutins used did not show similarity between them (Table 2), indicating that each β-conglutin has a specific behavior and the use of them separately was correct, instead of using the complete β-conglutin protein extract [163].

NLL conglutins β1, β3, and β6 inhibited the growth and viability of the three BC cell lines studied. This cytotoxic effect is followed by increased caspase-independent apoptosis. Other recent reports focusing on natural compounds for BC treatment have shown similar effects. Lupiwighteone is present in the root of Glycyrrhiza glabra, a medicinal herb [79], inhibits cell growth for MDA-MB-231 and MCF-7 cells and triggers caspase-independent apoptosis on both of them, whereas epithelial keranocytes were almost unaffected under the same conditions [79]. Another study shows that dioscin, a saponin natural product extracted from *Polygonatum zanlanscianense*, suppressed cell growth and induced caspase-independent cell death mechanisms in BC cells [208]. Since many cancer

cells have defects in caspase signaling, they can become resistant to conventional chemotherapy drugs that induce caspase-dependent apoptosis [209], so the induction of caspase-independent cell death could be an alternative pathway for overcoming cancer cell resistance [163].

Among other caspase-independent death cell mechanisms, ferroptosis, an iron-dependent cell death characterized by excess ROS-induced lipid peroxidation [210], has recently emerged as a new cell death mechanism related to the eradication of CSCs (resistant cells). In fact, several reports have showed a relationship between the inhibition of ferroptosis and the promotion of epithelial-to-mesenchymal transition (EMT), which prompts invasion and is related to the earliest stages of cancer and resistant cells [211]. In our study, β-conglutins reduced ferroptosis in the MCF-7 cell line and induced maintenance of ferroptosis basal levels in SK-BR-3 and MDA-MB-231 cells, indicating that other caspase-independent cell death could be responsible for the effects of these proteins [163].

Since neither inhibition nor any changes in ferroptosis are found in our study [163], treatment with β-conglutins could induce similar changes in reactive oxygen species (ROS) levels, as published before [151]. The modulation of ROS in cancer cells may represent a viable strategy in order to overcome drug resistance [212]. Aberrant regulation of redox homeostasis is found in cancer cells compared to normal ones [213]. In fact, other studies have shown that tumor cell lines, especially aggressive ones, such as MDA-MB-231, show higher levels of intracellular ROS compared to luminal or healthy breast cells [214], which is in concordance with our results[163]. A recent study with patients showed that the maintenance of chemotherapy-resistant cancer cells in TNBC is due to an increased mitochondrial oxidative phosphorylation and ROS, which is involved in the maintenance of CSCs [215]. Despite all of these data, the role of ROS levels in cancer including BC, remains controversial regarding a therapeutic approach [214]. Both ROS and DNA damage decreased after the β-conglutin treatment for

BC cells and maintained their levels for MCF-10A [163]. Increased ROS levels induce DNA mutations that can facilitate cancer metastasis [212], so β-conglutin proteins 1, 3, and 6 could prevent this scenario by inducing a reduction of both ROS and DNA damage, thus affecting viability and inducing cell death in BC cell lines, without affecting those levels in healthy epithelial cells [163].

The SIRT1/FoxO1 regulatory axis is a ROS-sensitive pathway implicated in BC progression and aggression [75–78]. This pathway of action was studied using natural compounds such as resveratrol, a dietary phenolic compound which reduced the effectiveness of paclitaxel, one of the usual chemotherapeutics agents in BC, and this reduction was mediated by up-regulation of the SIRT1/FoxO1 pathway in MDA-MB-231 and SK-BR-3 cells [78,163]. Other studies showed that SIRT1-mediated FoxO1 deacetylation is a key mechanism for multidrug resistance in BC cell lines [80]. Finally, a recent study about the use of an active compound naturally present in many vegetables and medicinal plants, isoalantolactone (IATL) and its anticancer properties in BC showed that this product induced caspase-independent apoptosis and could be related to a ROS-mediated downregulation of SIRT1 [216].

This dual pathway effect also appears in our study: SIRT1 and FoxO1 are up-regulated in MDA-MB-231, and down-regulated in MCF-7 and SK-BR-3. Whereas, no significant changes were found in the healthy epithelial cell line, MCF-10A, supporting our results describing its resistance to this treatment, at least at the doses used in our experiments [163]. Recent studies researched about the dual effect of the SIRT1/FoxO1 axis as tumor promotor or suppressor in different cancers [80], and p53 has emerged as a downstream effector of this axis in BC [80–82]. Previous results showed that in the MCF-7 cell line, with p53 wild-type, the activation of SIRT1 promoted invasion and migration on malignant cells by inhibiting p53 [217]. This matches our results as, in this cell line, the β-conglutins reduced SIRT1 levels which led to reduced cell growth and increased cytotoxicity[163]. On the other hand, other studies have found that high levels of

SIRT1 can inhibit tumorigenesis in BRCA-1 BC, which is usually a type of TNBC, and it is the phenotype of the MDA-MB-231 cell line, presenting higher levels of p53 mutations [218,219]. Finally, the cooperation between SIRT1 and p53 could be at the origin of genomic integrity and stability determining its role in cancer progression and aggressiveness [220]. One of the processes regulated by the SIRT1/FoxO1 pathway is autophagy. Recent studies have described this process as a precursor of apoptosis [85], and it can either inhibit or collaborate with apoptosis in tumor therapy. Our results showed that only in TNBC phenotype-like cells, MDA-MB-231, with mutant p53 gaining functionality, SIRT1 and FoxO1 increase is accompanied with autophagy induction [163]. Other natural compounds have already described this pathway of action for their anti-cancer effects, such as eugenol, a promising anti-cancer agent against TNBC and HER-2 positive BC (MDA-MB-231 and SK-BR-3, respectively), that targets the caspase pathway and induce autophagic cell-death [89]. As expected, inhibition of autophagy was observed in SKBR-3 cells after β-conglutin treatment since it induced downregulation of the SIRT/FoxO1 pathway in these cells. However, in the MCF-7 cells, the treatment induced inhibition of SIRT/FoxO1 but not changes in autophagy [163]. It was previously reported that the genic knock-out of SIRT1 reduced the proliferation, migration, and invasion of MCF-7 breast cancer cells [217]. In addition, a recent report showed that, for this specific cell line, MCF-7, the up-regulation of the SIRT1/FoxO1 pathway causes the induction of ferroptosis in a p53-dependent manner [221]. Those results suggest that, at least after the β1-conglutin treatment, the mechanism of action in MCF-7 could be induced by the regulation of the ferroptotic process instead of the regulation of autophagy observed for the other two BC cell lines [163]. Ultimately, it seems that the effect of the β-conglutin treatment is strongly linked to the status of p53. These proteins could modulate the duality of SIRT1/FoxO1 pathway, leading to an up or down-regulation of autophagy with different mutant p53 statuses or a ferroptosis down-regulation for p53 wt [163].

Regarding more advances stages of BC, as already introduced in the first part of this discussion chapter, the BMP pathway plays a crucial role not only in tumour initiation but in cancer progression. In fact, the BMP signalling alterations in BC are emerging to propose new therapeutic strategies for this disease [68]. As already mentioned, BMP2 has been shown to modulate the proliferation and differentiation of BC cells and promote the migration and invasion of Luminal A BC cells [70]. Other studies showed that BMP4 signalling enhanced the expression of stem cell genes and enhanced chemo-resistance and self-renewal in a TNBC model [71]. Our results showed that β1 and β3 significantly decrease both BMP2 and BMP4 genetic expression in a significant manner too, according to the early BC model results too. This could have a protective effect on healthy cells, preventing their malignant transformation. For MCF-7, β1 induced lower levels of BMP2 and BMP4 genetic expression. For SK-BR-3, no BMP2 detection was obtained, as already reported in [222] where BMP2 levels were significantly lower for SK-BR-3 cells than other BC cell lines. β1, 3 and 6 significantly decreased the levels of BMP4 in SK-BR-3 treated cells. Finally, for MDA-MB-231 cells both β1 and β3 caused a very low rate of BMP2 levels. The inhibition of BMP2 levels in BC cell lines could suggest an inhibition of BC progression and metastatic potential, as well as a lower EMT induction [222], and the lower levels of BMP4 in all BC cell lines could be related to a lower CSCs subpopulation and less self-renewal capacity, concretely for MDA-MB-231 as already reported [71,223].

Concerning BMP receptors, it has recently been showed that high BMPR1a gene expression correlate with decreased survival regardless of the molecular BC subtype, establishing BMP signaling through BMPR1a function as a tumor promoter [224]. On the other hand, the role of BMPR2 still remains controversial regarding BC prognosis, as some reports propose BMPR2 as a tumor-suppressor in mammary epithelia [225] but others showed elevated BMPR2 in breast tumors

cells compared to healthy tissue [226]. Our results suggest that β-conglutin proteins significantly decreased BMPR1a expression in all MCF-10A, SK-BR-3 and MDA-MB-231, which could be related to a tumor suppressing effect on BC and healthy cell lines. For MCF-10A, SK-BR-3 and MDA-MB-231 cells, β1 and β3 significantly decrease the levels of BMPR2. In concordance with our results, we assumed the lower BMPRII levels are beneficial for the inhibition of tumour growth and malignant properties, in concordance with evidence suggesting high levels of BMPRII in BC cells compared to normal ones [226]. Despite of that, the paper of BMPRII remains controversial and this lower level could potentially have other consequences not covered by this study.

In this context, as already described, the BMP pathway is closely related to stemness phenotype. In fact, BMP2 has been reported to facilitate epithelial-to-mesenchymal (EMT) transition and bone metastasis in BC, contributing to stem cell transformation and BC initiation [200]. Apart from that, a recent report showed that BMP4 enhanced EMT and stem cell properties in both mammary epithelial cell lines and BC cell lines, increasing the expression of EMT markers and activating Notch signalling, increasing the self-renewal capacity (sphere forming capacity) in MCF-10A and MDA-MB-231 [223]. The results about BMP expression on our BC model after treatments could suggest a regulation of stemness phenotype by the β-conglutins. On top of that, the SIRT1 signaling pathway plays a key role in the regulation of genes related to metastasis and stemness in BC [80]. In fact, Shi *et al*. demonstrated that SIRT1-centered circuitry regulates CSCs origination, related to distant-metastasis and drug-resistance in BC [77]. Other recent investigations suggest that autophagy, regulated by SIRT1/FoxO1 axis, plays a dual role in maintaining the activity of breast CSCs and could emerge as a therapeutic target in association with apoptosis [83]. All this evidence supports the relation between SIRT1 pathway and regulation of CSCs, resistance, and metastasis [163]. Our results showed that β1, β3, and β6 have anti-stemness

properties, reducing the number of CSCs and their phenotype in the three BC cell lines [163]. Interestingly, the mammosphere characterization showed that the capacity of self-renewal was only reduced in the TNBC phenotype-like cell line (MDA-MB-231) and the HER-2 positive cell line (SKBR-3), that are representative of BC tumors with worst prognosis and more aggressivity than the luminal A cell line, MCF-7 [34,30,42,227], in which the β-conglutins treatment had no significant effect. This could suggest that β-conglutins are only effective in terms of stemness regulation when autophagy is regulated in BC cell lines, being an effective CSCs targeted treatment for Luminal B and TNBC phenotype-like cell lines [163]. Finally, β-conglutins reduced stem-cell like properties in the healthy epithelial cell line too. This could be a preventive strategy against cell malignant transformation [163]. As described in recent studies, the epithelial-to-mesenchymal transition (EMT) induction in healthy MCF-10A cells contributed to acquisition of stem-like character, increasing CD44+ /CD24- percentage and mammosphere forming capacity [228]. Our treatment seems to generate the opposite effect, that could potentially be related to a prevention from EMT, directly related to malignant invasion and the earliest stages of cancer [228]. Figure 48 summarized both the process of obtention and the results obtained after their application in a normal BC cell model [163].

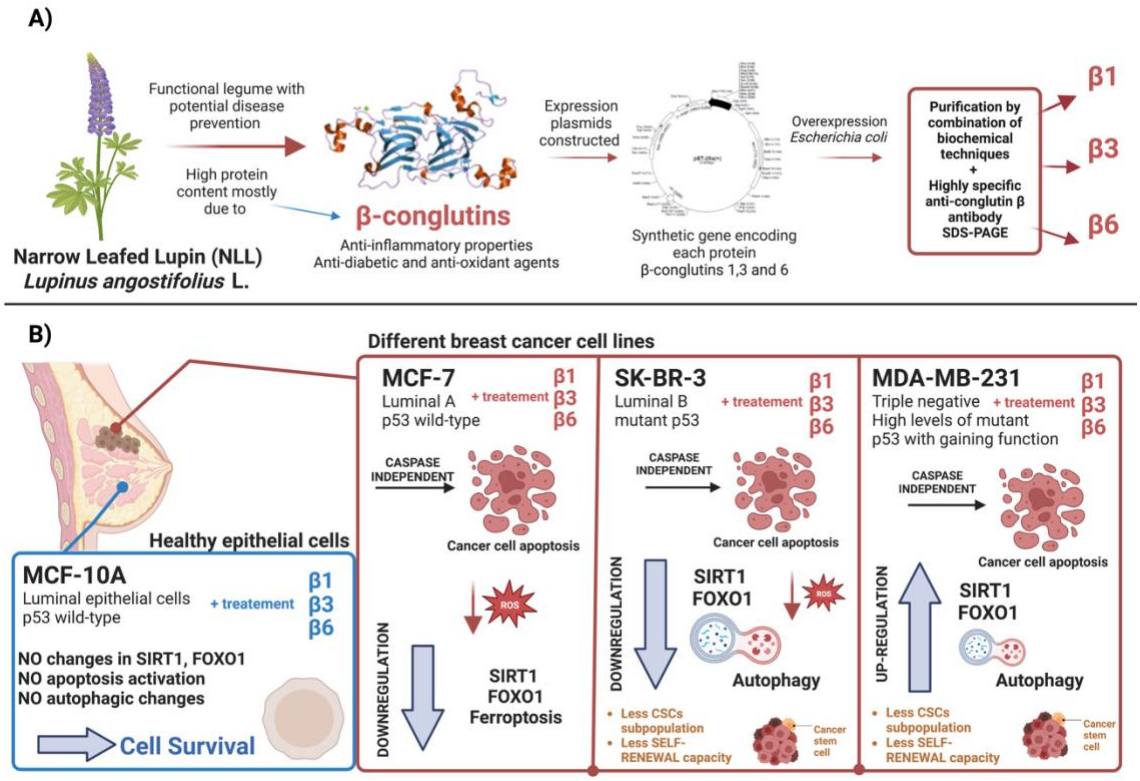


Figure 48: (**a**) Obtention, purification, and application of β-conglutin treatment in BC cell lines as a cytotoxic and anti-stemness agent against BC cell lines, preserving healthy epithelial cells. (**b**) Regulation of SIRT1/FoxO1 pathway and autophagy in a p53-dependent manner. Created with BioRender.com. From Escudero *et al*, 2023 [163].

Finally, the third and last part of this doctoral thesis had the aim to prove the potential radiosensitizing effect of β-conglutins in combination with radiotherapy (RT) or ionizing radiation therapy (IR therapy), based on all the results obtained for the simple β-conglutin treatment in both model, suggesting potential sensitizer effect for existing treatments, particularly targeting self-renewal capacity and CSCs.

RT is a highly selective and effective treatment modality in a large amount of tumours, including BC. The success of RT in eradicating the tumour depends mainly on the total dose of radiation administered, which is limited by the tolerance of the normal tissue surrounding the tumor [27,229]. Other limitations of

RT are related to the fact that normally, cancer treatments focus mainly on cancer cells without considering the role of the microenvironment in the regulation of growth and tumour metastasis [230,231] and forgetting about the proliferation and enhancement of CSCs within the tumour after radiation [232]. In fact, due to their intrinsically radioresistance, CSCs can survive conventional RT regimens and repopulate the tumour [233], contributing to tumor recurrence and metastasis. On top of that, IR has shown to enhance CSCs phenotype. All these evidence reports the fact that, beside the effective properties of RT for BC patients, improving survival rates and controlling the disease from early stages, one of the main limitations of this treatment could be the sided-induction of metastasis, cancer stem-cell phenotype and oncogenic metabolism by IR [97]. This is one of the reasons explaining the emergence of new radiosensitizers as a crucial players in order to enhance radiation effects while providing systemic disease control and improve oncologic outcomes in advanced and metastatic BC [234]. In clinical practice, synthetic radiosensitizers are commonly applied, but scientists have recently focused on using natural products as adjuvants in RT [235].

In this context, and after the obtained results for the simple treatment, the efficiency of β-conglutins in our BC cell model as potential radiosensitizers was tested with dose-response curves after colony formation assay. Our results showed a resistance to IR for MCF-10A cells, with no significant changes with the β-conglutin pre-treatment. This resistance has already been described in several reports [236] and is preserved with the combination of β-conglutins and IR at all the tested doses, providing a beneficial effect in terms of preservation of healthy epithelial cells.

A radiosensitizing effect of the β-conglutins was found for the three BC cell lines, depending on the molecular subtype. For MCF-7, Luminal A BC cells, β1 decreases significantly the survival fraction for doses > 4 Gy. On the other hand, β1 significantly reduced the survival fraction for SK-BR-3 (luminal B BC) cells at 4 Gy and β1, β3 and β6 reduced it even more for 6 and 8 Gy. Finally, for MDA-

MB-231, the TNBC phenotype cell line, the radiosensitizing effect was the most significant, with a high reduction of the survival fraction from 2 Gy with β1 and with the three β-conglutins for higher doses. Other natural compounds, such as curcumin, have shown similar properties as radiosensitizers for similar BC models [237]. Minafra *et al.* showed that curcumin-loaded lipid nanoparticles produce radiosensitazion in MCF-7 and even more efficiently for triple negative MDA-MB-231 cells, with anti-oxidant and anti-tumour effects, being radiosenitzing molecules against BC cells with a protective role against IR side effects [237]. Another natural compound, resveratrol, has shown a radiosensitizing effect on MCF-7 cells in combination with IR [238].

The cytotoxic effect of the combined therapy of β-conglutins and IR was studied by assessing apopotosis and caspase 3 levels at 4 Gy and 6 Gy, doses where the radiosensitizing effect was obtained in the three BC cell lines. Apoptosis was significantly increased for the three cell lines, depending on the β-conglutin treatment and the IR dose. Those higher apoptosic rates were not accompanied by a caspase 3 activation, so β-conglutins induce caspase-independent apoptosis not only in simple treatment but in combination with IR. The inhibition of executioner caspases as caspase 3 do not compromise the therapeutic potential of RT and its cytotoxic effect, as reported by Rodriguez-Ruiz *et al.*, and rather sensitize those cells to RT *in vitro* [239]. This result is in concordance with the sensitization and cytotoxic effect of β-conglutins through caspase-independent mechanisms. In this regard, various natural compounds have shown to exhibit their anti-cancer effect via induction of caspase-independent cell death [208,240]. On top of that, defects in signaling pathways leading to the activation of caspases are common in tumours, so many tumour cells can unexpectedly survive the activation of caspases, so caspase-independent cell death mechanisms are gaining interest among cancer researchers [241], and β-conglutins in combination with IR could be an interesting option in this field. Finally, while IR is known to activate caspase cascade resulted

in apoptosis in BC cells under normal conditions, but this caspase activation has also shown a stimulation of tumor cell re-population, leading to reduce the effectiveness of IR treatments long-term[242]. β-conglutins induced caspase-independent apoptosis could revert those non-desirable effects. Interestingly, in the treatments where no augmented apoptosis was found, an elevation of caspase 3 was observed. In fact, it has already been reported that, in some cases, the activation of executioner caspases like caspase 3 does not lead to death [243]. This augmentation could be have beneficial or non-beneficial aspects as the role of caspase 3 remains controversial regarding the approach in BC and RT [242].

As already discussed for the simple conglutin treatment, the modulation of ROS in cancer cells may represent a viable strategy in order to overcome drug or radio-resistance [212]. In this regard, our results showed a modulation of ROS levels depending on the treatment and IR dose, presenting lower ROS levels under the same conditions where higher apoptosis was induced with the combined therapy. In fact, IR is known to elevate intracellular ROS levels, that have a dual role as they can trigger cell death in a p53 dependent manner [244] but the elevation of ROS levels is also related to EMT transformation and carcinogenic transformation, being a potential initiating factor for malignant transformation [244]. In this context, antioxidant compounds are emerging as preventive agents for tumorigenesis, being also candidates for RT sensitazion through, among others, ROS modulation [235]. In some cases, β-conglutins combined with IR treatment induce peaks of ROS levels, like β1 at 4 Gy dose for MCF-7 or β3 at 4 Gy for SK-BR-3. As the role of ROS is dual in cancer context, those peaks could correspond to an accumulation of ROS leading to a shift of their carcinogenic roles into antitumor effects via induction of cell death such as necroptosis or ferroptosis [244]. The increase of ROS levels on those specific cases presented a significant radiosensitizing effect regarding colony forming assay, so ROS could enhance other caspase-independent cell deaths on those cases to obtain the lower survival rates without an apoptotic increase rate.

Other mechanism that is crucial for radioresponse and sensitizing agents is DNA damage and DDR. The major mechanism for radiation-induced cell killing is DNA damage, with double-strand breaks (DSBs) being the most lethal lesion [245]. The sensitivity of cancer cells to radiation largely depends on their ability to recognize and respond to DSBs [246]. However, some cancer cells, usually stem-cells, promote radioresistance and tumor cell survival through triggering the DDR and DNA repair after exposure to IR [247]. Because of that, targeting the DNA of tumour cells in order to radiosensitize them is a major area of research in cancer therapeutics, with strategies mainly involving two approaches: inducing DNA damage (like IR itself) or inhibiting the DDR [248]. In this context, we evaluated the DNA damage after the combined treatment of β-conglutins (24 hours pre-treatment) and exposure to IR, measuring γH2Ax by flow cytometry 1 hour after IR exposure. In fact, this technique has been widely used [249–252] for assessing both DNA damage and DDR, depending on the time of measurement of phosphorylated γH2Ax after treatment. In the case of the combined treatment, the effect on the pre-treatment of β-conglutins has been quantified more than 24 hours after its exposure, so the results of the γH2Ax quantification may gave us both information about the DNA damage caused by IR after 1 h of exposure and the modification of the DDR depending on the pre-treatment. In fact, is has been demonstrated that efficient repair of the DSBs requires a coordinated DDR including the phosphorylation of H2Ax histone, forming γH2Ax [250], so its quantification not only gives information about the number of DSBs but the efficiency of DDR after them. Our results showed that the pre-treatment of β-conglutins before IR exposure drastically reduced the γH2Ax population (depending on both the IR dose and the molecular subtype) in comparison with the non-treated IR control, meaning a significantly reduced DDR response after IR. For MCF-7, at 4 Gy, the three treatments induced a lower DDR response, but only β3 showed this effect at 6 Gy. For SK-BR-3, all treatment reduced DDR after 6 Gy exposure and, for MDA-MB-231, only β1, the most

effective β-conglutin in general terms for the TNCB cell line, reduced DDR in combination with 6 Gy exposure. Those results may support the role of β-conglutins as natural DDR inhibitors, reducing the DDR after IR exposure and preventing the DNA repair mechanisms of overcoming the effect of IR and inhibit or control tumor regrowth after treatment. In this context, other pharmacological DDR inhibitors proteins like ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR) kinases and poly (ADP-ribose) polymerase (PARP), have been recently developed to overcome tumor resistance in BC *in vitro* and *in vivo* and DDR inhibitors have showed a potentiation of fractionated therapy [248]. The combination of cytotoxic properties, apoptosis and caspase-independent death, modulation of ROS and DDR inhibition may all confer the antioxidant β-conglutins proteins their radiosensitizing role.

As previously studied, the SIRT1/FoxO1 regulatory axis is a ROS-sensitive pathway implicated in BC progression and aggression [75–78]. This pathway of action was studied using other natural compounds such as resveratrol or isoalantolactone (IATL) [78,216] showing both a down or up regulation of SIRT1-mediated FoxO1 as a mechanism of great interest in BC therapy. The dual effect of this pathway has been discussed with the simple β-conglutin treatment, showing a dual regulation of this pathway depending on p53 and the phenotype of the cell line. We also studied SIRT1 and FoxO1 expression after the combined β-conglutin and IR treatment, and the results showed some controversial results that could also support a dual role for radiosensitivity in BC. In MCF-7 at 4 Gy IR dose, the p53 wt Luminal A cell line, SIRT1 was down regulated after combined treatment and FoxO1 followed a similar pattern, ant a similar effect was observed for SK-BR-3 (luminal B, mutation of p53 without gaining function) at this dose. Interestingly, for MDA-MB-231 cells, the combined therapy with 4 Gy also reduced SIRT1 expression, but induced higher levels of FoxO1. For a

higher IR dose, 6 Gy, SK-BR-3 followed the same pattern, with a reduction of both SIRT1 and FoxO1, but MCF-7 and MDA-MB-231 showed an increase of SIRT1 for some β-conglutin treatments, with the same effect on FoxO1. Those results suggest a dual effect of SIRT1 on radiosensitivy in BC and the important role of their action through FoxO1 or not in this final outcome. In fact, recent papers have showed the importance of SIRT1 regulation in resistance to radiation and radiosensitivity. Zhang *et al*. demonstrated that targeting Sirt1 could be a promising therapy for IR radiosensitivity, as its knockdown suppressed tumorigenesis and improved radiosensitivity in BC cell lines [253]. Controversely, several reports have showed that the up-regulation of SIRT1 could cause radiosensitazion on BC cells and lower CSCs population, with an important role of IL-6 (implicated on the molecular action of β-conglutins) [254]. Regarding studies using natural radiosenstiziers, resveratrol treatment has showed a dual effect of the role of SIRT1in combination with RT [235]. Some reports point that resveratrol can act like a radioprotector due to the increase of SIRT1 levels by stimulating p53 activity [255]. On the other hand, its potential effect as a radiosensitizer have been demonstrated in BC cell lines despite its effect on SIRT1 activation [256]. Even if the dual role of SIRT1/FoxO1 pathway after β-conglutin treatment in BC and its relation with p53 has been explained and a lot of papers supported our hypothesis, research within SIRT1 as a radioprotective or radiosenstitizer gene and the potential effect of FoxO1 is very limited nowadays, as the role of this pathways still remains controversial and unclear in BC progression and prevention [257]. Our hypothesis, following the single treatment results, is that the final effect of β-conglutin treatment as a radiosensitizer is strongly linked, in this case, not only to the p53 state and the phenotypical characteristics of the cell line but also to the radiation dose and the radiosensitivity of each cell line. For SK-BR-3, β-conglutins reduce SIRT1 and FoxO1 strongly for 6Gy than for 4Gy, resulting in a more radiosensitive effect with the combined therapy at 6 Gy dose. For MCF-7, β1 is the only β-conglutin

providing significant radiosensitazion at long term, and due to the p53 state and the luminal subtype, β1 reduces SIRT1 at both radiation doses, although the subtle elevation of SIRT1 for the other β-conglutins should be studied in detail. Finally, for MDA-MB-231, which is the most radiosensitive cell line [258], the addition of IR after simple treatment reduced SIRT1 levels at moderate radiation dose (4 Gy) and increased them at higher radiation dose only for β1, in a very subtle way. Overall, the radiosensitizing effect of β-conglutins seems to be provided by a down-regulation of SIRT1/FoxO1 pathway, except for p53 gaining function cell line, MDA-MB-231, where the up/down regulation plays a critical and controversial role.

One of the controversial processes regulated by the SIRT1/FoxO1 pathway and related to IR is autophagy. The role of autophagy regarding RT is controversial as, in one hand, it has been reported that the inhibition of autophagy-involving genes results in tumour radiosensitazion *in vitro* but on the other hand that inhibition induced radioresponse *in vivo* [259]. In general terms, autophagy could provide an opportunity for cancer cells to survive in response to radiotherapy [260], but, controversially, recent evidence suggests that the irradiation-cell death might be involved in autophagy [261,262]. In other type of cancers, like glioblastoma cell, autophagy has been reported to radiosensitize cells [263] and other studies suggested that autophagy might be a radiation-induces cell death alternative in cancer, for those cells presenting apoptosis pathway defects [264]. It has also been demonstrated that p53 is involved in radiation-induced autophagic BC cell death [265]. Finally, a recent study demonstrated that the inhibition of autophagy sensitize BC cells to radiation [266]. All of those recent research provides controversial information regarding the role of autophagy in BC radioresponse. In this context, our results confirmed this dual role of autophagy in cell radiosensitazion, depending mainly on the cell line, the state of p53, the regulation of SIRT1/FoxO1 pathway and the stress cellular

context (ROS and different doses if IR). The western blot analysis showed that only in TNBC phenotype-like cells, MDA-MB-231, with mutant p53 gaining functionality, the effect exerted by β-conglutins and IR is accompanied with autophagy induction at 4 Gy, while only β6 seemed to increase autophagy in the combined therapy at 6 Gy. As expected regarding the simple treatment results, an inhibition of autophagy was observed in SKBR-3 cells after combined treatment and, in the MCF-7 cells, no autophagy inhibition or upregulation was detected, as both LC3B and p62 are reduced in this case. Autophagy could then exert its dual role by improving radiosensitazion of MDA-MB-231 cells with p53 gaining function and improving radiosensitazion of SK-BR-3 cells too, with a mutant p53, by inhibiting this same process. More research and experiments should be performed regarding the molecular mechanisms of SIRT1 regulation and autophagy in order to fully understand the radiosensitizer effect obtained in the three different BC cell lines.

Finally, CSCs are the main focus of the radiosensitizing therapies, as IR has demonstrated to enhance its phenotype and, despite its beneficial effects, being at the origin of tumour regrowth and metastasis as a late effect of IR [104,233,267]. Other studies have shown that the failure of RT might be attributed to the incomplete eradication of CSCs subpopulation [268,269]. The control of the CSCs phenotype acquisition and the reduction of this subpopulation would provide not only better clinical outcomes but a reduction on side effects and relapse after IR exposure. Our results showed that β-conglutins significantly reduced stemness in our BC model. Particularly, ALDH1+ population was decreased in MCF-7 and MDA-MB-231, CSCs markers showed a lower CD24low/CD44high population after the combined treatment and, more interestingly, the self-renewal capacity, assessed with the mammosphere assay, showed an inhibition of the spheres formation at 4 Gy and 6 Gy for MDA-MB-231 and MCF-7, and only at 6 Gy for β1 in SK-BR-3. This results are in concordance with the antistemness

properties of β-conglutins, demonstrated in several BC cell lines with the simple treatment in this doctoral thesis and could support the radiosensitizing effect of β-conglutins in BC, not only by apoptotis induction, ROS modulation, DDR inhibition and SIRT1/FoxO1/autophagy regulation, but being a precise trigger within the CSCs subpopulation, inhibiting their self-renewal capacity *in vitro* and preventing the acquisition of the stemness phenotype of single IR therapy.

CONCLUSIONS

1. Narrow-leafed lupin conglutins β5 and β7 are newly identified anti-inflammatory proteins since they inhibit the production of pro-inflammatory cytokines, iNOS expression and NO production, and regulate the antioxidant capacity of cells.

2. Mobile arm structural domain of β5 and β7 is involved in nutraceutical properties as compared to truncated forms.

3. NLL β-conglutin proteins 1, 3 and 6, which have never been studied in cancer before, can become a preventive agent of great interest in early stages of BC, regulating the BMP pathway and preventing malignant transformation and EMT enhancement in MCF-10A cells.

4. NLL β-conglutin proteins can also be an interesting cytotoxic, natural and anti-stemness treatment for early stages of BC *in vitro*, providing lower viability rates, lower luminal-like progenitors colonies, reducing BMP2, BMP4 and BMPR1B gene expressions, knocking out IDs and Smad6 expression, reducing the self-renewal capacity of basal-like and chemo-resistant BC cells and their CD10 phenotype.

5. NLL β-conglutin proteins can become a natural cytotoxic agent of great interest in BC cell lines, decreasing the viability of BC cells and increasing caspase-independent apoptotic levels.

6. NLL β-conglutin proteins showed anti-stemness potential in advanced BC cell lines, reducing the self-renewal capacity and the proportion of CSCs in TNBC and Luminal B HER2+ cell lines.

7. NLL β-conglutin proteins have also a radiosensitizing potential in combination with conventional RT. The combined therapy of β-conglutins and IR showed lower survival fraction, higher apoptotic rates and lower DDR in all BC cell lines, especially in the most aggressive phenotype, MDA-MB-231.

8. Combined therapy of NLL β-conglutin proteins and RT regulate and decreased CSCs subpopulation as well as self-renewal capacity, assuring the highest effectiveness of radiation and the prevention of the CSCs phenotype acquisition, at the origin of tumor regrowth, metastasis and relapse.

9. NLL β-conglutin proteins as a single treatment for BC could modulate the effect of SIRT1/FoxO1 pathway, depending on both the status of p53 and the tumor phenotype (luminal, HER-2 positive, or TNBC), a mechanism that could trigger cancer resistance and metastasis.

10. The mechanism of cell death ligated to the status of p53 after β-conglutin treatment and the regulation of BMP related genes seemed to be crucial for the final regulation of the phenotype and auto-renovation ability of CSCs in BC *in vitro*.

11. The mechanism of action of NLL β-conglutin proteins in combination with radiation remains controversial but is strongly linked to the effect of SIRT1/FoxO1 pathway, depending on the mentioned variables and IR dose, and the autophagy modulation. For this reason, more experiments should be performed to understand the final regulation of the phenotype and auto-renovation ability of CSCs in BC *in vitro* after this β-conglutins and IR combined treatment.

12. The use of NLL β-conglutin proteins in BC management can become a unique and novel strategy for treating BC at different stages and from multiple perspectives: prevention of the disease, treatment in early and advanced cases, sensitizing for radiotherapy and prevention of metastasis and relapse.

CONCLUSIONES

1. Las conglutinas de lupino de hoja estrecha β5 y β7 son proteínas antiinflamatorias recientemente identificadas, ya que inhiben la producción de citoquinas proinflamatorias, la expresión de iNOS y la producción de NO, y regulan la capacidad antioxidante de las células.

2. El dominio estructural del brazo móvil de β5 y β7 está involucrado en las propiedades nutracéuticas en comparación con las formas truncadas.

3. Las proteínas β-conglutinas 1, 3 y 6, que nunca se habían estudiado en cáncer, pueden convertirse en un agente preventivo de gran interés en etapas tempranas de CM, regulando la vía BMP y previniendo la transformación maligna y el aumento de TEM en células MCF-10A.

4. Las proteínas β-conglutinas también pueden ser un tratamiento citotóxico, natural y anti-*stemness* para las primeras etapas de CM *in vitro*, proporcionando tasas de viabilidad más bajas, colonias de progenitores de tipo luminal más bajas, reduciendo las expresiones génicas de BMP2, BMP4 y BMPR1B, reduciendo los niveles de IDs y la expresión de Smad6, reduciendo la capacidad de autorrenovación de las células de CM de tipo basal y quimiorresistentes y su fenotipo CD10.

5. Las proteínas β-conglutinas pueden convertirse en un agente citotóxico natural de gran interés en las líneas celulares de CM avanzado, disminuyendo la viabilidad de las células de CM y aumentando los niveles de apoptosis independientes de caspasa.

6. Las proteínas β-conglutinas mostraron potencial anti-*stemness* en líneas celulares de CM avanzado, reduciendo la capacidad de

autorrenovación y la proporción de CMCs en líneas celulares TN y Luminal B HER2+.

7. Las proteínas β-conglutinas también tienen un potencial radiosensibilizador en combinación con la RT convencional. La terapia combinada de β-conglutinas y RI mostró menor fracción de supervivencia, mayores tasas de apoptosis y menor respuesta al daño del ADN en todas las líneas celulares de CM, especialmente en el fenotipo más agresivo, MDA-MB-231.

8. La terapia combinada de proteínas β-conglutinas y RT regula y disminuye la subpoblación de CMC, así como la capacidad de auto-renovación, asegurando la máxima eficacia de la radiación y la prevención de la adquisición del fenotipo de CMC, que origina el crecimiento tumoral, la metástasis y la recidiva post RI.

9. Las proteínas β-conglutinas como tratamiento simple para el CM podrían modular el efecto de la vía SIRT1/FoxO1, dependiendo tanto del estado de p53 como del fenotipo tumoral (luminal, HER-2 positivo o TN), un mecanismo que podría modular resistencia a tratamiento y metástasis.

10. El mecanismo de muerte celular ligado al estado de p53 después del tratamiento con β-conglutinas y la regulación de genes relacionados con BMP parecen ser cruciales para la regulación final del fenotipo y la capacidad de auto-renovación de las CMC en CM *in vitro*.

11. El mecanismo de acción de las proteínas β-conglutinas en combinación con la radiación es controvertido, pero está fuertemente relacionado con el efecto de la vía SIRT1/FoxO1, dependiendo de las variables

mencionadas, la dosis de RI, y la modulación de la autofagia. Por esta razón, se deben realizar más experimentos para comprender la regulación final del fenotipo y la capacidad de auto-renovación de las CMC en CM *in vitro* después de este tratamiento combinado de β-conglutinas y RI.

12. El uso de proteínas β-conglutinas NLL en el manejo del esta enfermedad puede convertirse en una estrategia única y novedosa para tratar el CM en diferentes estadios y desde múltiples perspectivas: prevención de la enfermedad, tratamiento en casos tempranos y avanzados, sensibilización frente a radioterapia y prevención de metástasis y recidiva.

ANNEXES

A

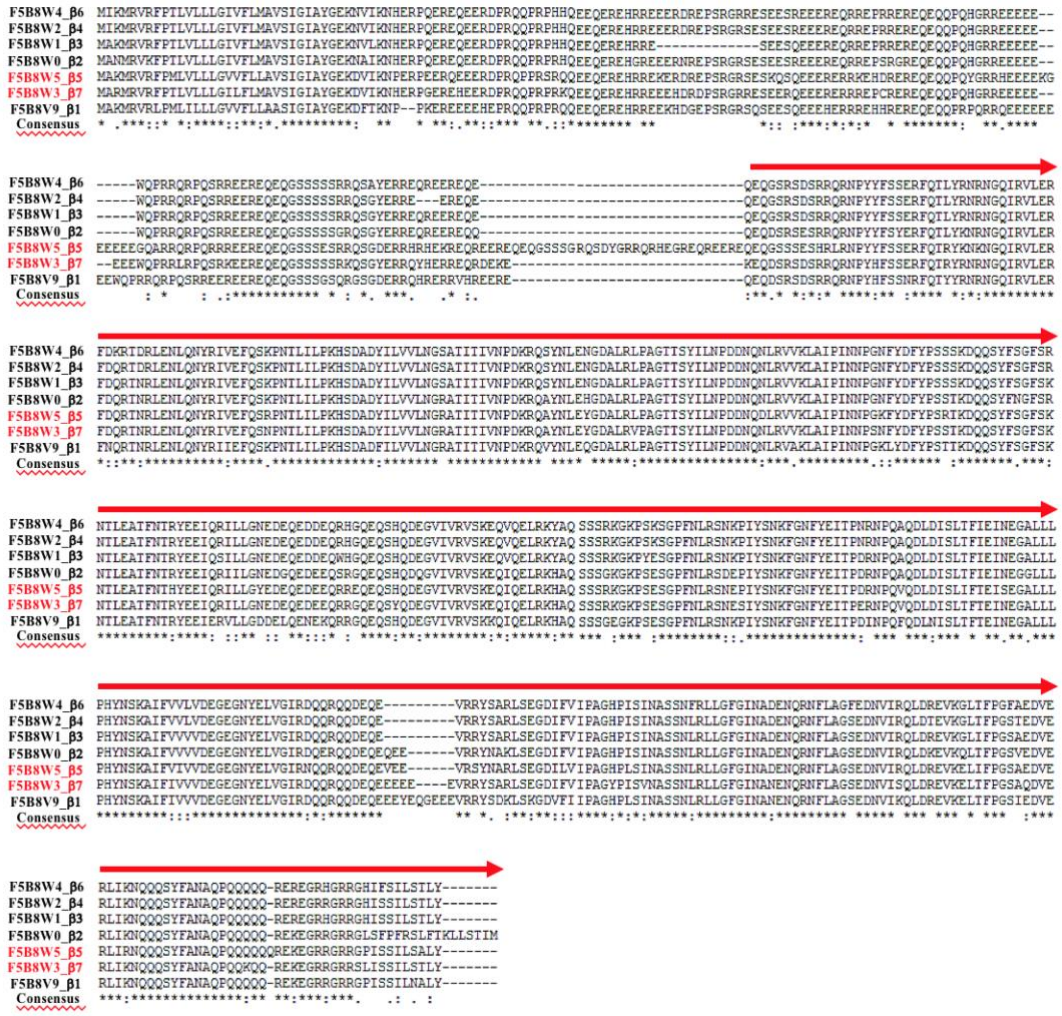


Figure S1 (A): Extensive *in silico* analysis of seed β-conglutins. The surface residues are involved in changes in 2-D structural elements (loops and coils) and 3D motives, and numerous micro-heterogeneities are present in fundamental residues directly involved in domains variability. Red lines in the alignment correspond to the globular domain, and the rest (non-marked) N-terminal is the domain of the mobile arm. (*continues in the following page*)

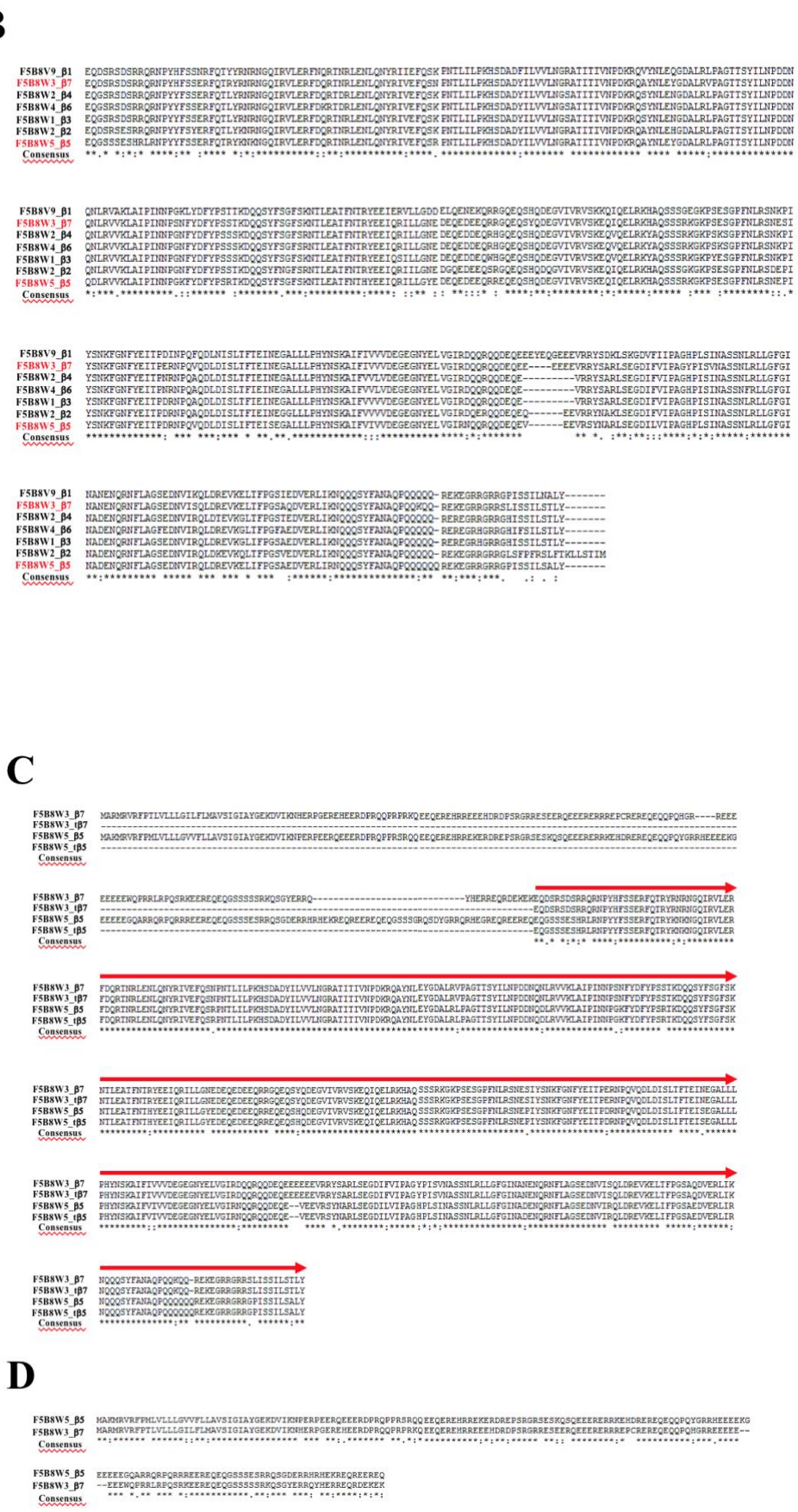


Figure S1 (B, C, D): **Extensive *in silico* analysis of seed β-conglutins.**

Table S1: Viability (%) assessment of human peripheral blood mononuclear cells (PBMC).

Viability was measured by MTT assay for isolated PBMC culture, conglutins β5 or β7, LPS, LPS + conglutins β5 or β7, LPS + truncated forms of conglutins β5 or β7 during 24 hours. Treatments including conglutin β5 or β7 (normal or truncated forms) were added at 10 μg, and LPS at 1 μg. Data represent mean ± SD from three independent experiments.

Samples	Viability
LPS	97.5 ± 3.5
Conglutin 5β	102.5 ± 2.3
Conglutin tβ5	105.0 ± 4.4
Conglutin 7β	99.0 ± 2.2
Conglutin tβ7	98.9 ± 2.5
Conglutin 5β + LPS	98.3 ± 2.1
Conglutin tβ5 + LPS	97.4 ± 3.5
Conglutin 7β + LPS	96.5 ± 5.6
Conglutin tβ7 + LPS	101.3 ± 2.4

Table S2: Table S2. Viability (%) assessment of HEPG2 cell culture.

Viability was measured by MTT assay for HEPG2 cell culture, conglutins β5 or β7, LPS, LPS + conglutins β5 or β7, LPS + truncated forms of conglutins β5 or β7 during 24 hours. Treatments including conglutin β5 or β7 (normal or truncated forms) were added at 10 μg, and LPS at 1 μg. Data represent mean ± SD from three independent experiments.

Samples	Viability
LPS	99.0 ± 1.5
Conglutin 5β	98.4 ± 3.7
Conglutin tβ5	97.0 ± 5.3
Conglutin 7β	99.0 ± 2.6
Conglutin tβ7	100.0 ± 4.3
Conglutin 5β + LPS	95.4± 4.7
Conglutin tβ5 + LPS	97.2 ± 5.5
Conglutin 7β + LPS	101.4± 3.6
Conglutin tβ7 + LPS	102.6± 5.2

GLOSSARY

AJCC: American Joint Committee on Cancer

ALDH1: aldehyde dehydrogenase 1

ARLI: age-related lobular involution

ATCC: American Type Culture Collection

BAA: BODIPY-aminoacetate

BAAA: BODIPY-aminoacetaldehide

BC: breast cancer

BCSCs: Breast Cancer Stem Cells

BER: base excision repair

BMI: body mass index

BMP: bone morphogenetic protein

BMPR1A, BMPR1B: BMP type IA/B receptor

BMPR2: BMP type II receptor

BRCA1, BRCA2: Breast Cancer 1,2

cDNA: complementary DNA

CBL: Caveolin

CT: Cycle Threshold

CSCs: Cancer Stem Cells

DCFH-DA: 2′7′-Dichlorofluorescein diacetate

DDR: DNA damage response

DEAB: Dietilbenzaldehide

DMEM: Dulbecco's Modified Eagle's Medium

DMEM-F12: Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham

DMSO: Dimethly sulfoxid

DNA: Deoxyribonucleic Acid

DSBs: DNA double-stand breaks

E-CFC: Epithelial Colony Formation Cell

ECM: extracellular matrix

EGF: Epidermal Growth Factor

EGFR: Epidermal Growth Factor Receptor

EMT: epithelial-to-mesenchymal transition

EPIC: European Prospective Study on Nutrition and Cancer

ER: Estrogens Receptors

FACS: Fluorescence-activated Cell Sorting

FBS: Fetal Bovine Serum

FGF: Fibroblast Growth Factor

FITC: Fluorescein Isothiocyanate

FOXO: Forkhead box

FS: survival fraction

Gy: Gray

H2AX: histone H2A variant

HbA1c: Glycated haemoglobin

HER2: Human Epidermal Growth Factor Receptor 2

HIF-1: Hypoxia-Inducible Factor 1

HR: homologous recombination

HRT: hormone replacement therapy

IARC: International Agency for Research on Cancer

iNOS: Inducible Nitric Oxyde Synthase

IL: Interleukin

IR: ionizing radiation

kDa: Kilo Dalton

LC3B (microtubule-associated protein 1 light chain 3, MAP1LC3)

LET: linear energy transfer

LPS: lipopolysaccharide

mRNA: messenger RNA

miRNAS: microRNAs

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide

NER: nucleotide excision repair

NHEJ: non-homologous end joining

NLL: Narrow Leafed Lupin

NO: Nitric oxide

OS: Oxidative stress

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate-Buffered Saline

PCR: Polymerase Chain Reaction

PI: Propidium Iodide

PR: progesterone receptor

P/S: penicillin/streptomycin

qPCR: Quantitative Polymerase Chain Reaction

RNA: Ribonucleic Acid

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

RT: Radiotherapy

RT: Room Temperature

RT: Reverse transcription

RT-qPCR: Real time Reverse Transcription PCR

SIRT: Sirtuin

SQSTM1/p62: sequesteome 1

T2D: Type 2 Diabetes

TGF-β: transforming growth factor-β

TME: tumour microenvironment

TNM: tumour-node metastasis

TNBC: triple negative breast cancer

UV: Ultraviolet Radiation

VEGF: vascular endothelial growth factor

WHO: World Health Organization

CURRICULUM VITAE

ACADEMIC ACHIEVEMENTS

Degree in Biomedical Engineering (Polytechnic University of Valencia, 2012-2016)

Master in Advances in Diagnostic and Therapeutic Radiology and Physical Medicine (University of Granada, 2016-2017)

PUBLICATIONS

Journal articles

- García-Costela M*, **Escudero-Feliu J*,** Puentes-Pardo JD, San Juán SM, Morales-Santana S, Ríos-Arrabal S, Carazo Á, León J. Circadian Genes as Therapeutic Targets in Pancreatic Cancer. Front Endocrinol (Lausanne). 2020 Sep

- Ríos-Arrabal S, Puentes-Pardo JD, Moreno-SanJuan S, Szuba Á, Casado J, García-Costela M, **Escudero-Feliu J**, Verbeni M, Cano C, González-Puga C, Martín-Lagos Maldonado A, Carazo Á, León J. Endothelin-1 as a Mediator of Heme Oxygenase-1-Induced Stemness in Colorectal Cancer: Influence of p53. J Pers Med. 2021 Jun 4;11(6):509.

- Aroca-Siendones MI, Moreno-SanJuan S, Puentes-Pardo JD, Verbeni M, Arnedo J, **Escudero-Feliu J**, García-Costela M, García-Robles A, Carazo Á, León J. Core Circadian Clock Proteins as Biomarkers of Progression in Colorectal Cancer. Biomedicines. 2021 Aug 6;9(8):967.

- **Escudero-Feliu J**, García-Costela M, Moreno-SanJuan S, Puentes-Pardo JD, Arrabal SR, González-Novoa P, Núñez MI, Carazo Á, Jimenez-Lopez

JC, León J. Narrow Leafed Lupin (*Lupinus angustifolius* L.) β-Conglutin Seed Proteins as a New Natural Cytotoxic Agents against Breast Cancer Cells. Nutrients. 2023 Jan 19;15(3):523.

- Lima-Cabello E*, **Escudero-Feliu J***, Peralta-Leal A, Garcia-Costela M, Nuñez MI, Siddique KHM, Singh KB, Leon J, Jimenez-Lopez JC. (2023). β-conglutins unique mobile-arm is a key structural feature involved in the molecular nutraceutics properties in narrow-leafed Lupin (*Lupinus angustifolius* L.) (*IN PEER-REVISION*)

Book chapters:

- Priego-Poyato, S., Rodrigo-Garcia, M., **Escudero-Feliu, J.,** … Jimenez-Lopez, J C. (2021). In: GRAIN AND SEED PROTEINS FUNCTIONALITY. Current Advances Research in Nutraceutical Compounds of Legumes, Pseudocereals and Cereals. Grain and Seed Proteins Functionality. IntechOpen London UK, doi:10.5772/intechopen.97782

CONTRIBUTIONS TO CONGRESSES:

1. **Julia Escudero Feliu**; Sara Moreno San Juan; Jose David Puentes Pardo; Anaïs Redruello; David Lopez; Angel Carazo; Josefa Leon. Chemotherapeutic effect of beta-conglutins extracted from blue lupine in breast cancer. III Precision Medicine Symposium ibs.GRANADA. Junta de Andalucía. 2022. Spain.

2. **Julia Escudero Feliu**; Elena Lima Cabello; Maria Garcia Costela; Josefa Leon; Jose Carlos Jimenez Lopez. Structural domain of narrow leafed lupine (Lupinus angustifolius) β-conglutin proteins involved in their nutraceutical properties and food security. 10th INTERNATIONAL

CONFERENCE ON LEGUME GENOMICS AND GENETICS. ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 2022.

3. **Julia Escudero Feliu**; Maria Garcia Costela; Sandra Rios Arrabal; Sara Moreno San Juan; Jose David Puentes Pardo; Josefa Leon; Jose Carlos Jimenez Lopez. Narrow-leafed lupine (Lupinus angostifolius L.) seed beta-conglutin proteins induce G0/G1 arrest and apoptosis in human colorectal cancer cells. IV National Congress of Young Researchers in Biomedicine. San Cecilio PTS Granada Clinical Hospital. 2021. Spain.

4. **Julia Escudero Feliu**; Maria Garcia Costela; Sandra Rios Arrabal; Sara Moreno San Juan; Jose David Puentes Pardo; Josefa Leon; Jose Carlos Jimenez Lopez. Narrow-leafed lupine (Lupinus angostifolius L.) seed beta-conglutin proteins induce G0/G1 arrest and apoptosis in human colorectal cancer cells. Congress of Biomedical Research Students (VII CEIBS). San Cecilio PTS Granada Clinical Hospital. 2021. Spain.

5. **Julia Escudero Feliu**; Maria Garcia Costela; Sandra Rios Arrabal; Sara Moreno San Juan; Jose David Puentes Pardo; Josefa Leon; Jose Carlos Jimenez Lopez. Narrow-leafed lupine (Lupinus angostifolius L.) seed beta-conglutin proteins induce G0/G1 arrest and apoptosis in human colorectal cancer cells. Congress of Biomedical Research Students (VI CEIBS). San Cecilio PTS Granada Clinical Hospital. 2020. Spain.

6. **Julia Escudero Feliu**; Maria Garcia Costela; Sonia Morales Santana; Sandra Rios Arrabal; Maria Isabel Nunez Torres; Josefa Leon; Jose Carlos Jimenez Lopez. Seed beta-conglutin proteins from Narrow-leafed lupine (Lupinus angostifolius L.) as functional foods and their role in cancer

prevention. 25th National Symposium for Applied Biological Sciences. Agro-BioTech. 2020. Belgium.

7. Maria Garcia Costela; **Julia Escudero Feliu**; Sandra Rios Arrabal; Sonia Morales Santana; Maria Isabel Nunez Torres; Josefa Leon; Jose Carlos Jimenez Lopez. Narrow-leafed lupine (Lupinus angostifolius L.) seed beta-conglutin proteins induce G0/G1 arrest and apoptosis in human colorectal cancer cells. 3rd International Legume Society (ILS3). Institute of Plant Genetics, Polish Academy of Sciences.. 2019. Poland.

8. **Julia Escudero Feliu**; Maria Garcia Costela; Sandra Rios Arrabal; Sara Moreno San Juan; Jose David Puentes Pardo; Josefa Leon; Jose Carlos Jimenez Lopez. Narrow-leafed lupine (Lupinus angostifolius L.) seed beta-conglutin proteins induce G0/G1 arrest and apoptosis in human colorectal cancer cells. I International Symposium of Precision Medicine. San Cecilio PTS Clinical Hospital. 2019. Spain.

9. Elena Lima Cabello; **Julia Escudero Feliu**; Maria Garcia Costela; Sonia Morales Santana; Sandra Rios Arrabal; Victor Alché; Josefa Leon; Alfonso Clemente; Juan de Dios Alché; Jose Carlos Jimenez Lopez. Narrow-leafed lupine (Lupinus angostifolius L.) seed beta-conglutin proteins as functional food to tackle inflammatory-related diseases. 6th International Conference on Food Digestion (INFOGEST). Zaidín Experimental Station. 2019. Spain.

10. Maria Garcia Costela; **Julia Escudero Feliu**; Sandra Rios Arrabal; Sonia Morales Santana; Maria Isabel Nunez Torres; Josefa Leon; Jose Carlos Jimenez Lopez. Beta-conglutin proteins from narrow-leafed lupine (Lupinus angostifolius L.) seeds with anti-colorectal cancer potential:

current evidences for their molecular mechanisms. XV International Lupine Conference. PROINPA Foundation. 2019. Bolivia.

11. **Julia Escudero Feliu**; Maria Garcia Costela; Sandra Rios Arrabal; Maria Isabel Nunez Torres; Josefa Leon Lopez. Role of hemoxygenase-1 in the manifestation of stem cell-like properties of colorectal cancer. I National Congress of PTS Researchers. Biomedical Research Center (CIBM). 2019. Spain.

12. Sandra Rios Arrabal; Maria Garcia Costela; **Julia Escudero Feliu**; Sonia Morales Santana; Jose Maria Ortega Rodríguez; Angel Carazo Gallego; Maria Isabel Nunez Torres; Josefa Leon Lopez. Role of Heme Oxygenase-1 in manifestation of colorectal cancer stem cell-like properties. 2nd Spanish Portuguese Meeting for Advanced Optical Microscopy (SPAOM). Zaidín Experimental Station. 2018. Spain.

REFERENCES

1. World Health Organization: Cancer. *World Health Organization: Cancer* https://www.who.int/news-room/fact-sheets/detail/cancer (2023).

2. Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, et al. Global Cancer Observatory: Cancer Today. Lyon: International Agency for Research on Cancer. *Global Cancer Observatory: Cancer Today. Lyon: International Agency for Research on Cancer* http://gco.iarc.fr/today/home (2020).

3. Sung, H. *et al.* Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA A Cancer J Clin* **71**, 209–249 (2021).

4. GLOBOCAN 2020 - Infogram. https://infogram.com/globocan-2020-1h9j6qg7xdp8v4g.

5. IARC: Home. https://www.iarc.who.int/.

6. Blackadar, C. B. Historical review of the causes of cancer. *World J Clin Oncol* **7**, 54–86 (2016).

7. Miller, K. D. *et al.* Cancer treatment and survivorship statistics, 2016. *CA: A Cancer Journal for Clinicians* **66**, 271–289 (2016).

8. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646–674 (2011).

9. Murgai, M., Giles, A. & Kaplan, R. Physiological, Tumor, and Metastatic Niches: Opportunities and Challenges for Targeting the Tumor Microenvironment. *Crit Rev Oncog* **20**, 301–314 (2015).

10. Wels, J., Kaplan, R. N., Rafii, S. & Lyden, D. Migratory neighbors and distant invaders: tumor-associated niche cells. *Genes Dev* **22**, 559–574 (2008).

11. Zheng, T., Lu, M., Wang, T., Zhang, C. & Du, X. NRBE3 promotes metastasis of breast cancer by down-regulating E-cadherin expression. *Biochim Biophys Acta Mol Cell Res* **1865**, 1869–1877 (2018).

12. Jemal, A. *et al.* Global cancer statistics. *CA Cancer J Clin* **61**, 69–90 (2011).

13. Fiorica, J. V. Breast Cancer Screening, Mammography, and Other

Modalities. *Clin Obstet Gynecol* **59**, 688–709 (2016).

14. Sun, Y.-S. *et al.* Risk Factors and Preventions of Breast Cancer. *International Journal of Biological Sciences* **13**, 1387–1397 (2017).

15. Majeed, W. *et al.* Breast cancer: major risk factors and recent developments in treatment. *Asian Pac J Cancer Prev* **15**, 3353–3358 (2014).

16. Karuturi, M. S. *et al.* Breast cancer and aging: Standing on the shoulders of a giant. *J Geriatr Oncol* **11**, 212–216 (2020).

17. Milanese, T. R. *et al.* Age-related lobular involution and risk of breast cancer. *J Natl Cancer Inst* **98**, 1600–1607 (2006).

18. Figueroa, J. D. *et al.* Standardized measures of lobular involution and subsequent breast cancer risk among women with benign breast disease: a nested case-control study. *Breast Cancer Res Treat* **159**, 163–172 (2016).

19. Association between local inflammation and breast tissue age-related lobular involution among premenopausal and postmenopausal breast cancer patients | PLOS ONE. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0183579.

20. Brewer, H. R., Jones, M. E., Schoemaker, M. J., Ashworth, A. & Swerdlow, A. J. Family history and risk of breast cancer: an analysis accounting for family structure. *Breast Cancer Res Treat* **165**, 193–200 (2017).

21. Anderson, K. N., Schwab, R. B. & Martinez, M. E. Reproductive risk factors and breast cancer subtypes: a review of the literature. *Breast Cancer Res Treat* **144**, 1–10 (2014).

22. Rojas, K. & Stuckey, A. Breast Cancer Epidemiology and Risk Factors. *Clin Obstet Gynecol* **59**, 651–672 (2016).

23. Horn, J. & Vatten, L. J. Reproductive and hormonal risk factors of breast cancer: a historical perspective. *Int J Womens Health* **9**, 265–272 (2017).

24. Hamajima, N. *et al.* Alcohol, tobacco and breast cancer--collaborative reanalysis of individual data from 53 epidemiological studies, including 58,515 women with breast cancer and 95,067 women without the disease. *Br J Cancer* **87**,

1234–1245 (2002).

25. Makarem, N., Chandran, U., Bandera, E. V. & Parekh, N. Dietary fat in breast cancer survival. *Annu Rev Nutr* **33**, 319–348 (2013).

26. Lee, K., Kruper, L., Dieli-Conwright, C. M. & Mortimer, J. E. The Impact of Obesity on Breast Cancer Diagnosis and Treatment. *Curr Oncol Rep* **21**, 41 (2019).

27. Golubicic, I., Borojevic, N. & Pavlovic, T. Risk factors for breast cancer: is ionizing radiation among them? *J BUON* **13**, 487–494 (2008).

28. Yeo, S. K. & Guan, J.-L. Breast Cancer: Multiple Subtypes within a Tumor? *Trends Cancer* **3**, 753–760 (2017).

29. Types of Breast Cancer | About Breast Cancer. https://www.cancer.org/cancer/breast-cancer/about/types-of-breast-cancer.html.

30. Maughan, K. L., Lutterbie, M. A. & Ham, P. S. Treatment of breast cancer. *Am Fam Physician* **81**, 1339–1346 (2010).

31. Dieci, M. V., Orvieto, E., Dominici, M., Conte, P. & Guarneri, V. Rare breast cancer subtypes: histological, molecular, and clinical peculiarities. *Oncologist* **19**, 805–813 (2014).

32. Stage 1 & Stage 2 Breast Cancer (Early Breast Cancer) | NBCF. *National Breast Cancer Foundation (NBCF) | Donate Online* https://nbcf.org.au/about-breast-cancer/diagnosis/stage-1-2-early-breast-cancer/.

33. Stages of Breast Cancer | Understand Breast Cancer Staging. https://www.cancer.org/cancer/breast-cancer/understanding-a-breast-cancer-diagnosis/stages-of-breast-cancer.html.

34. Olivares-Urbano, M. A. *et al.* Matrix metalloproteases and TIMPs as prognostic biomarkers in breast cancer patients treated with radiotherapy: A pilot study. *J Cellular Molecular Medi* **24**, 139–148 (2020).

35. Strehl, J. D., Wachter, D. L., Fasching, P. A., Beckmann, M. W. & Hartmann, A. Invasive Breast Cancer: Recognition of Molecular Subtypes. *Breast Care (Basel)* **6**, 258–264 (2011).

36. Cheang, M. C. U. *et al.* Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. *J Natl Cancer Inst* **101**, 736–750 (2009).

37. Yersal, O. & Barutca, S. Biological subtypes of breast cancer: Prognostic and therapeutic implications. *World J Clin Oncol* **5**, 412–424 (2014).

38. Frontiers | The Androgen Receptor in Breast Cancer. https://www.frontiersin.org/articles/10.3389/fendo.2018.00492/full.

39. Hicks, D. G. *et al.* The incidence of topoisomerase II-alpha genomic alterations in adenocarcinoma of the breast and their relationship to human epidermal growth factor receptor-2 gene amplification: a fluorescence in situ hybridization study. *Hum Pathol* **36**, 348–356 (2005).

40. Morrison, R. *et al.* Targeting the Mechanisms of Resistance to Chemotherapy and Radiotherapy with the Cancer Stem Cell Hypothesis. *Journal of Oncology* **2011**, 1–13 (2011).

41. CDCBreastCancer. How Is Breast Cancer Treated? *Centers for Disease Control and Prevention* https://www.cdc.gov/cancer/breast/basic_info/treatment.htm (2022).

42. Li, Y. *et al.* Dietary Natural Products for Prevention and Treatment of Breast Cancer. *Nutrients* **9**, 728 (2017).

43. Griñán-Lisón, C. *et al.* miRNAs as radio-response biomarkers for breast cancer stem cells. *Mol Oncol* **14**, 556–570 (2020).

44. DeSantis, C., Ma, J., Bryan, L. & Jemal, A. Breast cancer statistics, 2013: Breast Cancer Statistics, 2013. *CA A Cancer Journal for Clinicians* **64**, 52–62 (2014).

45. Cazzaniga, M. & Bonanni, B. Breast Cancer Chemoprevention: Old and New Approaches. *Journal of Biomedicine and Biotechnology* **2012**, 1–15 (2012).

46. Gangopadhyay, S., Nandy, A., Hor, P. & Mukhopadhyay, A. Breast cancer stem cells: a novel therapeutic target. *Clin Breast Cancer* **13**, 7–15 (2013).

47. Rosen, J. M. & Jordan, C. T. The increasing complexity of the cancer stem cell paradigm. *Science* **324**, 1670–1673 (2009).

48. Tsai, Y. C. *et al.* Contiguous patches of normal human mammary

epithelium derived from a single stem cell: implications for breast carcinogenesis. *Cancer Res* **56**, 402–404 (1996).

49. Kelly, P. N., Dakic, A., Adams, J. M., Nutt, S. L. & Strasser, A. Tumor growth need not be driven by rare cancer stem cells. *Science* **317**, 337 (2007).

50. Reya, T., Morrison, S. J., Clarke, M. F. & Weissman, I. L. Stem cells, cancer, and cancer stem cells. *Nature* **414**, 105–111 (2001).

51. Hernández-Camarero, P. *et al.* Revisiting the dynamic cancer stem cell model: Importance of tumour edges. *Crit Rev Oncol Hematol* **131**, 35–45 (2018).

52. Varela, I., Menendez, P. & Sanjuan-Pla, A. Intratumoral heterogeneity and clonal evolution in blood malignancies and solid tumors. *Oncotarget* **8**, 66742–66746 (2017).

53. Tang, D. G. Understanding cancer stem cell heterogeneity and plasticity. *Cell Res* **22**, 457–472 (2012).

54. Vermeulen, L., de Sousa e Melo, F., Richel, D. J. & Medema, J. P. The developing cancer stem-cell model: clinical challenges and opportunities. *Lancet Oncol* **13**, e83-89 (2012).

55. Ogawa, K. *et al.* Radiotherapy targeting cancer stem cells: current views and future perspectives. *Anticancer Res* **33**, 747–754 (2013).

56. Li, X. *et al.* Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. *J Natl Cancer Inst* **100**, 672–679 (2008).

57. Lawson, J. C., Blatch, G. L. & Edkins, A. L. Cancer stem cells in breast cancer and metastasis. *Breast Cancer Res Treat* **118**, 241–254 (2009).

58. Celià-Terrassa, T. *et al.* Epithelial-mesenchymal transition can suppress major attributes of human epithelial tumor-initiating cells. *J Clin Invest* **122**, 1849–1868 (2012).

59. Floor, S., van Staveren, W. C. G., Larsimont, D., Dumont, J. E. & Maenhaut, C. Cancer cells in epithelial-to-mesenchymal transition and tumor-propagating-cancer stem cells: distinct, overlapping or same populations. *Oncogene* **30**, 4609–4621 (2011).

60. Cancer stem cells, the epithelial to mesenchymal transition (EMT) and radioresistance: Potential role of hypoxia - ScienceDirect. https://www.sciencedirect.com/science/article/pii/S0304383512006660.

61. Cancer stem cells revisited | Nature Medicine. https://www.nature.com/articles/nm.4409.

62. Kaplan, R. N. *et al.* VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* **438**, 820–827 (2005).

63. Nigam, A. Breast cancer stem cells, pathways and therapeutic perspectives 2011. *Indian J Surg* **75**, 170–180 (2013).

64. Ginestier, C. *et al.* ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* **1**, 555–567 (2007).

65. Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J. & Clarke, M. F. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A* **100**, 3983–3988 (2003).

66. Jung, N., Maguer-Satta, V. & Guyot, B. Early Steps of Mammary Stem Cell Transformation by Exogenous Signals; Effects of Bisphenol Endocrine Disrupting Chemicals and Bone Morphogenetic Proteins. *Cancers* **11**, 1351 (2019).

67. Wang, R. N. *et al.* Bone Morphogenetic Protein (BMP) signaling in development and human diseases. *Genes Dis* **1**, 87–105 (2014).

68. Chapellier, M. *et al.* Disequilibrium of BMP2 Levels in the Breast Stem Cell Niche Launches Epithelial Transformation by Overamplifying BMPR1B Cell Response. *Stem Cell Reports* **4**, 239–254 (2015).

69. Laperrousaz, B. *et al.* Primitive CML cell expansion relies on abnormal levels of BMPs provided by the niche and on BMPRIb overexpression. *Blood* **122**, 3767–3777 (2013).

70. Jin, H. *et al.* BMP2 promotes migration and invasion of breast cancer cells via cytoskeletal reorganization and adhesion decrease: an AFM investigation. *Appl Microbiol Biotechnol* **93**, 1715–1723 (2012).

71. Sharma, R. *et al.* BMP4 enhances anoikis resistance and chemoresistance of breast cancer cells through canonical BMP signaling. *J Cell Commun Signal* **16**, 191–205 (2022).

72. Meek, D. W. Regulation of the p53 response and its relationship to cancer. *Biochem J* **469**, 325–346 (2015).

73. Song, H., Hollstein, M. & Xu, Y. p53 gain-of-function cancer mutants induce genetic instability by inactivating ATM. *Nat Cell Biol* **9**, 573–580 (2007).

74. Olive, K. P. *et al.* Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. *Cell* **119**, 847–860 (2004).

75. Hwang, J., Yao, H., Caito, S., Sundar, I. K. & Rahman, I. Redox regulation of SIRT1 in inflammation and cellular senescence. *Free Radic Biol Med* **61**, 95–110 (2013).

76. Yao, H. & Rahman, I. Perspectives on translational and therapeutic aspects of SIRT1 in inflammaging and senescence. *Biochem Pharmacol* **84**, 1332–1339 (2012).

77. Shi, L. *et al.* A SIRT1-centered circuitry regulates breast cancer stemness and metastasis. *Oncogene* **37**, 6299–6315 (2018).

78. Fukui, M., Choi, H. J., Wang, P. & Zhu, B. T. Mechanism underlying resveratrol's attenuation of paclitaxel cytotoxicity in human breast cancer cells: Role of the SIRT1-FOXO1-HER3 signaling pathway. *Cancer Treat Res Commun* **28**, 100386 (2021).

79. Won, Y.-S. & Seo, K.-I. Lupiwighteone induces caspase-dependent and -independent apoptosis on human breast cancer cells via inhibiting PI3K/Akt/mTOR pathway. *Food Chem Toxicol* **135**, 110863 (2020).

80. Dilmac, S. *et al.* SIRT1/FOXO Signaling Pathway in Breast Cancer Progression and Metastasis. *Int J Mol Sci* **23**, 10227 (2022).

81. Li, K. & Luo, J. The role of SIRT1 in tumorigenesis. *N Am J Med Sci (Boston)* **4**, 104–106 (2011).

82. Yang, H. *et al.* TP53 mutation influences the efficacy of treatment of

colorectal cancer cell lines with a combination of sirtuin inhibitors and chemotherapeutic agents. *Exp Ther Med* **20**, 1415–1422 (2020).

83. Han, Y. *et al.* Role of autophagy in breast cancer and breast cancer stem cells (Review). *Int J Oncol* **52**, 1057–1070 (2018).

84. Marsh, T. & Debnath, J. Autophagy suppresses breast cancer metastasis by degrading NBR1. *Autophagy* **16**, 1164–1165 (2020).

85. Wu, S. *et al.* Inhibition of macrophage autophagy induced by Salmonella enterica serovar typhi plasmid. *Front Biosci (Landmark Ed)* **19**, 490–503 (2014).

86. Dhingra, A., Alexander, D., Reyes-Reveles, J., Sharp, R. & Boesze-Battaglia, K. Microtubule-Associated Protein 1 Light Chain 3 (LC3) Isoforms in RPE and Retina. *Adv Exp Med Biol* **1074**, 609–616 (2018).

87. Klionsky, D. J. *et al.* Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* **12**, 1–222 (2016).

88. Kabeya, Y. *et al.* LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* **19**, 5720–5728 (2000).

89. Abdullah, M. L., Al-Shabanah, O., Hassan, Z. K. & Hafez, M. M. Eugenol-Induced Autophagy and Apoptosis in Breast Cancer Cells via PI3K/AKT/FOXO3a Pathway Inhibition. *Int J Mol Sci* **22**, 9243 (2021).

90. Chi, H.-C., Tsai, C.-Y., Tsai, M.-M., Yeh, C.-T. & Lin, K.-H. Roles of Long Noncoding RNAs in Recurrence and Metastasis of Radiotherapy-Resistant Cancer Stem Cells. *Int J Mol Sci* **18**, 1903 (2017).

91. Hendry, J. H. Radiation biology and radiation protection. *Ann ICRP* **41**, 64–71 (2012).

92. De Felice, F. *et al.* Relation between Hypofractionated Radiotherapy, Toxicity and Outcome in Early Breast Cancer. *Breast J* **23**, 563–568 (2017).

93. Vlashi, E. & Pajonk, F. Cancer Stem Cells, Cancer Cell Plasticity and Radiation Therapy. *Semin Cancer Biol* **0**, 28–35 (2015).

94. Valle, L. F. *et al.* Hypofractionated whole breast radiotherapy in breast conservation for early-stage breast cancer: a systematic review and meta-analysis

of randomized trials. *Breast Cancer Res Treat* **162**, 409–417 (2017).

95. Deshmukh, A. A. *et al.* Cost-effectiveness Analysis Comparing Conventional, Hypofractionated, and Intraoperative Radiotherapy for Early-Stage Breast Cancer. *J Natl Cancer Inst* **109**, (2017).

96. Hennequin, C. *et al.* [Radiotherapy of breast cancer]. *Cancer Radiother* **20 Suppl**, S139-146 (2016).

97. Induction of metastasis, cancer stem cell phenotype, and oncogenic metabolism in cancer cells by ionizing radiation - PMC. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5282724/.

98. Dörr, W. & Hendry, J. H. Consequential late effects in normal tissues. *Radiother Oncol* **61**, 223–231 (2001).

99. Olivares-Urbano, M. A., Griñán-Lisón, C., Marchal, J. A. & Núñez, M. I. CSC Radioresistance: A Therapeutic Challenge to Improve Radiotherapy Effectiveness in Cancer. *Cells* **9**, 1651 (2020).

100. Plasticity of Cancer Stem Cell: Origin and Role in Disease Progression and Therapy Resistance | SpringerLink. https://link.springer.com/article/10.1007/s12015-019-09942-y.

101. Steer, A., Cordes, N., Jendrossek, V. & Klein, D. Impact of Cancer-Associated Fibroblast on the Radiation-Response of Solid Xenograft Tumors. *Front Mol Biosci* **6**, 70 (2019).

102. Artacho-Cordón, F. *et al.* Matrix metalloproteinases: potential therapy to prevent the development of second malignancies after breast radiotherapy. *Surg Oncol* **21**, e143-151 (2012).

103. Zhou, Y.-C. *et al.* Ionizing radiation promotes migration and invasion of cancer cells through transforming growth factor-beta-mediated epithelial-mesenchymal transition. *Int J Radiat Oncol Biol Phys* **81**, 1530–1537 (2011).

104. Kim, R.-K. *et al.* Radiation promotes malignant phenotypes through SRC in breast cancer cells. *Cancer Sci* **106**, 78–85 (2015).

105. Sokolov, M. V. & Neumann, R. D. Human Embryonic Stem Cell Responses

to Ionizing Radiation Exposures: Current State of Knowledge and Future Challenges. *Stem Cells International* **2012**, e579104 (2012).

106. Sokolov, M. V., Dickey, J. S., Bonner, W. M. & Sedelnikova, O. A. gamma-H2AX in bystander cells: not just a radiation-triggered event, a cellular response to stress mediated by intercellular communication. *Cell Cycle* **6**, 2210–2212 (2007).

107. Dickey, J. S. *et al.* Intercellular communication of cellular stress monitored by gamma-H2AX induction. *Carcinogenesis* **30**, 1686–1695 (2009).

108. Sun, L. Radioresistance and Cancer Stem Cells: Survival of the Fittest. *J Carcinog Mutagen* **s1**, (2011).

109. Marie-Egyptienne, D. T., Lohse, I. & Hill, R. P. Cancer stem cells, the epithelial to mesenchymal transition (EMT) and radioresistance: Potential role of hypoxia. *Cancer Letters* **341**, 63–72 (2013).

110. Jiricny, J. The multifaceted mismatch-repair system. *Nat Rev Mol Cell Biol* **7**, 335–346 (2006).

111. Svilar, D., Goellner, E. M., Almeida, K. H. & Sobol, R. W. Base excision repair and lesion-dependent subpathways for repair of oxidative DNA damage. *Antioxid Redox Signal* **14**, 2491–2507 (2011).

112. Sengupta, S. & Harris, C. C. p53: traffic cop at the crossroads of DNA repair and recombination. *Nat Rev Mol Cell Biol* **6**, 44–55 (2005).

113. Liu, L., Lee, J. & Zhou, P. Navigating the Nucleotide Excision Repair Threshold. *J Cell Physiol* **224**, 585–589 (2010).

114. Kass, E. M. & Jasin, M. Collaboration and competition between DNA double-strand break repair pathways. *FEBS Lett* **584**, 3703–3708 (2010).

115. Mladenov, E. & Iliakis, G. Induction and repair of DNA double strand breaks: the increasing spectrum of non-homologous end joining pathways. *Mutat Res* **711**, 61–72 (2011).

116. Falck, J., Coates, J. & Jackson, S. P. Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. *Nature* **434**, 605–611 (2005).

117. Chumsri, S. & Shah, P. Radiation resistance of cancer stem cells as an

obstacle in cancer therapy. *Molecular and Cellular Pharmacology* **5**, 39–49 (2013).

118. Cojoc, M., Mäbert, K., Muders, M. H. & Dubrovska, A. A role for cancer stem cells in therapy resistance: cellular and molecular mechanisms. *Semin Cancer Biol* **31**, 16–27 (2015).

119. Survival of the Fittest: Cancer Stem Cells in Therapeutic Resistance and Angiogenesis-Reference-Cited by-全球学者库. http://globalauthorid.com/WebPortal/ArticleView?wd=972CA157D4962543D377C151BAC216F6C7AFAE121D20ECED40FF4424932A9E0D.

120. Woodward, W. A. *et al.* WNT/beta-catenin mediates radiation resistance of mouse mammary progenitor cells. *Proc Natl Acad Sci U S A* **104**, 618–623 (2007).

121. Barcellos-Hoff, M. H., Park, C. & Wright, E. G. Radiation and the microenvironment - tumorigenesis and therapy. *Nat Rev Cancer* **5**, 867–875 (2005).

122. Ghotra, V. P. S., Puigvert, J. C. & Danen, E. H. J. The cancer stem cell microenvironment and anti-cancer therapy. *Int J Radiat Biol* **85**, 955–962 (2009).

123. Borovski, T., De Sousa E Melo, F., Vermeulen, L. & Medema, J. P. Cancer stem cell niche: the place to be. *Cancer Res* **71**, 634–639 (2011).

124. Harrison, L. B., Chadha, M., Hill, R. J., Hu, K. & Shasha, D. Impact of tumor hypoxia and anemia on radiation therapy outcomes. *Oncologist* **7**, 492–508 (2002).

125. Bao, S. *et al.* Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* **444**, 756–760 (2006).

126. Keith, B. & Simon, M. C. Hypoxia-inducible factors, stem cells, and cancer. *Cell* **129**, 465–472 (2007).

127. Rich, J. N. Cancer stem cells in radiation resistance. *Cancer Res* **67**, 8980–8984 (2007).

128. (3) Current state of knowledge regarding the use of antiangiogenic agents with radiation therapy | Request PDF. https://www.researchgate.net/publication/51101738_Current_state_of_knowledge_regarding_the_use_of_antiangiogenic_agents_with_radiation_therapy.

129. Brunner, T. B., Kunz-Schughart, L. A., Grosse-Gehling, P. & Baumann, M.

Cancer stem cells as a predictive factor in radiotherapy. *Semin Radiat Oncol* **22**, 151–174 (2012).

130. Zheng, J. *et al.* Spices for Prevention and Treatment of Cancers. *Nutrients* **8**, 495 (2016).

131. Zhou, Y. *et al.* Dietary Natural Products for Prevention and Treatment of Liver Cancer. *Nutrients* **8**, 156 (2016).

132. Farvid, M. S. *et al.* Fruit and vegetable consumption in adolescence and early adulthood and risk of breast cancer: population based cohort study. *BMJ* i2343 (2016) doi:10.1136/bmj.i2343.

133. EPIC - European Prospective Investigation into Cancer and Nutrition. https://epic.iarc.fr/.

134. Elena Salamanca-Fernández, M. R.-B., María José Sánchez,-. La dieta como causa del cáncer: principales aportaciones científicas del Estudio Prospectivo. *NUTRICION CLINICA EN MEDICINA* 61–79 (2018) doi:10.7400/NCM.2018.12.2.5063.

135. Current Advances Research in Nutraceutical Compounds of Legumes, Pseudocereals and Cereals | IntechOpen. https://www.intechopen.com/chapters/76727.

136. Lambein, F., Travella, S., Kuo, Y.-H., Van Montagu, M. & Heijde, M. Grass pea (Lathyrus sativus L.): orphan crop, nutraceutical or just plain food? *Planta* **250**, 821–838 (2019).

137. Full article: Bean seeds: leading nutraceutical source for human health. https://www.tandfonline.com/doi/full/10.1080/19476337.2015.1063548.

138. Dwivedi, S. L. *et al.* Exploiting Phenylpropanoid Derivatives to Enhance the Nutraceutical Values of Cereals and Legumes. *Front Plant Sci* **7**, 763 (2016).

139. Cid-Gallegos, M. S. *et al.* Modification of In Vitro and In Vivo Antioxidant Activity by Consumption of Cooked Chickpea in a Colon Cancer Model. *Nutrients* **12**, 2572 (2020).

140. Alcázar-Valle, M. *et al.* Bioactive Compounds, Antioxidant Activity, and

Antinutritional Content of Legumes: A Comparison between Four Phaseolus Species. *Molecules* **25**, 3528 (2020).

141. Lima-Cabello, E. & Jiménez-López, J. C. Molecular perspective of the nutraceutical properties of narrow-leafed lupin (Lupinus angustifolius L.) seed β-conglutin proteins. *Legume Perspectives* **Issue 22**, 29–32 (2022).

142. Lucas, M. M. *et al.* The future of lupin as a protein crop in Europe. *Front. Plant Sci.* **6**, (2015).

143. The effects of lupin (Lupinus angustifolius) addition to wheat bread on its nutritional, phytochemical and bioactive composition and protein quality. https://www.infona.pl/resource/bwmeta1.element.elsevier-be65e310-2445-3eeb-886f-2e5a7ec7f4ab.

144. Yeheyis, L. *et al.* Crude protein, amino acid and alkaloid contents of annual sweet lupin (lupinus spp. l.) forages and seeds grown in ethiopia. *Experimental agriculture* (2012).

145. Kouris-Blazos, A. & Belski, R. Health benefits of legumes and pulses with a focus on Australian sweet lupins. *Asia Pac J Clin Nutr* **25**, 1–17 (2016).

146. Belski, R. The effects of lupin-enriched foods on body weight, body composition aand cardiovascular disease risk factors. (2010).

147. Dove, E. R. *et al.* Lupin and soya reduce glycaemia acutely in type 2 diabetes. *Br J Nutr* **106**, 1045–1051 (2011).

148. Jimenez-Lopez, J. C. Narrow-leafed lupin (Lupinus angustifolius L.) β-conglutin: A multifunctional family of proteins with roles in plant defence, human health benefits, and potential uses as functional food. *Legume Science* **2**, e33 (2020).

149. Lima-Cabello, E. *et al.* Narrow-leafed lupin (Lupinus angustifolius L.) seed β-conglutins reverse the induced insulin resistance in pancreatic cells. *Food Funct.* **9**, 5176–5188 (2018).

150. Lima-Cabello, E. *et al.* Ex vivo and in vitro assessment of anti-inflammatory activity of seed β-conglutin proteins from Lupinus angustifolius.

Journal of Functional Foods **40**, 510–519 (2018).

151. Lima-Cabello, E. *et al.* Narrow-leafed lupin (Lupinus angustifolius L.) β-conglutin proteins modulate the insulin signaling pathway as potential type 2 diabetes treatment and inflammatory-related disease amelioration. *Mol Nutr Food Res* **61**, (2017).

152. Foley, R. C. *et al.* Analysis of conglutin seed storage proteins across lupin species using transcriptomic, protein and comparative genomic approaches. *BMC Plant Biol* **15**, 106 (2015).

153. Foley, R. C. *et al.* Identification and characterisation of seed storage protein transcripts from Lupinus angustifolius. *BMC Plant Biol* **11**, 59 (2011).

154. Jiménez-López, J. C. Lupin (Lupinus angustifolius L.) b-conglutin proteins: Structure functional features, catalytic mechanism modeling and cross-allergenicity identification using protein threading and molecular docking methods. *Protein Science* **24**, 1–313 (2015).

155. Lima Cabello, E., Robles Bolívar, P., Alché Ramírez, J. de D. & Jiménez-López, J. C. Narrow-leafed lupin b-conglutin proteins epitopes identification and molecular features analysis involved in cross-allergenicity to peanut and other legumes. (2016) doi:10.13039/501100000780.

156. Duranti, M., Guerrieri, N., Cerletti, P. & Vecchio, G. The legumin precursor from white lupin seed. *European Journal of Biochemistry* **206**, 941–947 (1992).

157. Magni, C. *et al.* Combined 2D electrophoretic approaches for the study of white lupin mature seed storage proteome. *Phytochemistry* **68**, 997–1007 (2007).

158. Duranti, M., Consonni, A., Magni, C., Sessa, F. & Scarafoni, A. The major proteins of lupin seed: Characterisation and molecular properties for use as functional and nutraceutical ingredients. *Trends in Food Science & Technology* **19**, 624–633 (2008).

159. Jimenez-Lopez, J. C. *et al.* Lupin Allergy: Uncovering Structural Features and Epitopes of β-conglutin Proteins in Lupinus Angustifolius L. with a Focus

on Cross-allergenic Reactivity to Peanut and Other Legumes. in *Bioinformatics and Biomedical Engineering* (eds. Ortuño, F. & Rojas, I.) 96–107 (Springer International Publishing, 2015). doi:10.1007/978-3-319-16483-0_10.

160. Jimenez-Lopez, J. C. *et al.* Narrow-Leafed Lupin (Lupinus angustifolius) β1- and β6-Conglutin Proteins Exhibit Antifungal Activity, Protecting Plants against Necrotrophic Pathogen Induced Damage from Sclerotinia sclerotiorum and Phytophthora nicotianae. *Frontiers in Plant Science* **7**, (2016).

161. Singh, N. *et al.* Inflammation and Cancer. *Ann Afr Med* **18**, 121–126 (2019).

162. HepG2 Cell Culture. *HepG2 Transfection* https://hepg2.com/.

163. Escudero-Feliu, J. *et al.* Narrow Leafed Lupin (Lupinus angustifolius L.) β-Conglutin Seed Proteins as a New Natural Cytotoxic Agents against Breast Cancer Cells. *Nutrients* **15**, 523 (2023).

164. Suzuki, M., Bandoski, C. & Bartlett, J. D. Fluoride induces oxidative damage and SIRT1/autophagy through ROS-mediated JNK signaling. *Free Radic Biol Med* **89**, 369–378 (2015).

165. Nasrin, N. *et al.* JNK1 phosphorylates SIRT1 and promotes its enzymatic activity. *PLoS One* **4**, e8414 (2009).

166. Salminen, A., Kaarniranta, K. & Kauppinen, A. Crosstalk between Oxidative Stress and SIRT1: Impact on the Aging Process. *Int J Mol Sci* **14**, 3834–3859 (2013).

167. Sengupta, A., Molkentin, J. D., Paik, J.-H., DePinho, R. A. & Yutzey, K. E. FoxO transcription factors promote cardiomyocyte survival upon induction of oxidative stress. *J Biol Chem* **286**, 7468–7478 (2011).

168. Simmons, G. E., Pruitt, W. M. & Pruitt, K. Diverse roles of SIRT1 in cancer biology and lipid metabolism. *Int J Mol Sci* **16**, 950–965 (2015).

169. Vernaza, M. G., Dia, V. P., Gonzalez de Mejia, E. & Chang, Y. K. Antioxidant and antiinflammatory properties of germinated and hydrolysed Brazilian soybean flours. *Food Chemistry* **134**, 2217–2225 (2012).

170. Lima-Cabello, E., Alché, J. D., Morales-Santana, S., Clemente, A. &

Jimenez-Lopez, J. C. Narrow-Leafed Lupin (Lupinus angustifolius L.) Seeds Gamma-Conglutin is an Anti-Inflammatory Protein Promoting Insulin Resistance Improvement and Oxidative Stress Amelioration in PANC-1 Pancreatic Cell-Line. *Antioxidants (Basel)* **9**, 12 (2019).

171. Halban, P. A. *et al.* β-Cell Failure in Type 2 Diabetes: Postulated Mechanisms and Prospects for Prevention and Treatment. *The Journal of Clinical Endocrinology & Metabolism* **99**, 1983–1992 (2014).

172. Donath, M. Y. & Shoelson, S. E. Type 2 diabetes as an inflammatory disease. *Nat Rev Immunol* **11**, 98–107 (2011).

173. Wang, C., Guan, Y. & Yang, J. Cytokines in the Progression of Pancreatic β-Cell Dysfunction. *Int J Endocrinol* **2010**, 515136 (2010).

174. Rains, J. L. & Jain, S. K. Oxidative stress, insulin signaling, and diabetes. *Free Radic Biol Med* **50**, 567–575 (2011).

175. Tangvarasittichai, S. Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus. *World J Diabetes* **6**, 456–480 (2015).

176. Ighodaro, O. M. & Akinloye, O. A. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria Journal of Medicine* **54**, 287–293 (2018).

177. Boudjou, S., Oomah, B. D., Zaidi, F. & Hosseinian, F. Phenolics content and antioxidant and anti-inflammatory activities of legume fractions. *Food Chem* **138**, 1543–1550 (2013).

178. McDaniel, M. L., Kwon, G., Hill, J. R., Marshall, C. A. & Corbett, J. A. Cytokines and nitric oxide in islet inflammation and diabetes. *Proc Soc Exp Biol Med* **211**, 24–32 (1996).

179. Fujimoto, M. *et al.* A Role for iNOS in Fasting Hyperglycemia and Impaired Insulin Signaling in the Liver of Obese Diabetic Mice. *Diabetes* **54**, 1340–8 (2005).

180. de Mejia, E. G. & Dia, V. P. Lunasin and lunasin-like peptides inhibit

inflammation through suppression of NF-kappaB pathway in the macrophage. *Peptides* **30**, 2388–2398 (2009).

181. Coleman, J. W. Nitric oxide in immunity and inflammation. *Int Immunopharmacol* **1**, 1397–1406 (2001).

182. Gonzalez-Angulo, A. M., Morales-Vasquez, F. & Hortobagyi, G. N. Overview of Resistance to Systemic Therapy in Patients with Breast Cancer. in *Breast Cancer Chemosensitivity* (eds. Yu, D. & Hung, M.-C.) 1–22 (Springer, 2007). doi:10.1007/978-0-387-74039-3_1.

183. Song, Y. *et al.* Activation of FOXO3a reverses 5-Fluorouracil resistance in human breast cancer cells. *Experimental and Molecular Pathology* **105**, 57–62 (2018).

184. Jiang, L., Wang, P., Sun, Y.-J. & Wu, Y.-J. Ivermectin reverses the drug resistance in cancer cells through EGFR/ERK/Akt/NF-κB pathway. *J Exp Clin Cancer Res* **38**, 265 (2019).

185. Wu, A. *et al.* Characterization of mammary epithelial stem/progenitor cells and their changes with aging in common marmosets. *Sci Rep* **6**, 32190 (2016).

186. Clément, F., Zhu, H. H., Gao, W.-Q., Delay, E. & Maguer-Satta, V. Quantifying Epithelial Early Common Progenitors from Long-Term Primary or Cell Line Sphere Culture. *Current Protocols in Stem Cell Biology* **35**, 1E.7.1-1E.7.8 (2015).

187. Molyneux, G. *et al.* BRCA1 Basal-like Breast Cancers Originate from Luminal Epithelial Progenitors and Not from Basal Stem Cells. *Cell Stem Cell* **7**, 403–417 (2010).

188. Chapellier, M. & Maguer-Satta, V. BMP2, a key to uncover luminal breast cancer origin linked to pollutant effects on epithelial stem cells niche. *Molecular & Cellular Oncology* **3**, e1026527 (2016).

189. Dontu, G. *et al.* In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev* **17**, 1253–1270 (2003).

190. McLean, K. *et al.* Human ovarian carcinoma–associated mesenchymal stem cells regulate cancer stem cells and tumorigenesis via altered BMP

production. *J Clin Invest* **121**, 3206–3219 (2011).

191. Clément, F. *et al.* Long-term exposure to bisphenol A or benzo(a)pyrene alters the fate of human mammary epithelial stem cells in response to BMP2 and BMP4, by pre-activating BMP signaling. *Cell Death Differ* **24**, 155–166 (2017).

192. Milano, F. *et al.* Bone morphogenetic protein 4 expressed in esophagitis induces a columnar phenotype in esophageal squamous cells. *Gastroenterology* **132**, 2412–2421 (2007).

193. Ke, J., Wu, R., Chen, Y. & Abba, M. L. Inhibitor of DNA binding proteins: implications in human cancer progression and metastasis. *Am J Transl Res* **10**, 3887–3910 (2018).

194. Li, K. *et al.* ID2 predicts poor prognosis in breast cancer, especially in triple-negative breast cancer, and inhibits E-cadherin expression. *Onco Targets Ther* **7**, 1083–1094 (2014).

195. Nair, R., Teo, W. S., Mittal, V. & Swarbrick, A. ID Proteins Regulate Diverse Aspects of Cancer Progression and Provide Novel Therapeutic Opportunities. *Mol Ther* **22**, 1407–1415 (2014).

196. de Boeck, M. *et al.* Smad6 determines BMP-regulated invasive behaviour of breast cancer cells in a zebrafish xenograft model. *Sci Rep* **6**, 24968 (2016).

197. Maguer-Satta, V., Besançon, R. & Bachelard-Cascales, E. Concise Review: Neutral Endopeptidase (CD10): A Multifaceted Environment Actor in Stem Cells, Physiological Mechanisms, and Cancer. *Stem Cells* **29**, 389–396 (2011).

198. Louhichi, T., Saad, H., Dhiab, M. B., Ziadi, S. & Trimeche, M. Stromal CD10 expression in breast cancer correlates with tumor invasion and cancer stem cell phenotype. *BMC Cancer* **18**, 49 (2018).

199. Maguer-Satta, V., Chapellier, M., Delay, E. & Bachelard-Cascales, E. CD10: A tool to crack the role of stem cells in breast cancer. *Proceedings of the National Academy of Sciences* **108**, E1264–E1264 (2011).

200. Huang, P. *et al.* BMP-2 induces EMT and breast cancer stemness through Rb and CD44. *Cell Death Discov* **3**, 17039 (2017).

201. Boudreau, H. E., Casterline, B. W., Burke, D. J. & Leto, T. L. Wild-type and mutant p53 differentially regulate NADPH oxidase 4 in TGF-β-mediated migration of human lung and breast epithelial cells. *Br J Cancer* **110**, 2569–2582 (2014).

202. Motadi, L. R., Lekganyane, M. M. & Moela, P. RBBP6 expressional effects on cell proliferation and apoptosis in breast cancer cell lines with distinct p53 statuses. *Cancer Manag Res* **10**, 3357–3369 (2018).

203. Ghaleb, A., Padellan, M. & Marchenko, N. Mutant p53 drives the loss of heterozygosity by the upregulation of Nek2 in breast cancer cells. *Breast Cancer Research* **22**, 133 (2020).

204. Jordan, J. J. *et al.* Altered-Function p53 Missense Mutations Identified in Breast Cancers Can Have Subtle Effects on Transactivation. *Molecular Cancer Research* **8**, 701–716 (2010).

205. El-Masry, O. S., Brown, B. L. & Dobson, P. R. M. AMPK Activation of Apoptotic Markers in Human Breast Cancer Cell Lines with Different p53 Backgrounds: MCF-7, MDA-MB-231 and T47D Cells. *Asian Pac J Cancer Prev* **20**, 3763–3770 (2019).

206. Amaro, F. *et al.* β-Adrenoceptor Activation in Breast MCF-10A Cells Induces a Pattern of Catecholamine Production Similar to that of Tumorigenic MCF-7 Cells. *Int J Mol Sci* **21**, 7968 (2020).

207. Schratter, G. *et al.* GIRK1 triggers multiple cancer-related pathways in the benign mammary epithelial cell line MCF10A. *Sci Rep* **9**, 19277 (2019).

208. Kim, E.-A. *et al.* Dioscin induces caspase-independent apoptosis through activation of apoptosis-inducing factor in breast cancer cells. *Apoptosis* **19**, 1165–1175 (2014).

209. Devarajan, E. *et al.* Down-regulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance. *Oncogene* **21**, 8843–8851 (2002).

210. Yu, H., Guo, P., Xie, X., Wang, Y. & Chen, G. Ferroptosis, a new form of cell death, and its relationships with tumourous diseases. *J Cell Mol Med* **21**, 648–

657 (2017).

211. Chen, W. *et al.* Isoalantolactone induces apoptosis through ROS-mediated ER stress and inhibition of STAT3 in prostate cancer cells. *J Exp Clin Cancer Res* **37**, 309 (2018).

212. Cui, Q. *et al.* Modulating ROS to overcome multidrug resistance in cancer. *Drug Resist Updat* **41**, 1–25 (2018).

213. Dayem, A. A., Choi, H.-Y., Kim, J.-H. & Cho, S.-G. Role of oxidative stress in stem, cancer, and cancer stem cells. *Cancers (Basel)* **2**, 859–884 (2010).

214. Hecht, F. *et al.* Redox homeostasis of breast cancer lineages contributes to differential cell death response to exogenous hydrogen peroxide. *Life Sci* **158**, 7–13 (2016).

215. Lee, K.-M. *et al.* MYC and MCL1 Cooperatively Promote Chemotherapy-Resistant Breast Cancer Stem Cells via Regulation of Mitochondrial Oxidative Phosphorylation. *Cell Metab* **26**, 633-647.e7 (2017).

216. Isoalantolactone induces apoptosis in human breast cancer cells via ROS-mediated mitochondrial pathway and downregulation of SIRT1 - PubMed. https://pubmed.ncbi.nlm.nih.gov/27600429/.

217. Xu, Y., Qin, Q., Chen, R., Wei, C. & Mo, Q. SIRT1 promotes proliferation, migration, and invasion of breast cancer cell line MCF-7 by upregulating DNA polymerase delta1 (POLD1). *Biochem Biophys Res Commun* **502**, 351–357 (2018).

218. Wang, R.-H. *et al.* Interplay among BRCA1, SIRT1, and Survivin during BRCA1-associated tumorigenesis. *Mol Cell* **32**, 11–20 (2008).

219. Xu, X. *et al.* Genetic interactions between tumor suppressors Brca1 and p53 in apoptosis, cell cycle and tumorigenesis. *Nat Genet* **28**, 266–271 (2001).

220. Wang, R.-H. *et al.* Impaired DNA damage response, genome instability, and tumorigenesis in SIRT1 mutant mice. *Cancer Cell* **14**, 312–323 (2008).

221. Wang, D. *et al.* Novel pathways of fluoride-induced hepatotoxicity: P53-dependent ferroptosis induced by the SIRT1/FOXOs pathway and Nrf2/HO-1 pathway. *Comp Biochem Physiol C Toxicol Pharmacol* **264**, 109526 (2022).

222. Zhang, L., Shi, W., Wang, S., Wu, Y. & Tong, Z. *BMP-2 Promotes Breast Cancer Metastasis by Inducing EMT via PI3K/Akt Signaling Pathway*. https://www.researchsquare.com/article/rs-1964528/v1 (2022) doi:10.21203/rs.3.rs-1964528/v1.

223. Choi, S. *et al.* BMP-4 enhances epithelial mesenchymal transition and cancer stem cell properties of breast cancer cells via Notch signaling. *Sci Rep* **9**, 11724 (2019).

224. Pickup, M. W. *et al.* Deletion of the BMP receptor BMPR1a impairs mammary tumor formation and metastasis. *Oncotarget* **6**, 22890–22904 (2015).

225. Owens, P. *et al.* Disruption of bone morphogenetic protein receptor 2 (BMPR2) in mammary tumors promotes metastases through cell autonomous and paracrine mediators. *Proceedings of the National Academy of Sciences* **109**, 2814–2819 (2012).

226. Alarmo, E.-L., Kuukasjärvi, T., Karhu, R. & Kallioniemi, A. A comprehensive expression survey of bone morphogenetic proteins in breast cancer highlights the importance of BMP4 and BMP7. *Breast Cancer Res Treat* **103**, 239–246 (2007).

227. Fatehi, D., Soltani, A. & Ghatrehsamani, M. SRT1720, a potential sensitizer for radiotherapy and cytotoxicity effects of NVB-BEZ235 in metastatic breast cancer cells. *Pathology - Research and Practice* **214**, 889–895 (2018).

228. Lim, S. *et al.* SNAI1-mediated epithelial-mesenchymal transition confers chemoresistance and cellular plasticity by regulating genes involved in cell death and stem cell maintenance. *PLoS One* **8**, e66558 (2013).

229. Radiation Therapy for Cancer - NCI. https://www.cancer.gov/about-cancer/treatment/types/radiation-therapy (2015).

230. Tumor microenvironment complexity: emerging roles in cancer therapy - PubMed. https://pubmed.ncbi.nlm.nih.gov/22414581/.

231. Tumor microenvironment and nanotherapeutics - PMC. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3951160/.

232. Nguyen, G. H., Murph, M. M. & Chang, J. Y. Cancer stem cell radioresistance and enrichment: where frontline radiation therapy may fail in lung and esophageal cancers. *Cancers (Basel)* **3**, 1232–1252 (2011).

233. Gomez-Casal, R. *et al.* Non-small cell lung cancer cells survived ionizing radiation treatment display cancer stem cell and epithelial-mesenchymal transition phenotypes. *Mol Cancer* **12**, 94 (2013).

234. Bhat, V. *et al.* Radiotherapy and radiosensitization in breast cancer: Molecular targets and clinical applications. *Crit Rev Oncol Hematol* **169**, 103566 (2022).

235. Komorowska, D., Radzik, T., Kalenik, S. & Rodacka, A. Natural Radiosensitizers in Radiotherapy: Cancer Treatment by Combining Ionizing Radiation with Resveratrol. *International Journal of Molecular Sciences* **23**, 10627 (2022).

236. Baaße, A. *et al.* Radiation Sensitivity of Adipose-Derived Stem Cells Isolated from Breast Tissue. *International Journal of Molecular Sciences* **19**, 1988 (2018).

237. Minafra, L. *et al.* Radiosensitizing effect of curcumin-loaded lipid nanoparticles in breast cancer cells. *Sci Rep* **9**, 11134 (2019).

238. da Costa Araldi, I. C. *et al.* The in vitro radiosensitizer potential of resveratrol on MCF-7 breast cancer cells. *Chemico-Biological Interactions* **282**, 85–92 (2018).

239. Rodriguez-Ruiz, M. E. *et al.* Apoptotic caspases inhibit abscopal responses to radiation and identify a new prognostic biomarker for breast cancer patients. *Oncoimmunology* **8**, e1655964 (2019).

240. Lou, J. *et al.* Caspase-Independent Regulated Necrosis Pathways as Potential Targets in Cancer Management. *Frontiers in Oncology* **10**, (2021).

241. Mathiasen, I. S. & Jäättelä, M. Triggering caspase-independent cell death to combat cancer. *Trends in Molecular Medicine* **8**, 212–220 (2002).

242. Rahmanian, N., Hosseinimehr, S. J. & Khalaj, A. The paradox role of

caspase cascade in ionizing radiation therapy. *Journal of Biomedical Science* **23**, 88 (2016).

243. Khalil, H. *et al.* Caspase-3 Protects Stressed Organs against Cell Death. *Mol Cell Biol* **32**, 4523–4533 (2012).

244. Wang, Y. *et al.* The double-edged roles of ROS in cancer prevention and therapy. *Theranostics* **11**, 4839–4857 (2021).

245. Chen, Y. *et al.* LRRC31 inhibits DNA repair and sensitizes breast cancer brain metastasis to radiation therapy. *Nat Cell Biol* **22**, 1276–1285 (2020).

246. Santivasi, W. L. & Xia, F. Ionizing radiation-induced DNA damage, response, and repair. *Antioxid Redox Signal* **21**, 251–259 (2014).

247. Deng, S. *et al.* Targeting the DNA Damage Response and DNA Repair Pathways to Enhance Radiosensitivity in Colorectal Cancer. *Cancers (Basel)* **14**, 4874 (2022).

248. Wong, W.-K., Guerra Liberal, F. D. C. & McMahon, S. J. DNA Repair Inhibitors Potentiate Fractionated Radiotherapy More Than Single-Dose Radiotherapy in Breast Cancer Cells. *Cancers* **14**, 3794 (2022).

249. Tanaka, T., Halicka, D., Traganos, F. & Darzynkiewicz, Z. Cytometric Analysis of DNA Damage: Phosphorylation of Histone H2AX as a Marker of DNA Double-Strand Breaks (DSBs). *Methods Mol Biol* **523**, 10.1007/978-1-59745-190–1_11 (2009).

250. Collins, P. L. *et al.* DNA double-strand breaks induce H2Ax phosphorylation domains in a contact-dependent manner. *Nat Commun* **11**, 3158 (2020).

251. Moon, S.-H., Nguyen, T.-A., Darlington, Y., Lu, X. & Donehower, L. A. Dephosphorylation of γ-H2AX by WIP1: an important homeostatic regulatory event in DNA repair and cell cycle control. *Cell Cycle* **9**, 2092–2096 (2010).

252. Podhorecka, M., Skladanowski, A. & Bozko, P. H2AX Phosphorylation: Its Role in DNA Damage Response and Cancer Therapy. *J Nucleic Acids* **2010**, 920161 (2010).

253. Zhang, X., Li, Y., Wang, D. & Wei, X. miR-22 suppresses tumorigenesis and improves radiosensitivity of breast cancer cells by targeting Sirt1. *Biol Res* **50**, 27 (2017).

254. Masoumi, H., Soltani, A. & Ghatrehsamani, M. The beneficial role of SIRT1 activator on chemo- and radiosensitization of breast cancer cells in response to IL-6. *Mol Biol Rep* **47**, 129–139 (2020).

255. Li, J. *et al.* Radioprotective and Antioxidant Effect of Resveratrol in Hippocampus by Activating Sirt1. *International Journal of Molecular Sciences* **15**, 5928–5939 (2014).

256. Amini, P. *et al.* Resveratrol Induces Apoptosis and Attenuates Proliferation of MCF-7 Cells in Combination with Radiation and Hyperthermia. *Current Molecular Medicine* **21**, 142–150 (2021).

257. Rifaï, K., Idrissou, M., Penault-Llorca, F., Bignon, Y.-J. & Bernard-Gallon, D. Breaking down the Contradictory Roles of Histone Deacetylase SIRT1 in Human Breast Cancer. *Cancers (Basel)* **10**, 409 (2018).

258. Mahmoud, A. S. *et al.* The Detection of DNA Damage Response in MCF7 and MDA-MB-231 Breast Cancer Cell Lines after X-ray Exposure. *Genome Integrity* (2023) doi:10.14293/genint.14.1.001.

259. Ko, A. *et al.* Autophagy inhibition radiosensitizes in vitro, yet reduces radioresponses in vivo due to deficient immunogenic signalling. *Cell Death Differ* **21**, 92–99 (2014).

260. Autophagy-regulating microRNAs: potential targets for improving radiotherapy - PubMed. https://pubmed.ncbi.nlm.nih.gov/29971533/.

261. Kim, W. *et al.* Cellular Stress Responses in Radiotherapy. *Cells* **8**, 1105 (2019).

262. Levine, B. & Yuan, J. Autophagy in cell death: an innocent convict? *J Clin Invest* **115**, 2679–2688 (2005).

263. Zhuang, W. *et al.* Induction of autophagy promotes differentiation of glioma-initiating cells and their radiosensitivity. *Int J Cancer* **129**, 2720–2731

(2011).

264. Kim, K. W. *et al.* Autophagy for cancer therapy through inhibition of pro-apoptotic proteins and mammalian target of rapamycin signaling. *J Biol Chem* **281**, 36883–36890 (2006).

265. Cui, L. *et al.* Radiation induces autophagic cell death via the p53/DRAM signaling pathway in breast cancer cells. *Oncol Rep* **35**, 3639–3647 (2016).

266. Wang, B. *et al.* PLK1 Inhibition Sensitizes Breast Cancer Cells to Radiation via Suppressing Autophagy. *International Journal of Radiation Oncology, Biology, Physics* **110**, 1234–1247 (2021).

267. Gao, X. *et al.* Radiation-Induced Reprogramming of Pre-Senescent Mammary Epithelial Cells Enriches Putative CD44+/CD24−/low Stem Cell Phenotype. *Frontiers in Oncology* **6**, (2016).

268. Krause, M., Yaromina, A., Eicheler, W., Koch, U. & Baumann, M. Cancer stem cells: targets and potential biomarkers for radiotherapy. *Clin Cancer Res* **17**, 7224–7229 (2011).

269. Pawlik, T. M. & Keyomarsi, K. Role of cell cycle in mediating sensitivity to radiotherapy. *Int J Radiat Oncol Biol Phys* **59**, 928–942 (2004).

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