UNIVERSITÀ DEGLI STUDI DI PADOVA

Dipartimento di Ingegneria dell'Informazione DEI

Corso di Laurea Magistrale in Bioingegneria

Tesi di Laurea Magistrale

EPIGALLOCATECHIN-3-GALLATE IN BREAST AND COLON CANCER THERAPY: AN *IN VITRO* AND *IN VIVO* STUDY.

Relatore

Prof. Andrea Facchinetti

Correlatrice

Prof.ssa Ana Cristina Aguiar Santos

Laureanda: Arianna Parma

Matricola: 1159893

Anno Accademico: 2018-2019

This work was developed in collaboration with:

FFUC FACULDADE DE FARMÁCIA
UNIVERSIDADE DE COIMBRA

ICBR
Coimbra Institute for Clinical and Biomedical Research

Index

Index of Figures

Index of Tables

Abstract

Since ancient times plants have been used in treatments and cures for several pathologies. Worldwide population growth, high cost of some therapies, side effects, and the increasing resistance to synthetic pharmaceuticals are some of the reasons for a bigger search for natural products, to be used in therapeutics and prevention of a large array of humans' pathologies.

Green tea is the second most consumed drink in the world and the health benefits of green tea have been recognized in the past, in particular the protection against cardiovascular, neurodegenerative and cancer diseases. Green tea polyphenols, strong antioxidants, of which (—)-epigallocathechin-3-gallate (EGCG) is the most abundant, are considered the active components responsible for their anticancer properties. Studies have suggested a potential clinical activity for high doses of green tea in colorectal and breast cancer prevention. However, there are discrepancies between investigations and further controlled studies with adequate statistical treatment are needed.

In this context, this study will focus on the anti-tumor properties of this tea, applying them in a practical manner: adjuvant in the treatment of colon and breast cancer. The main goal of the work is to determine if green tea has a cytotoxic effect, due to the presence of the referred component (EGCG), over two human tumor cell lines – colon (WiDr) and breast (MCF-7) cancer –, its effectiveness in inhibiting their growth and if it can or not help potentiate specific chemotherapeutic agents, using well-defined clinical protocols.

In this context an experimental study has been designed, divided in *in vitro* and *in vivo* studies, conducted on these cell lines.

Several chemotherapy drugs with different actions were selected, and cell viability was determined by the MTT (bromide of 3-(4,5-dimethylthiazol,2-il)-2,5-diphenyl-tetrazolium) assay, which is based upon reduction of its tetrazolic ring through the formation of formazan crystals.

In vivo studies of tumor induction were made through subcutaneous cell injection of the referred cell lines in a non-orthotopic model, in nude mice (BALB/c). The animals were organized in groups to test the efficacy of oral administration of GTE powder (11.605 g/500mL).

The obtained *in vitro* studies' results indicated that the EGCG treatment may diminish the cell viability of these tumor cells compared to the control group and may potentiate the cytotoxicity of the tested drugs. The results of the *in vivo* studies, as well as the images provided by the easyPET-3D system, proved the effective decrease of the tumor's glandular structure in the animals following the defined protocol.

In conclusion, the aim is to further clarify if EGCG can be used as a natural added agent in the fight against colorectal and breast cancer, showing the experiments' results.

Chapter 1

1. Introduction

This chapter will describe, in general, all the performed work. Initially, the problem of cancer affecting the world's population will be addressed, referring to updated statistical data in this area, giving rise to this study. Afterwards, a natural compound useful in cancer fight will be presented. Furthermore, the work plan, the used methodologies and the project structure will be presented, along with a brief summary of what is covered in each chapter.

1.1 Contextualization and motivation

Oncology is a branch of medical science linked to prevention, diagnosis and treatment of both benign and malignant tumors. Every cell in the human body has a well-regulated system that controls its growth, maturity, reproduction and death. The names “tumor” and “cancer” are often used as synonyms, but this is incorrect. A neoplasm corresponds to an abnormal cell growth (faster than normal), continuing if left untreated. The term “tumor” is commonly used, even if it is not specific, when referring to a neoplasm. Actually, tumor means only a mass. A neoplasm can be benign (usually curable) or malignant (cancerous). A benign tumor is usually localized and does not reach other areas of the body. The majority responds well to treatment, but if left untreated, it can become large and have serious consequences. A malignant "tumor" or cancer invades the surrounding tissues, breaking down the defense barriers and often offering resistance to treatment, leading to metastasis and sometimes reappearing even after surgical removal. Malignant tumors are the second leading cause of death worldwide. Lung, colorectal, breast and prostate cancer are the cancers responsible for most of the deaths [1].

Based upon statistical data, predicting an increase in new cancer cases over the years, it is essential to find compounds that not only prevent this disease, but also manage to treat it more effectively [2].

In turn, there are references that, since prehistoric times, medicinal plants were used by almost all the civilizations of the globe. Today there are about 500.000 plant species on the planet and about 7.000 are or have been studied for medical applications. Furthermore, 25% of drug prescriptions currently contain compounds isolated from plants [2].

This study aims to relate one of the pathologies causing most of the deaths, with one very interesting plant, responsible for the most consumed infusion in the world, after water: tea. A practical example of the advantages of this compound is that, about 10 years ago, the Food and Drug Administration (FDA) approved the sale of sinecatechins (Veregen, Bradley/MediGene, AG, D82152 Planegg/Martinsried, Germany), a botanical drug used in the treatment of genital problems. Synechechin is an extract of green tea leaves from the *Camellia sinensis* plant, being the first herbal extract to be approved as a medicine for clinical therapy. The new drug, based on green tea, is now a real alternative to conventional therapy and shows how, thanks to the successful clinical trials of this tea and its catechins, this compound can lead to important beneficial formulations for health [2].

1.2 Study goals

The health benefits of green tea have been recognized since ancient times, in particular the protection against cardiovascular, neurodegenerative and cancerous diseases. The polyphenols of green tea, strong antioxidants, of which epigallocatechin-3-gallate (EGCG) is the most abundant, are considered the active components responsible for the anticancer properties. Different studies have suggested a potential clinical activity for high doses of green tea to prevent colorectal and breast cancers. However, there are discrepancies between investigations and further controlled studies with adequate statistical treatment are needed [].

In this context, this study aims to quantify the EGCG present in different brands of different green tea extracts, available on the market. It also aims to analyze the possible cytotoxicity of EGCG and its effectiveness in inhibiting the growth of two human tumor cell lines: colon (WiDr) and breast (MCF-7).

Recently, *in vitro* and *in vivo* studies have been conducted with the WiDr cell line and with the drugs used to treat colorectal cancer, by adding an EGCG-enriched extract. Similar *in vitro* and *in vivo* studies were planned with the MCF7 cell line. This project also aims to contribute to the evaluation of green tea extracts to achieve a possible enhancement of the effect of the drugs used in their therapy, trying, in the future, to reduce the drug dosages or the therapeutic cycles.

Additional information was obtained from an *in vivo* study, conducted on an immune-compromised animal model (BALB/c nude mice) injected subcutaneously (sc) with WiDr or MCF-7 cell lines, in a non-orthotopic model to test the administration of EGCG-enriched green tea extracts. In summary, it is intended to further clarify how far EGCG can be used as a natural added agent in the fight against colorectal and breast cancer.

1.3 Work structure

This work is divided into 5 chapters.

Chapter 1: Introduction

This chapter discusses the contextualization of the work, as well as its goals. It intends to briefly explain how this project is divided and what are the main topics addressed.

Chapter 2: Literature review

This chapter first discusses statistical data on colorectal and breast cancer, being the topics that have guided this study.

The stages of the referred tumors, and the different therapeutic regimens available for each of them are also explained. The cell lines chosen to perform this study (WiDr - colorectal cancer - and MCF-7 - breast cancer) are described, as well as the factors that led to their choice.

Since it was intended to associate this oncological pathology with natural agents and tumor inhibitors, this interrelation was addressed, explaining the possible benefits of using natural plants or their derivatives/extracts. In particular, the *Camellia sinesis* plant that produces green tea and the (-) - epigallocatechin-3-gallate (EGCG), the main responsible polyphenolic compound for the health benefits associated with the consumption of green tea, have been analyzed. The chemotherapeutic agents for colorectal and breast carcinoma were then described.

Finally, the animal model chosen to induce non-orthotopic colorectal and breast cancers, using the referred cell lines, was described.

Chapter 3: Material and methods

This chapter discusses the procedures concerning these cell lines, as well as their achievement, for the performance of reproducible and coherent experiments.

The green tea extracts studied and the analytical technique involved in determining the EGCG concentration present in each of them are described: high pressure liquid chromatography (HPLC).

The project has two distinct but converging parts: *in vitro* and *in vivo* tests. In the first case, the study procedures were explained and the study methodology of inducing cellular cytotoxicity was also described, explaining what the MTT test is (3-(4,5-dimethylthiazol-2-il)-2.5-diphenyltetrazolium). *In vivo* studies were performed both with WiDr and MCF-7 lines, and independently evaluated and analyzed. The features of the animal model used for the subcutaneous inoculation of tumor cells, the development of non-orthotopic tumors and their occurrence, obeying and respecting to the by-laws of animal studies, were also described.

Chapter 4: Results and Discussion

This chapter presents the results obtained with all the methods used in this project: *in vitro* and *in vivo* tests and their statistical analysis, using the SPSS and GraphPad Prism programs and, subsequently, their integrated critical analysis.

Chapter 5: Conclusion

This chapter mentions the main conclusions drawn from this study and how they can contribute to further study and to enhance knowledge in the area. Furthermore, proposals for future works and analysis are also suggested.

Chapter 2

2. Literature review

This chapter deals with the concepts used for the elaboration of the present study.

2.1 Tumor *vs* cancer

Tumor is an abnormal mass of tissue that results when cells divide more than they should or do not die when they should. Tumors may be benign (not cancer), or malignant (cancer), also called neoplasm [1,3].

Cancer designates different types of related diseases. In all types of cancer, some of the body's cells begin to divide without control and spread into surrounding tissues [3,4].

To better understand cancer, it is necessary to comprehend what happens to the cell in its normal life-time and how it can turn into a cancer cell. Human beings possess a huge variety of cell types in the body, most of which are replaced by new cells when they reach the end of the cell cycle. Thus, when cells become old or defective, they are destroyed and give rise to new cells with similar characteristics, in order to keep the organism functioning and healthy [5].

Sometimes the process of cell renewal does not take place correctly. Due to alterations in the genetic material, the cell loses the ability to control its growth, division and death. Hence, cancer is caused by changes to genes, being a genetic disease. These genetic changes can be hereditary or arise during a person's life due to errors that occur when cells divide or due to DNA damages caused by different environmental exposures (such as radiation, chemicals in tobacco smoke or food preservatives) [5].

When cancer develops, the orderly process of cells' growing and dividing breaks down. Cells become abnormal, and old or damaged cells survive when they should die, leading to cells' formation when they are not needed [3-5].

The process of carcinogenesis may be divided into three stages: initiation, promotion, and progression. The first stage of carcinogenesis, initiation, results from an irreversible genetic alteration, likely one or more simple mutations, transitions, and/or small deletions in DNA. The reversible stage of promotion involves changes in the expression of the genome mediated through

promoter-receptor interactions. The final irreversible stage of progression is characterized by instability and malignant growth [3-5].

Cancers are malignant tumors, which means they can spread into nearby tissues and, as these tumors grow, some cancer cells can break off and travel to distant places in the body through the blood or the lymphatic system and form new tumors far from the original one [3].

Cancer cells differ from normal cells in many ways that allow them to grow out of control and become invasive. One important difference is that cancer cells are less specialized than normal cells and, for this reason, cancer cells continue to divide without stopping, since they are able to ignore signals that normally tell cells to stop dividing or that begin the programmed cell death process (apoptosis) (**Figure 1**) [3].

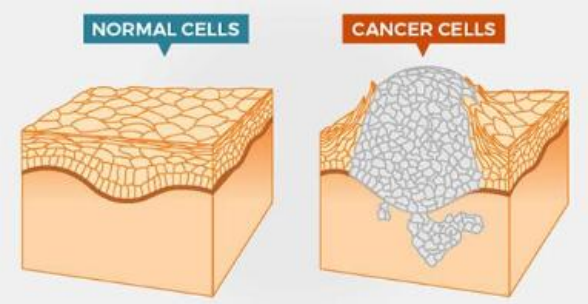


Figure 1: Cancer cells and normal cells. Cancer cells divide uncontrollably and spread into surrounding tissues (retrieved from [3]).

A cancer that has spread from the place where it first started to another site in the body is called metastatic cancer. The process by which cancer cells spread to other parts of the body is called metastasis (**Figure 2**) [3,4].

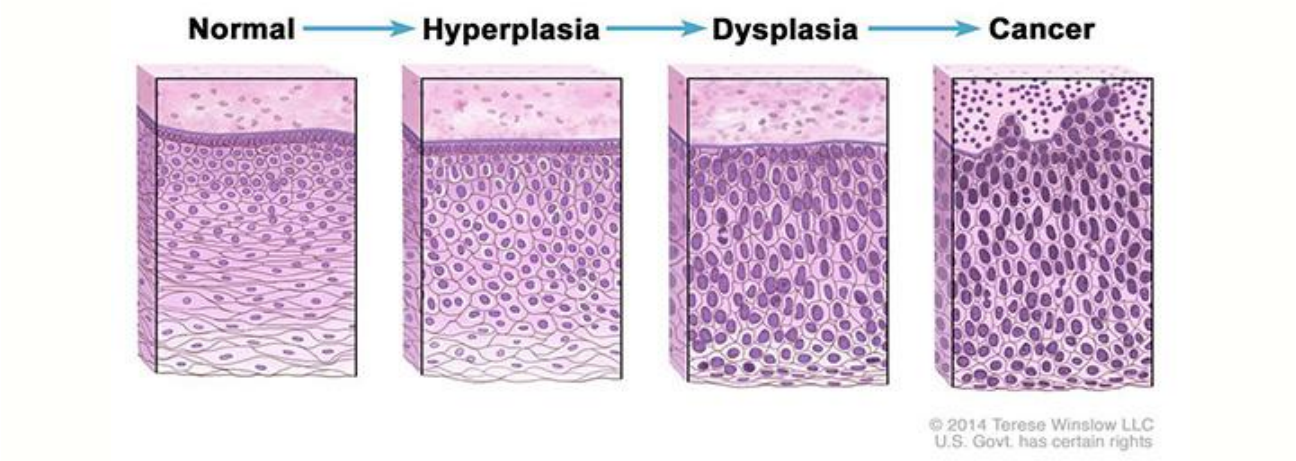


Figure 2: How normal cells become cancer cells: initially the cells go through abnormal changes called hyperplasia (an increase number of cells appearing normal under a microscope) and dysplasia (the

cells look abnormal) and those states may or not become a cancer. These abnormal cells can invade the nearby tissues and blood/lymphatic vessels (retrieved from [3]).

Cancer is a significant health problem worldwide, with very high incidences also in Europe. Nowadays it is the leading cause of death among the world's population (**Figure 3**) [6,7].

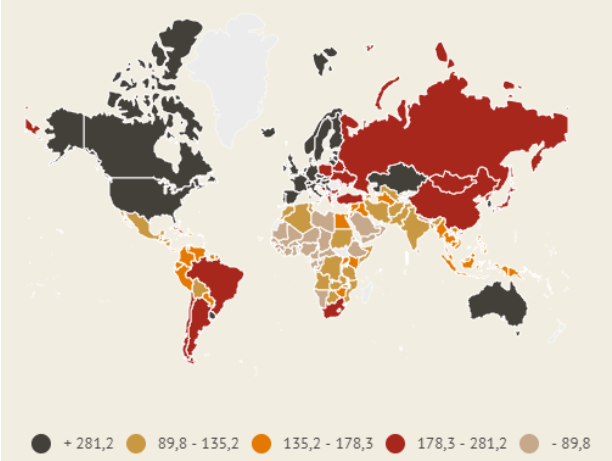


Figure 3: Study that reveals new cases of tumor depending on the total number of people diagnosed with these tumors (retrieved from [8]).

The International Agency for Research on Cancer (IARC) estimate that one-in-five men and one-in-six women worldwide will develop cancer over the course of their lifetime, and that one-in-eight men and one-in-eleven women will die from their disease. The factors appearing to be driving this increase, are a growing and ageing global population and an increase to the exposure to cancer risk factors linked to social and economic development [6].

In 2018, 18.1 million new cancer cases and 9.6 million cancer deaths worldwide were estimated (**Figure 4**) [6].

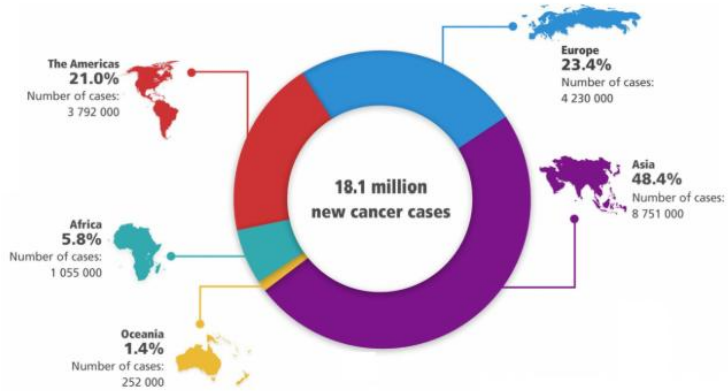


Figure 4: Global cancer incidence (adapted from [6]).

Europe data refers 23.4% of total cancer cases and 20.3% of deaths, representing around one quarter of the global total of cancer cases with some 3.7 million new patients per year and 1.9 million deaths each year (**Figure 5**). In Europe, cancer represents the second most important cause of death and morbidity. The most frequently diagnosed cancers are: lung, breast, prostate and colon types [8].

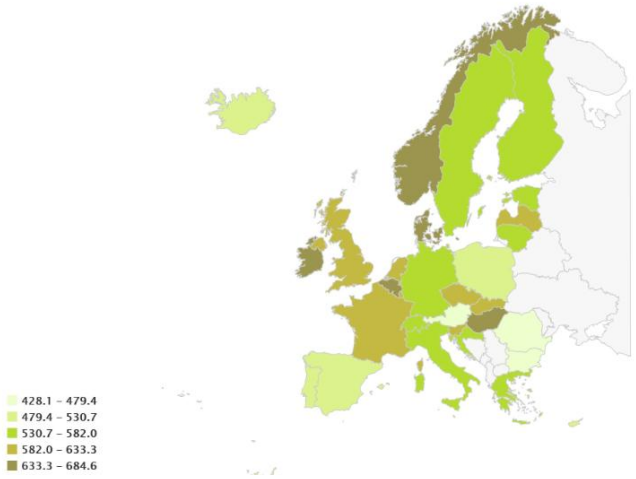


Figure 5: Estimated incidence in Europe by country, considering both sexes (adapted from [9]).

Considering Italy, in accordance with the data presented by AIOM (Italian Association of Medical Oncology) and AIRTUM (Italian Cancer Registry Association), every day more than 1.000 new cases of cancer are diagnosed.

About 373.300 new cancer diagnoses during the year are estimated, 52% among men and 48% among women. Considering the entire population, the most frequent tumors are the colorectal (13.7%) and breast (14.1%) cancer, followed by lung (11.1%) and prostate cancer (9.5% only in males) [10,11].

The good news is that overall recovery rates have improved over the past years thanks to the greater acceptance of screening campaigns, which allow the identification of the disease at an early stage, and the greater efficacy of the therapies [10,11].

Regarding Portugal, the country where this thesis project has been carried out, according to the data presented by the Portuguese League Against Cancer, cancer accounts for a quarter of deaths per year. Despite the increasing number of new people with cancer, care and survival with a good quality of life are more evident each time. The tumors causing the greatest number of deaths are colon, lung, breast and stomach cancer. As previously reported, the number of cases is always increasing and this may also be linked to the increase in average life expectancy, as this disease affects, in most cases, people of advanced age [8,12].

In this context, it is necessary to discover new natural or synthetic compounds that can effectively prevent and/or help to treat this disease.

In this project the focus has been on breast and colon cancer, two of the leading causes of death in the world population.

2.2 Breast Cancer

As mentioned above, cancer has emerged as the leading cause of morbidity and mortality in European populations. The most common diagnose among women worldwide is breast cancer, occurring with the highest incidence in developed countries including the EU, Australia, and the US, affecting approximately one in every ten women worldwide. It has been estimated that 12% of women will develop breast cancer over the course of their life [13,14].

The development of this type of cancer is caused by a gradual and lifelong accumulation of acquired (somatic) mutations and epigenetic changes, that affect mammary tissue cells and their precursors. Examining cancer cells under the microscope, the presence of chromosomal aberrations was observed. The somatic mutations in a cancer cell genome may include distinct classes of DNA sequence change, such as substitutions of one base by another, insertions or deletions of small or large segments of DNA and rearrangements [13-17].

A gene expression profiling of tumors has revealed five major molecular subtypes of breast cancer: basal-like, luminal A, luminal B, HER2+/ER–, and normal breast–like (**Figure 6**). The molecular differences result in distinct clinical outcomes and responses to treatment (**Figure 7**). These different subtypes are the explanation for the tumoral differences [18,19].

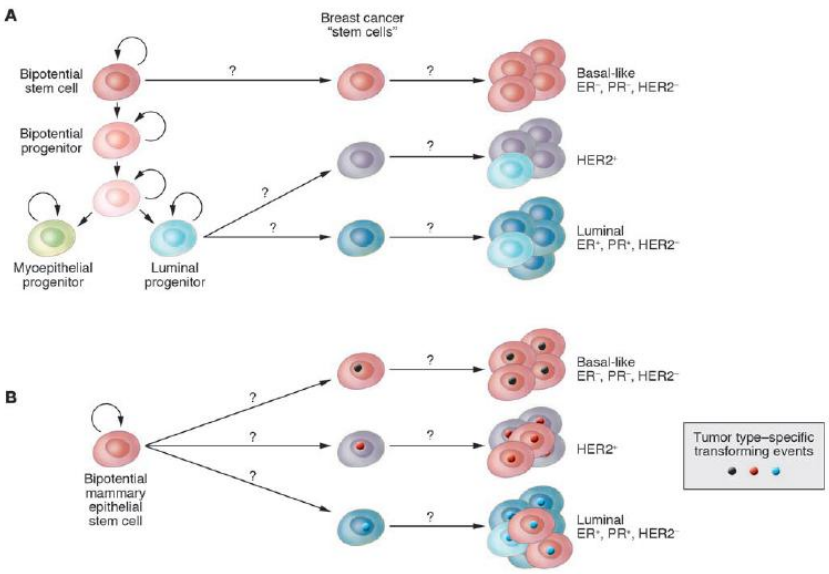


Figure 6: Hypothetical models explaining breast tumor subtypes. Cell of origin (**A**) and tumor subtype–specific transforming event (**B**) models (retrieved from [16]).

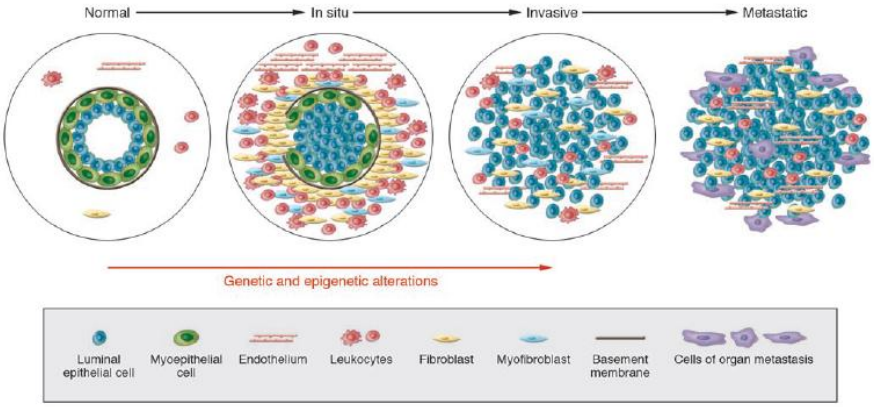


Figure 7: Hypothetical model of breast tumor progression. Schematic view of normal, *in situ*, invasive, and metastatic carcinoma progression (retrieved from [16]).

The molecular mechanism on the basis of hereditary changes that stimulate the development of breast cancer is not yet clear. However, it should be noted that most of the cancer susceptibility genes encode tumor suppressor proteins involved in DNA repair processes, especially the highly complex mechanisms of repair of DNA double-strand breakage [20].

2.2.1 Breast anatomy

The breast is an endocrine gland located on the front of the chest, consisting of glandular acini (alveoli), coated by cells that have the property of producing milk under the influence of some

hormones. Estrogen and progesterone induce changes in the glandular tissue depending on the period of the menstrual cycle. In the adult woman, the breast is made up of glandular tissue, connective and adipose tissue, that determine the size, shape and texture of the organ. The tissue is arranged in 15 to 20 lobes, each of which contains many lobules, terminating in clusters of rounded alveoli. The breast extends from the second rib above to the sixth rib below. Medially, it borders the lateral edge of the body of the sternum, and laterally it reaches the mid-axillary line [21-23].

At the apex of the breast there is the mammary areola, a pigmented skin area whose surface is characterized by the presence of modified sebaceous glands that, with their secretion, have the function of making the nipple soft and elastic. At the surface of the nipple, there are tubules from which the secretion product of the gland comes out, especially when it is sucked by the offspring (**Figure 8**) [21-23].

Considering the histology, two components can be considered [24]:

- structural component: the mammary stroma is responsible for supporting and protecting the mammary structure, dividing into an interlobular and intralobular stroma. The first one is mainly composed of dense connective and adipose tissue, which give its shape. The second one is composed of loose connective tissue, such as inflammatory cells or lymphocytes, resulting very sensitive to hormonal changes. The composition of the breast stroma changes over the years: it increases the adipose tissue and decreases the connective tissue
- functional component: the breast parenchyma, made up of epithelial cells, is responsible for the production and secretion of milk. The mammary lobules are circular contour structures, formed by the grouping of acini and ducts surrounded by the interlobular stroma. The berries and the ducts are composed of two types of covering cells. The inner lining is formed by epithelial cells, and the outer one by myoepithelial cells responsible for the contraction to expel the milk produced. The distribution of the tissues (dense and loose, adipose and epithelial connective tissue) depends on the hormonal cycle, age, nutrition and genetic factors.

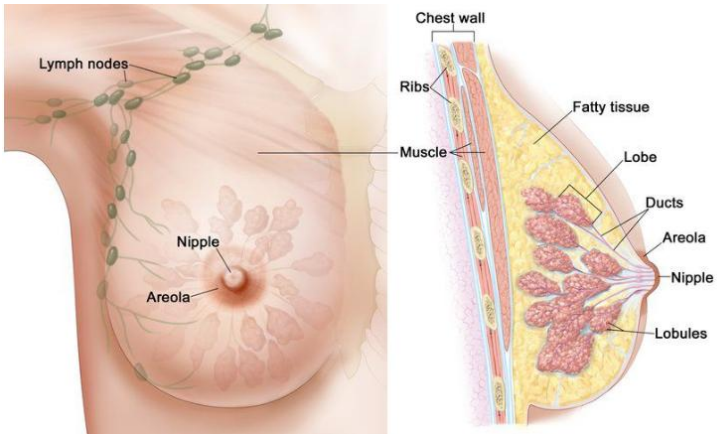


Figure 8: Anatomy of the breast (retrieved from [25]).

2.2.1 Breast changes

2.2.2.1 Benign

Most breast changes, such as breast lumps or breast pain, are not signs of breast cancer. The most common causes of breast lumps are breast cysts[1], fibroadenoma[2], and breast calcification. Situation like intraductal papilloma[3], fat necrosis[4], and benign phyllodes tumor[5] are less common causes for these breast lumps [26].

2.2.2.2 Cancer conditions

The condition in which malignant (cancer) cells multiply abnormally in the breast eventually spreading to the rest of the body, is denominated breast cancer. Most tumors develops at the terminal end of the lobular units of the breast parenchyma. Signs of breast cancer include a lump, bloody nipple discharge, or skin changes [27].

There are 4 main type of breast cancer (**Figure 9**) [27]:

- ductal carcinoma *in situ* (DCIS): develops in the duct cells not invading deeper or spreading through the body. Women diagnosed with DCIS have a high likelihood of being cured;

[1] Fluid-filled sac that can develop as the breasts change with aging.
[2] A lump that often develops during puberty, but which can occur at any age.
[3] A wart-like lump developing in one or more milk duct of the breast.
[4] A lump that forms itself when an area of fatty breast tissue is damaged.
[5] Smooth, firm lumps in the breast, which, occasionally, grows fairly quickly and can be quite large (40–50 mm). Sometimes the skin over the area can be inflamed.

- lobular carcinoma *in situ* (LCIS): occurs in the milk-producing lobule cells not invading or spreading to the rest of the body. However, women with LCIS have an increased likelihood of developing invasive breast cancer in the future;
- invasive ductal carcinoma: begins in the duct cells, then invading deeper into the breast, carrying the potential of metastasizing;
- invasive lobular carcinoma: begins in the milk-producing lobule cells, then progessing deeper into the breast, carrying the potential of spreading to the rest of the body (metastasizing). It is less common than invasive ductal carcinoma.

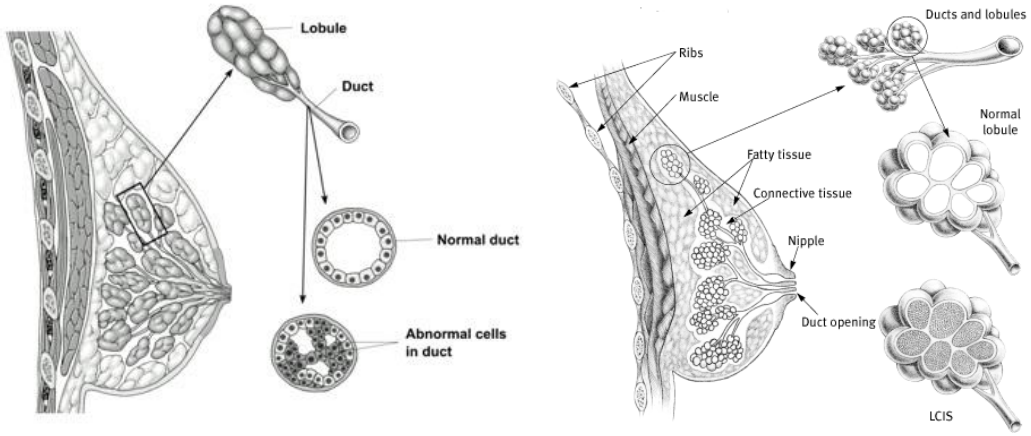


Figure 9: Cross section of the breast and scheme of DCIS and LCIS (retrieved from [28,29]).

2.2.3 Breast Cancer Classification

Breast cancer is a heterogeneous disease that presents different phenotypic characteristics at histological, molecular and clinical level, especially considering the course of the disease and the therapeutic response. Breast cancers with different histopathological and biological features exhibit distinct behaviors, leading to different treatment responses and should be approached by different therapeutic strategies [30].

The microarrays have led to a new way in deciphering breast cancer heterogeneity, and a subtyping system using prognostic multigene classifiers was developed. In this context, in order to make the best therapeutic decisions, it is important to group breast cancers into clinically relevant subtypes. The technologic development allows to correlate the profile of the genic expression to its clinical evolution and its therapeutics response [30-33].

The classification should consider some aspects such as the histological type, the state of the hormonal receptors, the HER2 gene expression and the staging. The so-called intrinsic molecular classification of human breast cancer includes basal-like, HER2-positive, luminal-A and luminal-B subtypes [30-33].

2.2.4 Histopathology

Histopathology and cytopathology are very important in the diagnosis of cancer, also providing information influencing prognosis and treatment [32-34].

Carcinomas are the most common malignant tumors of the breast, cancers derived from epithelial tissue. This means that they are mostly found in ducts or lobules, composed of granular tissue. Carcinomas have a wide range of subtypes that differ in terms of microscopic appearance and biological behavior. These specific tumor types are defined by their morphology, but are also linked to particular clinical, epidemiological, and molecular features [33,34].

It is important to distinguish between *in situ* and invasive carcinoma, in fact both types of carcinoma may possess similar symptoms and signs, although presentation as asymptomatic lesions detected during screening mammography is more common with *in situ* disease [34].

There are other histological types of breast carcinoma, such as tubular carcinoma, mutinous carcinoma, inflammatory breast cancer, invasive micropapillary carcinoma, metaplastic carcinoma, cystic adenoid carcinoma, and medullary carcinoma [31,35].

2.2.5 Breast Cancer Subtypes

Gene expression studies have identified several distinct breast cancer subtypes. These include two main subtypes of estrogen receptor (ER)-negative tumors, basal-like and human epidermal growth factor receptor-2 (HER2)-enriched, and two subtypes of ER-positive tumors, luminal A and luminal B. These subtypes differ markedly in prognosis and in the therapeutic targets they express [36-38].

There are five main intrinsic or molecular subtypes of breast cancer that are based on the genes a cancer expresses, which are presented in **Table 1**:

Table 1: Breast cancer's molecular subtypes (adapted from [39-43]).

Molecular subtypes of breast cancer	IHC status	Description
Luminal A	[ER+ and/or PR+] HER2-	It corresponds to around 54% of all breast cancers. Luminal tumors respond well to hormone therapy but poorly to conventional chemotherapy and luminal A tumors could be adequately treated with endocrine therapy. It is hormone-receptor positive (estrogen-receptor and/or progesterone-receptor positive), HER2 negative, and has low levels of the protein Ki-67, which helps control how fast cancer cells grow. Luminal A cancers are low-grade, tend to grow slowly and have the best prognosis of all breast cancer subtypes.
Luminal B negative HER2 **Luminal B positive HER2**	[ER+ and/or PR+] HER2- [ER+ and/or PR+] HER2+	It is hormone-receptor positive (estrogen-receptor and/or progesterone-receptor positive), and either HER2 positive or HER2 negative with high levels of Ki-67. Luminal B cancers generally grow slightly faster than luminal A cancers, and their prognosis is slightly worse. The risk of early relapse is high and it has greater resistance to therapy. Most luminal B cancers have high Recurrence Scores.
HER2 over-expression	[ER- and PR-] HER2+	Correspond to 10-15% of all breast cancers, shows high expression of the HER2 and proliferation gene clusters and low expression of the luminal cluster. In fact it is hormone-receptor negative (estrogen-receptor and progesterone-receptor negative) and HER2 positive. HER2-enriched cancers tend to grow faster than luminal cancers and can have a worse prognosis, but they are often successfully treated with targeted therapies aimed at the HER2 protein.
Basal-like	[ER- and PR-] HER2-	Its designation is due to some similarity in expression to that of the basal epithelial cells. Corresponds to about 15-20% of breast cancers. It is characterized by low expression of the luminal and HER2 gene clusters, hence being hormone-receptor negative (estrogen-receptor and progesterone-receptor negative) and HER2 negative, being called **Triple-negative**. This type of cancer is more common in women with *BRCA1* gene mutations, a gene responsible for tumor suppression. This type of cancer is more common in the premenopausal years and among Afro-American women. It carries a poor prognosis.
Normal-like	[ER+ and/or PR+] HER2-	It is similar to luminal A: hormone-receptor positive (estrogen-receptor and/or progesterone-receptor positive), HER2 negative, and with low levels of the protein Ki-67. Still, while normal-like breast cancer has a good prognosis, its prognosis is slightly worse than luminal A cancer's.

2.2.6 Histological grade

Histological tumor grade has become an indicator of prognosis in breast cancer. It is based upon the degree of differentiation of the tumor tissue, on how abnormal the tumor cells are and how the tumor tissue looks like under a microscope [44].

It indicates how quickly a tumor is growing and spreading. If the cells of the tumor are close to normal cells and tissue architecture, the tumor is called “well-differentiated” and tends to grow and spread slowly. On the other hand, “undifferentiated” tumors have abnormal-looking cells and may lose normal tissue structures. Considering these differences in microscopic appearance, a numerical “grade” is assigned to most cancers [45,46].

This system to grade breast tumors based upon the following features:

- tubule formation: how much of the tumor tissue has normal breast (milk) duct structures;
- nuclear grade: an evaluation of the size and shape of the nucleus of the tumor cells;
- mitotic rate: number of dividing cells, which is a measure of how fast the tumor cells are growing and dividing.

Each category gets a score between 1 and 3 (“1” means the cells and tumor tissue look normal, “3” means the cells and tissue look abnormal). The scores for the three categories are then added, reaching a total score of 3 to 9. Three grades are possible (**Table 2**) [47,48].

Table 2: Scarff-Bloom-Richardson grade system (adapted from [47,48]).

Grade	**Description**	**Score**
Grade 1	The lowest grade, with well-differentiated breast cells that appear normal and are not growing fast; cancer is arranged in small tubules.	3,4,5
Grade 2	An intermediate grade, with moderately-differentiated breast cells.	6,7
Grade 3	The highest grade, with poorly differentiated breast cells, that do not appear normal and tend to grow and spread more aggressively.	8,9

2.2.7 Staging

Cancer stage refers to the size and extent of the primary tumor and whether or not cancer cells have spread in the body. Cancer stage is based upon factors like the tumor size (**T**), the

spread of cancer to nearby lymph nodes (**N**), and the number of tumors present (number of metastasis)(**M**) [49].

The staging system used for breast cancer is the **TNM** staging system, which combines the three factors [49].

The breast cancer TNM classification is reported in **Tables 3** to **5**:

Table 3: Tumor (**T**) classification according to the American Joint Committee on Cancer Definition of Primary Tumor (adapted from [50 -52]).

Tumor category	**Tumor criteria**
TX	Primary tumor cannot be assessed.
T0	No evidence of primary tumor.
Tis	Tumor is present only in ducts or lobules (in situ) of the breast tissue and has not spread into the surrounding tissue of the breast.
T1	Tumor ≤ 2 cm in the greatest dimension.
T2	Tumor > 2 cm but ≤ 5 cm in the greatest dimension.
T3	Tumor > 5 cm in the greatest dimension
T4	Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or macroscopic nodules)

Table 4: Nodes (**N**) classification according to the American Joint Committee on Cancer Definition of Regional Lymph Nodes (adapted from [50-52])

Node category	**Node criteria**
NX	The lymph nodes can not be assessed (for example, if they were previously removed).
N0	There are64no cancer cells in any nearby nodes (no regional lymph node metastasis).
ITCs (Isolated Tumor Cells)	Small clusters of cancer cells less than 0.2 mm across or a cluster of fewer than 200 cells in one area of a lymph node (lymph nodes containing only isolated tumor cells are not accounted as positive lymph nodes).
N1	Micrometastasis or metastasis in 1-3 axillary lymph nodes, but the nodes are not stuck to surrounding tissues.
N1mi	One or more lymph nodes contain areas of cancer cells called micrometastasis that are larger than 0.2mm, or the nodes contain more than 200 cancer cells but are less than 2 mm in total size.
N2	There are cancer cells in the lymph nodes of the axilla, which are stuck to each other and to other structures (metastasis in 4-9 axillary lymph nodes).
N3	The cancer has spread to 10 or more axillary lymph nodes, or it has spread to the lymph nodes located under the clavicle, or above the superclavicular lymph nodes. The tumor may have also spread to the internal mammary lymph nodes.

Table 5: Metastasis (**M**) classification according to the American Joint Committee on Cancer Definition of Metastasis (adapted from [50-52]).

Metastasis category	Metastasis criteria
M0	There is no sign that the cancer has spread (no evidence of distant metastasis).
Mo(i+)	There is no sign of cancer on physical examination, scans or x-rays. Neverthless, cancer cells are present in blood, bone marrow, or lymph nodes far away from the breast cancer – the cells are found by laboratory tests.
M1	The cancer has spread to another part of the body (distant metastasis larger than 0.2 mm are detected).

The combination of the different variants of **T**, **N** and **M** determines the clinical stages. The stage provides a common way of describing the cancer, allowing doctors to plan the best treatments. There are 5 stages: stage **0**, which is noninvasive carcinoma ***in situ***, and stages **I** through **IV**, which are used for invasive breast cancer (**Table 6**).

Table 6: Classification of breast cancer stages (adapted from [52-56]).

Stage		Prognostic groups	Description
Stage 0		Tis, N0, M0	Carcinoma is only in the ducts and lobules (*in situ*) of the breast tissue and has not spread to the surrounding tissue of the breast. It is also called noninvasive cancer.
Stage I	**Stage IA**	T1, N0, M0	The tumor is small (< 2 cm), invasive, but has not spread yet to the lymph nodes.
	Stage IB	T0 or T1, N1mi, M0	Cancer has spread to the lymph nodes and the cancer in the lymph node is between 0.2 mm to 2 mm in size**.** There is either no evidence of a tumor in the breast or the tumor is ≤ 2 cm.
Stage II	**Stage IIA**	T0 or T1 or T2, N0 or N1, Mo	[**T0, N1, M0**]: there is no evidence of a tumor in the breast, but the cancer has spread to 1 to 3 axillary lymph nodes. [**T1, N1, M0**]: the tumor is ≤ 2 cm and has spread to the axillary lymph nodes. [**T2, N0, M0**]: the tumor is between 2 cm and 5 cm and has not spread to the axillary lymph nodes.
	Stage IIB	T2 or T3, N0 or N1, M0	[**T2, N1, M0**]: the tumor is between 2 cm and 5 cm and has spread to 1 to 3 axillary lymph nodes. [**T3, N0, M0**]: the tumor is > 5 cm but has not spread to the axillary lymph nodes.

Table 7: Classification of breast cancer stages (cont.) (adapted from [52-56]).

Stage		Prognostic groups	Description
Stage III	**Stage IIIA**	T0 to T3, N1 or N2, M0	[**T0,T1,T2** or **T3,N2,M0**]: the cancer of any size has spread to 4 to 9 axillary lymph nodes or to internal mammary lymph nodes, but not to other parts of the body. [**T3, N1, M0**]: the tumor is > 5 cm and has spread to 1 to 3 axillary lymph nodes.
	Stage IIIB	T4, N0 to N2, M0	The tumor has spread to the chest wall or caused ulceration of the breast or is diagnosed as inflammatory breast cancer. It may or may not have spread to up to 9 axillary or internal mammary lymph nodes.
	Stage IIIC	Any T, N3, M0	A tumor of any size that has spread to 10 or more axillary lymph nodes, the internal mammary lymph nodes, and/or the lymph nodes under the collarbone.
Stage IV		Any T, any N, M1	The tumor can be of any size and has spread to other organs, such as bones, lungs, brain, liver, distant lymph nodes, or chest wall.
Recurrent			Recurrent cancer is a cancer that has come back after treatment, and can be described as local, regional, and/or distant.

2.2.8 Risk factors

The increased risk for the development of female breast cancer is associated with age, reproductive factors, personal or family history of breast disease, genetic pre-disposition and environmental factors [57,58].

In fact, the risk of developing breast cancer increases with age: by using the SEER[6] database, the probability of a woman developing breast cancer is 1: 202 from birth to 39 years of age, 1:26 from 40-59 years, and 1:28 from 60-69 years [57,58].

A personal history of breast cancer or some breast disease are also significant risk factors for the development of a breast cancer or a second breast cancer [57,58].

Genetic predisposition alleles have been described in terms of clinical significance, conferring a 40%-85% lifetime risk of developing breast cancer [57,58].

[6] SEER – Surveillance, Epidemiology, and End Results Program of the National Cancer Institute.

A person lifestyle also influences the risk of breast cancer. Lifestyle factors include the excessive use of alcohol, obesity and physical inactivity. Consistent physical activity has been shown to reduce the risk of breast cancer conferring a 2-5% decrease of the risk [57,58].

Environmental factors are, for example, radiation exposure from various sources including medical treatment, and they increase the risk of breast cancer due to gene mutations [57,58].

2.2.9 Epidemology

Breast cancer is the most common cancer in women worldwide after skin cancer. It represents 16% of all cancers in women [59,60].

Worldwide, there were about 2.1 million newly diagnosed female breast cancer cases in 2018. The disease is the most frequently diagnosed cancer in the vast majority of the countries and it is also the leading cause of cancer death in over 100 countries [59,60].

All around the world the incidence of this cancer shows varied rates. These rates are low in less-developed countries and higher in the more-developed countries [59,60].

The average age at diagnosis is usually around 60 (**Figure 10**). The trend reflects a combination of demographic, social and economic development, including greater levels of obesity, physical inactivity, and increases in breast cancer screening and awareness. The primary risk factors for breast cancer are not easily modifiable since they derive from prolonged hormonal exposures [60].

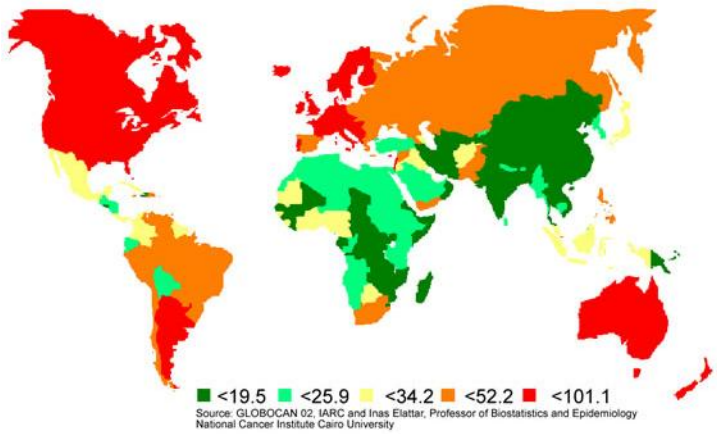


Figure 10: Breast cancer age-standardized incidence rate per 100.000 (retrieved from [59]).

2.2.10 Breast cancer treatment

Typically, treatment plans are based upon the type of breast cancer; its biology, behavior, and stage; the presence of known gene mutations; the patient's age; general health and other health conditions and personal preferences. In general, the more the breast cancer has spread, the more treatment you will likely need [61,62].

There are two types of treatments: *local treatments*, in which the tumor is treated without affecting the rest of the body, and *systemic therapies*, which use different drugs (chemotherapy, hormone therapy, targeted therapy) which will affect the whole body (**Table 7**) [61,62].

Table 8: Treatment of Breast Cancer by Stage (adapted from [63,64]).

Stage	**Therapeutic options**
Stage 0	**Surgery**: lumpectomy or mastectomy. **Radiation therapy**: after the surgery, the radiation attacks any abnormal cells that might have been missed. **Hormone therapy** : if the DCIS is hormone receptor-positive (HR+), this therapy may also help prevent more cancer from developing.
Stage I	**Surgery**: mastectomy or lumpectomy. **Radiation therapy**: necessary after lumpectomy. **Hormonal therapy**: prescribed for patients with hormone-receptor-positive (ER+) cancer. **Target therapy**: treat cancers that are HER2-positive (HER2+). **Chemotherapy**: to reduce the risk of recurrence.
Stage II	**Surgery**: mastectomy or lumpectomy. **Radiation therapy**: after surgery. **Hormonal therapy**: prescribed for patients with hormone-receptor-positive (ER+) cancer. **Target therapy**: treat cancers that are HER2-positive (HER2+). **Chemotherapy**: recommended.
Stage III	**Surgery**: total mastectomy. **Radiation therapy**: after surgery. **Hormonal therapy**: prescribed for patients with hormone-receptor-positive (ER+) cancer. **Target therapy**: treat cancers that are HER2-positive (HER2+). **Chemotherapy**: almost always recommended, necessary to reduce the cancer and/or treat cancer in the lymph nodes.
Stage IV	**Surgery**: total mastectomy. **Radiation therapy**: after surgery. **Hormonal therapy**: prescribed for patient with hormone-receptor-positive (ER+) cancer. **Target therapy**: treat cancers that are HER2-positive (HER2+) or cancers that are HER2-negative but have a *BRCA1* or *BRCA2* mutation. **Chemotherapy**: almost always recommended.
Recurrent	Treatment for recurrent breast cancer depends on where the cancer recurs and what treatments the patient has had before.

The most common surgical procedures are lumpectomy and total mastectomy. In the first one only the abnormal cells and some breast tissue are removed, while in the second one the entire breast is removed [63].

Radiotherapy is a technique that uses radiation to destroy cancer cells. Although radiation also damages normal cells, cancer cells are especially sensitive to its effects. This is a localized treatment, which means it only treats the area of the body it is aimed at. It is used as integral part of the breast cancer treatment, being usually applied after surgery. Its aim is to destroy any remaining cancer cells, reduce the recurrence and increase the possibility of survival. Radiotherapy can also be used before the surgery to reduce the size of the tumor, making it easier to be removed [64-67].

Chemotherapy (also called “chemo”) is a treatment which uses cytotoxic drugs, administered orally or intravenously, in order to kill cancer cells. It is a systemic therapy, which means it affects the whole body by going through the bloodstream. It can also affect some fast-growing healthy cells, like those of the skin, hair, intestines, and bone marrow. This is what causes some of the side effects from the treatment. Usually chemotherapy is used after surgery to reduce the risk of the cancer coming back, but, in some cases, it is given before surgery to reduce the cancer dimensions [68,69].

The drugs are different and are named according to their action or origin. In many cases, chemotherapy medicines are given in combination, which means the patient gets two or three different medicines at the same time (**Table 8**) [70].

Chemotherapy drugs attack all cells that are growing quickly (including cancer cells), so researchers have developed new types of drugs that target some of these cell changes. These targeted drugs are designed to block the growth and spread of cancer cells only. Targeted drugs sometimes work even when chemo drugs do not, can help other types of treatment to work better and have different side effects than chemo [68,69].

There are targeted therapies for HER2-positive breast cancer, for hormone receptor-positive breast cancer, for cancers with a*PIK3CA* gene mutation, and for women with *BRCA* gene mutations [71-73].

Table 9: Chemotherapy drugs for breast cancer (adapted from [70])

Class	Chemical name	Description
Anthracyclines	Doxorubicin Epirubicin Daunorubicin Mitoxantrone	Chemically similar to antibiotics, they damage the genetic material of cancer cells, which induces the cells dead. They are given intravenously.
Alkylating agents	Cyclophosphamid	Weaken or destroy breast cancer cells by damaging the cells' genetic material. They can be taken orally as a pill or given intravenously.
Antiangiogenesis targeted therapy	Bevacizumab	Blocks the growth of new blood vessels into a tumor, choking off the blood supply to cancer cells. It is given intravenously.
Antimetabolites	Fluorouracil Gemcitabine Methotrexate Capecitabine	Kill breast cancer cells by inhibiting enzymes involved in the synthesis of DNA and proteins, leading to cancer cell dead as it gets ready to divide. They are given intravenously, except Capecitabine which is taken orally as a pill.
Antitumor antibiotic	Mutamycin	kill cancer cells by damaging their genes and interfering with their re production. It is given intravenously.
Erythropoiesis stimulating agents	Epoetin alfa Darbepoetin alfa	Cause extra red blood cells production by the bone marrow, They are used to treat anemia that can be caused by chemotherapy. They are given intravenously or as an injection 1 to 3 times per week.
mTOR (mammalian target of rapamycin) inhibitor	Everolimus	mTOR is a kinase, a type of protein in the body that helps all cells (healthy and cancer cells) to get the energy they need, hence they help certain breast cancers grow. mTOR inhibitors work by interfering with the mTOR kinase. It is a pill taken orally, usually daily.
Platinum-based	Carboplatin	It weakens or destroys breast cancer cells by damaging the genetic material and making it hard for cells to repair any genetic damage.
Taxanes	Paclitaxel Docetaxel	Interfere with the ability of cancer cells to divide. They act by inhibiting cell mitosis: bind specifically to the microtubule's tubulin and prevent its depolymerization. The result is a significant decrease in free tubulin necessary for the formation of microtubules, with consequent arrest of cellular mitosis. They are given intravenously.
Vinca alkaloids	Vincristine Vinorelbine	Kill cancer cells by interfering with genes and stopping the cells from reproducing themselves. They are given intravenously.

2.3 Colon Cancer

Worldwide, colon cancer is the third most common type of cancer and it affects men and women almost equally. It affects about 140,000 people every year, mainly people over 40 years old, being the leading signs of colon cancer bleeding and changes to bowel habits [74].

Colon cancer is the malignant tumor of the large intestine, the result of the uncontrolled proliferation of one of the cells present in the layers of the inner wall of the large intestine. It develops from a "mad cell", located in the inner wall of one of the anatomical parts of the colon: ascending, transverse or descending colon, sigmoid or *rectum* [74-75].

The most common localization of colon cancer is the *rectum* (about 50% of cases), followed by the sigmoid (19-21% of cases), ascending colon (16%), transverse colon (8%) and descendant colon (6% of cases) [74-75].

The most common type of colon and rectal cancer is adenocarcinoma of the colon (95% of all cases). In the gastrointestinal tract, rectal and colon adenocarcinomas develop in the cells that are inside the large intestine. Types of colorectal cancer less common are primary colorectal lymphoma, gastrointestinal stromal tumors, leiomyosarcomas, carcinoid tumors and melanomas [74-75].

2.3.1 Anatomy of the Colon

The colon is the terminal portion of the digestive tract. It is shorter and with a larger diameter than the small intestine. It absorbs water, salt and some essential fat-soluble vitamins, before they are eliminated by the body [75,76].

The colon is divided into four sections: ascending colon, transverse colon, descending colon, and sigmoid (**Figure 11**) [76].

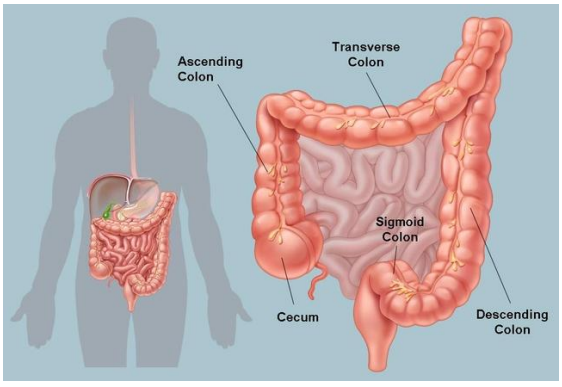


Figure 11: Colon anatomy (retrieved from [77])

2.3.2 Colon cancer grading and staging

The grade gives doctors an idea of how quickly a cancer may develop, while the stage of a cancer is how big it is and whether it has spread [78].

The grading system is the same one used for breast cancer [78-81]:

- **grade 1** (low-grade): the cancer cells tend to grow slowly and look similar to normal cells (well differentiated);
- **grade 2** (moderate-grade): the cancer cells look more abnormal;
- **grade 3** (high-grade): the cancer cells tend to grow more quickly and look very abnormal (poorly differentiated).

The staging system for colon cancer follows the tumor/node/metastasis (**TNM**) system of the American Joint Committee on Cancer (AJCC) [78-81].

"**T**" stands for the size of the tumor: how far the cancer grew into the wall (mucosa, submucosa, *muscularis propria*, subserosa and serosa) of the colon (**Table 9**) [78-81].

"**N**" stands for the spreading to nearby lymph nodes (**Table 10**) [78-81].

"**M**" stands for the spreading metastasis to distant sites, like lymph nodes or distant organs such as the liver or lungs (**Table 11**) [78-81].

Table 10: Primary tumor (**T**) (adapted from [78-81]).

Tumor category	**Description**
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma *in situ* (intraepithelial or intramucosal carcinoma)
T1	Tumor invades submucosa
T2	Tumor invades *muscularis propria*
T3	Tumor invades through the *muscularis propria* into the pericolorectal tissues
T4	Tumor invades the visceral peritoneum or invades or adheres to adjacent organ or structure
T4a	Tumor invades through the visceral peritoneum
T4b	Tumor invades or is adherent to other organs

Table 11: Lymph nodes (**N**) (adapted from [78-81]).

Node category	**Description**
NX	Regional lymph node cannot be assessed.
N0	No regional lymph node metastasis.
N1	Metastasis in 1-3 regional lymph nodes (tumor ≥ 0.2 mm) or any number of tumor deposits (in the subserosa or in the perirectal tissues) are present and all identifiable nodes are negative.
N1a	Metastasis in 1 regional lymph node.
N1b	Metastasis in 2-3 regional lymph nodes.
N1c	Tumor in the subserosa or in the perirectal tissues without regional metastasis.
N2	Metastasis in 4 or more regional lymph nodes.
N2a	Metastasis in 4-6 regional lymph nodes.
N2b	Metastasis in 7 or more regional lymph nodes.

Table 12: Metastasis (**M**) (adapted from [78-81]).

M category	**Description**
M0	No distant metastasis, no evidence of tumor in distant sites or organs.
M1	Metastasis to one or more distant organs, or peritoneal metastasis.
M1a	Metastasis confined to 1 organ, without peritoneal metastasis.
M1b	Metastasis to 2 or more organs, without peritoneal metastasis.
M1c	Metastasis to the peritoneal surface, alone or with organ metastases.

The combination of the different variants of **T**, **N** and **M** determines the clinical stages (**Table 12**) [78-81].

Table 13: Colon cancer stages (adapted from [78-81]).

Stage		**Prognostic groups**	**Description**
Stage 0		Tis, M0, N0	The earliest stage, known as carcinoma *in situ* or intramucosal carcinoma (**Tis**), it has not grown beyond the mucosa of the colon or *rectum*.
Stage I		T1 or T2, N0, M0	The cancer has grown through the mucosa into the submucosa (**T1**), and it may also have grown into the *muscularis propria* (**T2**). It has not spread to nearby lymph nodes (**N0**) or to distant sites (**M0**).
Stage II	Stage IIA	T3, N0, M0	The cancer has grown into the layers of the colon or *rectum* (has not gone through them) (**T3**), it has not reached nearby organs (**M0**) neither spread to nearby lymph nodes (**N0**).
	Stage IIB	T4a, N0, M0	The cancer has grown through the wall of the colon or *rectum* (**T4a**) but it has not yet spread to nearby lymph nodes (**N0**) or to distant sites (**M0**).
	Stage IIC	T4b, N0, M0	The cancer has grown through the wall of the colon or *rectum* and has grown into other nearby tissues or organs (**T4b**), but it has not yet spread to nearby lymph nodes (**N0**) or to distant sites (**M0**).
Stage III	Stage IIIA	T1 or T2, N1, M0	The cancer has grown into the submucosa (**T1**), and it may also have grown into the *muscularis propria* (**T2**). It has spread to 1 to 3 nearby lymph nodes (**N1**), and it has not spread to distant sites (**M0**).
		T1, N2a, M0	The cancer has grown into the submucosa (**T1**), it has spread to 4 to 6 nearby lymph nodes (**N2a**), but it has not spread to distant sites (**M0**).
	Stage IIIB	T3, N1/N1c, M0	The cancer has grown into the layers of the colon or *rectum* (**T3**), it has spread to 1 to 3 nearby lymph nodes (**N1**) or into areas of fat near the lymph nodes but not the nodes themselves (**N1c**). It has not spread to distant sites (**M0**).
		T2 or T3, N2a, M0	The cancer has grown into the *muscularis propria* (**T2**) or into the layers of the colon or *rectum* (**T3**). It has spread to 4 to 6 nearby lymph nodes (**N2a**), but it has not spread to distant sites (**M0**).

Table 14: Colon cancer stages (cont.) (adapted from [78-81]).

Stage		**Prognostic groups**	**Description**
Stage III	Stage IIIC	T1 or T2, N2b, M0	The cancer has grown into the submucosa (**T1**), and it may also have grown into the *muscularis propria* (**T2**). It has spread to 7 or more nearby lymph nodes (**N2b**), but it has not spread to distant sites (**M0**).
		T4a, N2a, M0	The cancer has grown through the wall of the colon or *rectum* (and the visceral peritoneum) but has not reached nearby organs (**T4a**). It has spread to 4 to 6 nearby lymph nodes (**N2a**), but it has not spread to distant sites (**M0**).
Stage IV	Stage IVA Stage IVB	T3 or T4a, N2b, M0	The cancer has grown into the layers of the colon or *rectum* (**T3**) or through the visceral peritoneum (**T4a**) but has not reached nearby organs. It has spread to 7 or more nearby lymph nodes (**N2b**). It has not spread to distant sites (**M0**).
		T4b, N1 or N2, M0	The cancer has grown through the wall of the colon or *rectum* and has grown into other nearby tissues or organs (**T4b**). It has spread to at least one nearby lymph node or into areas of fat near the lymph nodes (**N1** or **N2**). It has not spread to distant sites (**M0**).
		Any T, any N, M1a	The cancer may or may not have grown through the wall of the colon or *rectum* (**Any T**). It might or might not have spread to nearby lymph nodes. (**Any N**). It has spread to 1 distant organ (such as the liver or lung) or distant set of lymph nodes (**M1a**).
	Stage IVC	Any T, any N, M1b	The cancer might or might not have grown through the wall of the colon or *rectum* (**Any T**). It might or might not have spread to nearby lymph nodes (**Any N**). It has spread to more than 1 distant organ (such as the liver or lung) or distant set of lymph nodes (**M1b**).
		Any T, any N, M1c	The cancer might or not have grown through the wall of the colon or *rectum* (**Any T**). It might or might not have spread to nearby lymph nodes (**Any N**). It has spread to distant parts of the peritoneum (the lining of the abdominal cavity), and may or may not have spread to distant organs or lymph nodes (**M1c**).

2.3.3 Colon cancer pathogenesis

Colon cancer is a disease originating from the epithelial cells lining the colon of the gastrointestinal tract. One of the central aspects of colon cancer formation is the accumulation of

acquired genetic and epigenetic changes that transform normal glandular epithelial cells into invasive adenocarcinomas. The polyp to cancer progression sequence involves a step that initiates the formation of benign neoplasms; followed by a step that promotes the progression to more histologically advanced neoplasms; and then a step that transforms the tumors to invasive carcinoma (**Figure 12**) [82-85].

Genomic and epigenomic instability contribute to the formation of colorectal cancers. Some major forms of instability include [86]:

- chromosomal instability (CIN),
- microsatellite instability (MSI),
- non-MSI hypermutability,
- global DNA hypomethylation.

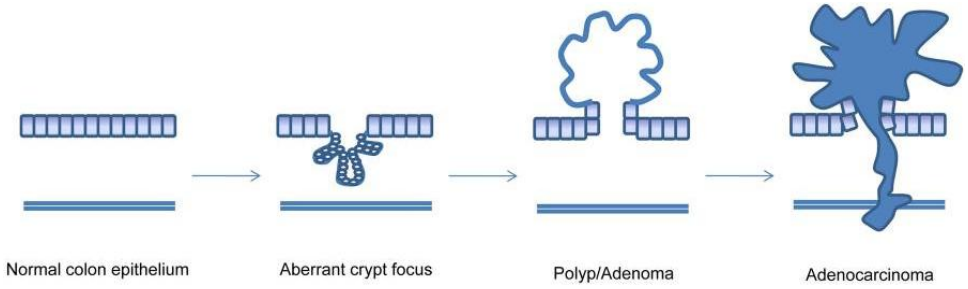


Figure 12: Schematic diagram of polyp --> colorectal cancer sequence (retrieved from [86]).

2.3.4 Risk factors

The risk of getting colorectal cancer increases with age, in fact more than 90% of cases occur in people who are 50 years old or older [87].

Other risk factors include having some inflammatory bowel disease, like Crohn's disease or ulcerative colitis, or other health problems such as type 2 diabetes or an inherited syndrome, causing gene changes (mutations) [87,88].

Another important point to take into account is the patient's family history: nearly 1 in 3 people who develop colorectal cancer have other family members who have had it [87,88].

People with a history of colorectal cancer in a first-degree relative (parent, sibling, or child) are at increased risk. The reasons for the increased risk are not clear in all cases. Cancers can "appear in the family" due to inherited genes, shared environmental factors, or some combination of these [87,88].

Lifestyle factors may also contribute to an increased risk of colorectal cancer. In fact, physical inactivity, an high-fat and low-fiber diet which is low in fruit and vegetables, an obesity condition, alcohol consumption and the use of tobacco very probably contribute to the increased risk of colon cancer [87,88].

Another thing influencing colon cancer risk is the race: Afro-Americans, for example, are more likely than other U.S. racial and ethnic groups to get colorectal cancer, however, the reason for this, is unknown [87,88].

2.3.5 Epidemology

Colorectal cancer is a major cause of morbidity and mortality throughout the world, accounting for over 9% of all cancer incidence. It is the third most commonly diagnosed cancer both in men and women. One in 22 men and one in 24 women will be diagnosed with colorectal cancer during their lifetime. There were over 1.8 million new cases in 2018 [89,90].

There is a large geographic difference in the global distribution of colorectal cancer. Colorectal cancer is mainly a disease of developed countries with a western culture worldwide mortality attributable to colorectal cancer of approximately half of the incidence [89-91].

The highest colon cancer incidence rates are found in parts of Europe, Australia and New Zealand, Northern America, and Eastern Asia (**Figure 13**) [89-91].

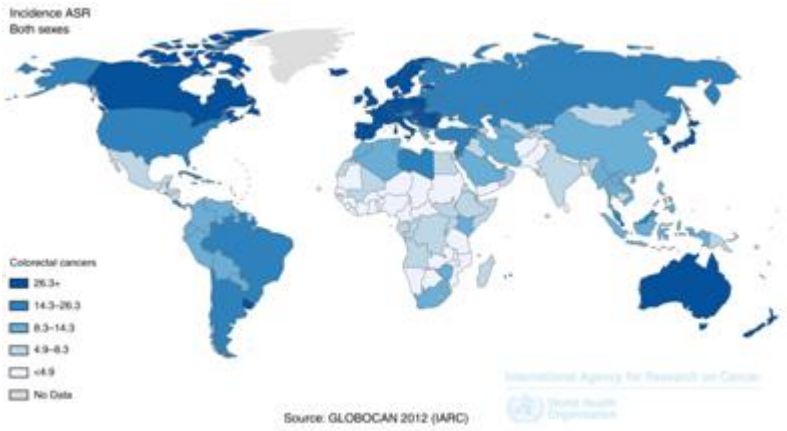


Figure 13: Colorectal cancer worldwide (retrieved from [92]).

2.3.6 Colon cancer treatment

The treatment plan for colorectal cancer does not include a perfectly standardized design, it must be modified and adjusted depending on the patient, considering the extent of the tumor and

its proliferation, as well as the risks it can pose to the patient's health, taking also into account other possible pathologies of the patient and any co-therapies already in progress. In this perspective, each patient must be considered as a unique case. Cancer treatment can include surgery, radiotherapy and/or chemotherapy. In most cases a combined pre and post-surgical therapy is performed depending on the patient. Drug combinations usually work better than single drugs since different drugs kill cancer cells in different ways (**Table 13**) [93,94].

Table 15: Treatment of colon cancer by stage (adapted from [93,94]).

Stage	**Therapeutic options**
Stage 0	**Surgery**: local excision or simple polypectomy. If the tumor is too large to remove by local excision, resection and anastomosis are performed.
Stage I	**Surgery**: resection and anastomosis.
Stage II	**Surgery**: resection and anastomosis. **Chemotherapy**: to reduce the risk of recurrence.
Stage III	**Surgery**: resection and anastomosis. **Chemotherapy**: recommended.
Stage IV	**Surgery**: resection and anastomosis. **Radiation therapy**: for patient who cannot have surgery or as palliative therapy. **Chemotherapy**: before surgery to shrink the tumor, after surgery, or both.
Recurrent	Treatment for recurrent colon cancer depends on where the cancer recurs (local excision, removal of parts of organs where the cancer has spread, chemotherapy and radiotherapy) and what treatments the patient has had before

Radiotherapy for colon cancer is less used than in other types of cancer. In fact, here are two major limitations regarding colon cancer: a poorly defined target (since colon is mobile), and the fact that dose-limiting structures surround the colon (i.e., large amount of small bowel, kidney, and liver) [95,96].

During the practical work resulting in the present thesis, some of the chemotherapeutical drugs were used according to the chemo strategies used in clinics (**Table 14**).

The chemo and radiotherapy protocols are increasingly specific for cancer cells, but they are each time less effective due to the chemo/radio resistance that individuals acquire [97].

Taking this into account, it is necessary to make people understand from young ages and at the level of education and family system, that lifestyle alterations help to prevent this dreadful pathology. The focus of several studies is on the use of alternative agents/helpers present in various plants, extracts or infusions, which are able to prevent or delay the onset, development and progression of the disease. The aim of these studies is to analyze the efficacy and limitations both in *in vitro* and *in vivo* tests.

Table 16: Applied chemotherapy drugs for colon cancer according to the chemo strategies used in clinics (adapted from [98,99]).

Regimen	Drug/Drug Combination	Dosages	Description
OX	Oxaliplatin	Oxaliplatin: 130 mg/m^2 IV in D1. Repeat the cycle every day for 21 days.	OX is used when colon cancer is in an advanced stage and as adjuvant therapy in patients who have had surgery.
5-FU	5-Fluorouracil	5-Fluorouracil: 1000 mg/m^2/day IV continuous from D1 to D5. Repeat the infusion in cycles 1 and 5 of chemotherapy.	5-FU is used when colon cancer is in an advanced stage and in combination with other drugs.
IRI	Irinotecan	Irinotecan: 350 mg/m^2 IV in 90 min. Repeat the cycle every 21 days.	IRI is used when colon cancer has metastasized or has not gotten better with other chemotherapy.
CAP	Capecitabine	Capecitabine: 1250 mg/m^2 VO 2 times per day, from D1 to D14. Repeat the cycle every 21 days, for 8 cycles in total.	CAP is used to treat stage III colorectal cancer in patients who have had surgery to remove the cancer and to treat patients ith metastatic colorectal cancer.
CET	Cetuximab	Cetuximab: initial dose 400 mg/m^2 IV in 2 h, followed by 250 mg/m^2 IV in 1 h weekly.	CET is used in patient in which cancer has metastasized and when cancer did not respond to oxaliplatin and irinotecan.
FOLFOX	5-Fluorouracil + Leucovorin + Oxaliplatin	Oxaliplatin: 100 mg/m^2 in 2 h in D1. Calcium folinate: 400 mg/m^2 IV in D1 with 2 h infusion. 5-Fluorouracil: 400 mg/m^2 IV in D1, followed by 2400 mg/m^2 IV continuous for 46 h in D1 and D2. Repeat the cycle every 14 days, for 12 cycles.	FOLFOX is a combination of drugs, used when cancer is in an advanced stage.
FOLFIRI	5-Fluorouracil + Leucovorin + Irinotecan	Irinotecan: 180 mg/m^2 IV in 90 min in D1. Calcium folinate: 400 mg/m^2 IV in D1 with 2 h infusion, before fluorouacil. 5- Fluorouracil: 400 mg/m^2 IV in D1, followed by 2400 mg/m^2 IV continuous for 46 h. Repeat the cycle every 14 days.	FOLFIRI is used when cancer is in an advanced stage.
CAPOX	Capecitabine + Oxaliplatin	Capecitabine: 1000 mg/m^2 VO 2 times per day, from D1 to D4. Oxaliplatin: 130 mg/m^2 IV in 2 h in D1. Repeat the cycle every 21 days.	CAPOX is used when cancer is in an advanced stage.
CAPIRI	Capecitabine + Irinotecan	Capecitabine: 1000 mg/m^2 VO 2 times per day, from D1 to D14, or from D2 to D15. Irinotecan: 250 mg/m^2 IV in D1. Repeat the cycle every 21 days.	CAPIRI is used when cancer is in an advanced stage.

2.4 *In vitro* model

2.4.1 MCF-7 cell line

The breast cancer cells used for the realization of the present work was the MCF-7 cell line. This cell line has been bought from the American Type Culture Collection (ATCC) (ATCC® HTB-22™, Rockville, Md., USA), a unique private and nonprofit resource dedicated to the collection, preservation and distribution of authentic cultures of living microorganisms, viruses, DNA probes, plants, and human and animal cells.

The MCF-7 cell line is a widely studied epithelial cancer cell line derived from breast adenocarcinoma, having the characteristics of differentiated mammary epithelium.

MCF-7 cells are used for *in vitro* and *in vivo* breast cancer studies as they have the ability to process estrogen in the form of estradiol via estrogen receptors in the cell cytoplasm. In fact, MCF-7 is an estrogen receptor (ER) positive, a progesterone receptor positive and HER2 negative cell line.

This cell line is noninvasive and represents an early-stage disease model. The breast cancer molecular subtype is Luminal A, specifically luminal A ductal carcinoma [100,101].

MCF-7 cells were established in 1973 by Dr. Soule and colleagues at the Michigan Cancer Foundation (hence the name). This cell line was isolated in 1970 for laboratory studies from the pleural effusion of a 69-year-old woman with metastatic disease. The patient was diagnosed with a benign tumor on the right breast and was then operated by mastectomy 10 years before the primary cell culture was started. Afterwards, the patient was submitted to a consecutive radical mastectomy of her left breast due to a malignant adenocarcinoma 4 years later. Just after she completed post-operative chest wall radiotherapy, a local recurrence in her left chest area was noticed. The recurrence was controlled by radiation and hormone therapy for three years. The patient was probably treated with high doses of the hormone and the disease was controlled three times longer than expected, since the tumor was hormone-responsive [102-105].

The cells from the pleural effusion grew initially in suspension and ultimately formed a monolayer on plastic, growing as a continuous culture. The popularity of the MCF-7 cell lines for breast cancer research reflects its fidelity to many aspects of breast cancer in the clinical setting, particularly in the management of postmenopausal women with hormone receptor–positive breast cancer [102-105].

One of the most important contributions of the MCF-7 cell line to breast cancer research has been its utility for the study of the estrogen receptor (ER), as this cell line expresses substantial

levels of ER mimicking the majority of invasive human breast cancers that express ER. These cells also express androgen, progesterone, and glucocorticoid receptors and have been a valuable model system to elucidate pathways of hormone response and resistance [102,103].

It was demonstrated that an anti-estrogen substance inhibited the growth of MCF-7 cells but the inhibition could be reversed by estrogen. The central focus of past studies with this cell line was to prove that estrogen directly stimulated tumor growth and that it is a suitable model for breast cancer investigations worldwide, for both *in vitro* and *in vivo* studies [106,107].

2.4.2 WiDr cell line

The colon cancer cell line used for the realization of the present work was the WiDr cell line. The WiDr cell line (ATCC® CCL-218 ™) was originally isolated in 1971 as a colon adenocarcinoma and established from a 78-year-old woman. However, through the identification method of the deoxyribonucleic acid (DNA) footprint it was shown that this cell line was derived from the HT-29 cell line, established as adenocarcinoma of the human colon in 1964. The phenotypes for some isozymes were identical for HT-29 and WiDr cell lines [108-110].

The WiDr cell line is tumorigenic in nude mice and expresses the carcinoembryonic antigen (CEA), the colon-specific antigen (CSAp) and transforms the growth factor beta and the epidermal growth factor (EGF). The cells quantity increases almost logarithmically during the culture period, making it a useful biochemical marker in immunohistopathology. Moreover, it has a short duplication time, comparing to other cancer cell lines - around 15 hours - and a confluence efficiency of 51%, showing that it easily propagates in culture with adherent characteristics. This cell line, when inoculated in immunosuppressed animals, develops histologically confirmed tumors with great efficacy. It is a well characterized cell line and it is used in many cancer and carcinogenesis studies, both in *in vitro* and *in vivo* studies [110,111].

2.5 New approaches in the fight against cancer - natural metabolites

Despite the progress made in the development of potent chemotherapy drugs, their toxicity to normal tissues and adverse side effects in multiple organ systems, as well as drug resistance, have remained the major obstacles for the successful clinical use. Cytotoxic agents decrease considerably the quality of life of cancer patients, manifested as acute complaints and also impacting on life of survivors for years after the treatment. Various plant-derived compounds

improve the efficiency of cytotoxic agents, decreasing their resistance, lowering and alleviating toxic side effects, and detoxifying the body of chemotherapeutics [112,113].

Microorganisms, plants, lichens, fungi and animals are able to produce a large quantity and variety of biologically active compounds, some of which have anticarcinogenic properties. In particular, plant metabolites exhibit a wide range of biological functions, including anti-cancer, analgesic, anti-inflammation, and anti-microbial activities and are emerging as new leads for anti-cancer drug development.
Bioactive compounds like polyphenols (flavonoids, stilbenes, phenolic acids), terpenoids (monoterpenoid, diterpenoids, tetraterpenoids), alkaloids and organosulfur compounds are secondary metabolites produced by different types of plants that are being studied regarding cancer treatment and prevention [114-116].

During the last few decades, a wide range of cytotoxic agents retrieved from plants, was chemically identified and their effectiveness in therapeutic cancer treatment was proved. Some compounds used in chemotherapy are vinca alkaloids (vinblastine, vincristine, vinorelbine, vinflunine), the taxanes (paclitaxel), and the camptothecin derivatives [114-116].

Some phytochemicals derived from food have been shown to be able to affect the efficacy of traditional chemotherapeutic agents, increase the cytotoxic activity of several drugs and reduce inflammatory responses [114-116].

Polyphenol classes (flavonoids, phenolic acids, lignans and stilbenes) are another important type of natural agents with good anticarcinogenic efficacy, that have been studied both in vitro and in vivo for many years [117,118].

The anticancer efficacy of natural polyphenols has largely been attributed to their potent antioxidant and anti-inflammatory activities as well as their abilities to modulate molecular targets and signaling pathways, which were associated with cell survival, proliferation, differentiation, migration, angiogenesis, hormone activities, detoxification enzymes and immune responses [117,118].

In this work, green tea, a popular drink in Asia, was considered. Catechins from green tea, in fact, can increase the therapeutic effect of drug-resistant tumors in animal studies, and the administration of green tea increases the concentration of this chemotherapeutic agent in tumor but not in normal tissue, enhancing its antitumor activity [115-118].

These natural compounds appear to act by interfering in multiple cellular signaling pathways, activating cell death signals, inducing apoptosis of cancer cells and blocking key events of tumor initiation and promotion, thus reversing the premalignant stage, without affecting negatively normal

cells. These agents may also prevent tumorigenesis by inhibiting or retarding tumor progression or by promoting cell differentiation [117-120].

The positive action of phytochemicals against tumors is described in the following figure (**Figure 14**).

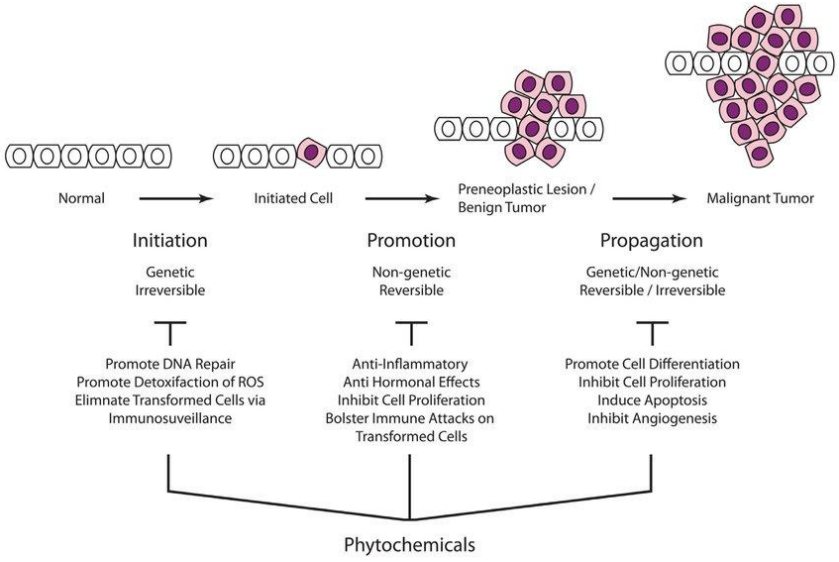


Figure 14: Carcinogenic process and phytochemicals effects (retrieved from [121]).

2.5.1 Green Tea

Tea, next to water, is the most popular beverage in the world. It is native of China and Southeast Asia and its plants have been cultivated for millions of years. Tealeaves were initially used as a medicine and later the tea became a drink [122].

Tea is made from the leaf of the plant *Camellia sinensis* (**Figure 15**). After harvesting, tea leaves begin a process of fermentation which involves the oxidation of flavonoids present in the leaves, caused by release of intracellular polyphenol oxidase. During oxidation, chemicals in the leaves are broken down by enzymes, resulting in darkening of the leaves and defining the aroma of tea. This oxidation process can be stopped by heating, which inactivates the enzymes. The amount of oxidation and other aspects of processing determine a tea's type [122,123].

Figure 15: *Camellia sinensis* (retrieved from [124])

Tea has been subjected to many scientific and medical studies to determine the extent of its long reported health benefits, as growing body of evidence from laboratory animal studies demonstrated that tea consumption has an inhibitory effect on carcinogenesis at different organ sites and tea drinkers may have a lower risk of developing heart disease and certain types of cancer [125].

There are three different types of tea - black tea, oolong tea and green tea.
The focus of this research is on green tea, which is made from unwilted leaves that are not oxidized, allowing the conservation of natural polyphenols, whose benefits for drinkers are highly studied in particular regarding the preventive effect on many pathological disorders [122,123;125].

Considering the chemical composition of tea, the list of components is large: polyphenols (30%), alkaloids (caffeine, theophylline and theobromine), amino acids (1-4% dry weight), fiber (26% dry weight), carbohydrates (5-7% dry weight), proteins (15-20% dry wight), lipids (5% dry weight), volatile organic compounds, fluoride, minerals and trace elements (5% dry weight) [126-127].

The polyphenols, a large group of plant chemicals, include flavonoids, flavans and phenolic acids. Green tea has mainly flavonoids, called catechins, that are thought to be responsible for the health benefits that have traditionally been attributed to tea, especially green tea. In green tea there are 4 types of catechines: epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC). One 200 mL cup of green tea supplies 140, 65, 28, and 17 mg of these polyphenols, respectively. The most active and abundant catechin in green tea is epigallocatechin-3-gallate (EGCG) [125-127].

Green tea polyphenols have been shown to have antioxidant properties against lipid peroxidation in phospholipid bilayers and in biological systems.

The mechanisms by which tea polyphenols may act include inhibition of mutagenesis, genotoxicity, transformation, cell proliferation, and angiogenesis. Each mechanism involves multiple potential molecular targets. Many studies have suggested that the polyphenol EGCG possesses the most potent antioxidative activity against all stages of multistage carcinogenesis [128].

The consumption of green tea is associated with a lower risk of cancer due to its important role in the reduction of free radicals that are capable of causing damage to the DNA, leadingto the carcinogenic process [128].

The inhibitory and antitumor effects of the green tea polyphenols have been confirmed in more than 20 tumor lines. This type of tea, in fact, has been shown to inhibit the carcinogenesis in different animal models of prostate, bladder, breast, colorectal, lung, skin, esophagus, and liver cancers. Green tea infusion as the sole source of fluid intake in mice, decreased for example the induced lung tumor incidence and tumor multiplicity [129-131].

Moreover, the inhibition of skin carcinogenesis by tea was investigated: it was found that oral administration of green tea reduced induced skin cancer incidence, tumor multiplicity, and tumor growth. The inhibitory effect of the polyphenols was associated with reduced expression of important factors in tumor growth, metastasis, and angiogenesis. Several *in vitro* studies also demonstrated that tea polyphenols can potently inhibit human breast cancer cells and tumor growth [130,131].

Considering the safety related to the consumption of this drink, the data show that there are no real concerns about the consumption of tea by normal people, hence, this does not lead to toxicity problems [129-131].

However, drinking large amounts of tea can cause problems. Tea, in fact, contains caffeine, and excessive caffeine intake can lead to nervousness, anxiety, abnormal heart rhythm. High caffeine consumption can also hinder calcium absorption, affecting the bone health and increasing the risk of osteoporosis. Green tea in elevated doses may also create problems if drunk when a person is taking stimulants, hormones, antibiotics or any drug, therefore it is necessary to pay attention to those factors [131].

Pharmacokinetic studies have shown that there is a correlation between the folic acid and the green tea at the level of intestinal absorption: the tea reduces the bioavailability of folic acid, so is

not recommended for pregnant women or with megaloblastic anaemia. Furthermore, evidence from some case reports suggests that vitamin K content in green tea may antagonize the anticoagulant effect of warfarin [132,133].

In any case, the information on the bioavailability of green tea compounds regarding human consumption: more studies on the absorption, distribution and metabolism of polyphenols in humans are needed, in order to understand the precise mechanisms of action as agents that can help in therapies [132,133].

2.5.2 EGCG: (-)-epigallocatechin-3-gallate

The natural product epigallocatechin-3-gallate (EGCG) is a polyphenolic constituent, precisely a catechin, present in high quantities in green tea.

Many studies have been done on EGCG since it has been suggested as a agent to decrease cholesterol levels, repress hepatic glucose production, and enhance insulin action, and to act as an antibiotic and anti-carcinogen. This compound has been shown to regulate dozens of disease-specific molecular targets. The exact mechanism of action of EGCG regarding a number of these effects is not entirely clear [134].

2.5.2.1 Structure of EGCG

EGCG has three aromatic rings (A, B, and D) that are linked together by a pyran ring (C) (**Figure 16**). The health-promoting function of EGCG is attributed to its structure. For example, the antioxidant activity of EGCG results from the transfer of a hydrogen atom or single-electron transfer reactions, involving hydroxyl groups of the B and/or D rings.

The pyrogallol-type structure of the B-ring induces apoptosis and possesses strong antioxidative activity undergoing autooxidation to form reactive oxygen species. The galloyl moiety (D-ring, gallate group) is the critical structure for the inhibition of fatty-acid synthase leading to cytotoxicity in human cancer cells. Both components B and D contribute to the exertion of biological activities related to the cell-surface receptor [135,136].

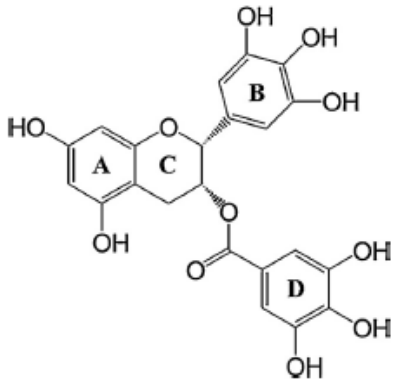


Figure 16: Chemical structure of epigallocatechin-3-gallate (retrieved from [136]).

2.5.2.2 Antitumor effects of EGCG

The anticancer property of EGCG is the focal point of researches. EGCG can inhibit tumorigenesis by reducing carcinogen activity. There are results suggesting that EGCG prevents obesity-related liver tumorigenesis by inhibiting the growth factors and attenuating chronic inflammation [138].

It can also restrain tumor proliferation by acting against angiogenesis (which provides oxygen and nutrients to tumor cells), and it also can inhibit pancreatic cancer tumor growth and metastasis. Moreover, it is able to inhibit tumor migration and penetration and induce tumor cell death via several mechanisms including capase-dependent apoptosis, capase-independent apoptosis, lysosomal membrane permeabilization-mediated cell death, and autophagy. It is widely accepted that hepatocyte growth factor (HGF) is involved in tumor migration and invasion, and EGCG has the capacity to suppress its activity [139-141].

In fact, most of the anticancer effects of EGCG play a role via several signal transduction pathways, but in general the mechanism of anticancer effect of EGCG is multiple and complicated [139-141].

2.5.2.3 Antioxidant and pro-oxidant effect

Antioxidation is a process of vital importance to the health of human body. On the basis of the chemical structure of EGCG, it results to be an antioxidant. The phenol rings in EGCG structure, in model chemical systems, act as electron traps and scavengers of free radicals, inhibit the formation of reactive oxygen species, and reduce the harm caused by oxidative stress. It is reported that EGCG can effectively inhibit oxidative stress induced by oxidative stress in blood platelets, and as an antioxidant it can improve the function of mitochondria. However, under typical cell culture conditions, green tea polyphenols are unstable and undergo auto-oxidative

reactions: there are some studies pointing out that high concentration of EGCG can cause self-oxidization and act as pro-oxidant by producing hydroxyl radicals, hydrogen peroxide, and quinonoid intermediates causing cytotoxicity. The pro-oxidative effects are apparently responsible for the induction of apoptosis in tumor cells, thus being very important for eliminating the growth of mutated cells and limiting tumor growth [142-145].

2.5.2.3 Bioavailability of EGCG

Considering the bioavailability of EGCG, the detailed pharmacokinetics of tea catechins in humans and rodents have been studied and the preclinical pharmacokinetic studies reported that EGCG has low oral bioavailability (2–13%) in both humans and rodents. There are multiple processes which contribute to the low bioavailability of EGCG, including low solubility in the gastrointestinal fluid, slow and hard absorption, fast metabolism and elimination system and wide tissue distribution.

Another factor that leads to a low bioavailability is the instability of EGCG in neutral or alkaline medium, since the basic medium favors its autooxidation that generates superoxide and hydrogen peroxide anions. In this way, many of its effects may be attributed to the production of Reactive Oxygen Species (ROS). Due to its low bioavailability, it is unlikely that high EGCG concentrations will be reached in the bloodstream of individuals who ingest only two or three cups of 200 mg of green tea a day [146].

Other clinical studies have examined the pharmacokinetic profile of EGCG oral administration in healthy subjects. The safety, tolerability, and pharmacokinetic properties of a single dose administration of EGCG ranging from 50 mg to 1600 mg were examined. It was demonstrated that only for oral doses > 1000 mg of EGCG, maximal plasma EGCG concentrations > 1 μM were observed, considering that the peak concentrations are reached between 1.3-2.2 h after consumption with a variation between 130-3.392 ng/mL [146,147].

It was also evaluated the safety and plasma kinetics of multiple-dose administration of EGCG (twice-daily over a four-week period). The results showed that EGCG intake at the doses of 400 and 800 mg established a peak serum concentration. However, an increase in EGCG bioavailability was only observed after chronic 800mg administration and a daily EGCG administration produced only minor gastrointestinal side effects [147].

Rats and mice were also studied and the distribution of EGCG was observed in multiple tissues. The highest concentration of EGCG was detected in the large intestine and significant concentrations of EGCG were also found in the kidneys, prostate, and lungs [148].

It was concluded that the EGCG reaches concentrations within values capable of potently and specifically inhibiting some important molecular targets, through the consumption of green tea or the dietary supplements rich in EGCG. These studies also suggest that frequent consumption of green tea aids in maintaining a high level of EGCG [146-149].

After these analyzes, the interest on EGCG was intensified and many studies about colorectal, breast, prostate, lung and other cancer cell lines were performed [150].

For example, studies with MCF-7 cell line have been performed. They showed there were not only significant changes in the growth of these cells (proliferation decreased, and cell death occurred after EGCG treatment using the necessary physiological levels), but even non-malignant epithelial cells were not affected. Based upon these conclusions, other studies were started using different cell lines in connection with the drugs used in chemotherapy, in order to try to analyze whether the effects of EGCG can be positive [151-153].

2.5.3 EGCG in cancer treatment

There are countless publications that analyze the preventive effect of EGCG in some neoplasms and, possibly, as a helper in treatment. Due to its molecular focus, different cell lines can be influenced both by the inhibition of enzymatic activities, oxidative and anti-oxidative actions, and by affinity for specific protein targets [154].

This agent has shown considerable anti-cancer effects in a variety of preclinical cell culture and animal model systems and, as mentioned before, EGCG can help to induce apoptosis, modulation of cellular proliferation and inhibition of angiogenesis and related mechanisms [154].

2.5.3.1 EGCG in breast cancer

Chao-You Huang et al. investigated the effects of EGCG on the apoptosis of the human MCF-7 cancer cell line and the underlying mechanism. MCF-7 cells were divided into control and EGCG groups. The proliferation of MCF-7 cells in the two groups was determined using MTT and apoptosis was examined using flow cytometry. Western blot analysis was used to analyze p53 (a protein that detects the presence of damaged DNA) and Bcl-2 (a protein that regulates cell death) expression levels and the silencing effect of specific siRNA. In conclusion, the results showed that EGCG suppressed the proliferation of human MCF-7 breast cancer cells and promoted apoptosis and the mechanism may be related to the p53/Bcl-2 signaling pathway [151].

Liang et al identified that EGCG inhibited the proliferation of breast cancer cells by repressing cycling-dependent protein activity and also inhibited the growth and metastasis of breast cancer cells by down-regulating the expression of VEGF (vascular endothelial grow factor) and MMP-9 (an enzyme involved in the degradation of the extracellular matrix) [155,156].

The effects of EGCG and other catechins on the cell cycle progression were also investigated. DNA flow cytometric analysis indicated that 30 μM of EGCG blocked cell cycle progression at G1 phase in asynchronous MCF-7 cells. EGCG also inhibited the activities of cyclin-dependent kinase 2 (Cdk2) and 4 (Cdk4) in a dose-dependent manner: as the cells were exposed to EGCG (30 μM) over 24 h, a gradual loss of both Cdk2 and Cdk4 kinase activities occurred. These results suggest that EGCG may exert its growth-inhibitory effects through modulation of the activities of several key G1 regulatory proteins such as Cdk2 and Cdk4 [155,156].

Another group of investigators, *Abd El-Rahman et al.*, performed an in vivo study in order to investigate the effect of EGCG on 3 groups of female rats, injecting them with mammary tumor metastasis and evaluating the effect of EGCG on angiogenesis and CSCs. As therapy, EGCG, paclitaxel or their combination were used. It was found that EGCG exhibited significant chemopreventive effects and anti-CSCs (*anti-cancer stem cells*) activity through several pathways, including a significant decrease in the size and number of tumors in rats. This is a significant improvement of the oxidative stress markers' alterations and a significant inhibition of CD44 (a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration), VEGF (Vascular endothelial growth factor), Ki-67 (a cellular marker for proliferation) and MMP-2 (a proteins involved in the breakdown of extracellular matrix) expression associated with a significantly increased expression of caspase-3 (a protein playing a central role in the execution-phase of cell apoptosis). Additionally, the combination of EGCG and paclitaxel significantly enhanced the later anticancer efficacy, and it was concluded that EGCG could be offered as a curative strategy to help cancer treatment [157].

2.5.3.2 EGCG in colorectal cancer

Kumar et al. tried to discuss the use of EGCG as a chemopreventive agent for prevention of colon carcinogenesis and present evidence for the efficacy and safety of this agent based on epidemiological, animal, in vitro studies and Phase I clinical trials. Epidemiological studies, performed in Shanghai using a large population-based case-control diagnosed cancer cases compared to controls, have demonstrated the cancer preventive properties of green tea polyphenols in this type of cancer. These findings provide evidence that green tea drinking may lower the risk of colorectal cancers [158,159].

Considering animal studies, the effects of green tea extract, given as drinking fluid, on the promotion/progression phase of colon carcinogenesis in rats, after induction of the neoplastic process, was determined. Adult rats were injected with colorectal cancer cells and then were randomly divided into two groups: one group received daily prepared aqueous solutions of green tea extracts; the control group received water. After six weeks, rats receiving green tea showed a 60% reduction in the number of colonic pre-neoplastic lesions and a significant decrease of cyclooxygenase (COX)-2 activity. Thus, the data demonstrated that green tea reduces COX-2 and suppresses the formation of colonic pre-neoplastic lesions [160].

Ju et al, using another animal model, investigated the effects of green tea and a high-fat diet on arachidonic acid metabolism and aberrant crypt foci (ACF) formation in an induced colon carcinogenesis mouse model. The female mice were maintained on either a high-fat (20% corn oil) or a low-fat (5% corn oil) diet. Two weeks after the injection, green tea was given as the drinking fluid and continued for 10 wk until the experiment was terminated. In the mice not receiving green tea, the high-fat diet significantly enhanced colonic levels of enzymes, but it did not significantly alter prostaglandin E2 levels and aberrant crypt foci formation. In treated mice with the high-fat diet, green tea treatment significantly decreased colonic levels of some enzymes and also decreased the number of aberrant crypt foci. The weights of fat pads were increased by the high-fat diet and decreased by green tea treatment. Therefore, this study shows that green tea inhibits ACF formation in mice on a high corn oil diet, suggesting its possible inhibitory effect on colon carcinogenesis in populations such as those in Western countries that consume high amounts of fat [158,161].

In vitro studies have highlighted that tea compounds reduce growth and/or induce apoptosis in several human cancer cell lines. In particular, EGCG has been shown to affect several signal transduction pathways in vitro in the colon cancer cell lines. The proteasome system plays an important role in the regulation of apoptosis and in the degradation of cellular proteins, and two of the proteasome functions are to allow tumor cell cycle progression and to protect tumor cells against apoptosis. Following these considerations, EGCG showed the strongest inhibitory activity against purified 20S proteasome, 26S proteasome of tumor cell extracts, and 26S proteasome in intact tumor cells, being able to help treating cancer [158,162].

In summary, the *in vitro* and *in vivo* studies indicate that EGCG is a promising chemopreventive agent, with several cellular effects, both genomic and non-genomic, which are critical elements required to effect chemoprevention [158,162].

2.6 Drugs used in chemotherapy – side effects

In recent years, cancer therapy has evolved a lot and is one of the most important aspects to reduce the mortality rate linked to cancer.
The problem is that chemotherapy drugs travel throughout the body and may have many adverse effects. Chemotherapy drugs, in fact, works on active cells: cells that are growing and dividing into more cells of the same type. Cancer cells are active, but it is the same for very active healthy cells. Hence, chemo drugs can also affect normal and healthy cells that are fast-growing and the damages produced to healthy cells causes side effects, like the known fatigue, hair loss, headache, easy bruising and bleeding, infection, anemia, nausea and vomiting, appetite changes, constipation and diarrhea. The normal cells most likely to be damaged by chemo are blood-forming cells of bone marrow, hair follicles, cells in the mouth, digestive tract, and reproductive system, but also cells of heart, kidneys, bladder, lungs, and nervous system [163,164].

Cardiotoxicity and other side effects limit the usefulness of treatments for cancer. The development and implementation of intensive anti-neoplastic treatments have greatly improved the prognosis of cancer patients but, despite the benefit of this type of therapy, the safety aspect must be taken into account [163-166].

Hence, all chemotherapy drugs have secondary effects and associated risks, and both are different from patient to patient. Consideration must be given to previous treatments, to any other medications the patient is taking (unrelated to cancer therapy), to the patient's age and to other individual characteristics such as particular health problems and temporary or permanent conditions. The oncological treatment is always very individualized and the doctor must consider each patient in a different way [167,168].

Especially due to the numerous undesirable effects of chemotherapy drugs, researchers have begun to look for biological compounds that could help in cancer therapy and, in this regard, EGCG is one of the natural compounds studied, both individually and compared to natural and carcinogenic cells, and in combination with drugs. In combination with chemotherapeutic agents, this natural compound can increase the healing effects and also decrease the toxicity [169].

The therapeutic modalities available for breast and colon cancer include surgery, radiotherapy and chemotherapy, studies have been conducted to try to associate chemotherapy and biological therapies [169].

In fact, as the effect of EGCG on malignant cells has been demonstrated, its effect has then begun to be studied in association with drugs used for different tumor cell lines, including colon and breast cancer cells. Despite advances in chemotherapeutic treatments, the acquisition of

resistance in the course of treatment causes failure of a successful clinical management. Considering this problem, in 2015, a study by *C. Hernandez et al*. with the MCF-7 cell line was performed: doxorubicin-sensitive and resistant MCF-7 cell lines were either first pre-treated with EGCG for 24 hours prior to doxorubicin treatment, or directly treated with doxorubicin. The doxorubicin-induced cytotoxicity was measured by the MTT assay and the results indicated a 20% increase in cell death of doxorubicin resistant cells that were first pretreated with EGCG, compared to cells that were treated with doxorubicin without EGCG pre-treatment. Those results suggest that pretreatment with EGCG could potentially enhance sensitivity to doxorubicin-induced cytotoxicity in doxorubicin-resistant MCF-7 breast cancer cells [170].

In this area there are several lines of investigation and it is hoped that they will soon have clinical applicability to improve chemotherapy efficacy and reduce the adverse side effects.

2.7 *In vitro* model

In vitro models are experimental models used when it is desired to evaluate the molecular, biochemical, cellular and histochemical behavior of normal or pathological tissues. These studies are performed with microorganisms, cells, or biological molecules outside their normal biological context, and they are done in labware such as flasks, test tubes and microplates. *In vitro* studies are conducted using components of an organism that have been isolated from their usual biological environment, enabling a detailed analysis. Microorganisms or cells can be studied in artificial culture media, and proteins can be examined in solutions. *In vitro* work simplifies the system under study, thus the investigator can focus on a small number of components. However, results obtained from *in vitro* experiments may not fully or accurately predict the effects on a whole organism [171].

Advances in Cell Biology have allowed an increase in the use of this type of experimental model. For example, cell cultures from cutaneous neoplasia have been applied in the study of the behavior of these tumors [171].

2.8 Animal Model

The development of experimental models became important as they aid in the understanding of natural biological phenomena, simulating situations of human disease. In medical science they allow the best knowledge of physiology, the etiopathogenesis of diseases, the action of medications or the effects of surgical interventions. Animal models can be used in all fields of

biological research. In the case of experimental models, it becomes important to conceptualize animal disease, to mimic the pathological mechanisms that are similar to those of a human disease. This animal model must necessarily allow the evaluation of natural, induced or behavioral biological phenomena that can be compared to the human phenomena in question [172].

Considering that they are "biological reagents", the results of the studies and experimental analyzes may be affected by the particular conditions of each of the selected species. Considering these aspects, the higher the uniformity of the animals regarding the environmental, genetic and experimental variables, the lower the number of animals to be used for the study to be valid [172].

Thus, animals used in experimentation can be classified according to sanitary status, condition, genotype and as experimental model. For example, considering an experimental model there are different types of animal models: induced models (experimental models in which the animal is submitted to a procedure capable of provoking a morbidity, in order to perform an experimental procedure); negative models (a specific disease does not develop, they include animals demonstrating lack of reactivity to a specific stimulus and its main application lies in studies on the mechanism of resistance to better understand its physiological bases); orphan models (describe the naturally occurring condition in non-human species, but has not yet been described in humans); adopted models (orphan models in which a similar disease in humans has been identified) [172,173].

The use of health, genetic and ethical rules to treat the animals is mandatory: animal experimentation, in fact, requires that ethical concerns speak louder than scientific interests. Each researcher must have a complete understanding of the animal model being used, and of the biology and behavior of that species. Researchers must also be aware of the importance of the work being conducted, and consider all the premises that justify each specific project based on a solid scientific background.

The law reinforces the idea that animal experiments must be based on ethical considerations and integrity-based assumptions. It is required that the animals satisfy standardized conditions, are maintained in controlled environments to meet the sanitary and genetic quality parameters and the number of animals used for the experience/study must be as low as possible [172,173].

In *in vivo* studies it is important to follow the principle of the three R's (***r**eduction, **r**eplacement and **r**efinement*). Each **R** stands for a principle for the ethical use of animals in experiments: **R**eduction is the application of methods that allow a reduced number of animals to be used in a protocol. This can be achieved by detailed planning of the experiments, guaranteeing that results will have statistical significance. The use of animals presenting the same or a similar genetic background also ensures a low fluctuation of the data, thereby reducing the number of

animals which is needed to be used in a study. **R**efinement consists in the application of methods that avoid animal suffering, such as: the use of anesthesia during a procedure and analgesic regimens for pain relief during recovery; the use of non-invasive techniques; housing conditions that provide a comfortable and safe environment and training the animal to cooperate with procedures. **R**eplacement is the major goal for the use of animals in science. It consists on the substitution of animals by other models, such as microorganisms or other invertebrates, cell cultures, organs or even cellular fractions. The ideal replacement would be a protocol conducted with no use of animals [174,175].

The mouse has long been the most widely used animal model for in vivo studies and for genetic research, as it has anatomical, physiological, biochemical and genetic characteristics similar to human beings. Like humans and many other mammals, mice naturally develop diseases that affect these systems, including cancer, atherosclerosis, hypertension, diabetes, osteoporosis and glaucoma. In addition, certain diseases that afflict humans but normally do not strike mice, such as cystic fibrosis and Alzheimer's, can be induced by manipulating the mouse genome and environment. Mice are also easy to manipulate, have a high reproduction rate and relatively inexpensive maintenance. Due to their small size, they can be kept in small spaces (cages) in appropriate installations,certified and approved by the animal welfare service [176].

The most common mice used in cancer research are the Balb/c nude mouse. The nude mouse was created in the laboratory between 1979 and 1980 in the National Institute of Health in the USA through a series of couplings. It derived from a strain with a genetic mutation that causes a deteriorated or absent thymus, resulting in an inhibited immune system due to a greatly reduced number of T cells. The phenotype is a lack of body hair, which gives it the "nude" nickname [177].

Given the characteristics of immunodeficiency of the lineage, it is used for immunological, infectious and oncological studies. Immunodeficiency makes these animals very vulnerable and this can be a limit for some types of research. Under normal conditions, nude mice have an average life time of 9 months, but if kept free from pathogenic agents, infections would occur with much less frequency and the life time could even reach two years [177-179].

It is important to know the basic principles of the anatomy of these rodents to be able to identify organs and collect samples and to perform oral and/or injectable administrations and small surgeries. The anatomy of this animal model is simple and can be represented schematically in the following figure (**Figure 17**).

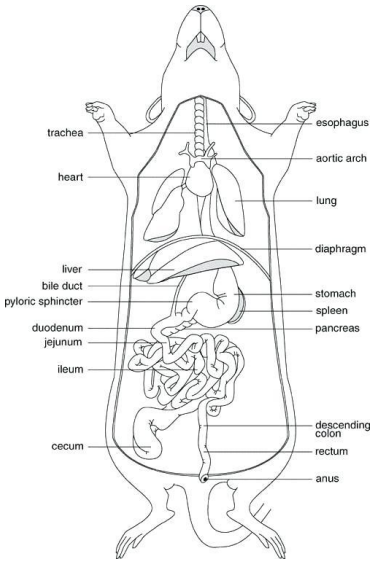


Figure 17: Representative diagram of the anatomy of the animal used for *in vivo* studies (adapted from [180]).

Chapter 3

3. Materials and Methods

This chapter will report on all the materials and methods used in this work. The used cell lines, the cell culture, the tea preparation, the animal studies and the realized experiences will be shown.

3.1 Cell lines

For the performance of this work, as mentioned in the previous chapter, two cellular lines were used: the cell line WiDr (ATCC® CCL-218™), derived from a human colorectal adenocarcinoma, and the cell line MCF-7 (ATCC® HTB-22™), derived from human breast adenocarcinoma. Both are adherent epithelial cells and have been acquired by the Institute of Biophysics of the Faculty of Medicine of the University of Coimbra (FMUC) to ATCC.
The MCF7 line retains several characteristics of differentiated mammary epithelium, including the ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes. The WiDr line is negative for Colon Antigen 3 expression, but positive for keratin by immunoperoxidase staining and expresses p53 antigen [181,182].

Cell lines were kept in the dry transport ice for the minimum time necessary for the transport and were grown for propagation. From the original sample, several aliquots were prepared (in culture DMEM medium with 10% of FBS and 10% of DMSO), identified and numbered. They were stored a few hours at -80°C (Thermo Scientific, Herafreeze HFUT Series, USA) and then transferred to a liquid nitrogen container at -190°C (ThermoNorma, Cryoplus 1, USA). The properly identified cryotubes were placed in storage racks, assuring the quality of the cell line stored with few passages. During short periods of time, some aliquots were kept at -80°C, in a dedicated freezer (Thermo Scientific, Herafreeze HFUT Series, USA), aviding the frequent opening of the liquid nitrogen container.

3.2 Cell Culture

Cell culture must take place in sterile and ventilated flasks, appropriate for adherent cells, kept in an incubator at a constant temperature of 37°C, with an atmosphere with 5% of CO_2 and 95% of humidity. All cell manipulations were performed in a special environment of the cell culture room, and inside a laminar flow chamber, in order to maintain the asepsis conditions, essential for the propagation and preservation of cells. For this procedure a specific equipment is essential: a

vertical laminar flow chamber (*Holten Lamin Air*, HB2448, USA), an incubator (*Binder*, Dias de Sousa S.A., Portugal), an inverted phase contrast optical microscope (CPOM, *Nikon Eclipse*, TS100, Japan), coupled to a digital photography system, and a centrifuge (*Heraeus Multifuge* 1L-R, USA) (**Figures 18,19**).

Figure 18: Images of the referred flow chamber, the CPOM and the centrifuge.

Figure 19: Images of the referred incubator.

The flow chamber is very important since it allows the work in sterile conditions, limiting contamination as much as possible. When an operator uses the flow chamber, he must ensure that everything entering the chamber and coming into contact with the cells is sterilized and sprayed with ethanol 75%. In fact, ethanol 75% destroys the cell membrane of bacteria by denaturing their proteins and dissolving their lipids, helping to maintain the environment sterile and aseptic.

Furthermore, a specific environment is required to allow cell adhesion and proliferation, in which the temperature is 37°C and the atmosphere is 95% humidified and contains 5% CO_2. This environment is guaranteed inside the incubator. Finally, the operator must make sure that all the material that came into contact with the cells is discarded and placed in special containers, for incineration.

3.2.1 Culture medium

The culture medium suggested by the cell line supplier is the *Eagle's Minimum Essential Medium* (EMEM) (ATCC® 30-2003™), with 2 mM of L-glutamine and 1% of non-essential amino acids (NEAA), fetal bovine serum (FBS) at 10% and 1% of antibiotics of pennicillin/estreptomycin (Pen/Strep), renewed two or three times a week.

However, the cell culture medium used in this study was the *Dulbecco's Modified Eagle Medium* (DMEM) (Gibco® 1x, 11966-025, UK), supplemented with 1% of L-glutamine (Biowest, L-glutamine 100x, 200mM, XO55O-100, USA), enriched with fetal bovine serum (FBS) (Sigma-Aldrich®, F7524, USA) at 10% and 1% of antibiotics Pen/Strep (Lonza Pen Strep, Amphotericin, B 100x, 17-745E, USA). The addition of serum to the culture medium is fundamental for the contribution of growth factors, hormones, chelating agents and detoxifying substances that induce the survival and proliferation of cultured cells (25 mL FBS in 500 mL DMEM).

DMEM contains approximately four times more vitamins than EMEN and two times more amino acids (as ferric nitrate and sodium pyruvate), and some supplementary amino acids (as serine and glycine).

After being prepared, the medium was sterilized by vacuum filtration in the laminar flow chamber. Then a test was carried out during at least 48 h – 72 h to check that there were no contaminations. If everything was correct, the culture medium stored in a refrigerator could be heated in a water bath thermostatized at 37 ° C (Tissue water bath, GFL, 1002, Germany) and supplemented with 5% FBS.

3.2.2 Cell thawing

Before being used, the cells were kept in a cryotube in the cryopreservation mixture with DMEM medium and 10% dimethylsulfoxide (DMSO) (SigmaAldrich, D2650, USA), either in the special freezer at -80°C or in the nitrogen chamber at -190°C.

The cryotube was transported and quickly defreezed by shacking it inside the thermostatized water bath at 37°C, containing a preservative – sodium azide (Sigma-Aldrich, S202, USA) to avoid fungi development.

In the laminar flow chamber, the cryotube volume was passed into a 15 mL Falcon (Sarsted, 62.554.502, Germany) and 2-3 mL of DMEM culture medium were added to inhibit the action of the

cryopreservative agent. The 15 mL tube was centrifuged at 1100 rpm (rotations per minute) for 5 minutes at 4 ° C (Thermo Scientific, Heraeus Multifuge 1L-R, Germany, certified by Certilab). Then, in the flow chamber, the supernatant was discarded, thus removing all the suspension medium. The obtained pallet was resuspended in 1 mL of DMEM medium and then the cell suspension passed into a 25 cm^2 culture flask (Corning, 430693, USA), adding about 15 mL of DMEM culture medium, an adequate amount to give the cell suspension enough nutrients to proliferate. Hence, the flask was placed in the incubator at 37°C and kept in a humidified and controlled environment. The culture medium was withdrawn and changed every 2/3 days, after checking the growth of the cells under the CPOM.

An important thing to consider is that the WiDr cells used require a time of 48 h for proper adhesion and proliferation, while the MCF-7 cells take 72 h. When plated every kind of manipulation with those cell lines must be performed after those periods of time, respectively.

3.2.3 Cell maintenance

Every day it was checked if cell proliferation and adhesion were progressing correctly and if it was necessary to change the culture medium. The flasks with the cells were observed under the CPOM, until they reached approximately 70-80% of confluence.

Cells were detached from the flat bottom flask by an enzymatic method (*TrypLE™ Express*, *Gibco by life tecnologies™*, Denmark) and transferred into new flasks or plated into culture plates, as necessary. The performed procedure is described in the following sub-section (**3.2.4**).

As the culture medium has a pH indicator (phenol red), it varies its color and shows a gradual transition from red to yellow as the pH value decreases from 7.4. In fact, when the pH value falls down, the color turns from red to yellow, and this means that the culture medium is reacting with the metabolic products of the growing and proliferating cells, which acidify it. Hence, the coloring of the culture medium serves as a visual indicator of its acidity, characteristic of the progression of the cell growth and proliferation. If the medium becomes really yellow, it means it is full of toxic products formed by the cells themselves. It is advised to change the medium when it is still orange to provide new nutrients to the cells, and allow them to continue to proliferate and grow until they have space. MCF-7 are very sensitive to low pH and to the lack of space and died very quickly. A daily check-up is mandatory.

If there was a need to change the culture medium, this procedure was done with an automatic pipettor (Gilson, Pipetting Aid 080333, France), coupled to a sterile glass Pasteur pipette (VWR, Pasteur Pipettes 612-1702, France), to aspirate all the culture medium present in the culture flask,

thus removing the cells in suspension and the medium full of metabolites. Then, another 15 mL of new culture medium were placed in the flask, and the flask was put back inside the incubator, after having been sprayed with 75% ethanol. The flasks were always checked under the microscope also to make sure there were no bacterial contaminations.

3.2.4 Cell detachment

As they grew, the cells adhered to the flat bottom of the flask and to each other. To maintain a good growth environment, it was necessary to ensure that the number of cells within the flask was such that growth was possible and did not prevent the normal development of the monolayer, considering that the cells respond to contact inhibition. In fact, contact inhibition is a signaling pathway activated by the glycocalyx present outside the cell membrane, which emits signals to contact with cells of the same type. These signals inhibit mitosis and lead to cell death. Cancerous cells typically lose this restrain signaling and thus divide and grow over each other in an uncontrolled manner even when in contact with neighbor cells, resulting in the invasion of surrounding tissues [183].

Nevertheless, the cell lines used in this work have special characteristic behaviors. WiDr cells can grow in piles, building up stalagmite alike structures. If the “mother” flask is kept for longer periods, little black dots become visible on its bottom. They stay glued together and this can explain the *in vivo* behavior of this tumor, which is capsulated, thus circumscribing colon cancer in the first stages. On the contrary, MCF-7 cells do not grow in heaps, leading to cell floats, and die relatively quick when they do not have enough free space to grow. If these floats drive quickly *in vivo* initiating metastasis, a situation that is common in mammary cancer.

Cell detachment can be done by mechanical or enzymatic methods. In this work, an enzymatic degradation was used, digesting the adhesion proteins using a protease. Proteases are necessary to break up the extracellular matrix, and thus obtaining individualized cells, aiming to transfer them to a new culture flask or to be plated onto culture plates. The non-specific proteolytic enzyme used was a recombinant enzyme present in *TrypLE™ Express* (*Gibco by life tecnologies™*, Denmark), which is specific for arginine and lysine, hydrolysing polypeptide chains in lysine-arginine radicals and forming ester and amide cleavage terminations. The reaction is similar to the one caused by trypsin, but without derivatives of animal origin, being more gently with the cells. This reaction disrupts the matrix, making impossible to bind the cell surface receptors, linked to the cytoskeleton and to the matrix, which forces the cells to reorganize their cytoskeleton. Due to the non-specificity of the enzyme, the cells were not left in its presence for much time, in order to avoid cell lysis (plasma membrane rupture leading to the destruction of the cell). The use of TrypLE is important mainly for MCF7 cells that are very sensitive [184,185].

The process was divided in a series of steps:

1. the culture medium was removed from the flask as described above;

2. 4 to 7 mL of TrypLE were added to the flask, depending on its size (25 or 75 cm^2);

3. typically cellular detachment occurred after 3-5 minutes for the MCF-7 cell line and after 6-8 minutes for the WiDr cell line;

4. after this period of time, the flasks were observed with the CPOM to check if most of the cells were in suspension;

5. once the cell layer was separated, the effect of TrypLE was neutralized by adding 1-2 mL of culture medium. Medium contains fetal bovine serum and since the serum possesses alpha1 anti-trypsin, this neutralized the action of the enzymatic agent;

6. the cells were suspended by soft pipetting, transferring the cell suspension into 15 mL sterile identified tubes, using a pipettor coupled with a sterile Pasteur pipette;

7. the tubes were centrifuged at 1.100 rpm, for 5 minutes at 4°C;

8. inside the flow chamber, the supernatant was discarded from the tube;

9. the cell pellet was re-suspended in 1 mL of culture medium;

10. the re-suspended pellet was placed in a new flask with about 15 mL of culture medium, the flask was then identified, sprayed with alcohol and placed in the incubator to continue proliferation;

11. for the experiments to evaluate cell proliferation in presence of several compounds, the necessary volume was completed for the appropriate concentration of cells with more culture medium. The homogenized cell suspension was then seeded in sterile multi-well plates according to the experiment design.

3.2.5 Cell counting

Both for the cryopreservation process and to start any cell feasibility study, it was necessary to know the number of available cells and select the number of cells needed (for example to be plated), thus that they do not reach the maximum confluence before the end of the experience or not to get a cell number too low in order to have a statistical value. To carry out this operation, the detachment process described above was performed, with the only difference that the cell pellet was not placed into a new flask but was re-suspended in 1 mL of culture medium. From the cell suspension 10 µl were then withdrawn and placed in a sterile 2 mL eppendorf (DeltaLab S.L., 4092.7N, Spain).

In order to proceed with the count of the approximate number of cells in the pellet, a vital dye, trypan blue (Sigma-Aldrich, T0776, USA) (Annexe III [A]), was used. This compound has the ability to cross the fragmented membrane of dead cells and, in this way, to mark the non-viable cells blue.

Considering this, 10 µl of trypan blue were added to the suspension previously placed in the 2 mL eppendorf, outside the laminar flow chamber. Subsequently, 10 µl of the well homogenized mixture were pipetted and placed in a Neubauer chamber (Marienfeld Neubauer improved bright-line, 0640030, German) under a glass lamella (RS, Cover Glass 100 PCS Thickness 0.13-0.17 mm, France) (**Figures 20** and **21**).

Figure 20: Neubauer chamber.

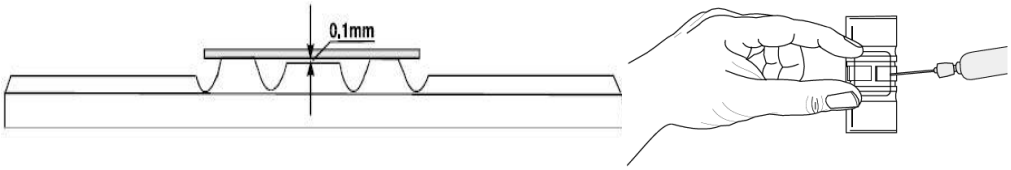


Figure 21: Neubauer chamber scheme and insertion of the suspension into the chamber, under the glass lamella (adapted from [186]).

The Neubauer chamber allows cell counting with the CPOM, after inserting the mixture into the chamber. This chamber contains a 3 mm^2 drawn grid, organized in 9 large squares (3 per line), of 1 mm^2 each. Each of the 4 large squares at the outer corners is divided into 16 squares of 0.25 mm^2 each (**Figure 22**). The large central square (which can be seen in entirely with the 10x objective), is divided into 25 medium squares with double or triple lines. Each of these 25 squares are divided again into 16 small squares with single lines, hence each of the smallest squares has an area of 1/400 mm^2. The glass cover is a squared glass 22 mm of width.

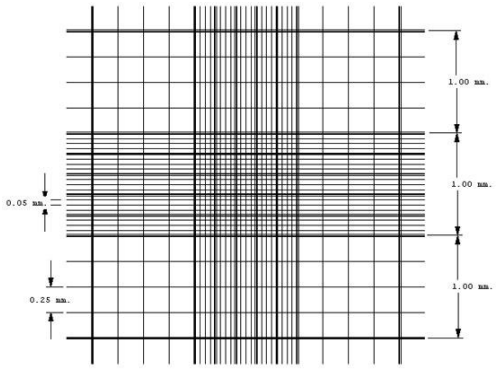


Figure 22: Neubauer chamber grid (retrieved from [186]).

In this work, cell concentration was determined using the 4 squares at the corners and calculating the average number of cells per quadrant. To carry out the insertion of the mixture under the lamella, the suspension must be well homogenized in order to avoid cellular

agglomerates. A serpentine trajectory should be followed over the scheme of the 16 squares of each quadrant and count the number of cells within each square. The cells over the lines of the squares are not considered (**Figure 23**).

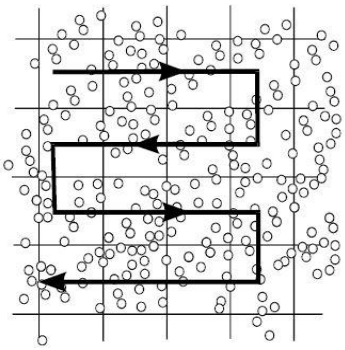


Figure 23: Cell counting scheme (retrieved from [186]).

Since the volume of each small square is 0.1 mm^3, taking into account the defined depth of 0.1 mm between the chamber and the lamella, the total number of cells in the quadrant allows to calculate how many cells are present in 0.0001 mL. This means that the total number per quadrant will be multiplied by 10^4. In this way it was possible to count the approximate number of cells that were then used for the *in vitro* and *in vivo* studies [186,187].

If the cells were not needed for further growth or for an experiment, they were frozen. Cryopreservation took place in specific cryotubes for this purpose, with a volume of 1.5 mL (SARSTED, 72380992, German). Sterile 10% DMSO (dimethylsulfoxide) (Sigma-Aldrich, dimethyl sulphoxide, D2650 5x5 mL, USA) was added to the cell suspension in culture medium. DMSO is a cryopreserving agent which prevents the water present in the cells to crystallize. In this way, the cells can survive to freezing at low temperatures. In fact, as the cell suspension was cooled below the freezing point, ice crystals are formed and the concentration of the solutes in the suspension increases. Intracellular ice can be minimized if water within the cell is allowed to escape by osmosis during the cooling process. However, as the cells lose water, they shrink in size and will quickly lose viability if they go beyond a minimum volume. The addition of cryoprotectant agents such DMSO allows to mitigate these effects [188].

The cryopreservation tubes with the cell suspension were correctly identified, marking the cell type, the number of cells, the date of cryopreservation and the number of passes. Later they were placed in a dedicated freezer, at -80°C.

3.3 Use of Green Tea Extract

The natural compound selected for this study was the (-) - epigallocatechin-3-gallate (EGCG), which is one of the most abundant constituents of green tea and is considered the main source for various health benefits, already mentioned previously. Studies on the national and international market were carried out in order to determine which teas and which extracts were the most marketed and consumed, trying to understand which ones held the highest concentration of this catechin.

3.3.1 Determination of EGCG concentrations

To achieve the goal of this work it was necessary to know the concentration for each brand in order to identify the extracts that had the highest concentration of EGCG, to verify if the data provided by the manufacturers were correct, and to assess whether the concentration was similar between all the lots. As it is known, the composition and quality of tea varies according to the season, the producing country, the climatic conditions and the processing of the leaves.

High-pressure liquid chromatography (HPLC) technique was applied to determine the concentration of EGCG more accurately, and to make sure that the value indicated by the manufacturer was correct some suppliers indicate the precise amount of EGCG in their extracts. HPLC is a technique that aims to separate, identify and quantify a sample into its components on the basis of its molecular structure and molecular composition [189].

In a simple way, this technique uses a pump to pass a mixture of solvent + sample to be analyzed at high pressure (400 atmospheres), through a column that contains an adsorbent material. All the components of the sample interact differently with the adsorbent material, causing different flow rates for the different components, in fact the components of the sample move through the column at different speeds, which are a function of specific physical interactions with the adsorbent. The time at which a specific substance elutes (emerges from the column) is called retention time and it is an identifying characteristic of a given sample. This process leads to the separation and the identification of the components of the sample as they flow out of the column, comparing them with known cone-shaped HPLC profiles for "pure" substances (**Figure 24**) [189,190].

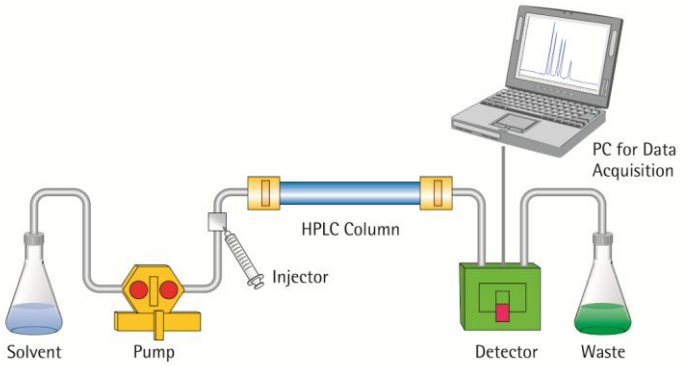


Figure 24: Typical setup of an HPLC system (retrieved from [190])**.**

The studied extracts of green tea were: in GTE powder (MyProtein, Green Tea Extract powder 500g, 1705807841, UK) and in the form of a capsule, MGTE (MyProtein, Mega Green Tea Extract^{TM}, 90 cap 450 mg, UK) e GTEE (ProHealth, Green Tea EGCG Extreme^{TM}, 100 veggie capsules, 390 mg EGCG cap, PH395, USA, certified by the Laboratory Purity&Potency) (**Figure 25**).

Figure 25: Tea extracts.

Data analysis was done with a dedicated software, and a graphical representation (chromatogram) was obtained with several peaks, related to the different components of each sample. This technique was performed 3 times for each sample to ensure the reliability of the results.

3.3.2 EGCG solutions prepared from the extracts

The HPLC protocol was used to determine the concentration of EGCG in the selected extracts. Capsules from different manufacturers, GTEE and MGTE, and GTE powder were used. According to supplier information, each GTEE capsule contained 390 mg of EGCG/capsule, for MGTE 180 mg of EGCG/capsule and GTE powder contained approximately 10% of EGCG.

To prepare the stock solutions, homemade sterile phosphate buffer saline (PBS) (Annexe III [B]) was used. PBS is a saline solution that contains sodium chloride, sodium phosphate and potassium phosphate. The buffer helps to keep the pH constant. The salt concentration matches that of the human body (isotonic). PBS has many uses especially since it is isotonic and non-toxic to cells. It can be used to dilute substances.

The aliquots of prepared stock solutions were duly identified and then frozen at -20°C, protected from light (surrounded by aluminum foil). Starting from these aliquots, previously filtered in a sterile environment as mentioned before, the necessary dilutions were prepared, in order to obtain the desired compound's concentrations for the different tests.

For the *in vivo* studies a solution was prepared using GTEE capsules. The capsules with EGCG extract were cut in half and the powder contained inside them was diluted in water to obtain a concentration of 24 mg/mL. This solution is 5 times more concentrated than that required to produce a plasma concentration of 0.1 µg/mL in a human [191,192].

An electronic analytical balance was used to weight the powder of the capsules (Radlag, AS 220/c/2, Germany). The powder was then placed in an Erlenmeyer Schott® flask and the previously measured volume of water was added. The solution was well homogenized to obtain a complete dissolution of the powder and then it was stored at 4°C, protected from light.

3.4 *In vitro* studies

In vitro tests allow to test cell lines in a standardized and reproducible form and to evaluate the toxicity of a drug or natural product in a controlled environment. Through various techniques, for example the absorbance test, it was possible to evaluate the cytotoxicity of the tested agents. After studying the physic-chemical properties of the compounds, the *in vitro* studies were the first to be carried out to try to determine and standardize experimental conditions and try to predict *in vivo* conditions.

All *in vitro* tests were carried out in a sterile environment, in standardized conditions and following well-defined protocols, in order to have more control over external variables. This methodology helps to decrease the number of individuals in *in vivo* studies, also in accordance with the 3**R**'s principle and the time taken for the study.

For the *in vitro* studies performed in this work, the WiDr cell line was used. The results of the MCF7 cell line were retrieved from a past work: for the present work there were not enough cells

for both *in vitro* and *in vivo* studies and no chemotherapeutic drugs, thus the choice was only to perform *in vivo* studies based on previous *in vitro* results.

3.4.1 General aims of *in vitro* studies

The *in vitro* tests had the general goal of testing the possible effect of the most abundant polyphenol present in green tea, EGCG, in a combined therapy with the agents used in chemotherapy, described in the literature, to treat colorectal cancer, using the WiDr cell line.
If EGCG enhances the action of chemotherapeutic agents, it would be possible to decrease the number of chemotherapy cycles or decrease its dosages, helping to reduce the undesirable side effects of drugs, that are extremely negative.

3.4.1.1 *In vitro* studies - WiDr cell line

Previous studies carried out with the WiDr cell line, at the Institute of Biophysics of the Faculty of Medicine of the University of Coimbra by the team, defined an optimized *in vitro* culture protocol. For these studies, 96 well culture plates, with an area of well 0.32 cm^2, were used (Costar, Corning Incorporation, 3596, USA).

The optimal number of cells per well was found to be 2.4×10^3 cells, in a volume of 150 µL DMEM per well. Adherence time had also been optimized: it was verified that the optimal time for the cells to adhere to the flat bottom plates was 48 h, in order to carry out the experiments (incubation at 37°C, 5% CO_2 and 95% humidity, as described). In this study, the daily consumption of EGCG was simulated, hence applications of the same volume of EGCG-rich solutions with predefined concentrations were made every 24 h. The effect of this compound has been tested for different replication schemes (1, 2, 3, 6, 9 and 12 applications) and with different EGCG concentrations (0.01, 0.1, 0.05, 1, 5 and 10 µg/mL) by previous students.

It was concluded that, for the WiDr cell line, the most relevant concentrations and applications were 0.5 and 5 µg/mL of EGCG for 3 applications.

In the present work several tests have been carried out for the referred situations, in order to increase the reliability of the results, always comparing them with the controls. The controls were wells in which no EGCG solution was applied, and which were exactly in the same culture conditions for the entire time of the experiment. This allowed to analyze cell viability with and without application of the compound, simultaneously.

In previous studies commercial tea infusions, such as Lipton, Twinning's and Pingo Doce, were also tested, preparing infusions with one, two or three bags at a time, but considering that teas also contain caffeine, it might not be convenient to all patients. Due to the large volume that would have to be drunk, patients with heart and/or kidney problems could be ruled out of the study. In this context, powdered or capsule EGCG enriched extracts were selected.

In the pursue of the proposed goal and to study the possibility of achieving a reduction of the chemotherapeutical doses or to reduce the number of therapeutic cycles, the effect of the different extracts together with some of these drugs was analyzed.

In this work it was analyzed if there can be a beneficial synergistic effect between EGCG infusions and chemotherapeutic agents. For this purpose cells were plated for incubation with each drug (clinical dosages were selected according to the literature and previous studies [193]), with tea extracts alone (0.5 and 5 µg/mL of EGCG), and a combination of drugs and extracts for the same concentrations, or no compound other than DMEM (controls). Several independent tests were performed for each condition in a series of wells per experiment, in order to increase the reliability of the results and their statistical value.

All the tested drugs were obtained through a protocol between the University Hospital of Coimbra, and the Institute of Biophysics (FMUC-ICBR/CIBB). These drugs are approved by the FDA (Food and Drug Administration) and the EMA (European Medication Agency), and used in chemotherapy for colorectal cancer treatment.

An illustrative diagram of a 96 well culture plate is presented here: each column corresponding to a single dosage, in order to increase the statistical value of the results (**Figure 26**).

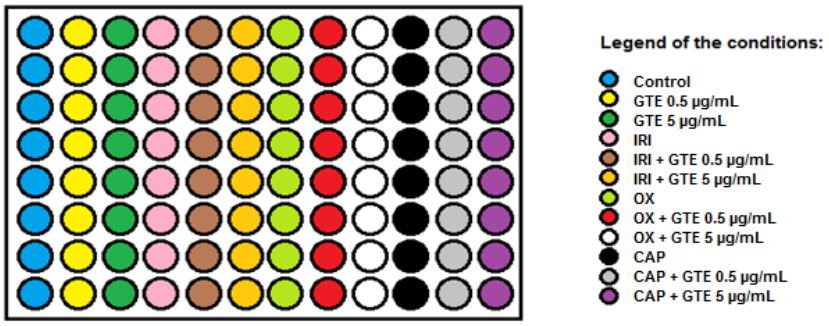


Figure 26: Illustrative diagram of a 96 well culture plate, containing the seeded cells and culture medium (in each well) and the other substances according to the selected distribution. These plates are used for the MTT tests to study the cytotoxicity of extracts, drugs and the combination of both.

3.4.1.2 *In vitro* studies – MCF7 cell line

Previous studies carried out with the WiDr cell line at the Institute of Biophysics of the Faculty of Medicine of the University of Coimbra by the team, defined an optimized *in vitro* culture protocol.

In these studies, 48 well culture plates with an area of well 1.1 cm^2 were used (Costar, Corning Incorporation, 3596, USA).

The optimal number of cells per well was found to be 7.2 x 10^3 cells, in a volume of 800 µL DMEM per well. Adherence time had also been optimized: it was ascertained that the best time for the cells to adhere to the flat bottom plates was 72 h, in order to carry out the experiments (incubation at 37°C, 5% CO_2 and 95% humidity, as described). In this study, the daily consumption of EGCG was simulated (chronic consumption was preferable if compared to acute consumption), hence applications of the same volume of EGCG-rich solution with predefined concentrations were made every 24 h. The effect of this compound has been tested for different replication schemes (2, 3, 4, 6 and 8 applications) and with different EGCG concentrations (0.5, 1, 5 and 10 µg/mL) by previous students, using GTE extract.

It was concluded that, for the MCF-7 cell line, the most relevant concentrations and applications were 0.5 and 5 µg/mL of EGCG for 6 applications.

Several tests have been carried out for these situations, comparing them with the controls (wells remaining in the same culture conditions for the entire time of the experiment), allowing to analyze cell viability with and without application of the compound, simultaneously.

In previous studies commercial tea infusions, such as Lipton, were also tested, preparing infusions with one, two or three bags at a time. Nevertheless considering that teas also contain caffeine, it might not be convenient for all patients. Due to the large volume that would have to be drunk, patients with heart and/or kidney problems could be ruled out of the study. In this context, a powdered EGCG enriched extract (GTE) was selected.

In the pursue of the proposed goal and to study the possibility of achieving a reduction of the chemotherapeutical doses or to reduce the number of therapeutic cycles, the effect of the GTE together with some of these drugs was analyzed.

For this purpose cells were plated for incubation with each drug (with the clinical dosages selected according to the literature and with previous studies [193,194]), with GTE tea extract alone (0.5 and 5 µg/mL of EGCG), and a combination of drugs and extracts for the same concentrations, or no compound other than DMEM (controls).

An illustrative diagram of a 48 well culture plate is presented here: each column corresponds to a single dosage, in order to increase the statistical value of the results (**Figure 27**).

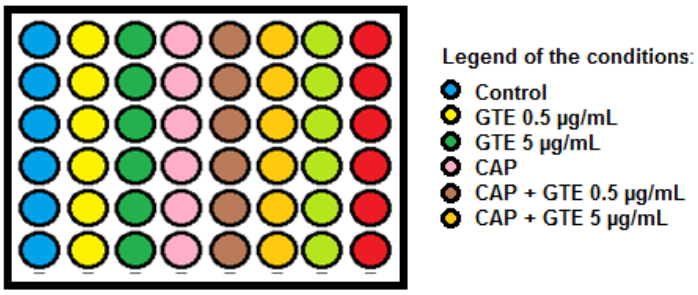


Figure 27: Illustrative diagram of a 48 well culture plate, containing the seeded cells and culture medium (in each well) and the other substances according to the selected distribution. These plates are used for the MTT tests to study the cytotoxicity of extracts, drugs and the combination of both.

3.4.2 Cell viability

After carrying out the studies previously described, with the seeded cells and stimuli application protocols, it was necessary to know how many cells died, and to quantify the cell viability. Measurement of cell viability and proliferation are the basis for numerous *in vitro* assays of a cell population's response to external factors. The MTT assay was used for this purpose.

3.4.2.1 MTT assay

The MTT Cell Proliferation Assay measures the cell proliferation rate and, conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. This is a colorimetric assay using the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The MTT is a yellow salt, due to the presence of a tetrazolyl ring. Viable cells contain NAD(P)H-dependent oxidoreductase enzymes which reduce the MTT reagent to formazan, by the dehydrogenases occurring in the mitochondria of living cells. Formazan is an insoluble crystalline product with a purple color. The concentration of these crystals is proportional to the number of living cells. The reduction of MTT depends on the cellular metabolic activity: cells with a low metabolism hardly reduce MTT, while rapidly dividing cells exhibit high rates of MTT reduction (**Figure 28**) [195,196].

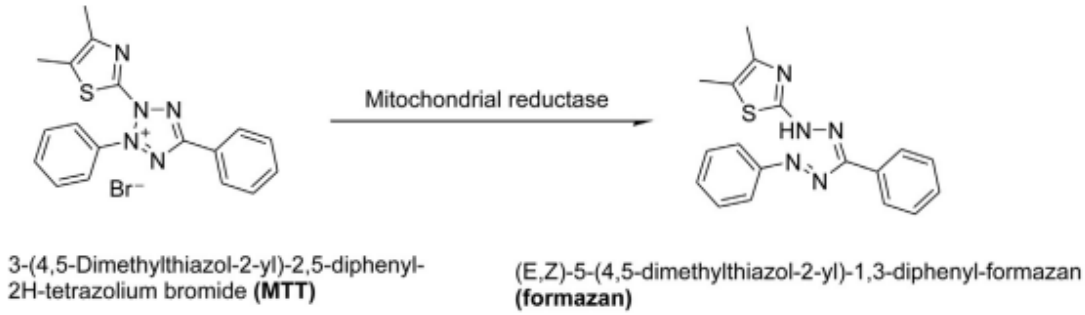


Figure 28: Illustrative scheme for the reduction of MTT molecule in formazan by living cells (retrieved from [197]).

The protocol to make the MTT test envisaged that, in aseptic conditions, the medium is totally withdrawn from each well and discarded. Subsequently, taking into account the volume used per well, new culture medium and 10% of MTT (Sigma, M2128, USA) were added. In this case, after removing the 150 mL (or 800 mL) present in the well, 135 mL (or 720 mL) of DMEM culture medium and 15 mL (or 80 mL) of MTT were used.

After this procedure, the culture plate was placed back in the incubator under the same conditions for 4 h and was then observed under the microscope. After this time, over the bench, the solution contained in each well was discarded and replaced by acidified isopropanol [a solution of HCl (VWR, 20255.420, EC) 0.04 N in isopropanol (Honeywell/Riedel-de Hean, 33539, Germany)] (Annexe III [C]), in a total volume of 150 mL. This last procedure, consisting in putting isopropanol in the plates, does not need to be performed inside the flow chamber, as the membrane of vital cells is destroyed by the action of the acidic isopropanol. The plate was then read to be quantified.

With a good homogenization, which occurred after about 15 minutes, the amount of formazan crystals, directly proportional to the number of viable cells, could be read by spectrophotometry (ELISA reader BioTek, Synergy HT, USA), coupled to a computer with the Gen5TM Biotek program (Bioteck Inc, USA). This optical method is colorimetric and allowed to quantify cell viability by reading at two wavelengths (570 and 620 nm).

3.5 *In vivo* studies

In vivo studies using an experimental animal model were carried out once the results of *in vitro* studies were favorable, in order to use the fewest animals possible. In this *in vivo* study the effect of GTEE in capsules was tested, after injecting tumor cell lines WiDr and MCF-7 into animal models.

The most widely used animal models in xenotransplantation experiences are nude mice (Foxn1) and mice with severe combined immunodeficiency (SCID), which possess mutations that

affect the immune system. The nude mice (Foxn1, Balb/c lineage) present an autosomal natural recessive mutation on chromosome 11, which causes a lack of hair growth and other alterations, such as disturbances in the formation of the thymus gland (in fact these mice are also called athymic nude mice) and consequently their immune system is inhibited and they are deficient in T cells. SCID mice have a spontaneous mutation that results to impair the recombination of antigen receptor genes leading to the arrest in the development of functional T and B cells [178,198].

Immunodeficient animals have also been developed from other species, such as, for example, the ATN rats (Rowett Nude Rats), a mutation of *Rattus norvegicus* [199].

The effect of the GTEE in capsules has already been studied in another experimental model: male RNU rat [216]. Some preliminary tests were done in nude mice in previous experiments for the WiDr cell line trying to establish a reliable tumor development protocol [200]. Hence, an aqueous solution of GTEE in capsules was given to the animals as a substituent of water, mimicking the protocol for rats. However, the nude mice do not like, and consequently do not drink, this beverage. In this *in vivo* study the effect of GTEE in capsules was tested, after injecting the WiDr tumor cell line in nude mice, using an alternative administration via called oral gavage.

Liquid compounds, in fact, may be administered directly into the stomach of mice and rats via oral gavage technique. In this procedure, a flexible cannula (abbocath for gavage feeding) (BD Insyte™ Autoguard™ Blood Control, 381034, 20G x 1.16 pol., 1.1 mm x 30 mm) is attached to a syringe and used to deliver the compound into the stomach. The correct cannula length is equal to the distance from the mouth to just beyond the last rib. The animal was gently restrained (grasping the animal by the loose skin of the neck and back) to immobilize the head but not such that the animal showed signs of distress. The animal was maintained in an upright (vertical) position and the gavage needle was passed along the side of the mouth. Following the roof of the mouth, the needle was advanced into the esophagus and toward the stomach. If resistance was encountered, needle position was altered, not entering the trachea. After the needle was passed to the correct length, the compound could be injected.

iCBR-FMUC/CIBB has a certified maintenance vivarium and has the authorization for breading some rat and mouse lineages, by a specialized staff. The animals were bread and maintained in a controlled environment (system of dedicated racks at 24°C, purified air, relative humidity of the air maintained between 40-60%, controlled light cycles and noise reduction). The animals were kept in disinfected cages (washed and dried with dedicated equipment, using non-foaming detergents and disinfectants) with a bed of sterilized corn cob, sterilized playful material and sterilized food.

The cages, all identified with a label containing indications regarding the lineage, the genus of the mice, the number of mice/cage and the date of birth, were placed in the racks connected to a

system of filtration of incoming and outgoing air in the referred certified installations. The solid food pellets, with the proper nutrients, was sterilized and the water acidified (pH 3-4) and made available through disinfected bottles with metal heads.

The tumor cells to be injected were cultured *in vitro* following the previously described culture protocol, as well as the following steps for the enzymatic detachment, centrifugation and vital staining with blue trypan for counting in the Neubauer chamber.

A non-orthotopic xenograft model was used and the cells were injected subcutaneously into the animal's right thigh.

The cells, after being detached and centrifuged, were aspirated with a 19G needle, 1,1x25 mm (T Terumo™ Neolus, NN-1925R, Belgium) coupled to a 1 mL syringe (T Terumo™ Syringe, U-100 insulin, SS + 01H1, Belgium), duly identified and in ice, in order to prevent cell degradation.

The subcutaneous injection into the mice was carried out with the same syringe, coupled with a 25G needle (BD Microlance^{TM}, 25G$^{5/8''}$, 0.5x16 mm, 300 600, Spain). This inoculation process took place inside a laminar flow chamber used for animal handling (Heraeus Holten, HBB 2448, USA) previously sterilized with ultraviolet light and cleaned with Virkon (Virkon S, 5 kg, A01576789, UK) and alcohol 75 % in accordance with the law in force [Law Decree No. 113/2013, of 7 August - national legislation transposing the European Directive 63/2010 / EC and duly authorized by the General Directorate of Veterinary Medicine and in agreement with the Agency for Animal Welfare (Órgão Responsável pelo Bem-Estar Animal - ORBEA - 17/2015)] (**Annexes I and II**).

For the subcutaneous injection, the animal was placed supine and the injection was made with extreme care so as not to reach the muscle fascia, not causing unnecessary pain to the animal and attaining a blood vessel. After the injection, the animal was put back in its cage.

The animals were monitored weekly to determine their weight (duly recorded in a notebook), using an animal scale (Seca, model 734, series 1/1, Germany), and to check if there were any alterations in the area of inoculation or somewhere else (conjunctivitis, wounds, etc.). As soon as a tumor mass was detected visually and by palpation, a photographic recording was made and the mass was measured (width and height) with a caliper (Wurth, stainless hardened, Germany). These measurements were performed weekly in the same controlled environment and by the same investigators.

3.5.1 WiDr cell line

In this study, Balb/c nude mice, about one month and a half old, were used. Male mice were injected with 3-4x10^6 WiDr cells, resuspended in 0.2 mL of culture medium. For this study, 15

Balb/c nude mice were used. The animals started taking EGCG enriched solutions when the tumor reached the size of 1 mm^2.

The animals were divided into two groups:

- group 1: animals injected with WiDr tumor cells, drinking water (control),

- group 2: animals injected with WiDr tumor cells, drinking water + GTEE extract by gavage once a day.

The occision of the animals was programmed when the tumor reached the maximum regulated proportions and performed by anesthesia overdose, having met all the requirements of the legislation in force [201].

3.5.2 MCF-7 cell line

Following the *in vitro* studies previously performed by other students [178], female nude mice were injected with MCF-7 cells (cultured, detached counted and resuspended according to a similar protocol as for the WiDr cells). The significant alteration is the 5-6x10^6 MCF-7 cells were injected sc. The MCF-7 cell line forms tumors in animal models only in the presence of estrogen. This administration/dosage procedure was carried out in accordance with the information gathered in the bibliographic review [202,203]. Thus, one week after the subcutaneous inoculation of MCF-7 tumor cells, a suspension of 2 mg of estradiol (Sigma, E8750, USA) was prepared, in 1 mL of sesame oil (Sigma, S3547, USA), previously filtered with 0.2 µm filters to an eppendorf in sterile conditions (inside the laminar flow chamber used to prepare cell suspensions). After vortexing to reach homogenization (Unimag ZX3 Vortex Mixer, D-82152, Germany), 0.3 mL of the solution were withdrawn with a 1 mL syringe coupled to a 19G needle. The s.c. administration was done with a 25G syringe, just like cell inoculation, but in the right upper quadrant of the dorsum of the animal.

The animals were controlled in the exact same way as for the WiDr protocol. Animals were divided in two groups:

- group 3: animals injected with MCF-7 tumor cells, drinking water (control),
- group 4: animals injected with MCF-7 tumor cells, drinking water + GTEE extract by gavage once a day.

3.6 Positron Emission Tomography – easyPET-3D

Positron emission tomography (PET) is a nuclear medicine imaging technique, used for clinical diagnostics, for example in early stage cancer detection, but also in preclinical studies, in order to develop and test new diagnostic and therapeutic agents, prior to clinical trials. It allows to obtain

information on the functional stage of the organs, detecting the positrons emitted by the radionuclide used [204].

The PET technique is based upon the detection of the radioactivity emitted by the organs, after the injection of a small amount of a radioactive tracer into the subject to be analyzed. A radiopharmaceutical is a substance composed by a radioactive atom linked to a chemical substance. The radiopharmaceutical will be taken up by the tissue/organ during its metabolic processes. The nucleus of the radioactive atom emits a positron, which collides with an electron in the tissue and in this process mass is converted into energy, in the form of two photons, with an energy of 511 keV each. The conventional PET system uses scintillation crystals positioned around the subject to detect the photons: crystals absorb photons and produce light which is then converted into an electrical signal (**Figure 29**) [204-206].

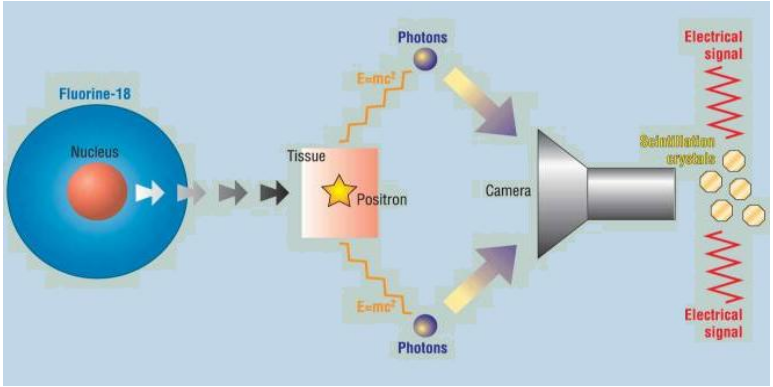


Figure 29: Scheme of the process that allows the generation of images (adapted from [204]).

The generated photons have opposite directions and they are detected by collinearly aligned detectors pairs, in coincidence. The pairs of detectors of a PET conventional system are built-in in a ring-like pattern. Only coincident events between the rings are acquired and generate valid data.

Hence, the obtained image is given by the location of the positron emission from the radionuclide absorbed by the subject's organs. Thanks to a suitable software based on specific reconstruction algorithms, it is possible to reconstruct an image of the emitting zones, starting from the energies and the directions of each pair of gamma rays, with the help of a computer, generating three-dimensional images [204-206].

By identifying body changes at the cellular level, PET may detect the early onset of disease before it is evident on other imaging tests, showing the function, but not anatomical details. For this purpose, hybrid systems were developed: PET + CT or PET + MRI.

Preclinical micro-PET systems play a key role in the evaluation of new drugs and therapies, as well as in the study of the biological origin of several human diseases through the imaging of

appropriate animal models. However, a pre-clinical micro-PET system costs between € 0.6 and € 1.2 M, limiting the access to research centers in the fields of nuclear medicine, medical imaging or radiopharmacy. This situation makes PET systems of this kind completely inaccessible to be purchased by universities or health schools.

The equipment used in this project is called easyPET-3D and it was developed by a collaborator team of the University of Aveiro (CENTRO-01-0247-FEDER-017823, SII & DT 17823; PTDC/bbb-img/4909/2014) (**Figure 30**).

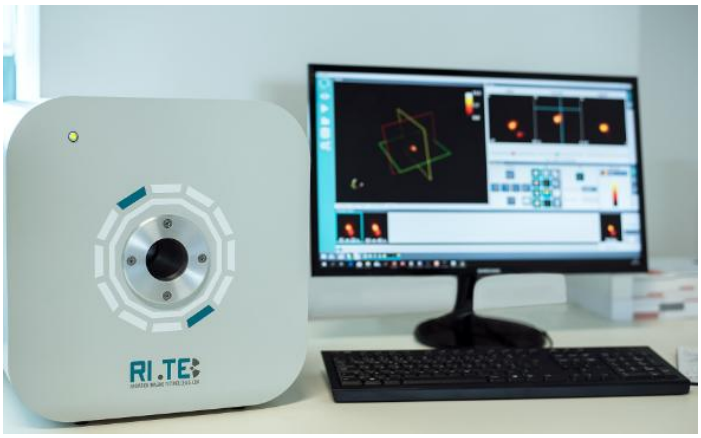


Figure 30: The easyPET-3D system.

The easyPET-3D technology is a new concept of PET, based upon an innovative PET image acquisition method, using only 2 sets of detectors that rotate intelligently according two axes, covering the same field of view of conventional PET systems - which use a complete detector ring, performing a full 2D image similar to the one obtained with a ring scanner (**Figure 31**).

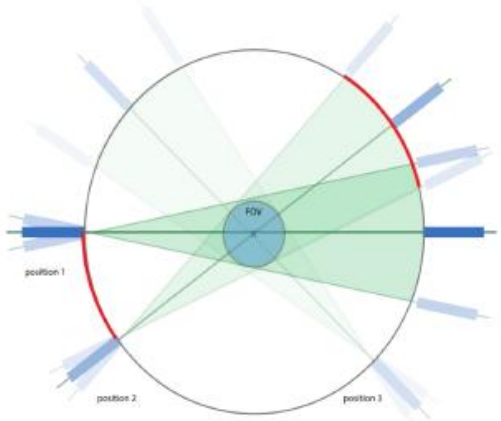


Figure 31: EasyPET-3D working principle scheme (retrieved from [207]).

In each position the scanner acquires a fanbeam projection: a fan rotation is used to cover the entire field of view (FoV). When a coincidence is detected between the two scintillation detectors, the line-of-response (LOR) is the line between the two front faces of the scintillation detectors.

The innovative method of image acquisition dramatically reduces the complexity and costs of production, allowing democratization of access and use of PET technology. The developed system has a completely innovative architecture, with a reduced number of components and, therefore, it is much simpler and economical. Simultaneously it has a high performance, particularly in terms of spatial resolution and uniformity, due to the intrinsic capacity of the method to eliminate uncertainty in the position associated with depth of interaction (DOI). The easyPET-3D, with a single pair of collinear crystals, can not detect the obliquely incident photons nor the photons undergoing Compton scattering in neighboring crystals. As a result, a good imaging capability without aberration effects and a high spatial resolution are expected. In addition, the original implementation of the acquisition method based upon two degrees of freedom ensures a uniform spatial resolution over all the FoV without the need to measure the DOI. One potential fragility of this concept is the reduced geometrical acceptance due to the use of only two detectors, which limits the slice sensitivity of the system. The basic idea is to exploit the absence of effects degrading the image quality to partially recover the events lost because of the system geometry [207-210].

The projected solution will enable the use of this PET system in pre-clinical environments, since due to its lower cost it will become accessible to a much wider range of institutions. The easyPET-3D allows an improvement of the educational offer for universities and health schools, by opening the possibility of teaching/learning how to perform the PET imaging. Pre-clinical tests using radioactive biomarkers in small animals (*in vivo*), in a controlled environment for image acquisition, can then be applied in very different areas of medicine using the easyPET-3D system developed by the team, especially in oncology and neuroimaging studies.

The radionuclide used in this work was the fluorodeoxyglucose (FDG), labelled with Fluoro-18 (^{18}F), a glucose analogue, which allows studying the metabolism of organs and tissues. The ^{18}F has a half-life of about 2 hours (109.8 min). Glucose is a simple sugar and it is used by the cells as the main source of energy. Considering that tumor cells have an altered metabolism and use an enormous amount of glucose to support their rapid expansion and proliferation, the FDG, being a glucose analogue, is accumulated in the tumor site, allowing its detection and visualization thanks to the PET system [211-213].

For the acquisition of the images a specific protocol was optimized by the team. The activity was measured and injected into the animal, and an hour (time required for the uptake of the radionuclide), the animal was anesthetized with a mixture of ketamine (Cetamine Hikama 50 mg/mL, Hikma PharmaceuticalsTM, Portugal) and chlorpromazine (Largactil, 5 mg/mL, Laboratórios Vitória, Portugal) in a proportion of 1:3. Ketamine is a medicine used primarily to induce and maintain anesthesia, acting as an inhibitor of NMDA activity (the main receptor responsible for measuring

the excitatory response of the central nervous system), which in turn is responsible for the increase in glutamate activity (one of the main neurotransmitters in the human body). Chlorpromazine, on the other hand, acts as a relaxant and tranquilizer without causing sedation, blocking the impulses generated by dopamine in the synapses.

For mice, 0.1-0.3 mL of anesthetic mixture diluted in equal parts (1:1) with saline were administered intraperitoneally (i.p.). After anesthesia, the animals were placed on the bed of the equipment to perform the study and were kept at a constant temperature of about 37°C, thanks to a water heating pump (Adroit Medical System, HTP-1500, USA) coupled to the easyPET-3D equipment. This pump allowed to maintain constant the temperature of the anesthetized animal that, otherwise, would suffer a body temperature lowering. The used animals are nude, hence the increased probability to body cooling which would implicate muscle contraction and shivering, situations that must be avoided for a good imaging acquisition.

The ^{18}F-FDG (ICNAS, IBA Cyclone, Portugal) was administered to the animals via intra-peritoneal (i.p.). Following this procedure, it was internalized by the cells through the usual glucose transporter, and it was not metabolized inside the cells, but converted into a form which was kept inside. Therefore, ^{18}F-FDG can be used to detect cells with high glucose consumption and consequently contain a large number of membrane transporters, as happens in fast-growing tumor cells, allowing the display of the tumor mass induced in the animals.

The radioactivity was transported from the hospital to iCBR using a protected lead case and all manipulations of the radioactivity took place in a special chamber, behind a lead protected shield. The ^{18}F-FDG dose to be injected in nude mice, for a single acquisition, was between 220 and 400 µCi and it was measured with a dose calibrator (CRC-15 W, Capintec, USA) in a 1 mL syringe (T Terumo™ Syringe, U-100 insulin, SS + 01H1, Belgium) coupled with a 25G needle (BD Microlance™, 25G, 5x8 "; 0.5x16 mm, 300600, Ireland), in accordance with the safety regulations for handling radioactive substances (Protector Shield Biodex, USA).

Not only the tumor, but also other organs, such as the bladder (which is an excretory organ), the heart and the brain are clearly identified with a PET system. Areas with inflammation also show a high glucose activity due to the increase in metabolism of the injured areas.

3.7 Histological tests

The histological technique consists of a set of steps to prepare a tissue for microscopic analysis, either for scientific research or pathological diagnosis. Procedures include material

collection, fixation, processing, inclusion, microtomy, and staining. In the case of calcified tissues, after fixation it is necessary to decalcify the tissue.

The collected samples were placed in cassettes (PrintMate ™ Embedding Cassette, A84810048) and, immediately afterwards, the procedure began, avoiding tissue degradation due to the action of the enzymes present in the cells. The first step was fixation: the cassettes were placed into a container with 10% buffered neutral formalin fixative which penetrates the tissue replacing all tissue water, thus preserving the sample.

After fixation, the sample was processed in order to impregnate the tissue in a solid and firm medium, allowing to obtain thin sections that could be stained and viewed under the optical microscope. The processing consisted of several steps. The first step was tissue dehydration: ethanol solutions of increasing concentration replaced water and fixative in the tissue. After this step, tissue was diaphanized: material was clarified, making it translucent, by removing ethanol and replacing it with a miscible fluid with an impregnation medium, the most commonly used xylene. In the impregnation process, the diaphanizing agent was replaced by the impregnating medium. At this stage, the samples were infiltrated by a substance with firm consistency (liquid paraffin), in order to acquire sufficient rigidity. At the end of the impregnation, a block containing the tissue was obtained, and was cut using microtome.

The cut material was then placed on the glass slide for drying and adhesion, and goes through processes that allowed to seal the cut.

The cuts coloring allowed to highlight the tissue components or to distinguish between different tissue structures. The staining process was hematoxylin-eosin, which is a simple technique allowing the differentiation of a large number of tissue structures. Hematoxylin is a basic dye that stains in dark blue/purple color the acidic components of the cell (such as DNA and RNA), staining the nucleus and ribosome-rich cytoplasm regions, while eosin is an acid dye that stains in pink color the basic components of the cell (such as cytoplasm and connective tissue fibers). To allow staining process, since the dyes are watery, deparaffinization and hydration of the cuts were performed.

After staining, the slides were dehydrated again and the procedure was repeated until the diaphanous agent. In order to protect the cut samples, the glass slides were covered with a coverslip glued with synthetic glue which, after drying, could be analyzed by an optical microscope. As it was coupled to a computerized system with an adequate software, the processing and recording of the obtained images in digital media was performed.

3.8 Statistic analysis

In order to compare between groups/conditions, the non-parametric Kruskal-Wallis test was used, due to the lack of Gaussian distributions. In Statistics, the Kruskal-Wallis test is a non-parametric test that does not assume that the populations follow Gaussian distributions, but it assumes that the shapes of the distributions are identical. It compares three or more unpaired or unmatched groups, aiming to verify if these groups come from the same population (or from populations with equal median). If the *p* value is low, the population medians are different. The null hypothesis says that “the distribution of MTT is the same across categories of substances”.

The aim of this test is to evaluate the differences in the values resulting from MTT. In order to assess the efficacy on cell viability of the different green tea extracts, adequate comparisons were performed with the respective controls of these tests. All *p* values were obtained using SPSS® *Statistics*, version 23 (*IBM Corporation, Armonk*, USA), and GraphPad Prism software, version 5, for the analysis of cytotoxicity data, analyzing the results with a significance level of 5%.

Results are presented using diagrams of extreme and quartile.

Chapter 4

4. Results and Discussion

This chapter presents and describes the results obtained using the methods described above and their discussion.

4.1 *In vitro* studies

4.1.1 MTT test

Several cell viability studies were performed to test the cytotoxicity of EGCG-enriched solutions for the WiDr tumor cell line, of the drugs used in colorectal cancer therapy (isolated and in some combination regimens) and of EGCG solutions co-administered with some of these drugs in the same therapeutic regimens. The specificity of EGCG for tumor cells has been confirmed in a previous study comparing to the normal cell line of CCD colon CCD 841 CoN [216]. Below there are the diagrams of the extremes and quartiles obtained for the MTT tests (tests carried out with 3 applications of EGCG every 24 h and a single pharmacological application on the first day; all carried out in 96-well plates with $2.4x10^3$ cells/well and DMEM to reach a volume of 150 µL).

4.1.2 WiDr cell line

4.1.2.1 Tea extracts

The green tea extracts enriched with EGCG used in the following *in vitro* experiments were: Green Tea EGCG Extreme (GTEE) capsules, and Mega Green Tea Extract (MGTE) and Green Tea Extract powder. The concentrations used for all 3 extracts were 0.5 µg/mL and 5 µg/mL. Those concentrations were selected in conclusion to another study in which 0.01, 0.1, 0.5, 1, 5, 10 µg/ml concentrations were tested [193].

The first tests were carried out considering these tea extracts. The graphs below, on the left (**Figure 32A**), show the percentage of cell viability in the presence of the extract compared with the control (which is considered with 100% viability) and on the right (**Figure 32B**) the comparison of the MTT values for the different extracts to the control. Considering the results provided by the

Kruskal-Wallis statistical test, the MGTE and GTE extracts show statistical significance comparing with control: the cell viability with the tea extracts is significantly lower than the one for the control, corresponding to the tumor cells only with culture medium (no other compounds). The GTEE extract, on the other hand, does not show statistical significance according to the Kruskal-Wallis test: the cell viability, in this case, is not much lower than the control. Everything can be observed in the following figure (**Figure 32**).

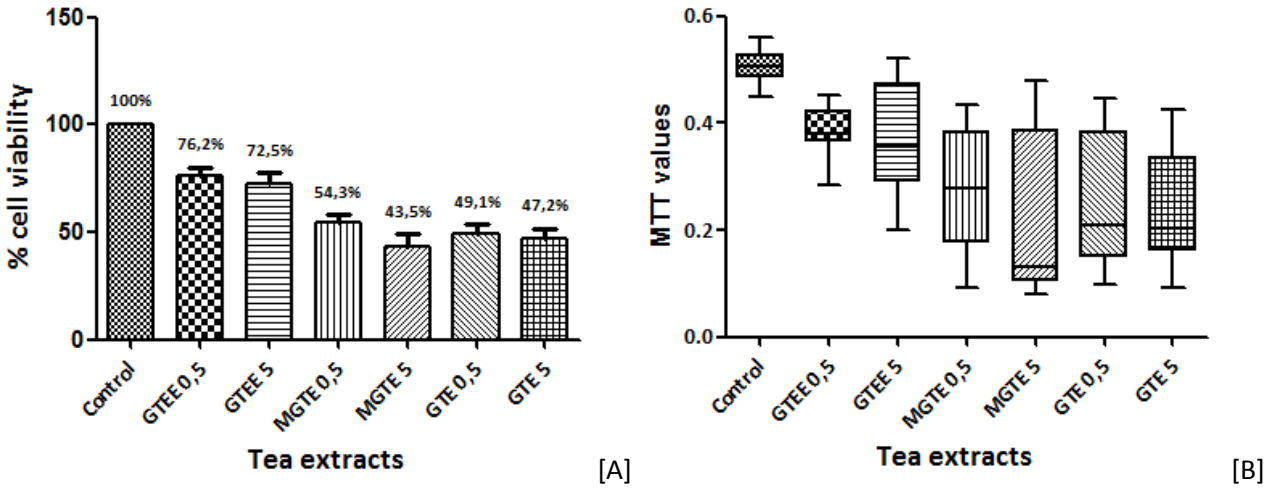


Figure 32: Graphs highlighting the percentage of cell viability [**A**] and the extremes and quartiles diagram [**B**] for MTT tests: controls and tea extracts (GTEE, MGTE, GTE) with 0,5 µg/mL and 5 µg/mL concentrations.

4.1.2.2 Capecitabine (CAP) (control *vs* drug *vs* drug+EGCG)

CAP monotherapy at a dosage of 350 ng/well does not induce a very marked decrease in cell viability compared to controls. However, when the drug was combined with an EGCG concentration [GTEE ([EGCG] = 0.5 and 5 µg/mL), MGTE ([EGCG] = 0.5 and 5 µg/mL), GTE ([EGCG] = 0.5 and 5 µg/mL)] a marked decline in cell viability, compared with controls and also to monotherapy, can be seen. The differences turned out to be statistically significant especially for GTEE 0.5 µg/mL and for GTE 5 µg/mL (**Figure 34**).

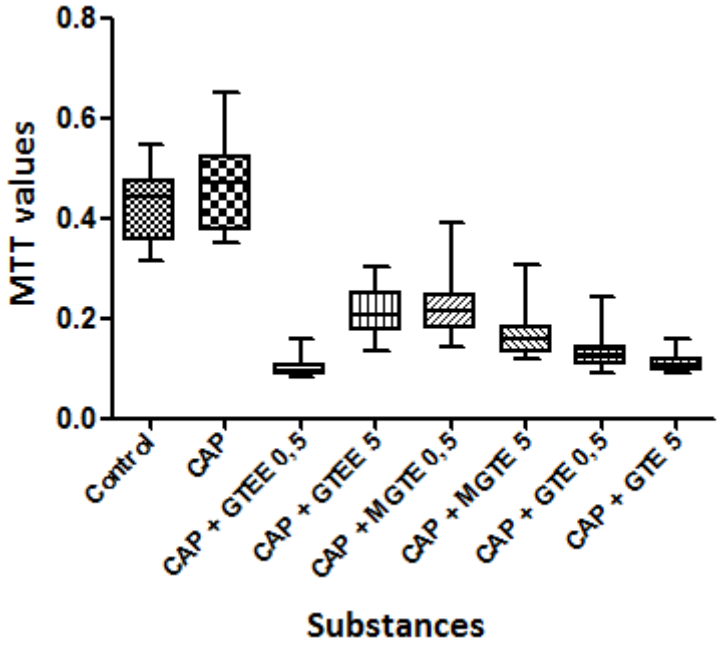


Figure 33: Extremes and quartiles diagrams for MTT tests: controls, CAP, CAP + GTEE 0.5 and 5 µg/mL, CAP + MGTE 0.5 and 5 µg/mL, CAP + GTE 0,5 and 5 µg/mL.

4.1.2.3 5-Fluoracil (5-FU) (control *vs* drug *vs* drug+EGCG)

Monotherapy performed with 5-FU at a dose of 112 ng/well induced a non-statistically significant reduction in cell viability compared with the control. The reduction, according to the Kruskal-Wallis test, became statistically significant when the tea extract were added GTEE ([EGCG] = 0.5 and 5 µg/mL), MGTE ([EGCG] = 0.5 and 5 µg/mL), GTE ([EGCG] = 0.5 and 5 µg/mL) compared with the control (**Figure 34A**). Furthermore, there was a statistically significant difference for the drug combined with GTEE ([EGCG] = 0.5 µg/mL) and GTE ([EGCG] = 0.5 and 5 µg/mL), compared to the monotherapy with this drug (**Figure 34B**). In the other cases the reduction of cell viability was not statistically significant if compared to the monotherapy drug.

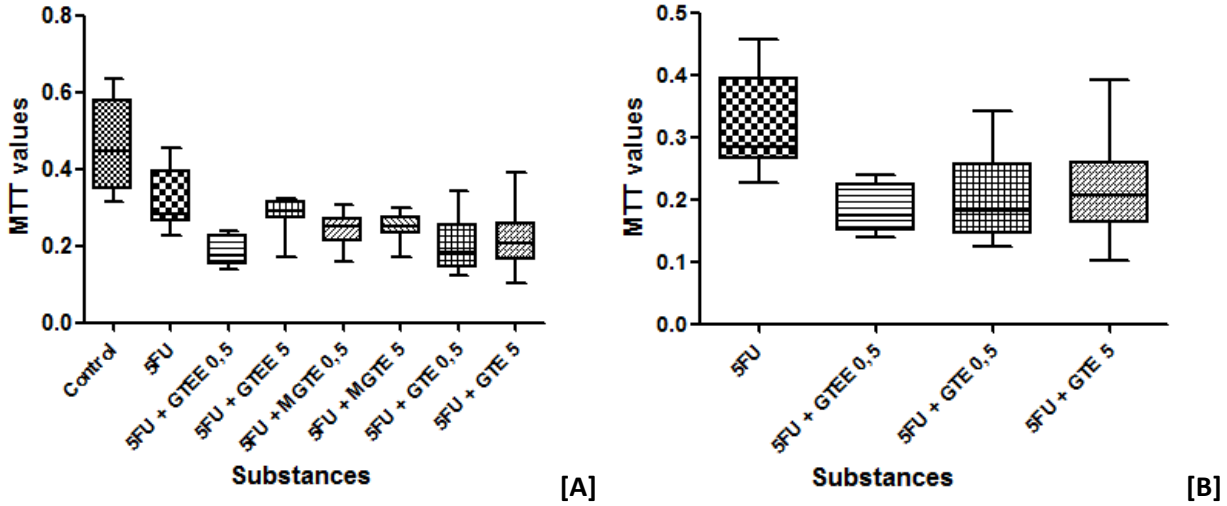


Figure 34: Extremes and quartiles diagrams for MTT tests: controls, 5-FU, 5-FU + GTEE 0.5 and 5 µg/mL, 5-FU + MGTE 0.5 and 5 µg/mL, 5-FU + GTE 0.5 and 5 µg/mL [**A**]; 5-FU + GTEE 0.5 µg/mL, 5-FU + GTE 0.5 and 5 µg/mL [**B**].

4.1.2.4 **Irinotecan (IRI)** (control *vs* drug *vs* drug+EGCG)

Monotherapy performed with IRI at a dosage of 98 ng/well induced a decrease in cell viability compared to controls. When GTEE ([EGCG] = 0.5 and 5 µg/mL), MGTE ([EGCG] = 0.5 and 5 µg/mL) or GTE ([EGCG] = 0.5 and 5 µg/mL) were added to the treatment, cell viability also decreased significantly in both cases compared to the control and to the drug alone. The Kruskal-Wallis test, in fact, showed a statistically significant difference in cell viability between control, monotherapy and combined monotherapy (**Figure 35A**) and between monotherapy and combined monotherapy (**Figure 35B**), without a particular preference among the brand of the extract.

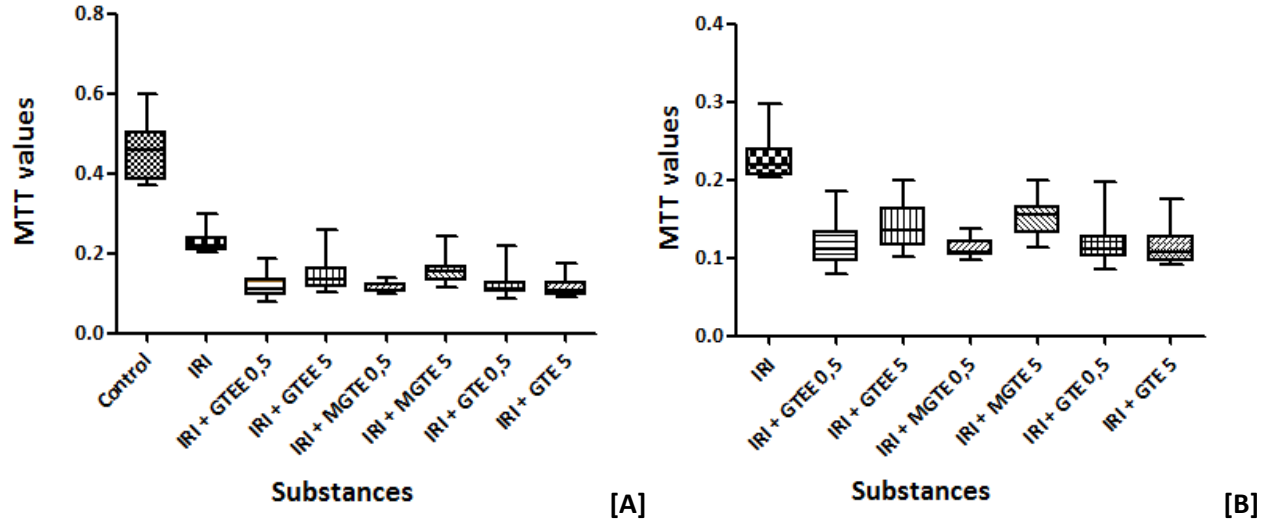


Figure 35: Extremes and quartiles diagrams for MTT tests: controls, IRI, IRI + GTEE 0.5 and 5 µg/mL, IRI + MGTE 0.5 and 5 µg/mL, IRI + GTE 0.5 and 5 µg/mL [**A**]; IRI, IRI + GTEE 0.5 and 5 µg/mL, IRI + MGTE 0.5 and 5 µg/mL, IRI + GTE 0.5 and 5 µg/mL [**B**].

4.1.2.5 Leucovorin (folinic acid) (FOL) (control *vs* drug *vs* drug+EGCG)

Monotherapy with FOL at a dose of 112 ng/well induces an increase in cell viability compared to the control. Combination therapies of this drug with GTEE ([EGCG] = 0.5 and 5 µg/mL), MGTE ([EGCG] = 0.5 and 5 µg/mL), GTE ([EGCG] = 0.5 and 5 µg/mL) have also been tested. For the lowest EGCG concentrations cell viability was significantly increased compared to controls (similar to monotherapy), but for the highest EGCG concentrations there was a slight decrease in the number of living cells (**Figure 36**). The fact that there is an increase and not a reduction in cell viability compared with the control is linked to the fact that this drug is used in therapy to reduce the toxic effects of other chemotherapy drugs and to preserve healthy cells.

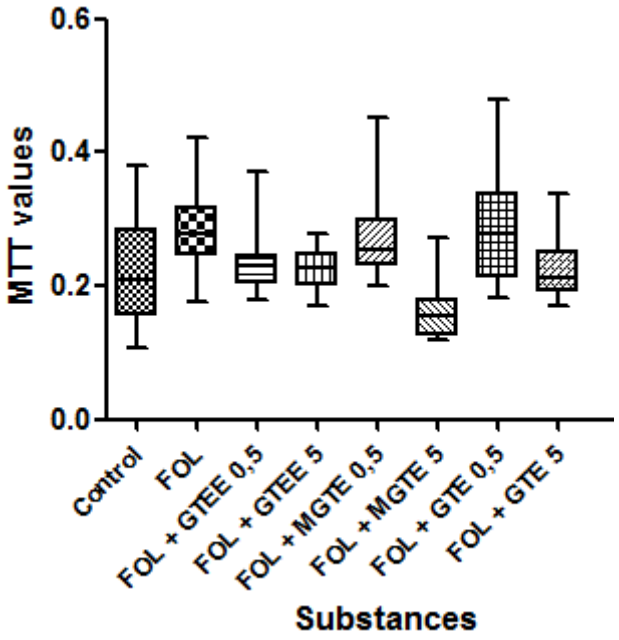


Figure 36: Extremes and quartiles diagrams for MTT tests: controls, FOL, FOL + GTEE 0.5 and 5 µg/mL, FOL + MGTE 0.5 and 5 µg/mL, FOL + GTE 0.5 and 5 µg/mL.

4.1.2.6 Oxaliplatin (OX) (control *vs* drug *vs* drug+EGCG)

OX monotherapy at a dose of 23.8 ng/well reduced cell viability compared to control, however, not very marked. It became higher when the compound GTEE ([EGCG] = 0.5 and 5 µg/mL), MGTE ([EGCG] = 0.5 and 5 µg/mL) or GTE ([EGCG] = 0.5 and 5 µg/mL) were added to the drug. MGTE and GTE combination therapy ([EGCG] = 5 µg/mL and 0.5 µg/mL), respectively, have not been shown to significantly reduce cell viability if compared to the drug alone, whereas GTEE ([EGCG] = 0.5 and 5 µg/mL), MGTE ([EGCG] = 0.5 µg/mL) and GTE ([EGCG] = 5 µg/mL) combination therapy produced a marked decay on cell viability when the same comparison were made (**Figure 37**).

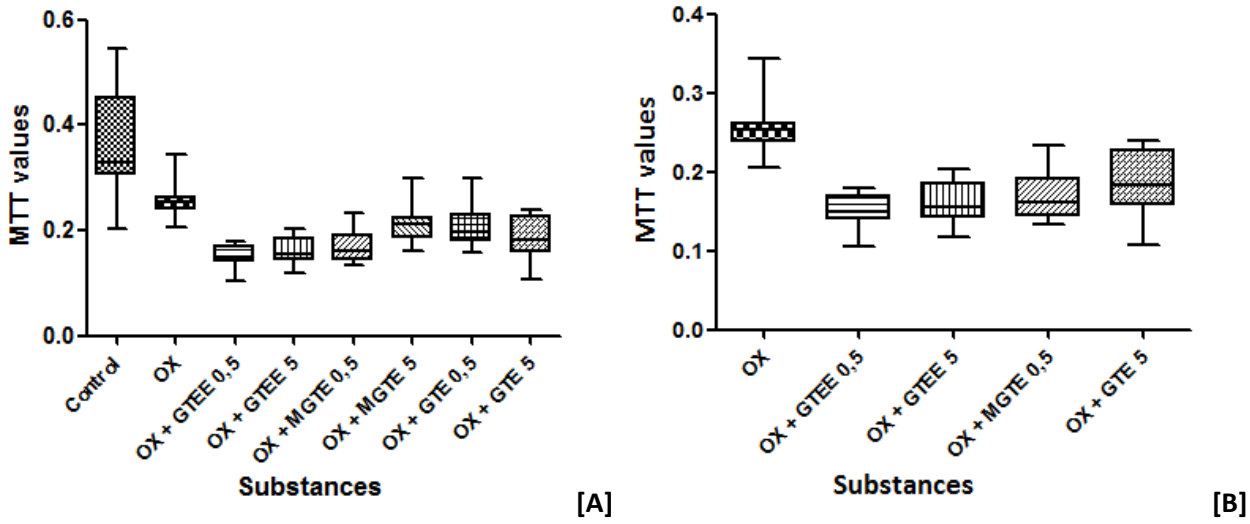


Figure 37: Extremes and quartiles diagrams for MTT tests: controls, OX, OX + GTEE 0.5 and 5 µg/mL, OX + MGTE 0.5 and 5 µg/mL, OX + GTE 0.5 and 5 µg/mL [**A**]; OX, OX + GTEE 0.5 and 5 µg/mL, OX + MGTE 0,5 µg/mL, OX + GTE 5 µg/mL [**B**].

4.1.2.7 Capecitabine + Oxaliplatin (CAPOX) combined regimen (control *vs* drug *vs* drug+EGCG)

CAPOX therapy at the studied doses (CAP = 560 ng/well, OX = 36.4 ng/well) reduced cell viability if compared to the control; however, the decrease in cell viability was not very marked (**Figure 38A**). It became larger when GTEE ([EGCG] = 0.5 and 5 µg/mL) or MGTE ([EGC G] = 0.5 µg/mL) were added to this combination of drugs (**Figure 38B**). On the other hand, GTE ([EGCG] = 0.5 and 5 µg/mL) and MGTE ([EGC G] = 5 µg/mL) combination therapy had not been shown to reduce viability compared to the combined regimen.

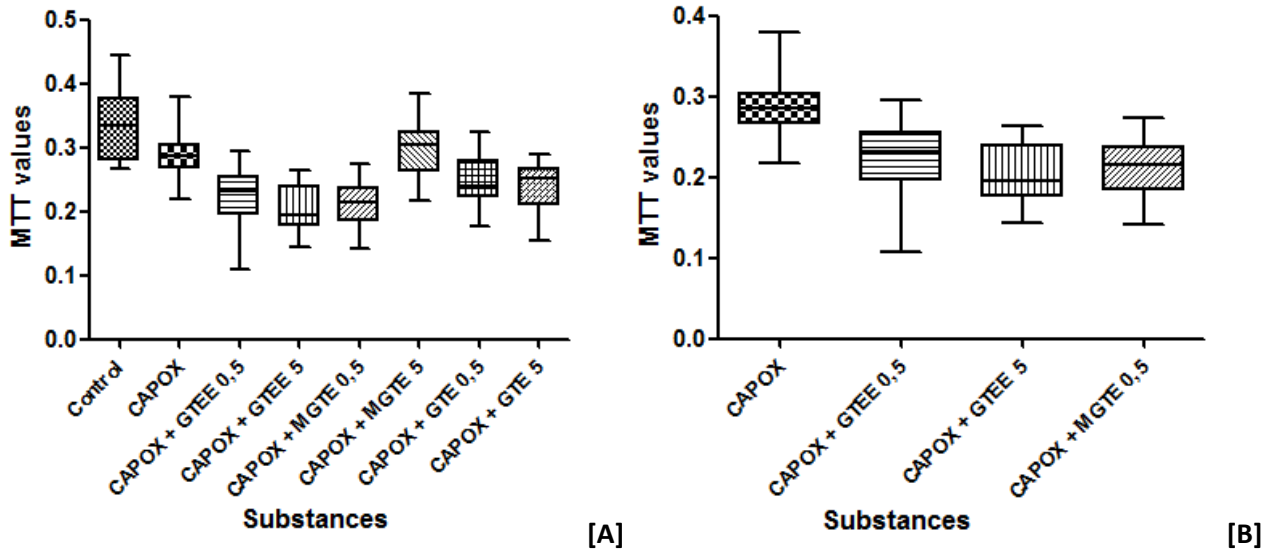


Figure 38: Extremes and quartiles diagram for MTT tests: controls, CAPOX, CAPOX + GTEE 0.5 and 5 µg/mL, CAPOX + MGTE 0.5 and 5 µg/mL, CAPOX + GTE 0.5 and 5 µg/mL [**A**]; CAPOX, CAPOX + GTEE 0.5 and 5 µg/mL, CAPOX + MGTE 0.5 µg/mL [**B**].

4.1.2.8 Capecitabine + Irinotecan combined regime (CAPIRI) (control *vs* drug *vs* drug+EGCG)

CAPIRI therapy at the studied dose (CAP = 560 ng/well, IRI = 70.0 ng/well) produced a decrease in cell viability comparable to the control. When GTEE ([EGCG] = 0.5 and 5 µg/mL), MGTE ([EGCG] = 0.5 and 5 µg/mL) or GTE ([EGCG] = 0.5 and 5 µg/mL) were added to the treatment, cell viability decreased more markedly than with the monotherapy (**Figure 39A**). In particular, the decrease in cell viability was greater with GTEE ([EGCG] = 0.5 and 5 µg/mL) or MGTE ([EGCG] = 0.5 µg/mL) combined with the chemotherapy drug. In fact, in these cases, the Kruskal-Wallis test showed a statistically significant difference in cell viability between control, therapy and combination therapy (**Figure 39B**).

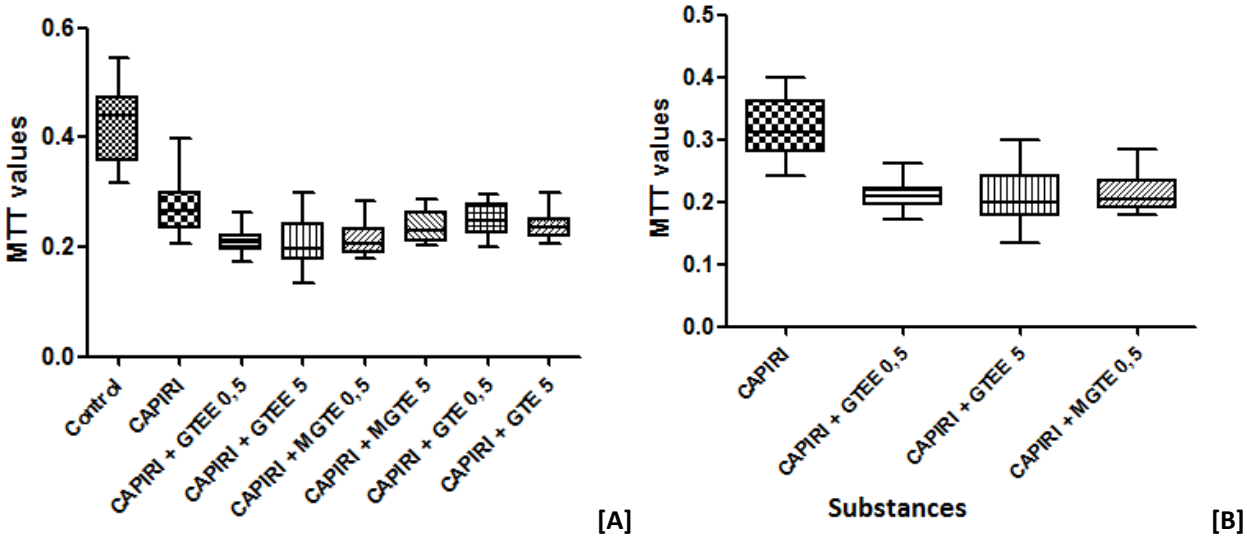


Figure 39: Extremes and quartiles diagrams for MTT tests: controls, CAPIRI, CAPIRI + GTEE 0.5 and 5 µg/mL, CAPIRI + MGTE 0.5 and 5 µg/mL, CAPIRI + GTE 0.5 and 5 µg/mL [**A**]; CAPIRI, CAPIRI + GTEE 0.5 and 5 µg/mL, CAPIRI + MGTE 0.5 µg/mL [**B**].

4.1.2.9 5-Fluorouracil + Leucovorin + Irinotecan combined regime (FOLFIRI) (control *vs* drug *vs* drug+EGCG)

Treatment with FOLFIRI at the studied dose (5-FU = 0.1 ng/well, FOL = 5.6 ng/well, IRI = 50.4 ng/well) induced a reduction in cell viability if compared with the control. When GTEE ([EGCG] = 0.5 and 5 µg/mL), MGTE ([EGCG] = 0.5 and 5 µg/mL) or GTE ([EGCG] = 0.5 and 5 µg/mL) were added to the treatment, cell viability decreased approximately similarly in all cases, and more markedly than the therapy only (**Figure 40A**). In particular, GTEE ([EGCG] = 5 µg/ml) and MGTE ([EGCG] = 0,5 µg/ml) were found to be the most effective in helping to reduce cell viability, as shown in **Figure 40B**, that highlights the percentages of cell viability compared to therapy alone, but anyhow, this greater reduction is not statistically significant.

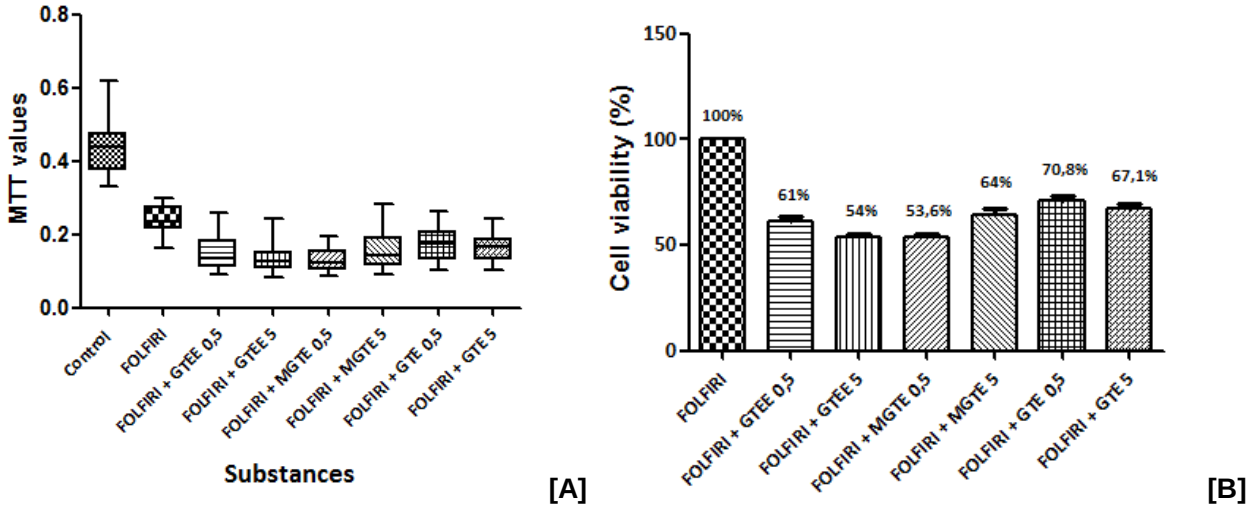


Figure 40: Extremes and quartiles diagrams for MTT tests: controls, FOLFIRI, FOLFIRI + GTEE 0.5 and 5 µg/mL, FOLFIRI + MGTE 0.5 and 5 µg/mL, FOLFIRI + GTE 0.5 and 5 µg/mL [**A**]; and graph that highlights the percentage of cell viability of the drug-extract combination compared to the drug alone [**B**].

4.1.2.10 5-Fluorouracil + Leucovorin + Oxaliplatin combined regime (FOLFOX) (control *vs* drug *vs* drug+EGCG)

Therapy performed with FOLFOX at the studied doses (FOL = 112 ng/well, 5-FU = 112 ng/well and OX = 23.8 ng/well) moderately reduced cell viability if compared with control. The addition of GTEE ([EGCG] = 0.5 and 5 µg/mL), MGTE ([EGCG] = 0.5 and 5 µg/mL) or GTE ([EGCG] = 0.5 and 5 µg/mL) to the therapy produced a decrease in cell viability approximately identical to the decrease produced by the drug alone. There were no statistically significant reductions in cell viability (**Figure 41**). GTEE ([EGCG] = 0.5 µg/mL) combination therapy reduced cell viability more than any other extract brands and concentrations, although this decrease was not statistically significant if compared to the decrease produced by the drug or by the drug combined with the other extracts.

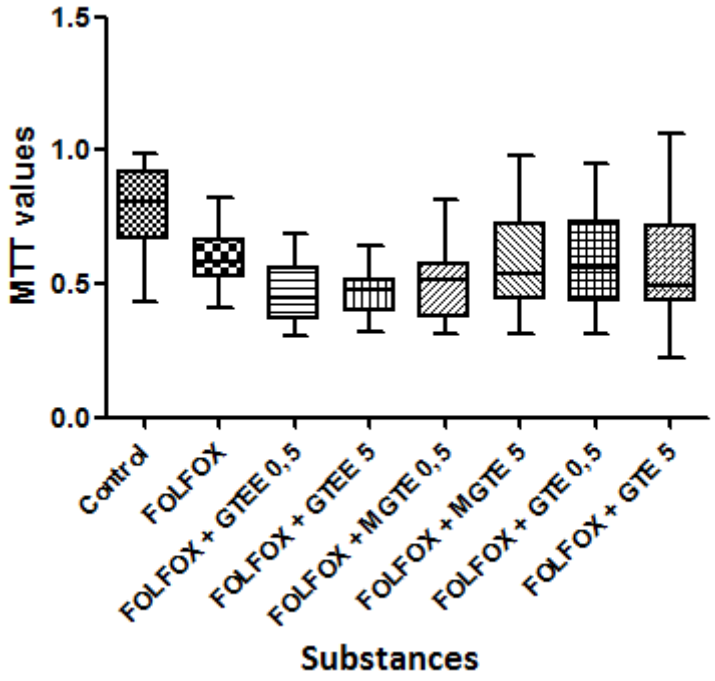


Figure 41: Extremes and quartiles diagrams for MTT tests: controls, FOLFOX, FOLFOX + GTEE 0.5 and 5 µg/mL, FOLFOX + MGTE 0.5 and 5 µg/mL, FOLFOX + GTE 0.5 and 5 µg/mL.

4.1.3 MCF-7 cell line

Due to the lack of new chemotherapy drugs for breast cancer to test, similar *in vitro* studies (done for WiDr) could not be performed for the MCF-7 cell line. The presented data, which were the basis for MCF-7 *in vivo* studies, have been coupled to the ones obtained in previous works.

Considering previous studies, for the MCF-7 cell line the number of applications giving statistically significant results was 6 applications (7 days).

4.1.3.1 Tea extract

The EGCG enriched green tea extract used in the following *in vitro* experiments was the Green Tea Extract (GTE) powder. The extract concentrations used were 0.5 µg/mL and 5 µg/mL.

The first tests were carried out with this tea extract. The graph below shows the comparison between the MTT values of the extract and the control. Considering the results provided by the Kruskal-Wallis statistical test, the GTE extract for a 5 µg/mL concentration showed statistical significance regarding the control ($p \leq 0.001$). For GTE extract with a 0.5 µg/mL concentration a $p \leq 0.01$ was determined. Cell viability with GTE 5 µg/mL is significantly lower than the control and than GTE 0.5 µg/mL (**Figure 42**).

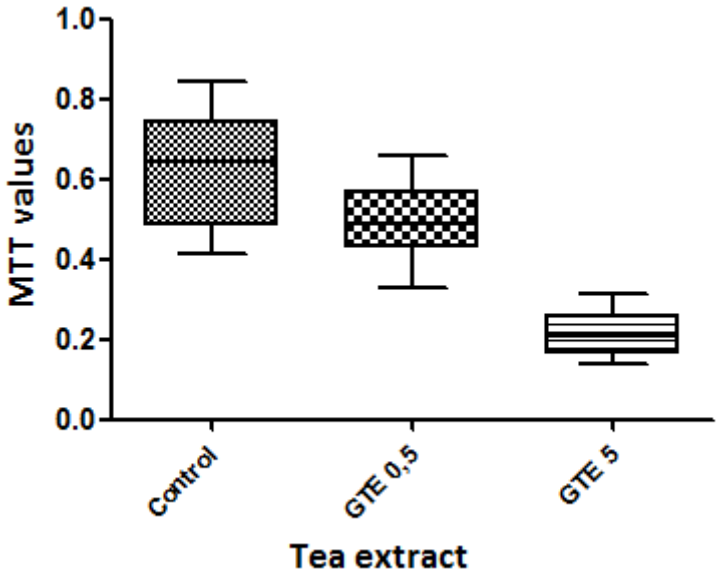


Figure 42: Extremes and quartiles diagram for MTT tests: controls, GTE 0.5 and 5 µg/mL.

4.1.3.1 Capecitabine (CAP) (control *vs* drug *vs* drug+EGCG)

Using monotherapy with this antimetabolite drug in a single application (1000 mg/m^2) for the 7-day incubation period, alone and in association with the GTE extract at different EGCG concentrations (0.5 and 5 µg/mL) were analyzed, for a total of 6 applications (7 days). Therefore, using the Kruskal-Wallis test, the control was compared with CAP and with CAP in association with the extract and then the drug in monotherapy was compared with the drug associated with the extract.

Statistical analysis showed a decrease in cell viability considering each substance compared to the control ($p \leq 0.001$) (**Figure 43A**). Furthermore, the association of CAP to GTE at both concentrations were more cytotoxic than CAP alone ($p \leq 0.01$), although the two associations showed no significant differences between them, thus having a similar effect ($p > 0.05$) (**Figure 43B**).

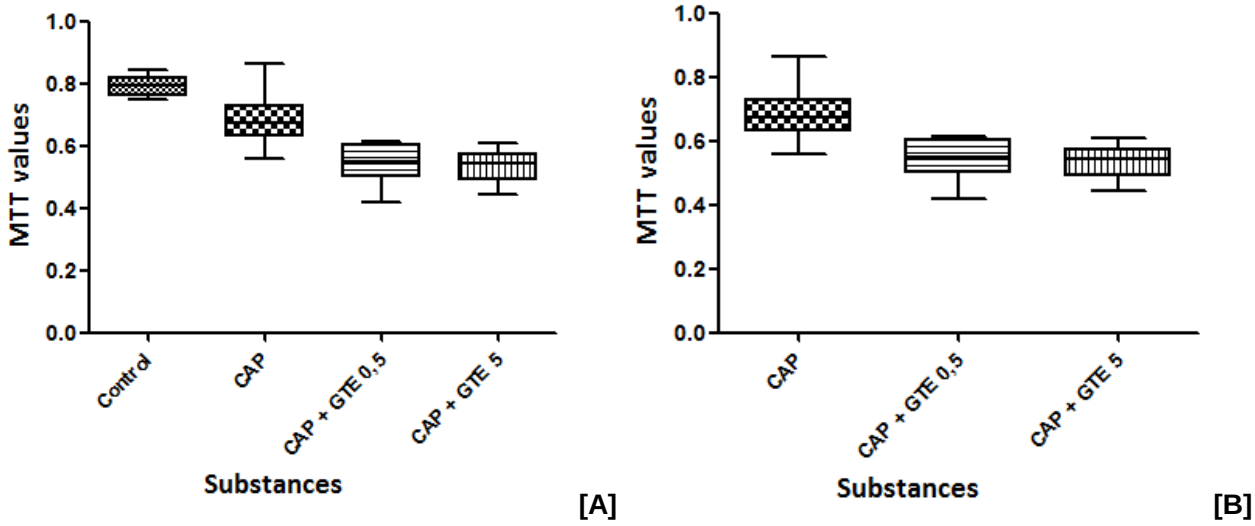


Figure 43: Extremes and quartiles diagrams for MTT tests: controls, CAP, CAP + GTE 0.5 and 5 µg/mL [**A**]; CAP, CAP + GTE 0.5 and 5 µg/mL [**B**].

4.1.3.2 Docetaxel (DOT) (control *vs* drug *vs* drug+EGCG)

Applying monotherapy with this taxane type of drug, performing a single application (60 mg/m^2) for the 7-day incubation period, its isolated action and its action in association with GTE extract at different EGCG concentrations (0.5 and 5 µg/mL) were analyzed. Therefore, using the Kruskal-Wallis test, comparison between groups was performed: the control was compared with the drug and with the combined therapy (DOT + extract) and then the drug in monotherapy was compared with the combined therapy.

The decrease in cell viability is statistically significant for all substances if compared with control ($p \leq 0.01$) (**Figure 44A**).

The associations DOT+GTE showed no significant differences from DOT alone ($p > 0.05$), although they appear to be more statistically effective. It will be necessary to increase the number of samples to see if differences arise.

Comparing the two associations DOT+GTE, a statistically significant difference was found ($p < 0.05$), being DOT+GTE 0.5 combination more cytotoxic than the one with the highest EGCG concentration, visually it also looks more cytotoxic than the others compared substances (**Figure 44B**).

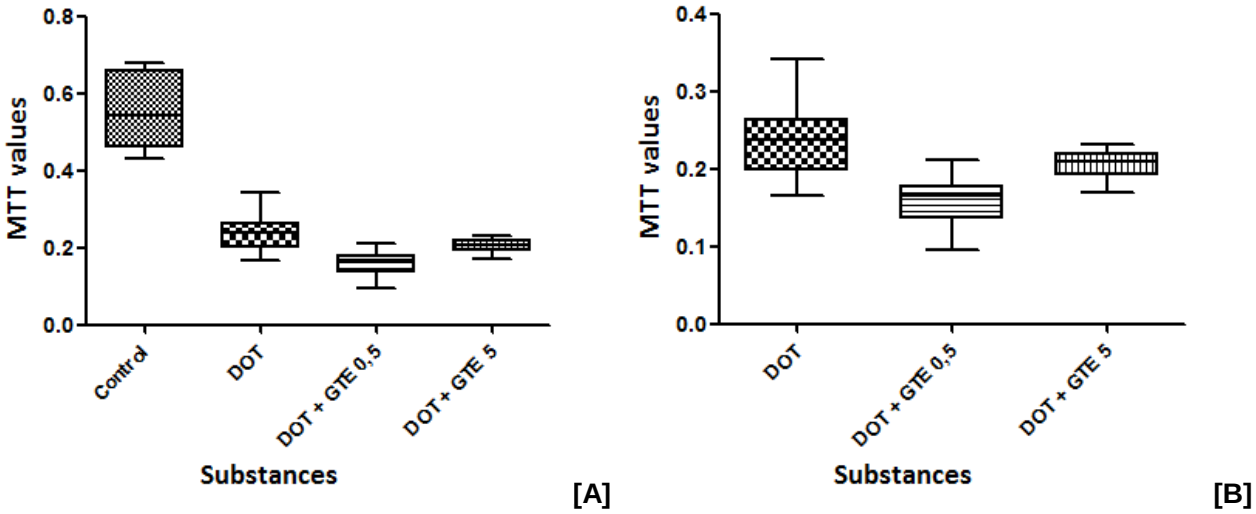


Figure 44: Extremes and quartiles diagram for MTT tests: controls, DOT, DOT+ GTE 0.5 and 5 µg/mL [**A**]; DOT, DOT + GTE 0.5 and 5 µg/mL [**B**].

4.1.3.3 Doxorrubicin (DOX) (control *vs* drug *vs* drug+EGCG)

Applying monotherapy with this anthracycline type of drug, for a single application (60 mg/m^2) for the 7-day incubation period, its isolated action and its action in association with GTE extract at different EGCG concentrations (0.5 and 5 µg/mL) were analyzed. Therefore, using the Kruskal-Wallis test, comparison groups were performed: the control was compared with the drug and with the combined therapy (DOX + extract) and then the drug in monotherapy was compared with the combined therapy.

The decrease in cell viability is statistically significant only for the comparison of substances with the control ($p \leq 0.01$) (**Figure 45A**).

For Doxorubicin, through statistical analysis, it was found there was no statistically significant difference between DOX and DOX associated with GTE ($p = 1.000$) as well as between DOX + GTE 0.5 and DOX + GTE 5 ($p = 0.273$) (**Figure 45B**). From the graph of the **Figure 45B** we can observe the combined therapy has increased the cytotoxicity only slightly compared to the DOX alone.

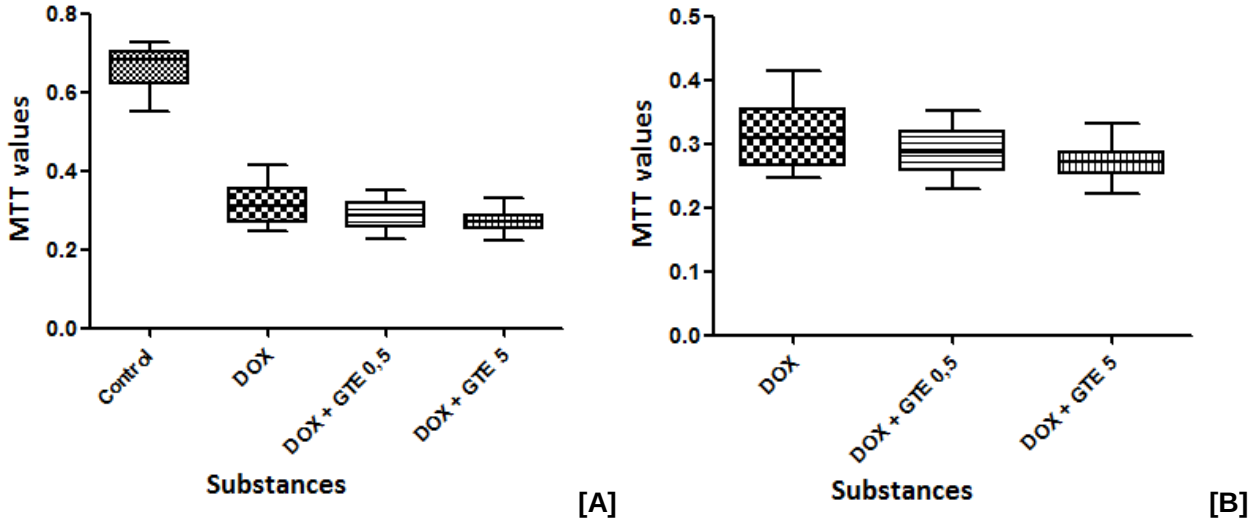


Figure 45: Extremes and quartiles diagram for MTT tests: controls, DOX, DOX + GTE 0.5 and 5 µg/mL [**A**]; DOX, DOX + GTE 0.5 and 5 µg/mL [**B**].

4.1.3.4 5-Fluoracil (5-FU) (control *vs* drug *vs* drug+EGCG)

Applying monotherapy with this anti-metabolite type of drug, performing a single application (500 mg/m^2) for the 7-day incubation period, its isolated action and its action in association with GTE extract at different EGCG concentrations (0.5 and 5 µg/mL) were analyzed. Therefore, using the Kruskal-Wallis test, comparison groups were performed: the control was compared with the drug and with the combined therapy (5-FU + extract) and then the drug in monotherapy was compared with the combined therapy.

The decrease in cell viability is statistically significant only when comparing substances with control ($p \leq 0.05$) (**Figure 46A**).

The association of 5-FU with GTE at different concentrations appear to be visually more effective than 5-FU alone, but the difference was not significant ($p > 0.5$) (**Figure 46B**). Maybe, a more significant response could be reached increasing the number of samples.

Comparing the two combinations, 5-FU + GTE 0.5 µg/mL and 5-FU + GTE 5 µg/mL, no significant differences were found ($p = 0.817$), being equally cytotoxic for the cell line.

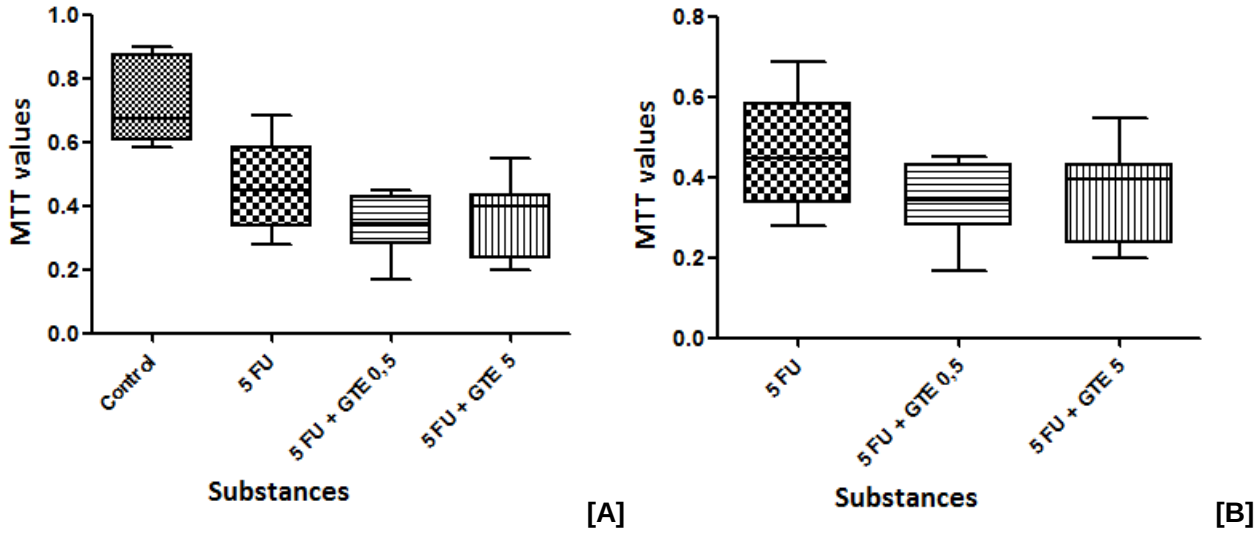


Figure 46: Extremes and quartiles diagram for MTT tests: controls, 5FU, 5FU + GTE 0.5 and 5 µg/mL [**A**]; 5FU, 5FU + GTE 0.5 and 5 µg/mL [**B**].

4.1.3.5 Paclitaxel (PAX) (control *vs* drug *vs* drug+EGCG)

Applying monotherapy with this taxane type of drug, performing a single application (175 mg/m^2) for the 7-day incubation period, its isolated action and its action in association with GTE extract at different EGCG concentrations (0.5 and 5 µg/mL) were studied. Therefore, using the Kruskal-Wallis test, comparison between groups was performed: the control was compared with the drug and with the combined therapy (PAX + extract) and the drug in monotherapy with the combined therapy.

The decrease in cell viability is statistically significant when comparing substances with the control ($p \leq 0.001$) (**Figure 47A**).

Paclitaxel assays showed that the PAX + GTE combination with EGCG, in both concentrations, showed higher cytotoxicity than PAX alone ($p \leq 0.01$), demonstrating that the combined therapy potentiated the cytotoxic effect of Paclitaxel, which in itself already had high cytotoxicity. Visually PAX + GTE 0.5 µg/mL combination and PAX + GTE 5 µg/mL combination showed the same cytotoxicity, in fact, no statistically significant differences were highlighted ($p > 0.05$) (**Figure 47B**). More tests will be needed to consolidate the results.

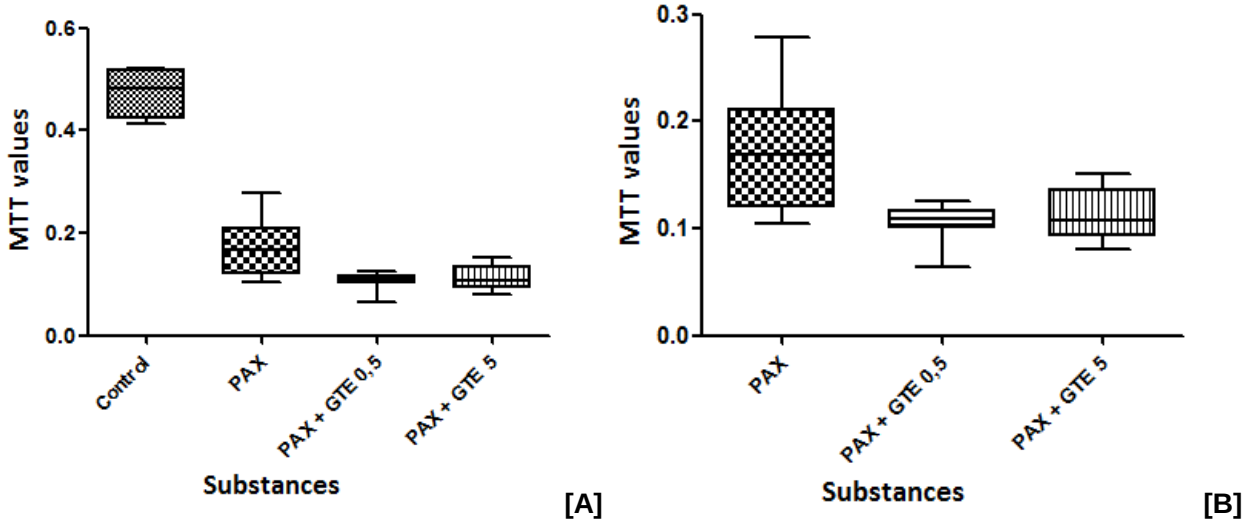


Figure 47: Extremes and quartiles diagram for MTT tests: controls, PAX, PAX + GTE 0.5 and 5 µg/mL [**A**]; PAX, PAX + GTE 0.5 and 5 µg/mL [**B**].

4.2 *in vivo* studies

4.2.1 Weight and tumor area monitoring of the animals in the study

As previously noted in Chapter 3, a colon cancer and a breast cancer cell line were injected into a non-orthotopic model: Balb/C nude mice (males for colon cancer and females for breast cancer). When the tumor developed and reached about 1 mm^2 size, the animals started the therapy with green tea extract. Animal weight and tumor size were monitored weekly. Two groups of animals were tested for both cell lines: an injected group drinking only water and an injected group drinking the tea extract. The animals weight control essentially helped to understand if the treatment was very intense, in fact in the literature it is described that the consumption of green tea could cause weight loss.

The graphs in **Figure 48** and **49** illustrate the variation concerning the tumor area.

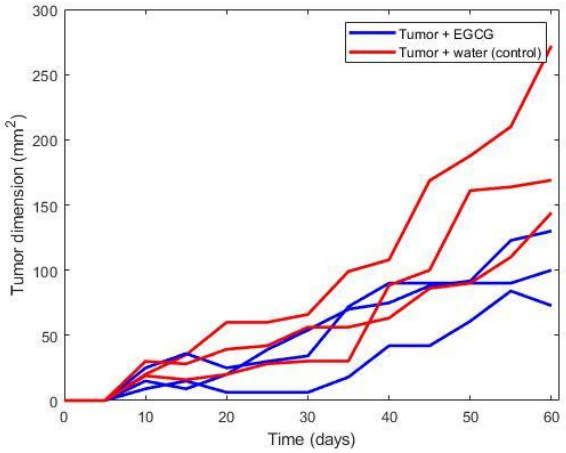


Figure 48: Trend of tumor size in animals injected with the WiDr cell line (groups 1-2).

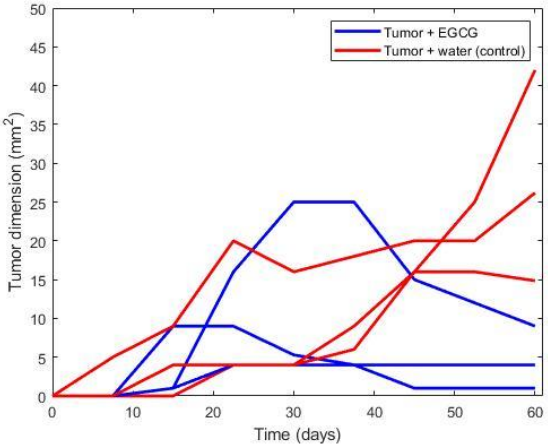


Figure 49: Trend of tumor size in animals injected With the MCF-7 cell line (groups 3-4).

Looking at these figures, it turns out that the tea extract therapy was effective and the tumor size was reduced if compared to the control (red lines). The tumor did not disappear, but its development slowed down.

The graphs in **Figure 50** and **51** illustrate the variation in animal weight.

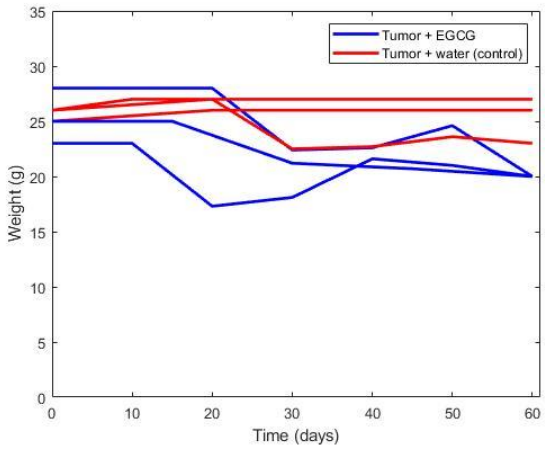


Figure 50: Trend of animal weight in animal injected with the WiDr cell line (groups 1-2).

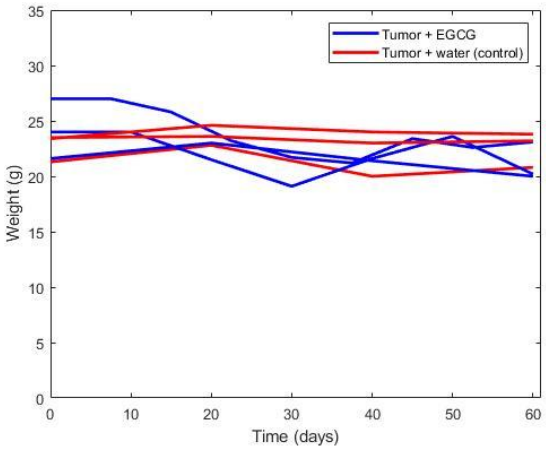


Figure 51: Tend of animal weight in animals injected with the MCF-7 cell line (groups 3-4).

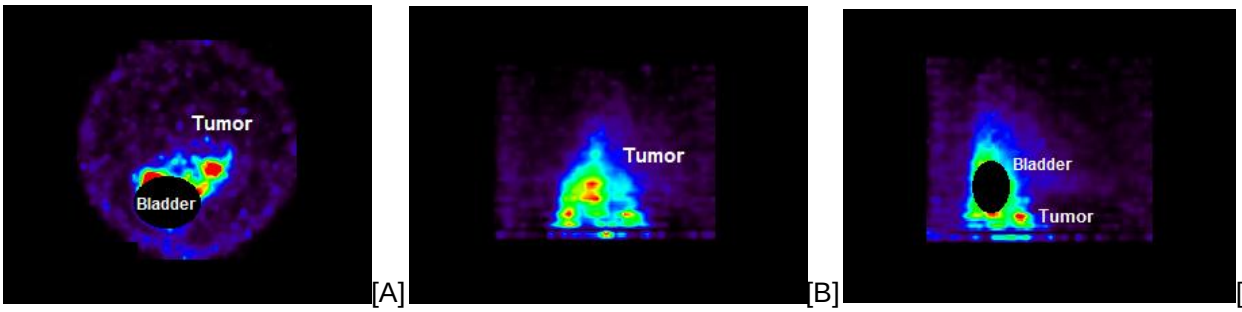
Considering the controls, the animals weight remained approximately constant for both cell lines. In treated mice, as shown in the literature, a reduction in weight was seen but this reduction was followed by a new increase in weight. In conclusion, there were no significant changes in animal weight from the start of treatment to necropsy.

4.2.3 Positron emission tomography (easyPET)

After the induction process of colon and breast cancer in the animal model (Balb/c nude mice), the tumors evolution was examined with the help of the easyPET-3D system, through the detection of ^{18}F-FDG radiopharmaceutical activity.

The aim was to perform PET acquisitions along the time of the experiment. Images of control animals have been performed applying a previously optimized protocol. Then each animal has been evaluated by imaging according to the data collected during the visual weekly observation. Another image has been acquired at key points of the animal model experiment: 1) before the tumor could be perceived externally perceiving, 2) during green tea “therapy”, and 3) post-“therapy”. This equipment was developed and tested by a team in collaboration with the University of Aveiro. The team has two funded projects: CENTRO-01-0247-FEDER-017823, SII and DT 17823; PTDC/bbb-img/4909/2014. The software is still being developed and optimized, but image acquisition, reconstruction and processing is already possible.

Animals have been studied using this micro PET system prototype but, as already mentioned, these animals (nude mice) are very sensitive and could easily undergo complications due to anesthesia, glucose reduction, lowering of body temperature, etc, hence every manipulation was performed with great care and attention. The image acquisition of this equipment (built with the technology designed by the team) has been very successful as it has allowed to detect the existence of very small tumors. Furthermore, it was also proven that tumors could be detected even when they were not yet visible externally. This means that it plays a very important role in the diagnosis and monitoring of tumor volume during therapy, in order to be able to evaluate its effects *in vivo*. The coupled software allowed to reconstruct and process the acquired images that can be shown in 3 planes: transverse, coronal and sagittal, as illustrated in the following **Figure 52**.



[A] [B] [C]

Figure 52: Example of easyPET-3D acquisition: transverse [**A**], coronal [**B**] and sagittal [**C**] plane.

This equipment was able to identify where the radionuclide was accumulated within the animal body and, in particular, which sites had the highest consumption of glucose. In the studied cases, the accumulation was highlighted over the dorsal area (as this is the point where the tumor was injected and developed), over the bladder (since ^{18}F-FDG has mainly by renal excretion), and

sometimes over the joints, if an inflammation (even though small) were present, showing an increase in glucose consumption similar to the other mentioned sites.

The presented images have been extrapolated using the Amide, which allows viewing the processed files. To highlight the tumor, the bladder area was obscured, when it was present in the image. The tumor images obtained were analyzed as follows: the region of interest containing the tumor was selected and the SUV was calculated. The results were then compared to evaluate the tumor progression.

The SUV ("standardized uptake value") is a dimensionless ratio used by nuclear medicine professionals to distinguish between "normal" and "abnormal" levels of uptake. Its largest use is in current clinical practice, being a relative measure of FDG uptake. The basic expression for SUV is

$$SUV = \frac{r}{\frac{a'}{w}}$$

where *r* is the radioactivity concentration measured by the PET scanner within a region of interest (ROI) previously identified, a' is the decay-corrected amount of injected FDG [µCi], and *w* is the weight of the patient [g], which is used for a distribution volume of tracer.

Hence, SUVs reflect the relative adsorption of FDG and higher SUVs are associated to greater regional metabolic activity. It may be useful as a measure to follow the metabolic activity of a tumor over time within the same patient and to compare different subjects within a research study under defined conditions.

For the following analysis, the SUV_Mean was considered. This value corresponds to the average value of all pixels inside the ROI, being an approximation of what would be the expected SUV in the tumor. SUVs > 1 are considered high values, corresponding to an abnormal glucose metabolism.

The following Figure (**Figure 53**) shows SUV means inside the ROIs enclosing the tumor. In this case, the acquisitions were performed when the tumors were not visible from the outside.

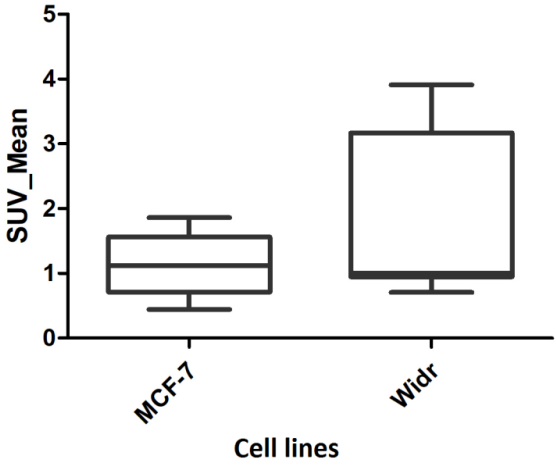


Figure 53: Extreme and quartile diagrams showing SUV mean values for MCF-7 and WiDr cell lines. In these mice tumor was not visible externally.

The results of **Figure 53** showed that the easyPET-3D system was able to detect the abnormal metabolism of tumor cells before the tumor mass was visible externally, by naked eye. This result is important because it shows the potential of this research tool. In fact, by identifying the tumor as soon as it develops (before causing visible anatomical changes), treatment can be started when the tumor is still in an early stage, contributing to a better success of the experiments. Furthermore, comparing the two tumor cell lines it is showed that MCF-7 takes longer to develop (lower SUVs in the same time interval), while the WiDr cell line is able to reach high SUVs (ex. SUV = 4) in shorter time (less than a month), proving that colon cancer is more aggressive than breast cancer in this animal species (Balb/c nude mice).

Figures 54,**55** show the mean SUVs over time, extrapolated from Amide, for control animals (injected with cancer cells, not receiving the treatment) and treated animals, for both cancer cell lines.

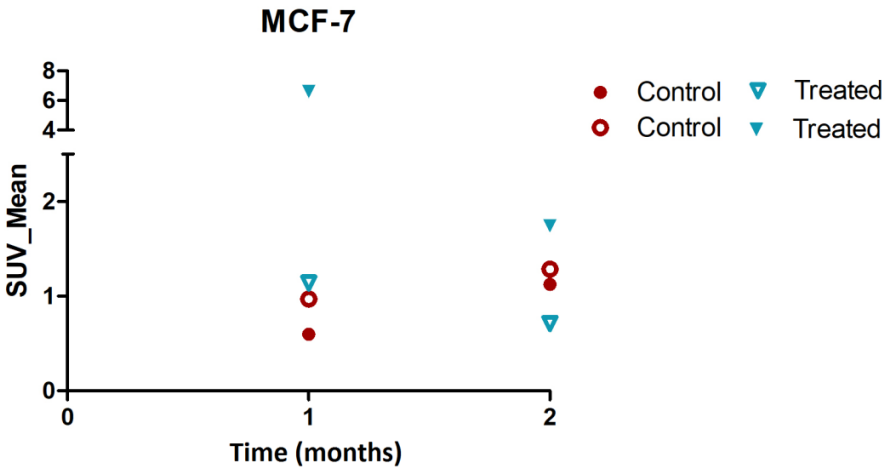


Figure 54: Graph showing the trend of SUVs in treated and untreated animals, for breast cancer cell line.

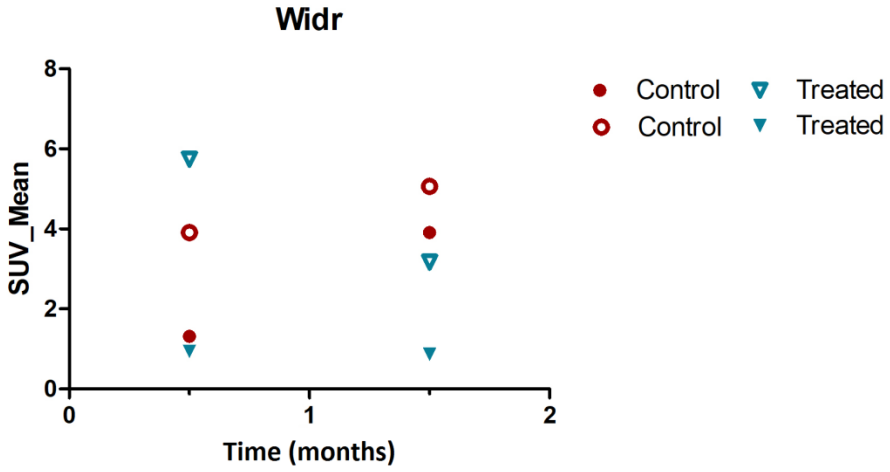


Figure 55: Graph showing the SUVs trend in treated and untreated animals, for colon cancer cell line.

Considering the above results, it was concluded that control animals showed an increasing SUVs trend over time, reflecting a constant growth of the tumor. On the other hand, the animals that received the EGCG compound showed a decreasing in SUVs trend, indicating a slowdown in tumor growth and the effectiveness of the treatment.

What matters most in this analysis is not the final value of the SUV means (since it could depend on the number of cells initially inoculated in the animal, on its age or on its metabolic characteristics), but the SUV means trend over time: its decrease, in fact, means a reduction in tumor activity over time, suggesting benefits of treatment.

These graphs also reinforce the idea that WiDr cells grow faster than MCF-7, being able to lead to a more dangerous oncological disease (higher SUVs in a shorter period of time). As for the MCF-7, it is easier to treat the tumor from an early stage because its development is slower, allowing an early detection.

To confirm the excellent performance of the easyPET-3D system in identifying even small activity accumulations, the acquisition of a tested animal is showed. In this case-study, in addition to the activity accumulation caused by the presence of the inoculated tumor, another site of high activity was detected, corresponding to the left knee joint (**Figure 56**).

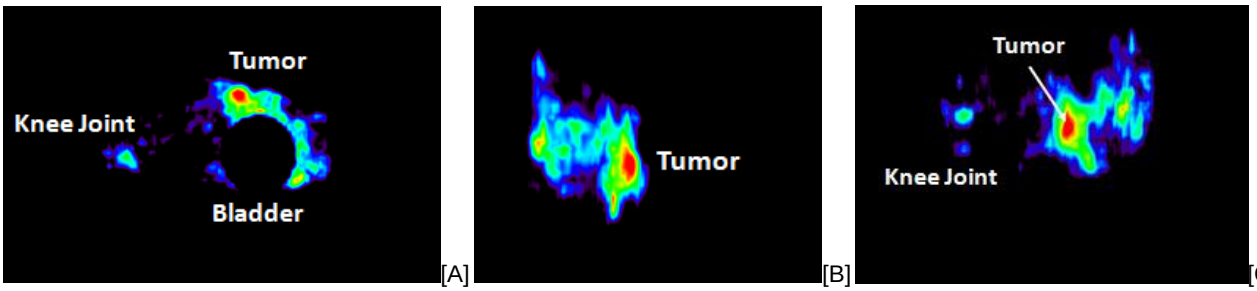


Figure 56: Positron emission tomography image obtained using the easyPET-3D system, observing the activity of ^{18}F-FDG radiopharmaceutical on 3 different planes: transverse [**A**]; coronal [**B**]; sagittal [**C**]. The bladder was darkened to highlight the tumor and the inflammation site.

Since glucose is present in larger amounts in tumor areas or in those with more intense muscle activity, the team tried to identify the cause of this abnormal activity. It has been noted that the mouse had knee edema. The fluid was removed from the knee and it was found to be inflammatory cells. After performing a rudimentary necropsy, it was observed that the mouse was born with a malformation of the right leg, having no fibula. The absence of this bone caused the mouse to make more strain on the opposite leg, causing inflammation and therefore accumulation of fluid in the left knee.

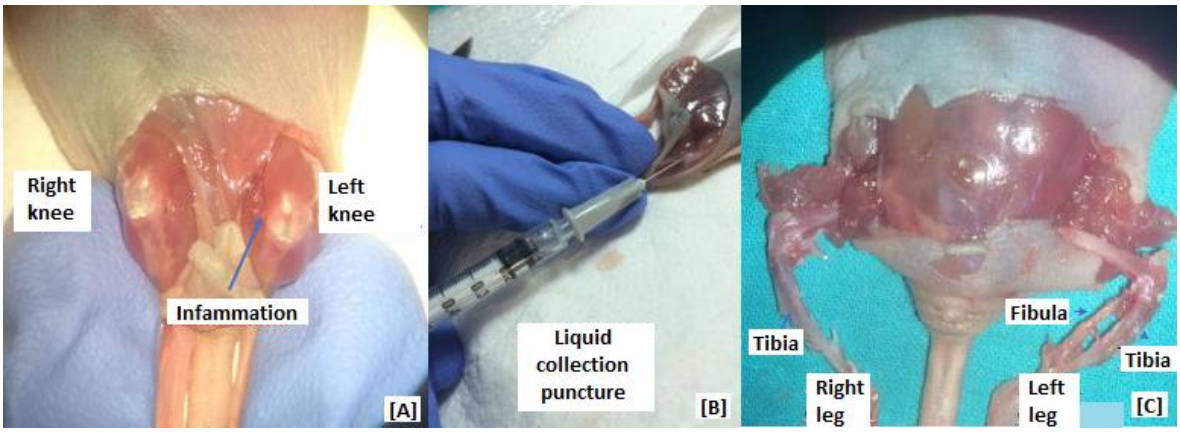


Figure 57: Illustrative image of the inflamed knee area [**A**]; representative figure of the puncture for accumulated liquid collection [**B**]; rudimentary necropsy identifying the malformation of mouse right leg.

Based on these results, it is noted that easyPET can capture gamma rays, producing sensitive and specific images to detect metabolic changes, allowing the diagnosis of a tumor mass even before it is visible externally. Moreover, thanks to the SUV calculation, the system allows to evaluate the progression/regression of the tumor even in a quantitative way.

This example confirms the excellent potential of this imaging research system.

4.2.3 Histological studies

At the end of the *in vivo* studies, animals were observed and tumors were collected for routine histological processing and observation under the optical microscope. The histological study was performed at the Experimental Pathology laboratory of the FMUC Department of Dentistry.

In all animals, macroscopic observation indicated that the main organs (stomach, intestine, lung, liver and kidney) were normal and no metastases developed. When analyzing the histological images, no differences were shown in areas surrounding the tumor, when compared with healthy animal tissues. This means neither tumor cell injection nor GTE green tea extract solution treatment altered architecture and physiology of tissues and organs.

Below are some routine optical microscopy images obtained from the samples of tumor (**Figure 58-60**).

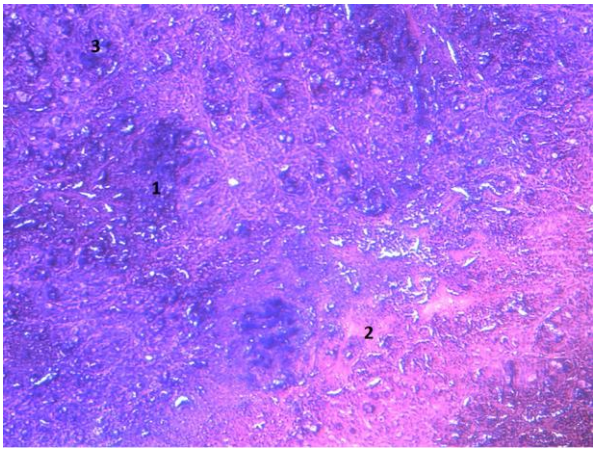


Figure 58: histological section of a WiDr tumor collected on 8 May 2019 (magnification of 200x). A glandular [**1**] and fibrous [**2**] tumor area and an inflammatory infiltrate [**3**] are identified. The dark blue spotted shaped are PMN (polymorphonuclear neutrophils).

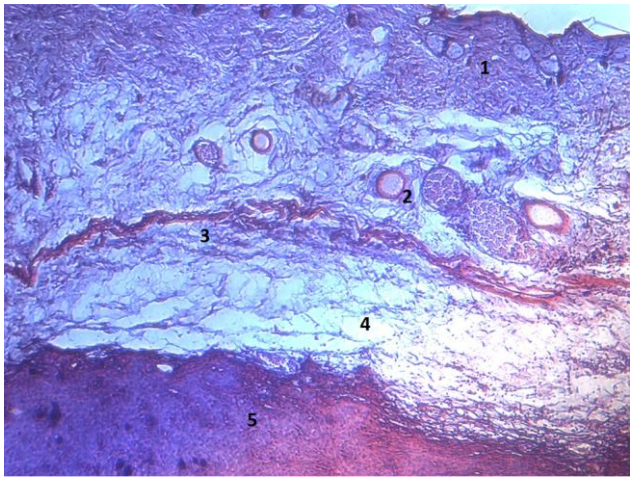


Figure 59: histological section of a MCF-7 tumor collected on 1st July 2019 (magnification of 200x). Muscular fibers longitudinally sectioned [**1**], muscular fiber circulary sectioned [**2**], tumor

remaining glandular area [**3**], thin skin layer [**4**], and fibrosis + fat cells (corresponding to the remaining tumor structures) [**5**] are identified.

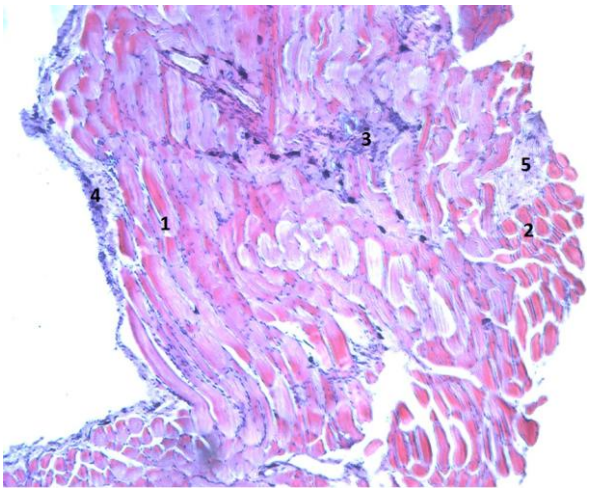


Figure 60: histological section of a WiDr tumor collected on 1st July 2019 (magnification of 200x). Dermis [**1**], blood vessels [**2**], muscular mucosa [**3**], subcutaneous areolar tissue [**4**] and tumor [**5**] are identified.

Tissues and structures in the area surrounding the tumor did not show alterations with respect to the tissues and structures of healthy animals, suggesting the non-aggressiveness of the treatment with tea extract.

Chapter 5

5. Conclusion

In this chapter the results presented in the previous sections are discussed and conclusions are drawn. The limits observed during this study are highlighted and future prospects for further research are indicated.

In *in vitro* studies, more specifically for the performed MTT tests, the cytotoxic potential of several monotherapy drugs and their combinations was studied, as well as their combination with GTEE ([EGCG] = 0.5 and 5 µg/mL), MGTE ([EGCG] = 0.5 and 5 µg/mL) and GTE ([EGCG] = 0.5 and 5 µg/mL). This extract, for each brand, has been shown to have a positive effect on cancer cells, decreasing their vitality. Furthermore, this extract has been shown to have a good synergy with most of the drugs tested: CAP, 5-FU, IRI, OX, PAX, CAPOX, CAPIRI and FOLFIRI. In these cases, when the extracts were added to the treatment, there was a significant reduction in biologically cell viability in at least one of the concentrations and in at least one of the brands. For FOLFOX there was a reduction of cell viability compared to the control when the drug was used on its own, however when the extracts were added to the treatment no more changes in vitality occurred. Compared to this drug, the extract seems to have no particular effects. In the case of FOL there was an increase in cell viability in monotherapy and in combination with all the extracts, with a small reduction in viability only with GTEE (EGCG] = 0.5 and 5 µg/mL) and MGTE ([EGCG] = 5 µg/mL). These results were expected since FOL is a drug used in combination with other chemotherapeutic drugs, not in monotherapy, to preserve healthy cells and to reduce the toxic effects of folic acid antagonist drugs, thus having a defense function and cell blood protection.

The problems that can occur for these types of studies may be due to the phase of the cell cycle in which the cells are found when therapy starts. In fact, since the cells used in these studies were collected from different culture flasks, the relationships between cells in the same cell division stage should be different, so the cytotoxic potential of drugs and combinations is different.

In the future, cytometric studies with apoptose/necrosis markers should be performed to identify the phase of the cell cycle, thus enabling to understand these differences, since the drugs act at different stages of cell development.

As for the *in vivo* tests, this type of tumor has been successfully inoculated in the lineage of Balb/c nude mice and green tea therapy has worked: a slowdown in tumor growth was initially verified and subsequently an effective reduction, although not very large, compared to the control

(drinking only water), in which the tumor grew faster and was never reduced. These animals were sacrified when the tumor reached the maximum allowed size and, through necropsy, it was verified that there were no metastases in other organs, nor other alterations suggesting that the therapy was harmful to the animal.

The results are promising and significant at biological level, being necessary to increase the number of tested animals in order to reinforce the statistics.

In these types of experiments, however, there are always so many variables that can lead to different and bad results: tumors can develop more or less depending on the age of the animal and the immunity of individuals acquired over time, over the generations, due to breading crossings with family. Along the generations, in fact, immune-compromised animals start to acquire some immunity and, thus starting to show some fur. In fact, some of the injected animals had some fur on their heads or around their eyes. This is probably why animals have different "response times".

Another factor that can lead to different results and developing speeds is the number of injected cells: the amount of cells will never be exactly the same from one animal to another.

Unlike previous studies, in this case the treatment with EGCG did not lead to an excessive decrease of the animal weight, allowing to continue the study, avoiding the interruption of the experiment for ethical and legal reasons. In fact, this animal species is very fragile and small (about 20 g), therefore even low weight losses could be significant. For this type of study, in order to proceed in the best way, it is mandatory that animal is as "strong" as possible to withstand the treatment. It would also be positive to increase the number of animals tested *in vivo,* trying to choose animals the most similar as possible.

With easy PET-3D equipment, it was possible to observe the tumor in mice before it was visible externally. In another parallel project it was also possible to observe distinct areas of the mice brain, revealing that the equipment is reliable, has good resolution and sensitivity. However, there are some limitations that the research group would like to improve. The acquisition tube only allows small animals to be studied (adult RNU rats are too large); the bed where mice are placed should be heated directly, taking into account the innate fragility of the species and the fragility acquired after anesthesia. This has been achieved heating the animal with a coupled external system. Furthermore, a CT or a MRI system should be combined with PET equipment in order to acquire morphological information for posterior images fusion, thus improving observation of the contours of the animal. This feature will allow to identify other morpho-anatomical structures, making the visualization of the tumor, inflammations or other organs more intuitive (the images acquired by this equipment need a trained observer). In any case, easyPET-3D has fulfilled all the previewed requirements.

At the end of the *in vivo* studies, or when animals had to be occized, major organs and tissues excised from the tumor injection area were collected to verify that there were no metastases. There were no anatomical differences between animals injected with cancer cells drinking only water and animals having continuous oral administration of green tea. Since no organ had abnormal differences nor alterations, it could be concluded that the treatment did not affect their morphology or functional. Observing the tumor masses of the treated animals and those drinking only water, it was possible to deduce that in the treated animals the tumor had reduced dimensions and less glandular areas (being replaced by fat and collagen fibers), revealing treatment effectiveness.

In the future, the number of replicates for *in vitro* studies should be increased in order to enhance statistical significance. Other drug dosages should be tested in cases where it was not possible to detect the existence of synergies between the EGCG extract and the drug; where the EGCG effect was really positive, future works should try to reduce the therapy drug dosage, as the drug has many side effects; new drugs should be tested; the phase of the cell cycle at the time of death should be identified to better understand the mechanisms of action of EGCG and the signaling chains in which it can act; immunohistochemical tests (the WiDr cell line produces a CEA antigen) should be performed to detect the expression of characteristic proteins using the antigen/antibody principle to easily identify cancer cells. Other antigens and markers of interest that can be used in sequence are: CD44, involved in cell proliferation and migration; Ki67, cell proliferation marker; suppressor genes (p53 and p21) and VEGF angiogenesis markers.

Acknowledgements

First of all, I would like to thank Dr. Ana Cristina Santos for her infinite teachings, for her dedication and for her immense helpfulness. She has always provided the best conditions inside and outside the laboratory, being always ready to help when needed. I realize how much time Professor has spent helping her students, and therefore my most sincere thanks.

Special thanks to Professor Bárbara Oliveiros for her co-guidance and assistance in data processing and statistical analysis, having always been helpfulness when I needed it.

Thanks to the great team of IBILI and Biotério, for their union and mutual help.

Thanks to Professor Andrea Facchinetti for his support in thesis writing.

Thanks to Patrícia Matos, Fabiana Ribeiro and, in particular, Mariana Lapo Pais, for their good company during the six months in Coimbra and for their constant help inside and outside the laboratory. Without a doubt, I couldn't have asked for better colleagues.

Thanks to my friends of Padua, “companions in adventures”, for these years spent together in joys and sorrows, satisfactions and disappointments, worries and lightheartedness moments. Thanks for everything we shared, you have been (and you will be forever) a fundamental and indispensable presence for me.

Thanks to my friends of Mantua, for having always been present for anything when I was at home.

Thanks to Cristina, it is useless to say why.

Finally, I want to thank all my family, especially Mom, Dad and Chiara, for the advice, the affection and the unconditional support they have always given me during my university life and for everything else, helping and guiding me in the best way. Without you I could have never overcome difficult times. Thanks will never be enough.

References

[1] NIH – National Cancer Institute, “NCI Dictionary of Cancer Terms” *[Online]*. Available: https://www.cancer.gov/publications/dictionaries/cancer-terms/def/tumor (Accessed: 06-Jun-2019).

[2] L. C. Tapsell *et al.*, «Health benefits of herbs and spices: the past, the present, the future», *Med. J. Aust.*, 2006, vol. 185, n. 4 Suppl, pp. S4-24.

[3] NIH – National Cancer Institute, “What Is Cancer?” *[Online]*. Available: https://www.cancer.gov/about-cancer/understanding/what-is-cancer (Accessed: 06-Jun-2019).

[4] American Cancer Society, “What is Cancer?” *[Online]*. Available: https://www.cancer.org/ (Accessed: 08-Jun-2019).

[5] C. Thompson, “Apoptosis in the pathogenesis and treatment of disease.”, *Science*, 1995, vol. 267, n. 5203, pp. 1456-62.

[6] Uicc.org, “New Global Cancer Data: GLOBOCAN 2018” *[Online]*. Available: https://www.uicc.org/news/new-global-cancer-data-globocan-2018 (Accessed: 08-Jun-2019).

[7] A. Jemal, F. Bray, I. Soerjomataram, J. Ferlay, L. A. Torre, R. L. Siegel, “Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.”, *CA Cancer J Clin.*, 2018, vol. 68, n. 6, pp. 394-424.

[8] Observador.pt, “Infografia. O cancro em Portugal e no mundo em 8 gráficos” *[Online].* Available: https://observador.pt/2016/02/04/infografia-cancro-portugal-no-mundo-8-graficos/ (Accessed: 08-Jun-2019).

[9] Ec.europa.eu, “European Commission, ECIS – European Cancer Information System” *[Online]*. Available: https://ecis.jrc.ec.europa.eu/ (Accessed: 08-Jun-2019).

[10] AIRC: Associazione Italiana per la Ricerca sul Cancro, “Le statistiche del cancro” *[Online]*. Available: https://www.airc.it/cancro/informazioni-tumori/cose-il-cancro/numeri-del-cancro (Accessed: 10-Jun-2019).

[11] Aiom – Associazione Italiana di Oncologia Medica, “I numeri del cancro in Italia 2018” *[Online]*. Available: https://www.aiom.it/i-numeri-del-cancro-in-italia/ (Accessed: 10-Jun-2019).

[12] Liga Portuguesa Contra o Cancro, “O que è o cancro?” *[Online]*. Available: http://www.lutacontraocancro.pt/ (Accessed: 10-Jun-2019).

[13] Y. Feng, M. Spezia, S. Huang, C. Yuan, Z. Zeng, L. Zhang, X. Ji, W. Liu, B. Huang, W. Luo, B. Liu, Y. Lei, S. Du, A. Vuppalapati, H. H. Luu, R. C. Haydon, T. C. He, G. Ren, “Breast cancer development and progression: Risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis”, *Genes Dis.*, 2018, vol. 5, n. 2, pp. 77–106.

[14] E. N. Imyanitov, K. P. Hanson, “Cancer, Mechanisms of breast cancer”, *Drug Discovery Today: Disease Mechanisms*, 2004, vol. 1, n. 2, pp. 235-245.

[15] M. Zhang, A. V. Lee, J. M. Rosen, “The Cellular Origin and Evolution of Breast Cancer”, *Cold Spring Harbor Prespect Med.*, 2017, vol. 7, n. 3, pp.27-28.

[16] K. Polyak, “Breast cancer: origins and evolution”, *J Clin Invest.*, 2007, vol. 117, n. 11, pp. 3155–3163.

[17] P. J. Stephens *et al*.," The landscape of cancer genes and mutational processes in breast cancer", *Nature,* 2012, vol. 486, n. 7403, pp. 400–404.

[18] X. Dai, T. Li, Z. Bai, Y. Yang, X. Liu, J. Zhan, B. Shi, "Breast cancer intrinsic subtype classification, clinical use and future trends", *Am J Cancer Res*., 2015, vol. 5, n. 10, pp. 2929–2943.

[19] HC. Pitot, "The molecular biology of carcinogenesis", *Cancer*, 1993, vol. 72, n. 3 Suppl., pp. 962-70.

[20] Z. Kleibl , V. N. Kristensen, "Women at high risk of breast cancer: Molecular characteristics, clinical presentation and management", *The Breast*, 2016, vol. 28, pp. 136-144.

[21] V. A. Cirolla, "Breast anatomy", *Oncological prevention*, 2017

[22] H. Ellis, V. Mahadevan, "Anatomy and physiology of the breast.", *Surgery (Oxford)*, 2013, vol. 31, n. 1, pp. 11-14.

[23] G. Ellis, H. Colborn, J. E. Skandalakis, "Surgical Embryology and Anatomy of the Breast and its Related Anatomic Structures." *Surgical Clinics of North America,* 1993, vol. *73,* n. 4, pp. 611–632.

[24] R. D.Cardiff, K. H.Allison, "Mammary Gland", *Comparative Anatomy and Histology: A Mouse and Human Atla*s, 2012, n. 4, pp. 41-52.

[25] Nbcf.org, National Breast Cancer Foundation, "Breast anatomy and how cancer starts" *[Online]*. Available: https://nbcf.org.au/about-national-breast-cancer-foundation/about-breast-cancer/what-you-need-to-know/breast-anatomy-cancer-starts/ (Accessed: 10-Jun-2019).

[26] BreastCancer.org, "Benign Breast Condition" *[Online]*. Available: https://www.breastcancer.org/symptoms/benign (Accessed: 11-Jun-2019).

[27] J. Hailes, "Common breast condition" *[Online]*. Available: https://jeanhailes.org.au/health-a-z/breast-health/common-breast-conditions (Accessed: 11-Jun-2019).

[28] Aboutcancer.com, "Lobular Carcinoma of the Breast" *[Online]*. Available: http://www.aboutcancer.com (Accessed: 11-Jun-2019).

[29] American Cancer Society, "Ductal Carcinoma In Situ (DCIS)" *[Online]*. Available: https://www.cancer.org/cancer/breast-cancer/understanding-a-breast-cancer-diagnosis/types-of-breast-cancer/dcis.html (Accessed: 11-Jun-2019).

[30] X. Dai, T. Li, Z. Bai, Y. Yang, X. Liu, J. Zhan, B. Shi, "Breast cancer intrinsic subtype classification, clinical use and future trends.", *Am J Cancer Res*., 2015, vol. 5, n. 10, pp. 2929-2943.

[31] H. P. Sinn, H. Kreipe, "Brief Overview of the WHO Classification of Breast Tumors", *Breast Care (Basel).*, 2013, vol. 8, n. 2, pp. 149–154.

[32] E. A. Rakha, A. R.Green, "Molecular classification of breast cancer: what the pathologist needs to know", *Pathology*, 2017, vol. 49, n. 2, pp. 111–119.

[33] R. Almarzooq, A. Alrayes, H. Alaradi, H. Abdulla, "Molecular subtypes of breast cancer", *Bahrain Medical Bulletin*, 2018, vol. 40, n. 4, pp. 222-225.

[35] A. C, Bateman, E. C. Shaw, "Breast pathology*." Surgery (Oxford),* 2016, vol. 34, n. 1, pp. 1–7.

[36] G. N. Sharma, R. Dave, J. Sanadya, P. Sharma, K. K Sharma, "Various types and management of breast cancer: an overview.", *J Adv Pharm Technol Res*., 2010, vol. 1, n. 2, pp. 109–126.

[37] AboutCancer.com, “Molecular subtypes of breast cancer” *[Online]*. Available: http://www.aboutcancer.com/breast_molecular_0610.htm (Accessed: 14-Jun-2019).

[38] A. R. Mwakigonja, N. E. Lushina, A. Mwanga, “Characterization of hormonal receptors and human epidermal growth factor receptor-2 in tissues of women with breast cancer at Muhimbili National Hospital, Dar es salaam, Tanzania”, *Infectious Agents and Cancer,* 2017, vol. 12.

[39] BreastCancer.org, “Molecular Subtypes of Breast Cancer” *[Online]*. Available: https://www.breastcancer.org/symptoms/types/molecular-subtypes (Accessed: 14-Jun-2019).

[40] M. Yanagawa, K. Ikemot, S. Kawauchi, T. Furuya, S. Yamamoto, M. Oka, A. Oga, Y. Nagashima, K. Sasaki, “Luminal A and luminal B (HER2 negative) subtypes of breast cancer consist of a mixture of tumors with different genotype”, *BMC Research Notes,* 2012, vol. 5, n. 376

[41] B. Tran, P. L. Bedard, “Luminal-B breast cancer and novel therapeutic targets”, *Breast Cancer Research,* 2011, vol. 13, n. 221.

[42] A. Llombart-Cussac, J. Cortés *et al*., “HER2-enriched subtype as a predictor of pathological complete response following trastuzumab and lapatinib without chemotherapy in early-stage HER2-positive breast cancer (PAMELA): an open-label, single-group, multicentre, phase 2 trial”, *Lancet Oncol.*, 2017, vol. 18, pp. 545–54.

[43] M. Valla, L. Vatten, *et al.*, “Molecular Subtypes of Breast Cancer: Long-term Incidence Trends and Prognostic Differences”, *Cancer Epidemology, Biomarkers&Prevention*, 2016 vol. 25, n. 12.

[44] E. A Rakha, J. Reis-Filho, F. Baehner, D. Dabbs, T. Decker, V. Eusebi, S. Fox, S. Ichihara, J. Jacquemier, S. Lakhani, J. Palacios, A. Richardson, S. Schnitt, F. Schmitt, P. H. Tan, G. Tse, S. Badve, I. Ellis, “Breast cancer prognostic classification in the molecular era: the role of histological grade”, *Breast Cancer Res.*, 2010, vol. 12, n. 4.

[45] B. Stenkvist, S. Westman-Naeser, J. Vegelius, J. Holmquist, B. Nordin, E. Bengtsson, E. Eriksson, “Analysis of reproducibility of subjective grading systems for breast carcinoma”, *Journal of Clinical Pathology*, 1979, vol. 32, pp. 979-985.

[46] H. J. G. Bloom and W. W. Richardson, “Histological Grading and Prognosis in Breast Cancer,A Study of 1409 Cases of which 359 have been Followed for 15 Years”, *Br J Cancer*., 1957, vol. 11, n. 3, pp. 359–377.

[47] cancer.gov, NIH – National Cancer Institute, “Tumor Grade” *[Online]*. Available: https://www.cancer.gov/about-cancer/diagnosis-staging/prognosis/tumor-grade-fact-sheet (Accessed: 14-Jun-2019).

[48] S. Edge, C. Compton, “The American Joint Committee on Cancer: the 7th Edition of the AJCC, Cancer Staging Manual and the Future of TNM”, *Annals of Surgical Oncology*, 2010, vol. 17, n. 6, pp. 1471-4.

[49] pathology.jhu.edu, “John Hopkins Medicine Pathology, Staging and Grade” *[Online]*. Available: https://pathology.jhu.edu/breast/staging-grade/ (Accessed: 16-Jun-2019).

[50] J. R. Benson, “The TNM staging system and breast cancer”, *The Lancet Oncology*, 2003, vol.4, n. 1, pp. 56-60.

[51] Cancer.net, “Breast Cancer: Stages” *[Online]*. Available: https://www.cancer.net/cancer-types/breast-cancer/stages (Accessed: 16-Jun-2019).

[52] BreastCancer.org, “Breast Cancer Stages” *[Online]*. Available: https://www.breastcancer.org/symptoms/diagnosis/staging (Accessed: 16-Jun-2019).

[53] Cancercenter.com, “Cancer Treatment - Breast Cancer, *[Online].* Available: https://www.cancercenter.com/cancer-types/breast-cancer/stages (Accessed: 16-Jun-2019).

[54] A. A. Fracchia, J. F. Evans, B. L. Eisenberg, “Stage III Carcinoma of the Breast: A Detailed Analysis”, *Ann Surg*., 1980, vol. 192 n. 6, pp. 705–710.

[55] T. Shien, “Impact of breast surgery in de novo stage IV breast cancer”, *Translational Cancer Research*, 2019, vol. 8, n. 2, pp. 118-119.

[56] H. Le-Petross, A. S Caudle, “Staging of Breast Cancer”, *Article*, 2015.

[57] A. M. Kabel, F. H. Baali, “Breast Cancer: Insights into Risk Factors, Pathogenesis, Diagnosis and Management”, *Journal of Cancer Research and Treatment*, 2015, vol. 3, n. 2, pp. 28-33.

[58] M. Melnichouk, H. Guo, A. M. Chiarelli, T. G. Hislop, M. J. Yaffe *et al.*, “Family history, mammographic density, and risk of breast cancer.”, *Cancer Epidemiol Biomarkers Prev*, 2010, vol. 19, n. 2, pp. 456-63.

[59] A. Mandal, “Breast Cancer Epidemiology”, *News Medical LifeScience [Online]*. Available: https://www.news-medical.net/health/Breast-Cancer-Epidemiology.aspx (Accessed: 16-Jun-2019).

[60] F. Bray, J. Ferlay, I. Soerjomataram, R. Siegel, L. A. Torre, A. Jemal, “Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries”, *CA Cancer J Clin.*, 2018, vol. 68, n. 6, pp. 394-424.

[61] Cancer.org, “Treating Breast Cancer” *[Online]*. Available: https://www.cancer.org/cancer/breast-cancer/treatment.html (Accessed: 16-Jun-2019).

[62] Cancer.net, “Breast Cancer: Types of Treatment” *[Online]*. Available: https://www.cancer.net/cancer-types/breast-cancer/types-treatment (Accessed: 16-Jun-2019).

[63] Webmd.com, “Stage 0 Breast Cancer Treatment Options” *[Online]*. Available: https://www.webmd.com/breast-cancer/stage-0-breast-cancer-treatment-options (Accessed: 17-Jun-2019).

[64] BreastCancer.org, “Options by Cancer Stage” *[Online]*. Available: https://www.breastcancer.org/treatment/planning/cancer_stage (Accessed: 16-Jun-2019).

[65] Y. Smith, “Radiotherapy for Breast Cancer”, *News Medical LifeScience [Online]*. Available: https://www.news-medical.net/health/Radiotherapy-for-Breast-Cancer.aspx (Accessed: 16-Jun-2019).

[66] Y. Lievens, U. Ricardi, P. Poortmans, D. Verellen, C. Gasparotto, C. Verfaillie, A. Cortese, “Radiation Oncology. Optimal Health for All, Together. ESTRO vision, 2030”, *Radiotherapy and Oncology,* 2019, vol. 136, pp. 86-97.

[67] G. Chen, G. Sharp, S. Mori, “A review of image-guided radiotherapy”, *Radiological Physics and Technology*, 2009, vol. 2, n. 1, pp. 1-12.

[68] CancerResearchUK.org, “Chemotherapy” *[Online]*. Available: https://www.cancerresearchuk.org/about-cancer/cancer-in-general/treatment/chemotherapy/how-chemotherapy-works (Accessed: 16-Jun-2019).

[69] R. Page and C. Takimoto, “Principles of chemotherapy”, Chapter 3, pp. 21-37.

[70] BreastCancer.org, “Chemotherapy medicines” *[Online]*. Available: https://www.breastcancer.org/treatment/chemotherapy/medicines (Accessed: 16-Jun-2019).

[71] Cancer.org, “Target Therapy for Breast Cancer” *[Online]*. Available: https://www.cancer.org/cancer/breast-cancer/treatment/targeted-therapy-for-breast-cancer.html (Accessed: 16-Jun-2019).

[72] R. Amodio, M. Zarcone, R. Cusimano, I. Campisi, C. Dolcemascolo, A. Traina, B. Agostara, N. Romano, “Target Therapy in HER2-Overexpressing Breast Cancer Patients”, *A Journal of Integrative Biology,* 2011, vol. 15, n. 6.

[73] V. V. Padma, “An overview of targeted cancer therapy”, *BioMedicine*, 2015, vol. 5, n. 4, pp. 1-6.

[74] LAColon.com, “Colon Cancer”, *[Online]*. Available: https://lacolon.com/conditions (Accessed: 16-Jun-2019).

[75] Cancer.gov, NIH – National Cancer Institute, “Colorectal Cancer” *[Online].* Available: https://www.cancer.gov/types/colorectal (Accessed: 16-Jun-2019).

[76] Gray’s Anatomy

[77] M. Hoffman, “Picture of the Colon”, *Human Anatomy [Online]*. Available: https://www.webmd.com/digestive-disorders/picture-of-the-colon#1 (Accessed: 18-Jun-2019).

[78] Cancer.org, American Cancer Society, “Colorectal Cancer Stages” *[Online]*. Available: https://www.cancer.org/cancer/colon-rectal-cancer/detection-diagnosis-staging/staged.html (Accessed: 18-Jun-2019).

[79] S. Rafiyath, “Oncology, Colon Cancer Staging”, *Drugs & Diseases*, 2019.

[80] Radiopaedia.org, M. Thurston, F. Gaillard et al., “Colorectal cancer (staging)” , *[Online]*. Available: https://radiopaedia.org/articles/colorectal-cancer-staging-1 (Accessed: 18-Jun-2019).

[81] E. Weisenberg, “Colon tumor, TNM staging of colorectal carcinoma (AJCC 8th edition)”, *UW Medicine Pathology*, 2017.

[82] C. N. Arnold, A. Goel, H. E. Blum, C. R. Boland, “Molecular Pathogenesis of Colorectal Cancer, Implications for Molecular Diagnosis”, *Cancer – Review Article*, 2005, vol. 104, n. 10, pp. 2035-2047.

[83] K. Bardhan, K. Liu, “Epigenetics and Colorectal Cancer Pathogenesis, *Cancers (Basel)*., 2013, vol. 5, n. 2, pp. 676–713.

[84] M. Mustafa, J. Menon, M.,Illzam, MJ.Shah, A. M.Sharifa, “Colorectal Cancer: Pathogenesis, Management and Prevention”, *Journal of Dental and Medical Sciences*, 2016, vol. 15, n. 5, pp. 853-861.

[85] M.Ponz, L. A.Percesepe, “Pathogenesis of colorectal cancer”, *Digestive and Liver Disease*, 2000, vol. 32, n. 9, pp. 807-821.

[86] W. M. Grady, S. D. Markowitz, “The molecular pathogenesis of colorectal cancer and its potential application to colorectal cancer screening”, *Dig Dis Sci*., 2015, vol. 60, n. 3, pp. 762–772.

[87] Cancer.org, American Cancer Society, “Colorectal Cancer Risk Factors” *[Online]*. Available: https://www.cancer.org/cancer/colon-rectal-cancer/causes-risks-prevention/risk-factors.html (Accessed: 19-Jun-2019).

[88] Cdc.gov, CDC: Centers for Disease Control and Prevention, “Colon Cancer, What Are the Risk Factors for Colorectal Cancer?” *[Online]*. Available: https://www.cdc.gov/cancer/colorectal/basic_info/risk_factors.htm (Accessed: 19-Jun-2019).

[89] F. A. Haggar, R. P. Boushey, “Colorectal Cancer Epidemiology: Incidence, Mortality, Survival, and Risk Factors”, *Clin Colon Rectal Surg*., 2009, vol. 22, n. 4, pp. 191–197.

[90] R. L. Siegel, K. D. Miller, A. Jemal, “Cancer statistics”, *CA: A Cancer Journal for Clinicians*, 2018, vol. 68, n. 1, pp. 7-30.

[91] F. Bray, J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, A. Jemal, “Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries”, *CA: A Cancer Journal for Clinicians*, 2018, vol. 68, n. 6, pp. 394-424.

[92] H. Brenner, C. Chen, “The colorectal cancer epidemic: challenges and opportunities for primary, secondary and tertiary prevention”, *British Journal of Cancer*, 2018, vol. 119, pp. 785–792.

[93] Cancer.org, “Treatment of Colon Cancer, by Stage” *[Online]*. Available: https://www.cancer.org/cancer/colon-rectal-cancer/treating/by-stage-colon.html (Accessed: 21-Jun-2019).

[94] Cancer.gov, NIH, National Cancer Institute, “Treatment Options for Colon Cancer” *[Online]*. Available: https://www.cancer.gov/types/colorectal/patient/colon-treatment-pdq#_162 (Accessed: 21-Jun-2019).

[95] A. Agranovich, E. Berthelet, “Radiotherapy for colorectal cancer”, *BCMJ- Clinical Articles*, 2000, vol. 42 , n. 3, pp. 139-141.

[96] WebMD.com, “Chemotherapy for Colorectal Cancer” *[Online]*. Available: https://www.webmd.com/colorectal-cancer/chemotherapy#1 (Accessed: 21-Jun-2019).

[97] W. A. Hammond, A. Swaika, K. Mody, “Pharmacologic resistance in colorectal cancer: a review”, *Ther Adv Med Oncol*., 2016, vol. 8, n. 1, pp. 57–84.

[98] Cancer.gov, NIH – National Cancer Institute, “Drugs Approved for Colon and Rectal Cancer” *[Online]*. Available: https://www.cancer.gov/about-cancer/treatment/drugs/capecitabine (Accessed: 21-Jun-2019).

[99] CancerTherapyAdvisor.com, Cancer therapy advisor, “Colon Cancer Treatment Regimens” *[Online]*. Available: https://www.cancertherapyadvisor.com/home/cancer-topics/gastrointestinal-cancers/colon-cancer-treatment-regimens/ (Accessed: 21-Jun-2019).

[100] mcf7.com, “MCF-7” *[Online]*. Available: http://www.mcf7.com/ (Accessed: 10-Jul-2019).

[101] J. E. Welsh, “Chapter 40 - Animal Models for Studying Prevention and Treatment of Breast Cancer.“, *Animal Models for the Study of Human Disease,* 2013, pp. 997-1018.

[102] S. Comsa, A. M. Cimpean, M. Raica, “The Story of MCF-7 Breast Cancer Cell Line: 40 years of Experience in Research”, *Anticancer Research*, 2015, vol. 35, n. 6, pp. 3147-3154.

[103] A. V. Lee, S. Oesterreich, N. E. Davidson, “MCF-7 Cells—Changing the Course of Breast Cancer Research and Care for 45 Years”, *J Natl Cancer Inst.,* 2015, vol. 107, n. 7.

[104] H. D. Soule, J. Vazquez, A. Long, S. Albert, M. Brennan, “A Human Cell Line From a Pleural Effusion Derived From a Breast Carcinoma”, *J Natl Cancer Inst*., 1973, vol. 51, n. 5, pp. 1409-1416.

[105] A. S. Levenson, V. C. Jordan, “MCF-7: The First Hormone-responsive Breast Cancer Cell Line”, *Cancer Research*, 1997, vol. 57, pp. 3071-3078.

[106] M. Saceda, M. E. Lippman, P. Chambon, R. L. Lindsey, M. Ponglikitmongkol, M. Puente, M. Martin , “Regulation of the Estrogen Receptor in MCF-7 Cells by Estradiol”, *Molecular Endocrinology*, 1988, vol. 2, n. 12, pp. 1157–1162.

[107] E. R. Locke, H. D. Soule, “Estrogen Receptor in a Human Cell Line (MCF -7) from Breast Carcinoma”, *The Journal of Biological Chemistry*, 1973, vol. 2, n. 17, pp. 6251~6253.

[108] T. R. Chen, D. Drabkowski, R. J. Hay, M. Macy, W. Peterson, “WiDr is a derivative of another colon adenocarcinoma cell line, HT-29.”, *Cancer Genet Cytogenet*, 1987, vol. 27, n. 1, pp. 125-134.

[109] accegen.com, ACCEGEN Biotechnology, “WiDr” *[Online]*. Available: https://www.accegen.com/HumanTumor/1338.html (Accessed: 15-Jul-2019).

[110] P. Noguchi, R. Wallace, J. Johnson, E. M. Earley, S. O'Brien, S. Ferrone, M. A Pellegrino, J. Milstien, C. Needy, W. Browne, J. Petricciani, “Characterization of the WIDR: a human colon carcinoma cell line.”, *In Vitro*., 1979, vol. 15, n. 6, pp. 401-408.

[111] Altogenlabs.com,” Xenograft Model, Altogen labs” *[Online]*. Available: http://altogenlabs.com/xenograft-models/colon-cancer-xenograft/widr-xenograft-model/ (Accessed: 15-Jul-2019).

[112] K. Sak, “Chemotherapy and Dietary Phytochemical Agents”, *Chemother Res Pract*., 2012.

[113] S.-A. Shin, S. Y. Moon, W.-Y. Kim, S.-M. Paek, H. H. Park, C. S. Lee, “Structure-Based Classification and Anti-Cancer Effects of Plant Metabolites.”, *Int J Mol Sci*., 2018, vol. 19, n. 9, p. 2651.

[114] Y. Zhou, J. Zheng, Y. Li, D.-P. Xu, S. Li, Y.-M. Chen, H.-B. Li, “Natural Polyphenols for Prevention and Treatment of Cancer”, *Nutrients*., 2016, vol. 8, n. 8.

[115] E. Martens, “Useful Anti-Cancer Agents of Natural Product Origin”, *Cancer Studies and Therapeutics*, 2017.

[116] M. L. Seca, C. G. A. Pinto, *“*Plant Secondary Metabolites as Anticancer Agents: Successes in Clinical Trials and Therapeutic Application”, *Int J Mol Sci*., 2018.

[117] S. Fulda, T. Efferth, “Selected Secondary Plant Metabolites for Cancer Therapy”, *Modern Research on Chinese Materia Medica*, 2015.

[118] M. J. Nirmala, A. Samundeeswari P. D. Sankar, “Natural plant resources in anti-cancer therapy-A review.”, *Research in Plant Biology*, 2011, vol. 1, n. 3, pp. 1-14.

[119] A. D. Kinghorn *et al*., “Discovery of Anticancer Agents of Diverse Natural Origin.”, *International Jurnal of Cancer Research and Treatment, Anticancer Research*, 2016, vol. 36, n. 11, pp. 5623-5637.

[120] F. Aurélien, C. Moumbock, L. Simoben, W. S. Wessjohann, S. Günther, F. Ntie-Kang, “Computational Studies and Biosynthesis of Natural Products with Promising Anticancer Properties”, *Natural Products and Cancer Drug Discovery*, 2017.

[121] R. Kotecha, A. Takami, J. L. Espinoza, “Dietary phytochemicals and cancer chemoprevention: a review of the clinical evidence.”, *Oncotarget*., 2016, vol. 7, n. 32, pp. 517–529.

[122] teaguardian.com , “Green Teas: A (very) Brief History” *[Online]*. Available: https://www.teaguardian.com/what-is-tea/green-tea-history/ (Accessed: 29-Jun-2019).

[123] J. Dwyer, J. Peterson, “Tea and flavonoids: where we are, where to go next.”, *Am J Clin Nutr*., 2013, vol. 98, n. 6, pp. 1611–1618.

[124] J. Schweikart, “Camelia Sintesis: Chà verde e saùde”, *[Online]*. Available: https://www.cha-verde.net/camellia-sinensis/ (Accessed: 30-Jun-2019).

[125] S. A. Thasleema, “Green Tea as an Antioxidant- A Short Review.”, *J. Pharm. Sci. & Res*., 2013, vol. 5, n. 9, pp. 171–173.

[126] K. Min, T. K. Kwon, “Anticancer effects and molecular mechanisms of epigallocatechin-3-gallate”, *Integr. Med. Res*., 2014, vol. 3, pp. 16–24.

[127] N. Harold, P. Graham, “Green Tea Composition, Consumption, and Polyphenol Chemistry”, *Preventive Medicine*, 1992, vol. 21, pp. 334-350.

[128] Q. P. Dou, “Molecular Mechanisms of Green Tea Polyphenols.”, *Nutr Cancer*., 2009, vol. 61, n. 6, pp. 827–835.

[129] D. Chen, S. B. Wan, H. Yang, J. Yuan, T. H. Chan, Q. P. Dou, “EGCG, Green Tea Polyphenols and their synthetic analogs and prodrugs for human cancer prevention and treatment.”, *Adv Clin Chem*., 2011, vol. 53, pp. 155–177.

[130] M. Kampa, A.-P. Nifli, G. Notas, E. Castanas, “Polyphenols and cancer cell growth”, *Reviews of physiology, Biochemistry and Pharmacology*, pp. 79-113.

[131]Livestrong.com, K. Nina, “The Negative Effects of Green Tea” *[Online].* Available: https://www.livestrong.com/article/442179-the-negative-effects-of-green-tea/ (Accessed: 01-Jul-2019)

[132] R. Johnson, S. Bryant, A. L.Huntley, “Green tea and green tea catechin extracts: An overview of the clinical evidence.”, *Maturitas*, 2012, vol. 73, n. 4, pp. 280-287.

[133] T. J. Smith, “Green Tea Polyphenols in drug discovery - a success or failure?” *Expert Opin Drug Discov*, 2011, vol. 6, n. 6, pp. 589–595.

[134] D. G. Nagle, D. Ferreira, Y.-D. Zhou, “Epigallocatechin-3-gallate (EGCG): Chemical and biomedical perspectives.”, *Phytochemistry*, 2006, vol. 67, n. 17, pp. 1849–1855.

[135] K. Min, T. K. Kwon, “Anticancer effects and molecular mechanisms of epigallocatechin-3-gallate.”, *Integr. Med. Res*., 2014, vol. 3, pp. 16–24.

[136] D. Mereles and W. Hunstein, “Epigallocatechin-3-gallate (EGCG) for Clinical Trials: More Pitfalls than Promises?”, *Int. J. Mol. Sci.*, 2011, vol. *12*, pp. 5592-5603.

[138] L. Elbling, I. Herbacek, R.-M. Weiss et al., “Hydrogen peroxide mediates EGCG-induced antioxidant protection in human keratinocytes.”, *Free Radical Biology and Medicine*, 2010, vol. 49, n. 9, pp. 1444–1452.

[139] Y. C. Lim, H. Y. Park, H. S. Hwang et al., “(−)-Epigallocatechin-3-gallate (EGCG) inhibits HGF-induced invasion and metastasis in hypopharyngeal carcinoma cells.”, *Cancer Letters*, 2008, vol. 271, n. 1, pp. 140–152.

[140] C. Chu, J. Deng, Y. Man, Y. Qu, “Green Tea Extracts Epigallocatechin-3-gallate for Different Treatments.”, *BioMed Research International*, 2017, vol. 2017, n. 2, pp. 1-9.

[141] G.-J. Du, Z. Zhang, X.-D. Wen, C. Yu, T. Calway, C.-S. Yuan, C.-Z. Wang, “Epigallocatechin Gallate (EGCG) Is the Most Effective Cancer Chemopreventive Polyphenol in Green Tea”, *Nutrients*., 2012, vol. 4, n. 11, pp. 1679–1691.

[142] C. A. Rice-Evans, N. J. Miller, and G. Paganga, “Structure-antioxidant activity relationships of flavonoids and phenolic acids.”, Free *Radical Biology and Medicine*, 1996, vol. 20, no. 7, pp. 933–956.

[143] L. Elbling, R.-M. Weiss, O. Teufelhofer et al., “Green tea extract and (–)-epigallocatechin-3-gallate, the major tea catechin, exert oxidant but lack antioxidant activities.”, *The FASEB Journal*, 2005, vol. 19, no. 7, pp. 807–809.

[144]G.-X. Li, Y.-K. Chen, Z. Hou *et al*., “Pro-oxidative activities and dose-response relationship of (−)-epigallocatechin-3-gallate in the inhibition of lung cancer cell growth: a comparative study in vivo and in vitro,” *Carcinogenesis*, 2010, vol. 31, no. 5, pp. 902–910.

[145] J. D. Lambert, R. Elias, *“*The antioxidant and pro-oxidant activities of green tea polyphenols: A role in cancer prevention.”, *Archives of Biochemistry and Biophysics*, 2010, vol. 501, n. 1, pp. 65-72.

[146] H. H. Chow, I. Hakim, D. Vining, J. A. Crowell, J. Ranger-Moore, W. Chew, C. A. Celaya, S. Rodney, Y. Hara, D. Alberts, “Effects of dosing condition on the oral bioavailability of green tea catechins after single-dose administration of Polyphenon E in healthy individuals.”, *Clin Cancer Res*., 2005, vol. 11, n. 12, pp. 4627–4633.

[147] D. G. Nagle, D. Ferreira, Y.-D. Zhou, “Epigallocatechin-3-gallate (EGCG): Chemical and biomedical perspectives”, *Phytochemistry*, 2006, vol. 67, n. 17, pp. 1849–1855.

[148] S. Kim, M.-J. Lee, J. Hong, C. Li, T. J. Smith, G.-Y. Yang, D. N. Seril, C. S. Yang, “Plasma and Tissue Levels of Tea Catechins in Rats and Mice During Chronic Consumption of Green Tea Polyphenols”, *Nutrition and Cancer*, 2000, vol. 37, n. 1, pp. 41–48.

[149] J. Kanwar, M. Taskeen, I. Mohammad, C. Huo, T. H. Chan, Q. P. Dou, “Recent advances on tea polyphenols”, *Front Biosci (Elite Ed)*, 2012, vol. 4, pp. 111–131.

[150] J.-M. Yuan, C. Sun, L. M. Butler, “Tea and Cancer Prevention: Epidemiological Studies.”, *Pharmacol. Res*., 2011, vol. 64, n. 2, pp. 123–135.

[151] C.-Y. Huang, Z. Han, X. Li, H.-H. Xie, S.-S. Zhu, “Mechanism of EGCG promoting apoptosis of MCF-7 cell line in human breast cancer”, *Oncology letters,* 2017, pp. 3623-3627]

[152] L.-P. Xiang, A. Wang, J.-H. Ye, X.-Q. Zheng, C. A. Polito, J.-L. Lu, Q.-S. Li, Y.-R. Liang, “Suppressive Effects of Tea Catechins on Breast Cancer”, *Nutrients*., 2016, vol. 8, n. 8, p 458.

[153] L. Zeng, J. P. Holly, and C. M. Perks, “Effects of Physiological Levels of the Green Tea Extract Epigallocatechin-3-Gallate on Breast Cancer Cells.”, *Front Endocrinol (Lausanne)*, 2014, vol. 5, n. 61, pp 1-11.

[154] I. Rady, H. Mohamed, M. Rady, I. A. Siddiqui, H. Mukhtar, “Cancer preventive and therapeutic effects of EGCG, the major polyphenolin green tea”, *Egyptian Journal of Basic and Applied Sciences*, 2018, vol. 5, pp. 1-23.

[155] Y.-C. Liang, S.-Y. Lin-Shiau, C.-F. Chen, J.-K. Lin, “Inhibition of Cyclin-Dependent Kinases 2 and 4 Activities as Well as Induction of Cdk Inhibitors p21 and p27 During Growth Arrest of Human Breast Carcinoma Cells by (2)-Epigallocatechin-3-Gallate”, *Journal of Cellular Biochemistry*, 1999, vol. 75, pp. 1–12.

[156] M. R. Sartippour, Z.-M. Shao, D. H. Perrin, B. Liping, Z. Canhui, L. Lee, E. Wen, L. Vay, L. Go, M. N. Brooks, “Green Tea Inhibits Vascular Endothelial Growth Factor (VEGF) Induction in Human Breast Cancer Cells”, *The Journal of Nutrition*, 2002, vol. 132, n. 8, pp. 2307–2311.

[157] S. Abd El-Rahman, G. Shehab, H. Nashaat, “Epigallocatechin-3-Gallate: The Prospective Targeting of Cancer Stem Cells and Preventing Metastasis of Chemically-Induced Mammary Cancer in Rats.” *The American Journal of the Medical Sciences,* 2017, vol. 354, n. 1, pp. 54–63.

[158] N. Kumar, D. Shibata, J. Helm, D. Coppola, M. Malafa, “Green tea polyphenols in the prevention of colon cancer.”, *Frontiers in Bioscience*, 2007, vol. 12, pp. 2309-2315.

[159] B.-T. Ji, W.-H. Chow, A. W. Hsing, J. K. McLaughlin, Q. Dai, Y.-T. Gao, W. J. Blot, J. F. Fraumeni, “Green Tea consumption and the risk of pancreatic and colorectal cancers”, *Int. J. Cancer*, 1997, vol. 70, pp. 255–258.

[160] N. Metz, A. Lobstein, Y. Schneider, F. Gosse, R. Schleiffer, R. Anton, F. Raul, “Suppression of azoxymethane-induced preneoplastic lesions and inhibition of cyclooxygenase-2 activity in the colonic mucosa of rats drinking a crude green tea extract.”, *Nutr Cancer*, 2000, vol. 38, n. 1, pp. 60-64.

[161] J. Ju, Y. Liu, J. Hong, M.-T. Huang, A. H. Conney, C. S. Yang, “Effects of Green Tea and High-Fat Diet on Arachidonic Acid Metabolism and Aberrant Crypt Foci Formation in an Azoxymethane-Induced Colon Carcinogenesis Mouse Model*.” Nutrition and Cancer,* 2003, vol. 46, n. 2, pp. 172–178.

[162] S. Nam, D. M. Smith, Q. P. Dou, “Ester Bond-containing Tea Polyphenols Potently Inhibit proteasome Activity in vitro and in vivo.”, *The Journal of Biological Chemistry*, 2001, vol. 276, n. 16, pp.322-330.

[163] cancer.org, “Treatments and side effects” *[Online]*. Available: https://www.cancer.org/treatment/treatments-and-side-effects/treatment-types/chemotherapy/chemotherapy-side-effects.html (Accessed: 05-Jul-2019).

[164] K. A. Conklin, “Chemotherapy-Associated Oxidative Stress: Impact on Chemotherapeutic Effectiveness.”, *Integrative Cancer Therapies*, 2005, vol. 3, pp. 294-300.

[165] A. Schlitt, K. Jordan, D. Vordermark, J. Schwamborn, T. Langer, C. Thomssen, “Cardiotoxicity and Oncological Treatments.”, *Dtsch Arztebl Int.*, 2014, vol. 111, n. 10, pp. 161–168.

[166] M. S. Aslam, S. Naveed, A. Ahmed, Z. Abbas, I. Gull, M. A. Athar, “Side Effects of Chemotherapy in Cancer Patients and Evaluation of Patients Opinion about Starvation Based Differential Chemotherapy.”, *Journal of Cancer Therapy*, 2014, vol. 5, pp. 817-822.

[167] G. Kruse, “Personalized chemotherapy dosing treatment improves responses, minimizes side effects.”, *MD Anderson Cancer Center*, 2016.

[168] M. Verma, “Personalized Medicine and Cancer.”, *J Pers Med*., 2012, vol. 2, n. 1, pp. 1–14.

[169] Z. P. Chen, J. Schell, C. T. Ho, K. Y. Chen, “Green tea epigallocatechin gallate shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counterparts”, *Cancer Letters*, 1998, vol. 129, n. 2, pp. 173-179.

[170] C. Hernandez, K. Singh, L. Ponnusamy, “Re-sensitization to Doxorubicin-induced cytotoxicity in drug-resistant breast cancer cells by green tea polyphenol.”, *Sacnas National Conference – The Diversity in STEM Conference*, 2015.

[171] J. P. Mather, “In vitro models”, *Stem Cells*., 2012, vol. 30, n. 2, pp. 95-99.

[172] L. M. Ferreira, B. Hochman, M. V. J. Barbosa, “Experimental models in research”, *Acta Cir. Bras*., 2005, vol. 20, n. 2, pp. 28-34.

[173] M. A. Koch, “Experimental Modeling and Research Methodology”, 2006, *The Laboratory Rat,* pp.587–625.

[174] M. L. Andersen, M.F. Winter, “Animal models in biological and biomedical research - experimental and ethical concerns”, *An. Acad. Bras. Ciênc*., 2017, vol.91, n. 1.

[175] P. Flecknell, “Replacement, reduction and refinement.“, *ALTEX*., 2002, vol. 19, n. 2, pp. 73-78.

[176] G. Spencer, “Background on Mouse as a Model Organism”, *National Human Genome Research Institute*, 2002.

[177] Pelleitier, S. Montplaisir , “The nude mouse: a model of deficient T-cell function.”, *Methods Achiev Exp Pathol*., 1975, vol. 7, pp. 149-66.

[178] I. Szadvari, O. Krizanova, P. Babula, “Athymic Nude Mice as an Experimental Model for Cancer Treatment”, *Physiol. Res*., 2016, vol. 65, n. 4, pp. 441-453.

[179] J. E. Belizário, “Immunodeficient Mouse Models: An Overview”, *The Open Immunology Journal*, 2009, vol. 2, pp. 79-85.

[180] “Mouse Dissection Labs” *[Online]*. Available: http://helferphoto.com/editor/?

[181] Atcc.org, “ATCC WiDr (ATCC® CCL-218™)” *[Online]*. Available: http://www.lgcstandards-atcc.org/products/all/CCL-218.aspx?geo_country=pt (Accessed: 20-jul-2019).

[182] Atcc.org, “ATCC, MCF7 (ATCC® HTB-22™)” *[Online]*. Available: http://www.lgcstandards-atcc.org/products/all/HTB-22.aspx?geo_country=pt (Accessed: 20-jul-2019).

[183] D. Ribatti, “A revisited concept: Contact inhibition of growth. From cell biology to malignancy.”, *Exp Cell Res*., 2017, vol. 359, n. 1, pp. 17-19.

[184] ThermoFisher.com, “ThermoFisher Scientific, TrypLE™ Select Enzyme (1X), no phenol red” *[Online]*. Available: https://www.thermofisher.com/order/catalog/product/12563011 (Accessed: 20-Jul-2019).

[185] ResearchGate.net, “What is the trypsin mechanism in cell culture?” *[Online]*. Available: https://www.researchgate.net/post/What_is_the_trypsin_mechanism_in_cell_culture (Accessed: 20-Jul-2019).

[186] Editorial Team, “Manual Cell Counting With Neubauer Chamber”, Hematology, 2019.

[187] Cell Counting with Neubauer Chamber Basic Hemocytometer Usage, Technical Note - Neubauer Chamber Cell Counting -, Oscar Bastidas, https://mafiadoc.com/cell-counting-with-neubauer-chamber-celeromics_5bb24da2097c47b27f8b466d.html]

[188] Atcc.org, “Animal Cell Culture Guide, Cryopreservation” *[Online]*. Available: http://www.lgcstandards-atcc.org/Documents/Marketing_Literature/Animal_Cell_Culture_Guide/Cryopreservation.aspx?geo_country=pt (Accessed: 20-Jul-2019).

[189] Editorial Team, “High Performance Liquid Chromatography (HPLC) : Principle, Types, Instrumentation and Applications.”, *Biochemistry*, 2019.

[190] Laboratory Journal (2014), HPLC Analysis, The Role of Ultrapure Water, Katrin Töppner, Dirk Hansen, Elmar Herbig]

[191] Ullmann, Haller, Decourt, Girault, Spitzer, Weber, “Plasma-Kinetic Characteristics of Purified and Isolated Green Tea Catechin Epigallocatechin Gallate (EGCG) after 10 Days Repeated Dosing in Healthy Volunteers.”, *International Journal for Vitamin and Nutrition Research*, 2004, vol. 74, n. 4, pp. 269–278.

[192] J. Qiao, C. Gu, W. Shang, J. Du, W. Yin, M. Zhu, W. Wang, M. Han, W. Lu, “Effect of green tea on pharmacokinetics of 5-fluorouracil in rats and pharmacodynamics in human cell lines *in vitro.”, Food and Chemical Toxicology*, 2011, vol. 49, n. 6, pp. 1410-1415.

[193] C., H., “Green Tea Epigallocatechin-3-gallate (EGCG) – A natural agent in the fight against colorectal cancer”, 2016.

[194] C. M., “EGCG* do chá verde – Um agente natural contra o cancro da mama”, 2018.

[195] M. Barnabe, “Cell viability assays: MTT assay application and protocol, (2017)” *[Online]*. Available: https://blog.quartzy.com/2017/05/01/cell-viability-assays-mtt-protocol (Accessed: 21-Jul-2019).

[196] J. Van Meerloo, G. J. Kaspers, J. Cloos, “Cell Sensitivity Assays: The MTT Assay.” *Cancer Cell Culture,* 2011*.*

[197] V. Kuete, O. Karaosmanoğlu, H. Sivas, “Medicinal Spices and Vegetables from Africa*:* Therapeutic Potential Against Metabolic, Inflammatory, Infectious and Systemic Diseases.”, *Anticancer Activities of African Medicinal Spices and Vegetables*, 2017, n. 10, pp. 271-297.

[198] S. Van Heukelum, Beyond Aggression: Characterising the Phenotype of the BALB/cJ Mouse, 2016.

[199] M. Pelleitier, S. Montplaisir, “The nude mouse: a model of deficient T-cell function.”, *Methods Achiev Exp Pathol*., 1975, vol. 7, pp. 149-66.

[200] F. O. A, “EGCG* do chá verde –um agente natural contra o cancro colo-retal”, 2018.

[201] “Subsidiary Legislation 439.13, Protection of Animals for Scientific Purposes Regulations”, *Legal Notice*, 2013.

[202] E. Ingberg, A. Theodorsson, E. Theodorsson, J. O. Strom, “Methods for long-term 17β-estradiol administration to mice.”, *Gen Comp Endocrinol*., 2012, vol. 175, n. 1, pp. 188-93.

[203] S. M. Shafie, F. H. Grantham, “Role of hormones in the growth and regression of human breast cancer cells (MCF-7) transplanted into athymic nude mice.”, *J Natl Cancer Inst*., 1981, vol. 67, n. 1, pp. 51-56.

[204] A. Berger, “Positron emission tomography”, *BMJ*., 2003, vol. 326, n. 7404, p. 1449.

[205] S. I. Ziegler, “Positron Emission Tomography: Principles, Technology, and Recent Developments.”, Nuclear Physics A, 2005, vol. 752, pp. 679–687.

[206] Hopkinsmedicine.org, “Positron Emission Tomography” *[Online].* Available: https://www.hopkinsmedicine.org/health/treatment-tests-and-therapies/positron-emission-tomography-pet (Accessed: 21-Jul-2019).

[207] P. M. M Correia, J. Menoita, A. L. M Silva, N. Romanyshyn, J. F. C. Veloso, “An EDUGATE simulation toolkit based on the educational easyPET”, *easyPET*, 2018.

[208] M. Caccia, I. F. Castro, P. M. M. Correia, C. Mattone, L. M. Moutinho, R. Santoro, A. L. M. Silva, J. F. C. Veloso, “easyPET: a novel concept for an affordable tomographic system”, *Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment,* 2017, vol. 845, pp. 644-647.

[209] Projeto-easypet.net, “EasyPET” *[Online].* Available: http://projeto-easypet.net/#projeto (Accessed: 18-Jul-2019).

[210] V. Arosio, M. Caccia, I. F. Castro, P. M. M. Correia, C. Mattone, L. M. Moutinho, R. Santoro, A. L. M. Silva, J. F. C. Veloso, “The EasyPET: A novel concept for an educational cost-effective positron emission 2D scanner”, *Conference Paper*, 2016.

[211] E. García-Toraño, V. Peyrés, M. M. RotetaIbarra, “The half-life of ^{18}F”, *Applied Radiation and Isotopes*, 2010, vol. 68, n. 7–8, pp. 1561-1565.

[212] L.-F. de Geus-Oei, T. Ruers, C. Punt, J. W. Leer, F. Corstens, W. Oyen, “FDG-PET in colorectal cancer”, *Cancer Imaging*, 2006, vol. 6, pp. 71–81.

[213] K. Kawada, M. Iwamoto, Y. Sakai, “Mechanisms underlying ^{18}F-fluorodeoxyglucose accumulation in colorectal cancer”, *World J Radiol*., 2016, vol. 8, n. 28, pp. 880-886.

Annexe I - ORBEA statement

Biotério IBILI – Sub-Unidade I – FMUC

ORBEA

PARECER 17/2015

Título do projeto: PET com sistema inovador de leitura rápida para correção de DOI

Investigador Responsável: João Filipe C. A. Veloso

O Órgão para o Bem Estar Animal – ORBEA, do Biotério IBILI – Sub-Unidade I da Faculdade de Medicina da Universidade de Coimbra, após análise do projeto acima mencionado, deu o seu parecer favorável.

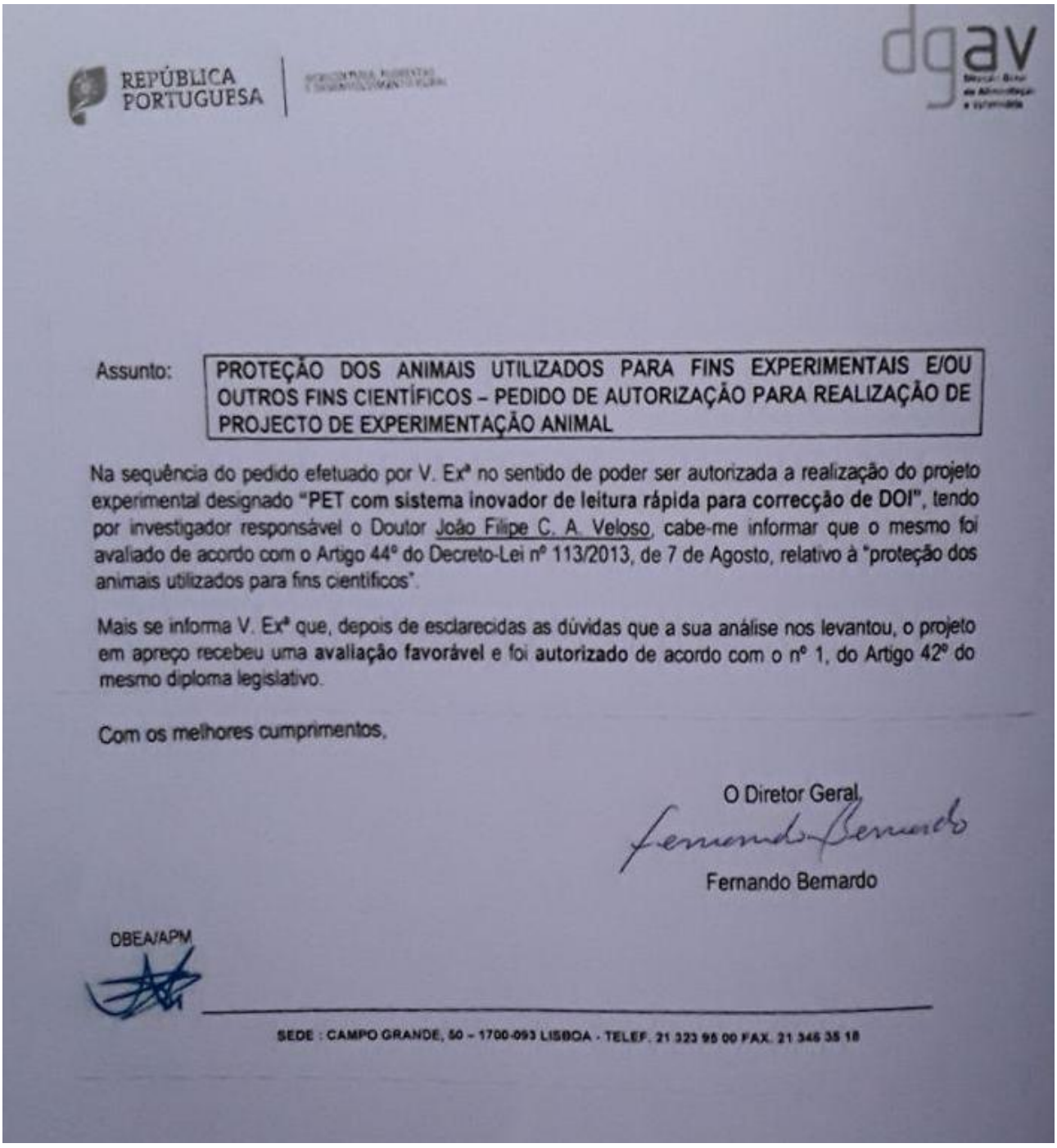
Coimbra, 24 de Julho de 2015

A Diretora do Estabelecimento

A Veterinária Designada

Faculdade de Medicina da Universidade de Coimbra
Pólo III da Universidade de Coimbra | Azinhaga de Santa Comba, Celas
3000 548 Coimbra, Portugal
Telefone: 239480040 | Fax: 239480258

Annexe II - DGAV statement



REPÚBLICA PORTUGUESA

dgav

Assunto: PROTEÇÃO DOS ANIMAIS UTILIZADOS PARA FINS EXPERIMENTAIS E/OU OUTROS FINS CIENTÍFICOS – PEDIDO DE AUTORIZAÇÃO PARA REALIZAÇÃO DE PROJECTO DE EXPERIMENTAÇÃO ANIMAL

Na sequência do pedido efetuado por V. Exª no sentido de poder ser autorizada a realização do projeto experimental designado "PET com sistema inovador de leitura rápida para correcção de DOI", tendo por investigador responsável o Doutor João Filipe C. A. Veloso, cabe-me informar que o mesmo foi avaliado de acordo com o Artigo 44º do Decreto-Lei nº 113/2013, de 7 de Agosto, relativo à "proteção dos animais utilizados para fins científicos".

Mais se informa V. Exª que, depois de esclarecidas as dúvidas que a sua análise nos levantou, o projeto em apreço recebeu uma **avaliação favorável** e foi **autorizado** de acordo com o nº 1, do Artigo 42º do mesmo diploma legislativo.

Com os melhores cumprimentos,

O Diretor Geral,

Fernando Bernardo

Fernando Bernardo

OBEA/APM

SEDE : CAMPO GRANDE, 50 – 1700-093 LISBOA - TELEF. 21 323 95 00 FAX. 21 346 35 18

Annexe III

[A] Trypan blue

The procedure consists of preparing 4 g trypan blue in 100 mL distilled water.

[B] PBS

The procedure consists of adding to a sterile 500 mL Schott bottle (Duran, 00361668, Germany) 7.4 g of NaH_2PO_4, 3.6 g of NaCl, 0.215 g of KH_2PO_4 and 500 mL of ultrapure water. At the end, the pH is adjusted to 7.4 and the vial is closed and labeled.

[C] Acid isopropanol

The procedure consists of adding to a sterile 500 mL Schott bottle (Duran, 00361668, Germany) 1.75 mL of fuming hydrochloric acid at 37% and 498.25 mL of 2-propanol at 99,5%.