UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO

HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY CHARACTERIZATION OF MAMMARY LESIONS CHEMICALLY-INDUCED BY 7,12-DIMETHYLBENZ(A)ANTHRACENE AND 1-METHYL-1-NITROSOUREA IN FEMALE SPRAGUE-DAWLEY RATS

Thesis in Veterinary Sciences

ANTONIETA MARIA ALVARADO MUÑOZ

Advisors:

Professor Doutor Bruno Jorge Antunes Colaço

Professora Doutora Paula Alexandra Martins de Oliveira

Professor Doutor António Cabrita

Vila Real, November 2017

UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO

HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY CHARACTERIZATION OF MAMMARY LESIONS CHEMICALLY-INDUCED BY 7,12-DIMETHYLBENZ(A)ANTHRACENE AND 1-METHYL-1-NITROSOUREA IN FEMALE SPRAGUE-DAWLEY RATS

Thesis in Veterinary Sciences

ANTONIETA MARIA ALVARADO MUÑOZ

Advisors:
Professor Doutor Bruno Jorge Antunes Colaço
Professora Doutora Paula Alexandra Martins de Oliveira
Professor Doutor António Cabrita

Composition of the Jury

Professor Doutor Jorge Manuel Teixeira de Azevedo
Professora Doutora Maria da Conceição da Cunha e Vasconcelos Peleteiro
Professor Doutor Rui Pedro Brás Martins Faísca
Professora Doutora Anabela Gouveia Antunes Alves
Professora Doutora Isabel Cristina Ribeiro Pires
Professora Doutora Helena Maria Vala Correia
Professor Doutor Bruno Jorge Antunes Colaço

Vila Real, November 2017

Original thesis presented by Antonieta María Alvarado Muñoz at the University of Trás-os-Montes and Alto Douro, to obtain the doctor's degree on Veterinary Science

This work was partially funded by
Operational Competitiveness and Internationalization Programme, under Project POCI-01-0145-FEDER-016728, National Funds by FCT-Portuguese Foundation for Science and Technology, under the project PTDC/DTP-DES/6077/2014 and Consejo de Desarrollo Científico, Humanístico y Tecnológico CDCHT, under the project 008REV2015

To My Fathers, Brothers, Nephews and Nieces. My Family

"Whatever the mind….. can conceive and believe, it can achieve"

W. Clement Stone (1902-2002)

ACKNOWLEDGEMENTS

Many people made possible the realization of this thesis, therefore, I want to express my sincerely acknowledgement *to*:

Professor Bruno Jorge Antunes Colaço, PhD, whom, without knowing me trusted and believed in my ability to carry out this project. He became my counselor and truly a good friend during this difficult journey. Thank you very much for your teaching, advices, explanations, conversations and contributions to my professional growth.

Professor Paula Alexandra Martins de Oliveira, PhD, I do not have enough words to express my sincere gratitude for providing me with the tools to undertake this work. Thank you for the teaching and for supporting me during the most difficult moments of this process. Without your help, I could not have moved forward. Again, many thanks.

My Professor and Mentor, Victoria Del Rosario Colmenarez, I want to thank you for the knowledge transmitted through all these years, not only in science but in courage and wisdom, and for your help to make it possible for me to be here.

Professor António Cabrita, PhD, for your contribution to this work and for your good disposition to complete this thesis.

Professor Ana Calado, PhD, for your contribution to this work, for your good disposition and the tools you provided me to complete part of this thesis.

Professor Rita Ferreira, PhD, for the contribution, good disposition and the tools provided to this work.

Laboratório de Histologia e Anatomía Patológica of the Universidade de Trás-os-Montes e Alto Douro, for the good disposition for processing the tools for this thesis.

Professor Aura Antunes Colaço, PhD, for contributing to this thesis, by offering your support, whenever I needed it.

Professor Jorge Colaço, PhD, for your support with the use of statistics to complete this work.

Professor Carlos Lopes, PhD, for the contribution and suggestions in the histopathological analysis.

Rui Gil Da Costa, PhD, thank you very much for you time, help, knowledge and dedication to make possible the realization of a chapter of this thesis. Thank you too for showing me your the beautiful city of Oporto, for every meal and conversation and for your sincere friendship.

My good friend Ana Faustino Rocha, because I never met any person who gave me her disinterested friendship, like the one you offered me; because I learned too much from you. Thank you very much, without your help I would not be writing this thesis. I hope the future will bring us together in big projects. I wish you great success.

My friend Ana Catharina Batista, for giving me a space in your life. Thanks for listening my complaints, for being my travel companion and for your friendship.

My true friends in the distance: Yulman Cortez, my old friend always attentive with me; Yaritza Salas, thanks for your help when I need it. German Vargas, thanks for every word of encouragement, really you helped me.

My Portuguese friends, particularly Magda and Tânia, thanks for listening to my problems, my histories and my laughs.

My siblings: Hugo, Gloria, Federico and Eduardo for being my support, I hope that this work can be proof to my nephews and nieces that all of they want in their lives it is possible.

My father Hugo Federico Alvarado Ochoa and my mother Gloria Madelón Muñoz Ojeda. Thanks for making me the person I am, everything that I have accomplished in my live it is because of both of you, thanks.

RESUMO

As neoplasias de mama quimicamente induzidas pelos agentes carcinogénicos 7,12-dimetilbenz (a) antraceno (DMBA) e 1-metil-1-nitrosureia (MNU) em ratos têm sido frequentemente utilizadas como modelo para o estudo do cancro de mama humano, devido às semelhanças em termos da sua histopatologia e dependência hormonal. O receptor de estrogénio α (ERα), o receptor de progesterona (PR) e a proteína Ki-67 são considerados bons marcadores de prognóstico e fatores preditivos no cancro da mama. Este estudo teve como objetivo avaliar o comportamento biológico das lesões da mama induzidas pela MNU e pelo DMBA em ratos do sexo feminino da estirpe Sprague-Dawley, através da imunoexpressão dos marcadores de prognóstico ERα, PR e Ki-67, de modo a identificar o modelo mais adequado para o estudo do cancro da mama na mulher. Neste estudo também foram avaliados os efeitos da prática de exercício físico durante 35 semanas na imunoexpressão dos fatores de prognóstico ERα e Ki-67, no índice de atividade mitótica (MAI), e no desenvolvimento de metástases em neoplasias mamárias induzidas pela MNU em ratos do sexo feminino da estirpe Sprague-Dawley.

Para este estudo, um grupo de animais do sexo feminino da estirpe Sprague-Dawley recebeu uma única dose do agente carcinogénico DMBA (65 mg/kg) por gavage aos 50 dias de idade. Outro grupo de animais foi dividido aleatoriamente em quatro grupos experimentais: MNU sedentário, MNU exercitado, controlo sedentário e controlo exercitado. Aos 50 dias de idade, os animais dos grupos MNU receberam uma única injeção intraperitoneal do agente carcinogénico MNU (50 mg/kg). Os animais dos grupos controlo receberam uma injeção intraperitoneal do veículo (solução salina a 0,9%). No dia seguinte à administração, os animais dos grupos exercitados (grupos MNU e controlo) foram treinados num tapete rolante a uma velocidade de 20 m/min, 60 min/dia, 5 dias/semana durante 35 semanas. Os animais do grupo DMBA foram sacrificados 27 semanas após a administração do agente carcinogénico, enquanto os animais dos grupo MNU e controlo foram sacrificados 35 semanas após da administração do agente carcinogénico. As neoplasias mamárias induzidas pelos agentes carcinogénicos foram classificados por histopatologia; os órgãos das cavidades torácica e abdominal foram avaliados para o estudo das metástases. A expressão dos marcadores de prognóstico ERα, PR e índice de proliferação Ki-67 (Ki-67 PI) foi avaliada por imuno-histoquímica. O ERα-H-Score e o MAI também foram avaliados.

Assim, para um dos estudos desta tese foram analisadas 28 neoplasias mamárias induzidas pela MNU no grupo sedentário e 16 neoplasias mamárias induzidas pelo DMBA. Em cada neoplasia mamária foi identificado mais de um padrão histológico, assim apenas os carcinomas mamários foram analisados de modo a uniformizar as amostras. Todos os carcinomas mamários induzidos pela MNU e pelo DMBA foram positivos para o ERα e o PR (ER^+/PR^+), com maior expressão do ERα quando comparado com a expressão do PR. O peso das neoplasias, a expressão do ERα, do PR, o Ki-67 PI e o MAI foram mais elevados nos carcinomas mamários induzidos pela MNU quando comparados com os carcinomas induzidos pelo DMBA. Foram observadas diferenças estatisticamente significativas entre os grupos para o ERα, o PR e o MAI ($p<0,05$).

No trabalho seguinte foram analisadas e comparadas as neoplasias mamárias induzidas pela MNU nos grupos exercitados e sedentários. Todas as neoplasias de ambos grupos foram positivas para o ERα com um H-score ≥20. Embora não tenham sido observadas diferenças estatisticamente significativas no ERα H-Score, no Ki-67 PI e no MAI entre os grupos, o valor absoluto do ERα H-Score foi maior no grupo exercitado, enquanto o Ki-67 PI e o MAI foram mais elevados no grupo sedentário. Finalmente, no estudo das metástases observou-se que os animais exercitados não desenvolveram qualquer metástase, enquanto que duas metástases pulmonares foram observadas no grupo sedentário. À semelhança dos carcinomas mamários, as metástases foram positivas para o ERα e para o PR, indicando uma elevada sensibilidade hormonal.

Os elevados valores do KI-67 PI e do MAI em carcinomas mamários induzidos pela MNU são indicadores de uma maior agressividade destes carcinomas quando comparados com os induzidos pelo DMBA, sugerindo uma pior resposta à terapia e um pior prognóstico. Estes dados sugerem que o modelo de cancro da mama em ratos induzidos pela MNU é aconselhado em protocolos experimentais que tenham como objectivo o estudo de carcinomas mamários mais agressivos dentro do grupo dos carcinomas mamários positivos para ambos os recetores hormonais (ER^+/PR^+). As neoplasias do grupo MNU exercitado foram menos proliferativas e mais diferenciadas quando comparadas com as neoplasias mamárias do grupo MNU sedentário, sugerindo que a prática de exercício físico durante um longo período de tempo teve efeitos positivos na carcinogénese mamária. Finalmente, foi possível concluir que a prática de exercício físico durante um longo período de tempo diminuíu o risco de formação de metástases do cancro da mama, este fenómeno ocorreu na presença de níveis de estrogénio

aumentados, com lesões primárias e metastáticas sensíveis a hormonas esteróides, indicando que este efeito foi independente da estimulação hormonal. A prática de exercício físico durante um longo período de tempo parece ser benéfica para doentes com cancro de mama, através do aumento da diferenciação das neoplasias mamárias, da diminuição da proliferação e da inibição da formação de metástases.

Palavras-chave: receptor de estrogénio, receptor de progesterona, Ki-67, índice de atividade mitótica, rato, MNU, DMBA, neoplasia da mama, carcinogénese química, tapete rolante.

ABSTRACT

Chemically-induced mammary tumors in rats by the carcinogens 7,12-dimethylbenz(a)anthracene (DMBA) and 1-metil-1-nitrosourea (MNU) are the most widely used models for studies on mammary human carcinogenesis because of their similar histopathology and hormone dependence. Estrogen receptor α (ERα), progesterone receptor (PR) and Ki-67 protein are considered strong prognostic and predictive markers in breast cancer. This study aimed to assess the biological behavior of MNU and DMBA-induced mammary tumors in female Sprague-Dawley rats, through the immunoexpression of prognostic markers ERα, PR and Ki-67, in order to know the model that best suits to woman breast cancer. The effects of 35 weeks of exercise training on the immunoexpression of prognostic factors ERα, Ki-67 and the mitotic activity index (MAI), and the development of metastasis in mammary tumors MNU-induced in female Sprague-Dawley rats were also evaluated.

For this study, a group of animals received a single dose of the carcinogen DMBA (65 mg/kg) by gavage at 50 days of age. Other group of animals was randomly divided into four experimental groups: MNU sedentary, MNU exercised, control sedentary and control exercised. The MNU groups received a single intraperitoneal injection of the carcinogen MNU (50 mg/kg) at 50 days of age. Control groups received an intraperitoneal injection of the vehicle (saline solution 0.9%). After this, animals from exercised groups (MNU and control groups) were exercised in a treadmill at a velocity of 20 m/min, 60 min/day, 5 days/week for 35 weeks. Animals from DMBA group were sacrificed 27 weeks after the carcinogen administration, while the animals from MNU and control groups were sacrificed 35 weeks after the carcinogen administration. Mammary tumors induced by both carcinogens were histologically classified; the organs from thoracic and abdominal cavities were evaluated for the metastasis study. The expression of the prognostic markers ERα, PR and Ki-67 proliferation index (Ki-67 PI) were assessed by immunohistochemistry. ERα H-Score and MAI were also evaluated.

Thus for one of the studies of this these, 28 MNU-induced mammary tumors from sedentary group and 16 DMBA-induced mammary tumors were analyzed. More than one histological pattern was identified in each mammary tumor, so in order to homogenize the samples only mammary carcinomas were analyzed. All MNU and DMBA-induced mammary

carcinomas were ERα and PR positive (ER^+/PR^+), with a higher expression of ERα when compared with PR. Tumor weight, the expression of ERα, PR, Ki-67 PI and MAI were higher in MNU-induced mammary carcinomas when compared with the DMBA-induced ones. Statistically significant differences between groups were observed for ERα, PR and MAI ($p<0.05$).

In the following study, MNU-induced mammary tumors in exercised and sedentary groups were analyzed and compared. All neoplasms from both groups were ERα-positive with an H-score ≥20. Although statistically significant differences were not found in the ERα H-score, Ki-67 PI and MAI between groups, the absolute value of ERα H-score was higher in exercised group, while the Ki-67 PI and MAI were higher in sedentary group. Finally, in the metastasis study, it was observed that exercised animals did not develop any metastasis while two pulmonary metastases were observed in the sedentary group. Like the mammary carcinomas, their metastases were positive for ERα and PR, indicating high hormonal sensitivity.

The higher KI-67 PI and MAI in MNU-induced mammary carcinomas was indicative of a higher aggressiveness of these carcinomas when compared with the DMBA-induced ones, suggesting a worse response to the therapy and a worse prognosis. These data suggest that the rat model of MNU-induced mammary tumors is advised in experimental protocols aiming to study more aggressive mammary carcinomas within the group of double-positive mammary tumors (ER^+/PR^+). Tumors from MNU exercised group were less proliferative and more differentiated when compared with mammary tumors from MNU sedentary group, suggesting that long-term exercise training had positive effects on mammary carcinogenesis. Finally, it was possible to conclude that long-term exercise training may decrease the risk of mammary cancer metastasis; this occurred in the presence of enhanced oestrogen levels, with hormone-sensitive primary and metastatic lesions, indicating that the effect was hormone-independent. The practice of exercise training during a long period of time seems to be beneficial for breast cancer patients, through the increase of mammary neoplasms differentiation, decrease of proliferation and inhibition of metastasis.

Keywords: estrogen receptor, progesterone receptor, Ki-67, mitotic activity index, rat, MNU, DMBA, mammary tumours, chemical carcinogenesis, treadmill.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND SYMBOLS

AB	Alveolar buds
ANOVA	Analysis of variance
b.w.	body weight
DADS	Diallyl disulfide
DHA	Docosahexaenoic acid
DMBA	7,12-dimethylbenz(a)anthracene
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
EPA	Eicosapentaenoic acid
ER	Estrogen/oestrogen receptor
ERα	Estrogen/oestrogen receptor α
ERβ	Estrogen/oestrogen receptor β
ERs	Estrogen/oestrogen receptors
FMPA	9α-Fluoromedroxyprogesterone Acetate
H&E	Hematoxylin and eosin
HPF	High power field
4-HPRCG	*N*-(4-hydroxyphenyl)retinamide-C- glucoronide
HSD	Honestly significant difference
IDP	Intraductal proliferation
i.p.	Intraperitoneal injection
i.v.	Intravenous injection
Ki-67PI	Ki-67 proliferation index
Kg	Kilogram
m	Metre
MGA	Megestrol acetate

Mito-T	Mito-Tempol
ml	Millilitre
min	Minute
MNU	1-metil-1-nitrosurea
MPA	Medroxyprogesterone acetate
NAC	*N*-acetyl-L-cysteine
NDGA	Nordihydroguaiaretic acid
NK	Natural killer
N-EL	Norgestrel
N-ONE	Norethindrone
OR	Odds ratio
PCNA	Proliferating cell nuclear antigen
PEITC	Phenethylisothiocyanate
PI	Proliferation index
PR	Progesterone receptor
PUFA	n-3 polyunsaturated fatty acids
p.o.	Oral administration
rBMP-2	Recombinant bone morphogenetic protein-2
RNAs	Ribonucleic acids
rUCMS	Rat umbilical cord matrix stem cells
SAC	S -all1cysteine
S.D.	Standard deviation
s.c.	Subcutaneous injection
TEB	Terminal end bud
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

GENERAL INTRODUCTION

Breast cancer is the second most frequent human cancer and the most common cancer among women worldwide. In 2002, approximately 1.15 million of women were diagnosed with breast cancer, representing 23% of all cancers (Parkin *et al.*, 2005). Since then, the number of breast cancer cases was increasing and in 2012 approximately 1.67 million of new cases were diagnosed in women, representing 25% of all cancers (Ferlay *et al.*, 2015). Although the breast cancer incidence has increased globally, its incidence varies across the world regions being higher in less developed regions when compared with developed ones. Despite that the number of breast cancer deaths is high in developing countries, the number of cancer cases with a favorable prognosis is higher than the less developed (Curado, 2011; Ferlay *et al.*, 2015; Vrdoljak *et al.*, 2016) . In South America, the breast cancer incidence was of 4.3% for the period between 1998-2002 and increased to 6.91% in 2012 (Curado, 2011; Ferlay *et al.*, 2015). An incidence of 59 *per* rate of 100.000 inhabitants with a 19% of mortality was registered in the Center and Eastern Europe (Vrdoljak *et al.*, 2016). The cancer statistics in Portugal follow the tendency of the developed countries, the breast cancer is the most frequent cancer among women being considered the first cause of cancer death in 2002 with a mortality of 17% (Bastos *et al.*, 2007).

Despite the inexistence of a true incidence rate or registries in domestic animals, mammary cancer is the most frequently diagnosed cancer in female intact dogs and approximately 50% of them are malignant (Merlo *et al.*, 2008; Salas *et al.*, 2015).

Breast cancer incidence is related with a combination of different factors, including genetic mutations, family history, ethnicity, endocrinology, diet, and exposition to ionizing radiation or chemical compounds that can induce conformational changes in the mammary gland leading to the development of breast cancer disease (Russo and Russo, 1996b). Approximately 90% of breast cancer deaths occur due to metastatic dissemination. Indeed, the distinct characteristics of metastasis and their location makes difficult their detection, evaluation and treatment by conventional methods (Mori *et al.*, 2001; Cummings *et al.*, 2014).

Breast cancer is not a simple pathological entity but rather a very complex group of heterogeneous neoplasms emerging from epithelial and/or mesenchymal component of mammary gland tissue (Polyak, 2011; Dai *et al.*, 2016). The terminology employed can vary from “breast cancer” or “breast tumor” in women to “mammary cancer” or “mammary tumor” in animals mainly due to the interspecies biological and anatomical differences (Russo and Russo, 1996a).

Since the breast cancer is considered a global public health problem, animal models have been used for several years for a better understanding of this disease and they are still considered valuable tools to study several aspects of human breast carcinogenesis, like histopathology, treatment, control and prevention (Thompson, 1997). Mammary tumor development in rats can be induced through the administration of hormones, chemical carcinogens and exposition to physical agents (Russo, 2015), being the model of chemically-induced mammary cancer the most frequently used. Indeed, the rat mammary tumors induced through the administration of the established chemical carcinogens DMBA and MNU are very similar to those developed by humans (Russo and Russo, 1996b). The spectrum of mammary lesions induced by both carcinogens in female rats is influenced by animal age, carcinogen dose and route of administration (Nandi *et al.*, 1995; Thordarson *et al.*, 2001).

Estrogen receptor (ER) and PR are nuclear proteins with an important role in the mammary gland development, controlling proliferation and development of epithelial alveolar cells of the mammary gland (Obr and Edwards, 2012). Both receptors are well-established as prognostic and predictive factors for breast cancer. The hormone-dependency of breast tumors, established by their positivity or negativity for ER and PR (ER^+/PR^+, ER^+/PR^-, ER^-/PR^+, ER^-/PR^-) is crucial for the treatment as it determines significantly the most adequate therapeutic approach (Dai *et al.*, 2016). Ki-67 protein is another important marker related with the proliferation of tumor cells, being the expression of its receptor considered a prognostic factor in breast cancer (Scholzen and Gerdes, 2000). In conjunction, hormone receptors and Ki-67 are important prognostic factors in early stage of breast cancer (Haroon *et al.*, 2013). The immunoexpression of ER and low levels of Ki-67 is suggestive of a good response to hormonal therapy and a positive prognostic (Arpino *et al.*, 2015). Recently, it was demonstrated that the genetical and immunohistochemical expression of these markers in chemically-induced mammary tumors in female rats is similar to that observed in women breast cancer. Given this similarity, the immunohistochemical study of prognostic markers in rats can be transposed to women (Russo, 2015).

Several therapeutic approaches have been studied in rat models of mammary cancer, including surgery, chemotherapy, hormonal therapy, radiation and immunotherapy. Additionally to this, also the effects of lifestyle on mammary carcinogenesis namely practice of a physical activity, have been evaluated in rat models. Previous researches on this field pointed different theories in an attempt to explain the protective effects of exercise training on

mammary cancer: it increases detoxification of chemical carcinogens reducing the DNA-damage and increases DNA repair preventing the initiation (the first stage of carcinogenesis) and reducing the promotion the otherwise initiated cells (the second stage of the carcinogenesis); additionally it reduces the serum levels of some growth factors, reducing the occurrence of errors during the cell replication (Rundle, 2005). Recently, it was demonstrated that exercise can modulate the immune response increasing immune cell infiltration in the tumoral microenvironment, specifically natural killer cells (NK cells), leading to an increase in the cytokines secretion and inhibition of tumor growth (Pedersen *et al.*, 2016), and can modulate the permeability of blood vessels reducing the extravasations of potential metastatic cells (Wolf *et al.* 2015). Despite this, the exact mechanism as the exercise training reduces the risk of cancer development in the different stages of carcinogenesis is not entirely elucidated.

All the previously described works conducted to define the aims of the present thesis. In this way, it was intended to study the mammary tumors chemically-induced in female rats through the administration of the carcinogen agents MNU and DMBA with an emphasis on the histological patterns, immunoexpression of hormone receptors and proliferation markers, in order to establish the most adequate one to study woman breast cancer. It was also intended to evaluate the effects of exercise training on the development and metastization of mammary tumors MNU-induced, and on the immunoexpression of the prognostic factors described above.

AIMS

The present work had as main purposes:

- Evaluate the immunoexpression of the prognostic factors ERα, PR, Ki-67 and MAI, in MNU and DMBA-induced mammary tumors in female Sprague-Dawley rats.

- Evaluate the effects of lifelong exercise training on the immunoexpression of ERα, Ki-67PI and MAI in MNU-induced mammary tumors in female Sprague-Dawley rats.

- Evaluate the effect of lifelong exercise training on the development of breast cancer metastasis, as well as a characterization of those metastatic lesions in MNU-induced mammary tumors in female Sprague-Dawley rats.

CHAPTER 1

EXPERIMENTAL MAMMARY CARCINOGENESIS RAT MODELS

THE CONTENT OF THIS CHAPTER WAS PUBLISHED IN:

Alvarado, A.; Faustino-Rocha, A.; Colaço, B. and Oliveira P. (2017). Experimental mammary carcinogenesis - Rat models. *Life Sciences*. 173(15):116-134. (IF 2.685; Q2). (Full Paper).

1. EXPERIMENTAL MAMMARY CARCINOGENESIS - RAT MODELS

1.1. Introduction

Breast cancer remains as one of the most frequent cancers among women, affecting approximately one out of ten women worldwide (Liska *et al.*, 2000). According to the World Health Organization, in 2012 breast cancer was responsible for the death of more than half a million of women around the world (WHO, 2015). In this way, a better knowledge about the cancer biopathology is important to the development of new preventive and therapeutic strategies to fight this disease.

Since early times, animal models have been used by researchers in order to better know the anatomy and physiology of the human body. Aristotle (384-322 B.C.), who is considered one of the most important thinkers, used animals to study intern differences among species. His studies were well documented and spread to other countries, contributing to the use of animal models as a research tool in several European and Arabian countries (Ericsson *et al.*, 2013). Since then, animal models have been frequently used and they have had a great importance in biomedical research. Proofing this is the fact that over the last century, all Nobel prizes in the field of physiology and medicine used animals as models of diseases (Badyal and Desai, 2014). Nowadays, animal models still represent an important tool to study several diseases, including cancer. They allow the researchers to better understand many aspects of this disease, such as the etiology, the pathogenesis, the progression, the genetic and molecular basis, and the development and evaluation of several therapeutic approaches that may improve the quality of life and lifespan of oncologic patients (Clarke, 1996; Liska *et al.*, 2003; Iannaccone and Jacob, 2009; Faustino-Rocha *et al.*, 2015a).

1.2. How to select an animal model?

Several animal species, such as fishes, rabbits, rats, mice, dogs, non-human primates and large animals may be used as models, and the researchers should be able to choose the most adequate model to answer to their questions/hypotheses (Conn, 2013). An ideal animal model of human diseases should be simple, not expensive and similar to Human as much as possible (Fagundes and Taha, 2004). When confronted with the need to choose an animal model, the researchers should take into consideration the following aspects: the aim of the study, available species, advantages and disadvantages of each species, accommodation

expenses, manipulation, required equipment and ethical considerations (Van der Gulden *et al.*, 1999; Fagundes and Taha, 2004). Mice (*Mus musculus*) and rats (*Rattus norvegicus*) are among the species more frequently used in research protocols performed in the European Union Comission (2013). Indeed, when compared with other species, they have some advantages, namely their physiology and genetic are well known, they are small animals, easy to accommodate and manipulate, they are relatively cheap, their use is easily approved by legislation on the protection of animals used for scientific purposes, and the most important one, they are mammals and have many similarities with humans, like anatomy, physiology, genetic and biochemistry (Cardiff, 2007; Iannaccone and Jacob, 2009).

1.3. Rat as a model of mammary cancer

The occurrence of mammary tumors (mammary fibroadenoma) in female rats was described by the first time by Mceuen in 1938 after the daily vaginal application of a solution of estrone in corn oil for two years and an half (Mceuen, 1938). Since then, the female rat has been continuously used as a model of mammary cancer and nowadays it constitutes one of the most frequently used animal models to study mammary carcinogenesis (Cardiff, 2007). Indeed, the mammary cancer in female rats resembles that of women in several features, namely in its hormone responsiveness, histology, biochemical properties, molecular and genetic characteristics. Additionally to this, when compared with mice, the rats provide a higher quantity of blood and tissue samples for posterior studies (Mullins *et al.*, 2002; Hoenerhoff *et al.*, 2011).

1.4. Rat mammary gland anatomy and histology

The female rat has two mammary chains (right and left) with six mammary glands with a nipple each one: three pairs in thoracic region (extended to the cervical region) and three pairs in abdominal-inguinal region (Maeda *et al.*, 2000). The mammary glands of each mammary chain are usually numbered by the nipple from one to six in the cranio-caudal direction and the gland tissue of the thoracic region are smaller when compared with those of the abdominal-inguinal region (Hvid *et al.*, 2011). Inversely to that happens in women, the rat mammary glands are poorly developed and they can be identified externally only by the presence of the nipple. Rat mammary glands are greatly vascularized by the branches of

several arteries, namely superficial cervical, thoracic internal and external, external pudendal and axillary (Maeda *et al.*, 2000) (Figure 1.1).

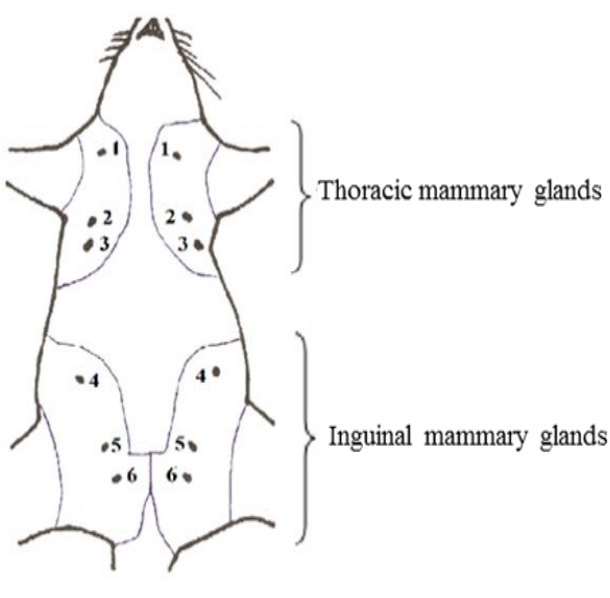

Figure 1.1. Schematic representation of anatomic location of mammary glands in female rat.

Microscopically, the rat mammary gland has a tubuloalveolar conformation, being composed by a group of branched tubular ducts and alveolar buds (Lucas *et al.*, 2007). It consists of two main tissues: the parenchymal and the stromal tissue (connective, adipose and vascular network tissues) (Nandi *et al.*, 1995). When compared with mice mammary gland, the rat mammary gland tissues have a higher stromal and parenchymal component (Mullins *et al.*, 2002; Hoenerhoff *et al.*, 2011). Like in women, the rat mammary gland tissue is hormone dependent and grows during estrous cycle and pregnancy (Hvid *et al.*, 2012).

The development of rats' mammary gland occurs through different phases. An extensive development occurs by the day 21, being this phase characterized by the differentiation of the epithelium into terminal end bud units that correspond to the bulbous end of the branch lactiferous duct (Colditz and Frazier, 1995; Lucas *et al.*, 2007). The duct is composed by a layer of luminal epithelial cells (ductal epithelial cells) fated to form the walls of the ductal lumen with outer of myoepithelial cells and the basement membrane. Terminal end bud is composed by multi-layered of preluminal epithelial cells (also called body cells) surrounded by a layer of pluripotent stem cells (also called cap cells) that are progenitors of

mioephitelial cells. Both body cells and cap cells are very proliferative (Figure 1.2A) (Hinck and Silberstein, 2005; Cardiff, 2007; Manivannan and Nelson, 2012). Terminal end bud proliferates dichotomously into alveolar buds and terminal ductules with continuous branching ducts and ductules that drain into the duct of the nipple (Figure 1.2B) (Hvid *et al.*, 2012). Each alveolar buds and duct have one layer of simple epithelial cells surrounded by a layer of myoepithelial cells and the basement membrane, supported by the stroma. Near to 50-55 days of age, the alveolar lobules are formed from the alveolar buds in both non-pregnant and pregnant rats (Lucas *et al.*, 2007; Hvid *et al.*, 2012).

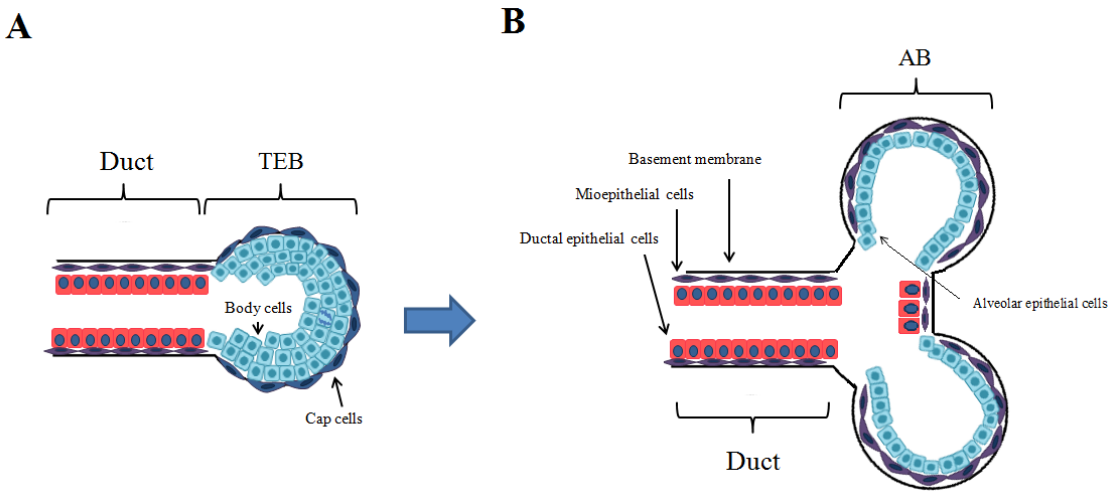

Figure 1.2. A. Schematic representation of the development of female rat mammary gland. The mammary gland is composed by several terminal end bud (TEB) units. **B**. At the puberty, these units differentiate dichotomously into alveolar buds (AB).

As mentioned above, the rat mammary gland tissue is hormone dependent and consequently the secretory structure is influenced by the sexual maturity (reached at about 6 weeks of age), estrous cycle and pregnancy (Sengupta, 2013). Estrogen and growth hormone are considered the main hormones responsible for the ductal elongation. Despite these, the proliferation, branching and differentiation of mammary gland may be influenced by other hormones, such as progesterone and thyroid hormones. When the pregnancy occurs, the progesterone is the main responsible for the exponential growth of the mammary gland, and the prolactin is responsible for the mammary gland alveologenesis and the development of specialized epithelium for milk production (Radisky *et al.*, 2003).

1.5. Rat mammary carcinogenesis

Carcinogenesis is a multistep process that may progress over many years. It consists of distinct but also linked phases (initiation, promotion, progression and metastization) in which different molecular and cellular alterations occur (Figure 1.3). The **initiation** is characterized by the spontaneous or induced irreversible DNA-damage that leads to the conversion of a normal cell into an initiated one. The **promotion** is considered a relatively lengthy and reversible process during which the initiated cells grow and divide in an uncontrolled way as a result of accumulated abnormalities, originating a population of preneoplastic cells. The promotion may be changed by the administration of chemopreventive agents that can affect the tumor growth rates. A fast increase in tumor size occurs during the **progression** and in this phase the preneoplastic cells may convert into neoplastic ones as a consequence of additional genetic alterations. In this step, the tumor may become malignant and possibly metastatic. The tumor **metastization** is a complex process during which the cancer cells spread from the primary tumor to discontiguous organs, through blood or lymphatic system. The ability to metastasize is exclusive of the malignant neoplasms, however it is worth to note that not all malignant neoplasms metastasize (Oliveira *et al.*, 2006, 2007; Eickmeyer *et al.*, 2012; Siddiqui *et al.*, 2015; Oliveira, 2016).

The faster expansion of the mammary gland epithelium that occurs during the rat puberty (45-55 days of age) is considered the key-point for the initiation of carcinogenesis (Sternlicht, 2006). The rat mammary tumors may emerge from epithelial cells located in duct, terminal end bud and alveolar buds. However, due to the high mitotic/proliferation index of pluripotent cells from terminal end bud (cell cycle takes approximately 13 hours) that conduct to the ductal elongation, ramification and cell differentiation, when compared with the proliferation index in the alveolar buds (approximately 34 hours), the terminal end bud is the most common site of mammary carcinogenesis initiation in female rats (Sternlicht, 2006). As it is commonly known, the pluripotent cells also called stem cells are very susceptible to DNA changes resulting in the easy formation of preneoplastic and neoplastic cells, which give rise to the primary tumor and potential generation of metastatic cells (Eickmeyer *et al.*, 2012) (Figure 3). As the cell cycle in terminal end bud is shorter when compared with alveolar buds, the time to repair the DNA damages is shorter, contributing to the higher susceptibility of terminal end bud for carcinogenesis initiation (Colditz and Frazier, 1995).

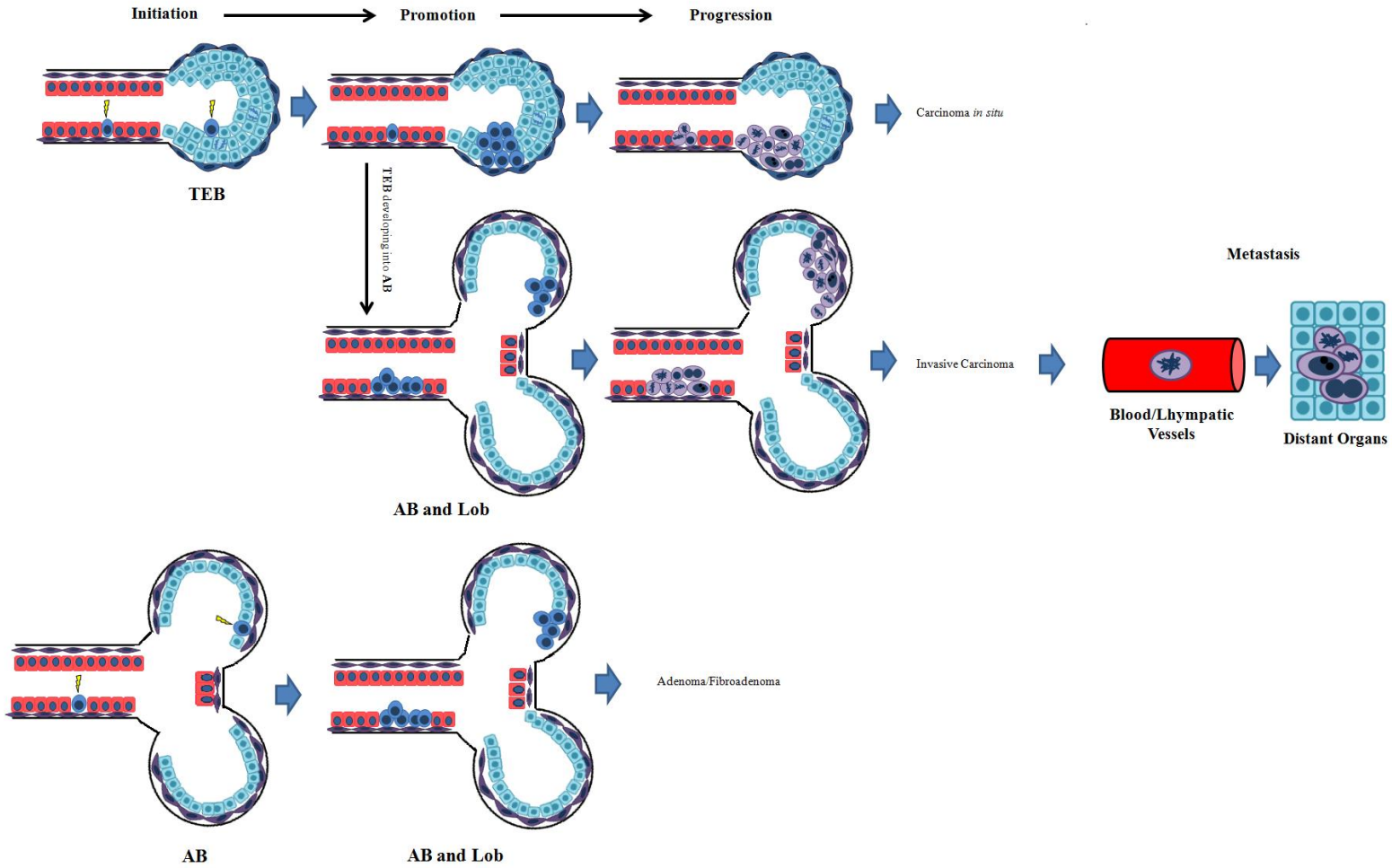

Figure 1.3. Schematic representation of stages of carcinogenesis in mammary parenchyma. When the carcinogen agent is administered between 45 and 60 days of age (puberty), the initiation occurs in terminal end bud (TEB). Then the mammary carcinogenesis may continue in these structures originating intraductal proliferation and finally *in situ* carcinomas. The initiated TEB may develop dichotomously to AB and lobules. The carcinogenesis in these structures ultimately originates invasive carcinomas that may metastasize to distinct organs. When the carcinogen agents are administered in animals after puberty, in which the TEB already developed to AB and lobules, the carcinogenesis initiates in AB and lobules originating mainly benign mammary lesions (adenoma and fibroadenoma).

1.6. Rat strains for the study of mammary carcinogenesis

A wide range of rat strains are available to study mammary carcinogenesis. Due to the distinct genetic background among them, a different susceptibility to develop mammary tumors and tumors' heterogeneity are observed (Luzhna *et al.*, 2015). The strain of the Norway rat is the most frequently used in laboratory assays (Maeda *et al.*, 2000). During the first studies, the outbred strains of Sprague-Dawley and Wistar female rats demonstrated to be more sensitive to chemical carcinogen agents when compared with other strains, namely inbred Marshall and August rats (Boyland and Sydnor, 1962). More recent studies of chemically-induced mammary carcinogenesis showed that the number of mammary tumors developed by carcinogen-exposed female Wistar rats was lower when compared with female Sprague-Dawley rats, suggesting that the last strain is more sensitive to carcinogen exposition (Gal *et al.*, 2011). Although inbred female Fisher 334 rats have also been widely used as a model of mammary carcinogenesis, the Sprague-Dawley and Wistar rats remain as the most used strains in assays of chemically-induced mammary carcinogenesis due to the higher susceptibility of mammary tissue to the initiation of carcinogenesis after carcinogen exposition (Russo and Russo, 1996b; Rudmann *et al.*, 2012). Additionally to the above described immunocompetent strains, the nude rat strain (rnu/rnu rat) is also frequently used in the study of mammary carcinogenesis. This nude rat, which has an autosomal recessive mutation known as *rnu*, was backcrossed with different strains and consequently a high number of congenic strains characterized by hairlessness and congenital absence of thymus were generated. Due to the absence of cell-mediated immunity (immunocompromised animals), these strains are usually used as xenograft models in which human breast cancer cells are transplanted into rats (Schuurman *et al.*, 1992; Rudmann *et al.*, 2012; Marchesi, 2013). The ACI strain that is the result of a cross between August and Copenhagen Irish rats was identified several years ago as a sensitive strain for mammary tumors development after a combined estrogen administration and radiation exposition (Holtzman *et al.*, 1982; Shellabarger *et al.*, 1983). Due to the estrogens susceptibility and morphological similarities with human mammary tumors, the ACI strain is considered an ideal model to study estrogen-induced human breast carcinomas (Ravoori *et al.*, 2007). Since several rat strains are available, the researchers should select the strain according to the aims of the research protocol.

1.7. Rat models of mammary carcinogenesis

The extensive experimental research in the field of mammary carcinogenesis over years has conducted to the development of several rat models. Distinct approaches involving rats for modelling mammary cancer such as the induction of mammary tumors development by the administration of carcinogenic substances, irradiation or hormone administration, implantation of cancer cells, or use of genetically engineered animals have been established (Russo and Russo, 1996b; Shull *et al.*, 1997; Bartstra *et al.*, 1998; Smits *et al.*, 2007). Several chemical and natural compounds that are routinely used by humans, and some of them are even included in the human daily diet, like milk, apple, grape, fish oil, vitamin C, were tested in rodent models of mammary carcinogenesis in order to assess their effects on mammary carcinogenesis and in an attempt to transpose the results from these models to humans. For this, distinct rat models of mammary carcinogenesis that will be described below have been used. During the experiments, the tested compounds were administered through different routes and the researchers concluded that the tested compounds inhibited, promoted or had no effect on mammary carcinogenesis. The studies may be consulted in detail in Table 1.1.

1.8. Spontaneous tumors

The use of rat strains that spontaneously develop mammary tumors plays an important role in the design of experimental protocols. Although the rat mammary gland is the second organ more frequently affected by the development of spontaneous neoplasms after the pituitary gland, it is age-dependent occurring mainly after the first year of age (Mcmartin *et al.*, 1992; Oishi *et al.*, 1995; Son *et al.*, 2010; Ikezaki *et al.*, 2011). As the experimental protocols usually occurs during relatively short periods of time (before the first year of age of animals), the development of spontaneous mammary tumors will not interfere with the interpretation of the effects of a carcinogen agent. The mammary fibroadenoma (frequency from 18.9 to 57.0%), followed by adenocarcinoma (frequency from 8.8 to 16.8%) and adenoma (3.5 to 7.0%) are the spontaneous mammary neoplasms more frequently identified in female Sprague-Dawley rats (Chandra *et al.*, 1992; Mcmartin *et al.*, 1992; Kaspareit and Rittinghausen, 1999). Our research team has performed some studies in the field mammary carcinogenesis using the model of mammary cancer MNU-induced in female Sprague-Dawley rats. Unexpectedly, in the last experiment, a high-grade undifferentiated mammary carcinoma was identified in a seven-week-old female rat belonging to the control group of the

experiment (the animal did not receive any drug) (Faustino-Rocha *et al.*, 2016a, 2016b). To our knowledge, no previous reports had described a spontaneous mammary tumor in such a young rat.

1.9. Induced tumors

Although the rat spontaneous mammary tumors are uncommon, their development may be easily induced in immunocompetent rats through the administration of chemical carcinogenic agents, hormone administration or exposure to physical agents (Russo and Russo, 1996b).

1.9.1. Chemical induction

From the chemical point of view, a carcinogenic is any compound able to induce the development of cancer in living tissues (Oliveira *et al.*, 2007). The first studies on chemically-induced mammary tumors in rats were described by Howell in 1963 (Howell, 1963). In experimental assays using animals for the study of chemical carcinogenesis, a simple, relatively fast and safe method for the administration of the carcinogenic agent is required (Arcos, 1995; Oliveira *et al.*, 2006).

The induction of rat mammary tumors by chemical carcinogens may be hormone dependent or independent. In the case of the hormone dependent mammary tumors, the first step of the carcinogenesis (initiation) greatly depends on the age of animals at the time of the chemical carcinogen administration (Nandi *et al.*, 1995; Russo and Russo, 1996b; Thordarson *et al.*, 2001). The chemical carcinogenesis is maximal when the carcinogen agent is administered between 45 and 60 days of the age (animals in sexual maturity), which coincides with the time of active differentiation of terminal end bud to alveolar buds (Russo and Russo, 1996b) (Figure 1.3). It was also previously described that the ovariectomy after the chemical carcinogen administration may reduce the incidence of mammary tumors in 96%. However, although hormone dependent mammary tumors depend on estrogen hormones in an initial phase of the carcinogenesis, later they may progress to a more aggressive phenotype without estrogen dependency and expression of the respective receptors (Thordarson *et al.*, 2001).

An adequate rat model of mammary carcinogenesis should exhibit histopathological features and genetic alterations similar to those described in women mammary tumors, the initial or intermediate lesions (preneoplastic lesions) should simulate the different steps of the

carcinogenesis, the tumors should originate specifically from the mammary gland tissue, a higher incidence (higher than 60%) should be obtained in a relatively short period of time (latency period lower than six months), and the assay should be reproducible (Noble and Cutts, 1959; Russo and Russo, 1996b; Barros *et al.*, 2004; Wagner, 2004; Steele and Lubet, 2010). Until now, only two chemical carcinogenic agents for mammary carcinogenesis with these characteristics are well established: DMBA and MNU (Medina, 2007).

The DMBA is a classical polycyclic aromatic hydrocarbon commonly used to the induction of mammary tumors in rodents (Russo and Russo, 2000; Currier *et al.*, 2005; Al-Dhaheri *et al.*, 2008; Cortés-García *et al.*, 2009). When administered by gavage at 50-56 days of age, in a single dose ranging from 10 to 100 mg *per* kg of body weight, it induces the development of a high number of mammary tumors (Al-Dhaheri *et al.*, 2008). To exert its carcinogenic effects, the DMBA must be previously bioactivated by the cytochrome P-450/P1-450 monooxygenase enzyme systems mainly located in the liver. The metabolites are mono- and dihydroxymethyl and they can also be metabolized to their corresponding dihydrodiols, phenols and other oxidation products (Russo *et al.*, 1982). The generated epoxides interact with the DNA generating the transversions A:T for T:A and G:C for T:A, through two mechanisms: the formation of adducts DMBA-adenine and DMBA-guanine, and the loss of purines by spontaneous lysis of the complex between the DMBA epoxide and DNA purines target (Cortés-García *et al.*, 2009).

Inversely to the DMBA, the MNU is a direct alkylating agent that does not require the metabolic activation in order to induce irreversible changes in DNA (Doctores *et al.*, 1974; Murray *et al.*, 2009). It acts as carcinogen by methylation of the guanine nucleosides, promoting the GGA to GAA transitional mutation in H-ras codon 12 encoding glutamic acid in place of glycine (Sukumar *et al.*, 1983; Lu *et al.*, 1998; Murray *et al.*, 2009). A single intraperitoneal (i.p.) injection of this carcinogen agent at 50 days of age, at a standard dose of 50 mg *per* kg of body weight induces mammary tumors in all susceptible animals (100% of incidence) (Murray *et al.*, 2009; Faustino-Rocha *et al.,* 2015a; Alvarado *et al.*, 2016; Faustino-Rocha *et al.*, 2016c). The MNU may also be administered by other ways, such as subcutaneous (s.c.), intravenous (i.v.) and gavage. However, the induction rate of mammary tumors by MNU depends on administration route and dose, being higher when MNU is intraperitoneally injected (Lu *et al.*, 1998).

Among the studies performed in order to evaluate the effects of several compounds on mammary carcinogenesis using the rat model of mammary cancer DMBA or MNU-induced, not all of them had positive effects on mammary carcinogenesis. Although the majority of them inhibited mammary carcinogenesis, some compounds like bisphenol A, copper, resveratrol, folic acid, iron, Methyl-amoorain, milk, estrone sulfate, Phenethylisothiocyanate, among others described in sections 1.1.1. and 1.1.2. of the Table 1.1., promoted mammary carcinogenesis.

1.9.2. Hormone induction

As mentioned before, the development and maintenance of rat mammary tissue is hormone-dependent (Sengupta, 2013). Estrogens, which are involved in the normal development of mammary gland, have the ability to induce a tumorigenic response when administered in combination with chemical carcinogens or exposure to physical carcinogenic agents like irradiation (Russo and Russo, 1998).

The epithelial mammary tissue from ACI rats is particularly susceptible to estrogen administration, a proliferation and transformation of the epithelial cells from mammary gland is observed after the exposition to this hormone (Ravoori *et al.*, 2007). Shull *et al*. performed an experimental assay with this strain in which 17β-estradiol pellets were implanted into ovary-intact ACI rats inducing mammary tumors development (mainly comedo carcinomas) in all exposed animals with a mean latency of 145 days (Shull *et al.*, 1997). The susceptibility of mammary gland to other steroid hormones, such as 2-hydroxyestradiol, 4-hydroxyestradiol, 16-hydroxyestradiol, or 4-hydroxyestrone was different, with no induction of mammary tumors development when these steroid hormones were administered at a similar dose of 17β-estradiol (Turan *et al.*, 2004).

1.9.3. Physical induction

The first studies describing the susceptibility of the human mammary gland to ionizing radiation (gamma rays) of Hiroshima and Nagasaki atomic bombs clearly demonstrated the carcinogenic effects of this kind of radiation. Additionally, these studies also demonstrated that the adolescent women's breast tissue is more sensitive to ionizing radiation than that of older women (Mcgregor *et al.*, 1977). The rat mammary gland is susceptible to distinct types of radiation, namely x-rays, neutron, gamma radiation and magnetic fields. They may be used

alone or in combination with chemical carcinogens or hormones to induce mammary carcinogenesis in these animals (Russo and Russo, 1996b; Thompson and Singh, 2000). Inversely to that observed in women, the incidence of neoplasms in Sprague-Dawley female rats exposed to X-rays was not different among the different aged groups. The mammary lesions identified were histologically classified as adenocarcinoma and fibroadenomas (Holtzman *et al.*, 1982).

1.10. Implantation of cancer cells

Additionally to the techniques described above to the induction of mammary tumors, tumor cells may be directly implanted in rats for tumor development. Depending on the origin of the cancer cells, these models may be classified as xenograft or syngeneic. In the xenograft models, the tumor cells are derived from human mammary tumors and are implanted in immunocompromised animals (Kim *et al.*, 2004b). Since no more rat strains are available, the rnu/rnu nude rat described above is the most frequently used for this kind of mammary cancer modelling (Marchesi, 2013; Yang *et al.*, 2014). In the syngeneic models, the tumor cells had origin in laboratory animals genetically similar to those in which tumor cells will be implanted, avoiding the use of immunocompromised animals and the costs associated with their maintenance. The syngeneic models are useful to study the interaction between immune system and cancer development (Khanna and Hunter, 2005). Both xenograft and syngeneic models may be orthotopic if the mammary tumor cells are implanted in the tumor site of origin (mammary fat pad), or heterotopic when the tumor cells are implanted in a different place (subcutaneously, intraperitoneally, intramuscular) (Vargo-Gogola and Rosen, 2007; Sano and Myers, 2009).

1.11. Genetically engineered models

Advances in molecular biology and the ability to create genetically modified animals has completely changed our ability to understand the molecular mechanisms and cellular pathways underlying biologic processes and disease states, including cancer (Doyle *et al.*, 2012; Vandamme, 2014). Genetically modified animals are organisms whose genetic material was changed by adding (transgenic), modifying (knock-in) or removing (knock-out) DNA sequences (Forabosco *et al.*, 2013). Although the number of genetically engineered mice is significantly higher when compared with the number of genetically engineered rats, the

genetically modified rats have become an excellent model to study aspects of molecular etiology of mammary cancer (Smits *et al.*, 2007; European Commission, 2010). In the specific case of mammary carcinogenesis, the genetic engineered rats are excellent models to evaluate the role of *ras*, *neu*, *BRCA1* and *BRCA2* genes on the malignant progression of mammary tumors (Mullins *et al.*, 2002; Zan *et al.*, 2003; Cotroneo *et al.*, 2007; Smits *et al.*, 2007; Hoenerhoff *et al.*, 2011).

1.12. Pathogenesis of rat mammary cancer, sample collection and histological evaluation

As previously described, mammary tumors in rats may arise from both parenchyma and stroma cells, and they may be composed of a single histological type or a combination of distinct patterns. Russo and Russo (Russo and Russo, 2000) established a complete and useful histological classification for chemically-induced rat mammary tumors taking into account their histogenesis and behavior was established. In Figure 1.4 and Figure 1.5 may be observed the spectrum of lesions developed by Sprague-Dawley female rats administered with the carcinogen agent MNU during an assay performed by our research team in order to evaluate the effects of long-term moderate exercise training on mammary carcinogenesis (Faustino-Rocha *et al.*, 2015b; Alvarado *et al.*, 2016; Faustino-Rocha *et al.*, 2016c). In general, the carcinogenesis initiates within 14 days after the administration of the carcinogen agent with an enlargement of terminal end bud. After initiation, the carcinogenesis may follow two distinct pathways: one in which benign lesions such as cysts, adenomas, alveolar hyperplasias and fibroadenomas originate from alveolar buds, and another one in which terminal end bud originates malignant lesions (intraductal carcinomas) with distinct histological patterns, such as papillary, cribriform and comedo (Russo and Russo, 1996a, 1998). Usually, chemically-induced mammary tumors are detected by palpation at 7^{th} to 10^{th} week after carcinogen administration and the number of tumors increases over time (Russo *et al.*, 1982; Faustino-Rocha *et al.*, 2013b, 2015a, 2016c). At sacrifice, animals should be skinned/scalped and the skin should be carefully evaluated under a light in order to detect small mammary tumors not previously detected by palpation (Faustino-Rocha *et al.*, 2013b, 2013c; Lopes *et al.*, 2014; Faustino-Rocha *et al.*, 2016c). The fixation of mammary tumors in buffered formalin followed by routine staining with hematoxylin and eosin (H&E) and observation in a light microscope is the most frequently used method to classify the chemically-induced rat mammary tumors.

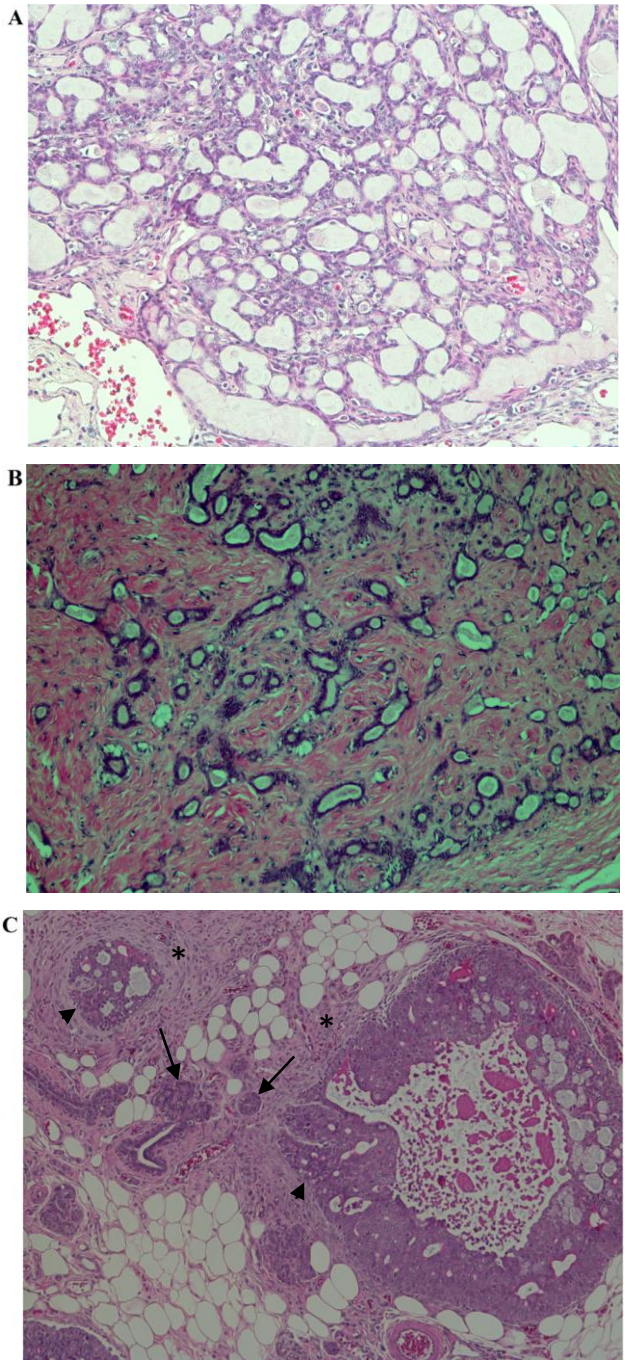

Figure 1.4. Microscopic images of the distinct benign histological patterns of mammary tumors chemically-induced in female rats. **A.** Adenoma (H&E, 200X magnification), **B.** Fibroadenoma (H&E, 100X magnification), **C.** An intraductal proliferation with a proliferation of the epithelial cells (three cell layers) is seen in terminal ductal structures (arrows). *In situ* cribriform carcinomas involving epithelial proliferation with a solid pattern and formation of secondary lumens is also observed (arrowheads). Both lesions exhibited a desmoplastic reaction of the stroma and neovascularization (asterisks) (H&E, 100X magnification).

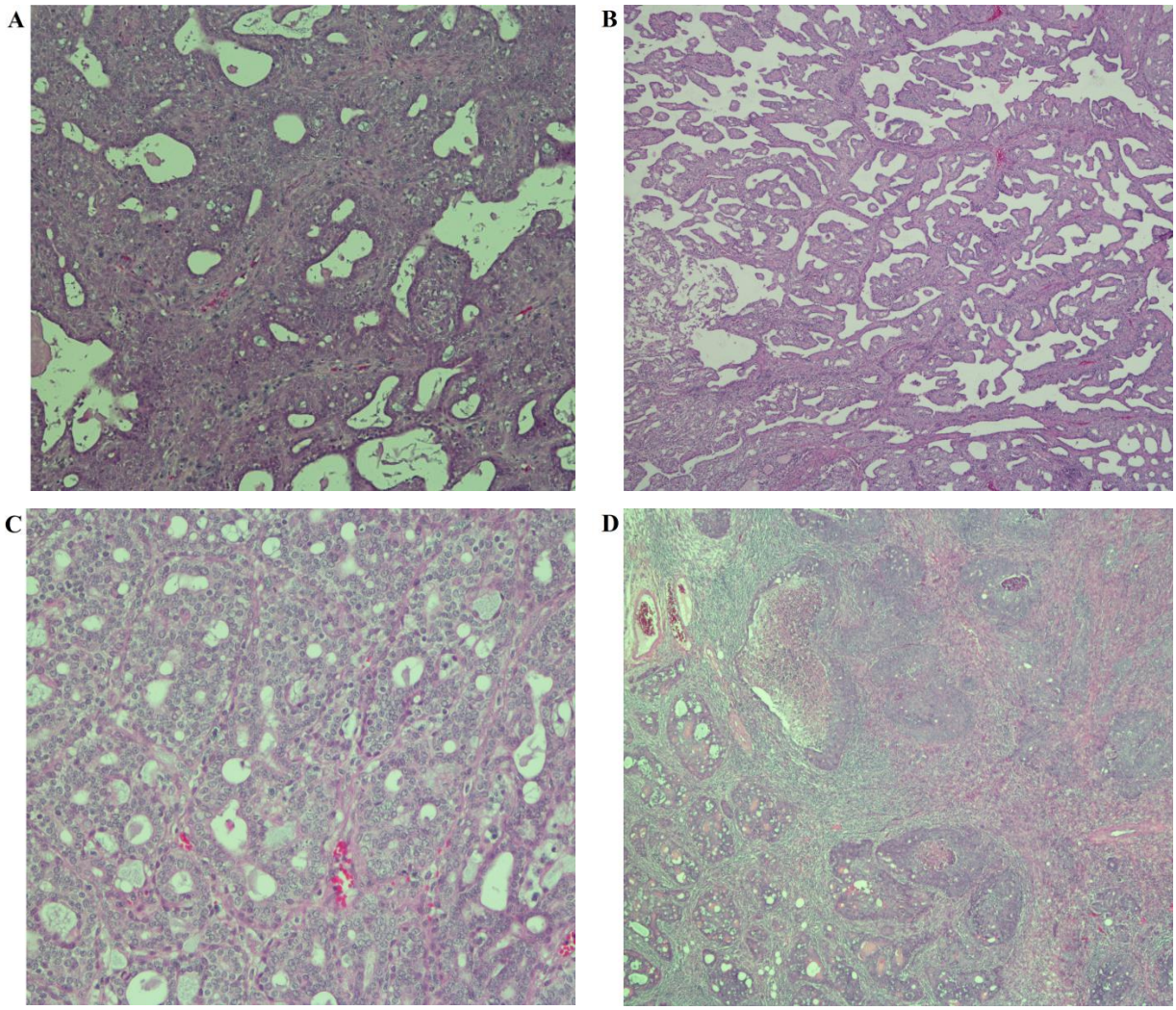

Figure 1.5. Microscopic images of the distinct malignant histological patterns of mammary tumors chemically-induced in female rats. **A.** Tubular carcinoma (H&E, 100X magnification), **B.** Papillary carcinoma (H&E, 40X magnification), **C.** Cribriform carcinoma (H&E, 200X magnification), **D.** Comedo carcinoma (H&E, 40X magnification).

1.13. Imaging modalities for diagnosis and monitoring of mammary cancer

The detection of breast alterations and the response of mammary tumors to pharmacological and non-pharmacological therapeutic strategies may be non-invasively evaluated through the use of different imaging techniques. Although several imaging modalities are already approved or being tested for the evaluation of women mammary gland, not all of them are used in rodent models of mammary carcinogenesis. Despite this, a lot of imaging modalities namely mammography (Karathanasis *et al.*, 2008, 2009), ultrasonography (Faustino-Rocha *et al.*, 2013a, 2016e), computed tomography (Liu *et al.*, 2008a), magnetic resonance imaging (Song *et al.*, 2009; Budde *et al.*, 2012), magnetic resonance spectroscopy (Stubbs *et al.*, 1989, 1990; Lyng *et al.*, 1993), positron emission tomography (Yang *et al.*, 1993; Ito *et al.*, 2006; Wu *et al.*, 2015), thermography (Kirubha *et al.*, 2012; Faustino-Rocha *et al.*, 2016c, 2013c), and diffusion-weighted imaging (Zhai *et al.*, 2013) have been used in experimental protocols using rat models of mammary carcinogenesis.

Mammography provides an adequate visualization of soft tissue abnormalities and the detection of subtle calcifications (Karellas and Vedantham, 2008). However, it has some disadvantages, namely radiation imposition, limited dynamic range, contrast characteristics and granularity (Nishikawa *et al.*, 1987). Ultrasonography provides a panoramic high resolution image of entire mammary gland and it is very useful in the differentiation between benign and malignant solid breast lesions, and also to perform several eco-guided procedures, namely needle aspiration and core-needle biopsies (Gordon and Goldenberg, 1995; Kolb *et al.*, 1998; Moss *et al.*, 1999; Kaplan, 2001; Kolb *et al.*, 2002; Corsetti *et al.*, 2006). Beyond the B mode, the use of Color Doppler, Power Doppler, Pulsed Doppler, B Flow and Contrast-enhanced ultrasound allows the detection and characterization of blood flow in mammary tumors (Kubota *et al.*, 2002). When compared with other imaging modalities, ultrasonography has some advantages, namely it is a real-time exam, it does not impose radiation, and the apparatus are portable and less expansive. However, it is operator-dependent and some problems related with posterior acoustic shadowing may occur (Kubota *et al.*, 2002). Computed tomography provides a complete view of the mammary gland without tissue overlapping that occurs in mammography (Lindfors *et al.*, 2010; Kalender *et al.*, 2012). However, it is expensive, non-portable, and imposes high dose of radiation (Fred, 2004). Magnetic resonance imaging is the most sensitive imaging modality for breast cancer detection, identifying breast alterations that are occult on other imaging modalities (Harms,

1998; Morris, 2002). The use of contrast agents provide a three-dimensional anatomical image and information about tumor angiogenesis. It is also very useful in determining the spread of cancer to the chest wall and the cancer recurrence after surgical excision (Orel and Schnall, 2001). The cost, requirement of contrast agents injection for functional imaging, no detection of calcifications, and long-time required for scanning are the main disadvantages of this imaging modality (Wiberg *et al.*, 2002). Magnetic resonance spectroscopy is a non-invasive imaging modality, it does not require contrast injection and demonstrates improved sensitivity and specificity when used as an adjunct to breast magnetic resonance imaging (Meisamy *et al.*, 2005; Bartella *et al.*, 2007). Positron emission tomography is one of the most recent imaging modalities. It has as the main disadvantages the costs, the lower sensitivity in detecting some breast tumors due to their small size, metabolic activity, histological type (benign mammary lesions will be negative on positron emission tomography scan), microscopic tumor growth and proliferation. The lack of evidences demonstrating clear advantages when compared with other imaging modalities has limited its use (Avril *et al.*, 2001). Thermography is a non-invasive technique that creates a temperature map of the breasts based on infrared radiation. However, it is proved to have a low sensitivity and no advantages when compared with mammography for breast cancer diagnosis (Gershon-Cohen and Haberman, 1964; Feig *et al.*, 1977). Diffusion-weighted imaging is a non-invasive technique that evaluate breast tissue microstructure on the basis of random thermal motion (brownian motion) of water molecules (Woodhams *et al.*, 2011; Partridge and McDonald, 2013). According to previous studies, it has no advantages when compared with other imaging modalities, and consequently its use is limited (Castillo *et al.*, 2000; Koh and Collins, 2007). Our research team has used the thermography and ultrasonography to monitor not only the growth but also the vascularization of mammary tumors MNU-induced in female Sprague-Dawley rats (Faustino-Rocha *et al.*, 2013a, 2013b, 2013c, 2016c, 2016e) (Figure 1.6).

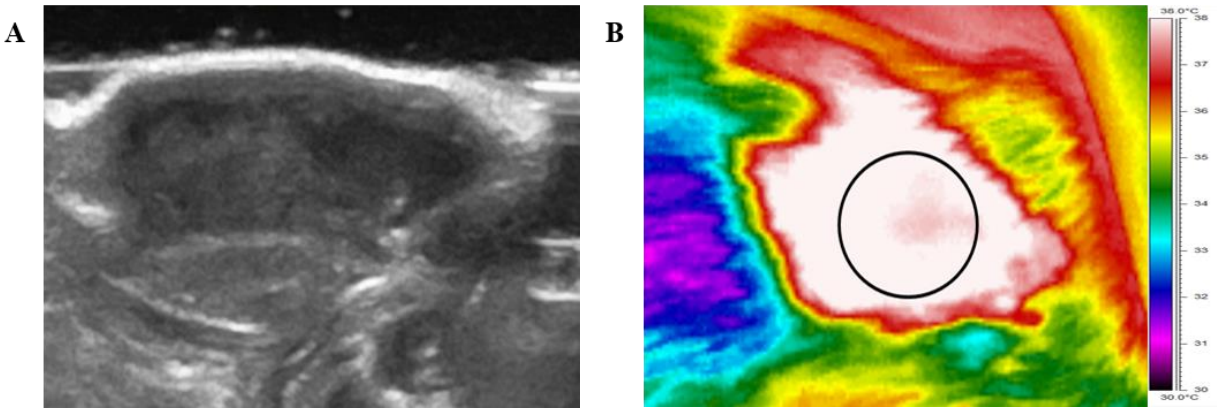

Figure 1.6. MNU-induced rat mammary tumors in Sprague-Dawley female rats evaluated by **A,** ultrasonography B-mode and **B,** thermography (the tumor is delimited by the black circle).

1.14. Conclusion

We have reviewed experimental data related to the rat models of mammary cancer. Although several animal models are available for mammary cancer research, no one of them is perfect but they provide an initial point to study the Human mammary carcinogenesis and evaluate the potential role of new preventive and therapeutic strategies. The researchers should be able to select the model that best suits the aims of their studies after considering the advantages and disadvantages of each one.

Despite the specify of the DMBA and MNU in the induction of tumor development in the mammary gland when administered at a given age, dose and route, the researchers could not control the development of primary tumors in other organs. However, the model of chemically-induced mammary tumors in female rats continues being used due to its advantages when compared with other models, the mammary tumors are easily induced, several carcinogen agents are available, the experimental protocols are well-defined (dose, route, age of administration, high incidence rate, short latency period), the animals develop a high number of tumors, the mammary tumors are similar to those found in humans in terms of histology, hormone dependency, expression of estrogen receptors and genetic alterations, and they allow the researchers to study the different stages of mammary carcinogenesis (benign, pre-neoplastic and neoplastic lesions).

Table 1.1. *In vivo* studies using different rodent models of mammary cancer to assess the efficacy of several therapeutic strategies.

Model/gender and animal strain	Drugs and/or compounds evaluated	Dose/treatment	Therapeutic effects (Ref.)
1.1.1. DMBA-induced mammary tumors			
♀ Sprague-Dawley rats	Allyl isothiocyanate	Gavage (10, 20 and 40 mg/kg/day, 3 times/week for 16 weeks)	Exerted chemopreventive effects at a doses of 20 and 40 mg/kg (Rajakumar *et al.*, 2015)
	Apple extract	Gavage (0, 3.3, 10 and 20 mg/kg b.w. for 26 weeks)	Inhibited mammary carcinogenesis (Liu *et al.*, 2009)
	Arthrospira platensis (Spirulina)	Diet supplementation (1% for 12 months)	Inhibited mammary carcinogenesis (Ouhtit *et al.*, 2014)
	Asiaticoside	2 weeks before and 8 weeks after DMBA administration or 5 weeks after DMBA administration (i.p., 200 µg/animal, 2 times/week)	Inhibited mammary carcinogenesis (Al-Saeedi, 2014)
	Atrazine	Diet supplementation (5, 50 and 500 ppm for 34 weeks)	Enhanced tumor growth (Ueda *et al.*, 2005)
	Azadirachta indica	Diet supplementation (10-12.5% for 2 weeks)	Inhibited mammary carcinogenesis (Tepsuwan *et al.*, 2002)
	Bamboo extract	Diet supplementation (0.5% for 13 weeks)	Inhibited mammary carcinogenesis (Lin *et al.*, 2008)
	5,6-benzoflavone (5,6-BF) Indole-3-carbinol (I3C) Diindolylmethane (DIM)	5,6-BF (diet supplementation, 165, 550 and 1650 ppm, for 14 days) I3C (gavage, 180 mg/kg b.w. for 14 days) DIM (gavage, 20 and 180 mg/kg b.w. for 14 days)	5,6-BF and I3C were highly effective on mammary tumors inhibition; DIM had minimal effects (Lubet *et al.*, 2011)

Bisphenol A	Gavage (0, 25 and 250 µg/kg b.w. for 130 days)	Increased number of tumors and decreased latency period (Lamartiniere *et al.*, 2011)
Broccoli sprouts	Gavage (1 mL for 5 days)	Inhibited mammary carcinogenesis (Fahey *et al.*, 1997)
4'-bromoflavone	Diet supplementation (2000 and 4000 mg/kg diet for 2 weeks)	Inhibited mammary carcinogenesis (Song *et al.*, 1999)
Buckwheat protein extract	Diet supplementation (38.1% for 61 days)	Inhibited mammary carcinogenesis (Kayashita *et al.*, 1999)
Calotropis procera protein Cyclophosphamide	*Calotropis procera* (0.2 mg/kg/b.w., gavage for 20 days) Cyclophosphamide (0.2 mg/kg/b.w., i.p. for 20 days) *Calotropis procera* + Cyclophosphamide (0.1 mg/kg/b.w., oral for 20 days+ 0.1 mg/kg/b.w., i.p. for 20 days)	Inhibited mammary carcinogenesis (Samy *et al.*, 2012)
3-carbamoyl-2,2,5,5-tetramethylpyrroline-1-oxyl (Pirolin) 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one (Quercetin)	i.p. (10 mg/kg, for 14 days)	Acted as cytoprotector and inhibited tumor growth (Tabaczar *et al.*, 2015)
Celecoxib	Diet supplementation (500 ppm and 1500 ppm or 15 weeks)	Inhibited mammary carcinogenesis (Jang *et al.*, 2002)
Chorionic gonadotropin	i.p. (100 IU, for 5, 10, 20 and 40 days)	Induced cell death (Srivastava *et al.*, 1997)
Cimicifuga racemosa extract.	Gavage (0.714, 7.14 and 71.4 mg/kg b.w. for 6 weeks)	No significant effects were observed (Freudenstein *et al.*, 2002)
Cisplatin Nordihydroguaiaretic acid (NDGA)	After tumor development: NDGA (10 mg/kg, i.p., for 5 days) followed by cisplatin (7.5 mg/kg, i.p., single dose)	Reduced kidney toxicity and improved anti-breast cancer activity (Mundhe *et al.*, 2015)

Cloudy apple juice	Gavage (10 mL/kg/b.w., for 28 days before DMBA administration)	Decreased blood levels of biochemical liver and kidney markers (Szaefer *et al.*, 2014)
Conjugated linoleic acids	Diet supplementation (1.0 or 2.0%) before, after, or before and after the DMBA administration for 23 weeks	Inhibited mammary carcinogenesis (Białek *et al.*, 2016)
Copper Resveratrol	Copper (42.6 mg/kg diet, gavage for 100 days) Copper + Resveratrol (42.6 mg/kg diet + 0.2 mg/kg b.w., gavage for 100 days)	Promoted tumor growth (Skrajnowska *et al.*, 2013)
Cow's milk	Oral (for 21 days after birth)	Reduced risk of mammary tumors development (Nielsen *et al.*, 2011)
Curcumin Dibenzoylmethane	Curcumin (diet supplementation, 0.2 and 1% for 14 days) Dibenzoylmethane (diet supplementation, 0.5 and 1% for 14 days)	Inhibited mammary carcinogenesis (Singletary *et al.*, 1998)
Diethylstilbestrol	s.c. (1 µg/animal, 0-14, 0-5 or 6-14 days after birth)	The administration from 0 to 14 days after birth inhibited mammary carcinogenesis (Yoshikawa *et al.*, 2008)
2,2'-diphenyl-3,3'-diindolylmethane	Gavage (5 mg/kg b.w., each two days for 21 days)	Inhibited mammary carcinogenesis (Bhowmik *et al.*, 2013)
Eicosapentaenoic acid (EPA) Docosahexaenoic acid (DHA)	Gavage (0.5 mL for 20 weeks)	Inhibited mammary carcinogenesis (Noguchi *et al.*, 1997)
Endostatin	s.c. (20 mg/kg for 28 dyas)	Inhibited mammary carcinogenesis (Perletti *et al.*, 2000)
Enterolactone	Gavage (1 and 10 mg/kg b.w. for 50 days)	Inhibited mammary carcinogenesis (Saarinen *et al.*, 2002)
9α-Fluoromedroxyprogesterone Acetate (FMPA)	Gavage (30 and 120 mg/kg for 3 weeks)	Inhibited mammary carcinogenesis (Murata *et al.*, 2006)
Folic acid	Diet supplementation (5, 8 and 10 mg/kg diet, after tumor development, for 12 weeks)	Promoted the mammary tumors progression (Manshadi *et al.*, 2014)

Ganoderma lucidum	Gavage (500 mg/kg b.w. for 16 weeks)	Inhibited mammary carcinogenesis (Deepalakshmi and Mirunalini, 2013)
Genistein Daidzein	Genistein + Daidzein (gavage, 20 mg + 20 mg/kg, for 16 weeks)	Protected the structural integrity of cell surface and membranes (Pugalendhi *et al.*, 2011)
Grape seed extract	Grape seed extract (diet supplementation, 1.25 and 5% for 135 days)	No protective effects were observed (Kim *et al.*, 2004a)
Green tea polyphenol Black tea polyphenol	Green and black tea polyphenols (oral administration, 0.1% for 28 weeks)	Both polyphenols inhibited mammary carcinogenesis (Roy *et al.*, 2011)
Iodine Potassium iodide	Iodine (0.05%, 0.07%) Potassium iodide (0.05%, 0.1%) Iodine + Potassium iodide (0.05% + 0.05%; 0.05% + 0.1%)	The combination of Iodine + Potassium iodide (0.05% + 0.05%) exerted antineoplastic effects (Soriano *et al.*, 2011)
β-ionone	Diet supplementation (9, 18 and 36 mmol/kg for 24 weeks)	Inhibited mammary carcinogenesis (Liu *et al.*, 2008b)
Iron	s.c. (50 μmol/kg, twice a week, for 53 weeks)	Promoted mammary carcinogenesis (Diwan *et al.*, 1997)
Isoflavone	Diet supplementation (100, 500 or 1000 mg/kg diet, for 24 weeks)	Inhibited mammary carcinogenesis (decreased tumor incidence, mean number of tumors per animals and increase tumor latency) (Ma *et al.*, 2014)
Kalpaamruthaa	Gavage (100, 200, 300, 400 and 500 mg/kg b.w., for 14 days)	Inhibited mammary carcinogenesis (Veena *et al.*, 2006)
Low-fat milk (1%) Artificial milk Estrone sulfate solution (0.1 μg/mL)	Oral administration for 20 weeks	Low-fat milk and estrone promoted mammary carcinogenesis (Qin *et al.*, 2004)
Magnetic fields	50-Hz twice a week, for 18 weeks	Promoted mammary carcinogenesis (Fedrowitz *et al.*, 2004)

Medroxyprogesterone acetate (MPA) Norgestrel (N-EL) Norethindrone (N-ONE) Megestrol acetate (MGA)	MPA, N-EL, N-ONE, MGA (s.c. implant, 60 days)	N-EL inhibited tumor growth, MGA did not change tumor growth (Benakanakere *et al.*, 2010)
Melatonin	Drinking water (25 µg/mL for 9 weeks)	Inhibited mammary carcinogenesis (Cos *et al.*, 2006)
	Gavage (10 mg/kg for 15 days and 6 months)	Inhibited mammary carcinogenesis (Clarke, 1996)
Methyl-amoorain (methyl-25-hydroxy-3-oxoo-lean-12-en-28-oate)	Gavage (0.8, 1.2 and 1.6 mg/kg b.w., 3 times/week for 18 weeks)	Promoted mammary carcinogenesis (Mandal *et al.*, 2013)
Methylseleninic acid	Diet supplementation (2 ppm for 22 weeks)	Antineoplastic effects (Ip *et al.*, 2000)
Milk Estrone sulfate	Milk (oral administration for 20 weeks) Estrone sulfate (100 ng/ml for 20 weeks)	Both promoted mammary carcinogenesis (Ma *et al.*, 2007)
Morin (3,5,7,2',4'-pentahydroxyflavone)	Gavage (50 mg/kg b.w., thrice a week, for 15 weeks)	Beneficial effects as chemopreventive agent (Nandhakumar *et al.*, 2012)
N-(4-hydroxyphenyl)retinamide-C-glucoronide (4-HPRCG)	Diet supplementation (2 mmol/kg diet for 28 days)	Inhibited mammary carcinogenesis (Alshafie *et al.*, 2005)
N-acetyl-L-cysteine (NAC) Anethole trithione Phenethylisothiocyanate (PEITC) Miconazole	NAC (diet supplementation, 4000 and 8000 ppm for 100 days) Anethole trithione (diet supplementation, 200 and 400 ppm for 100 days) PEITC (diet supplementation, 600 and 1200 ppm for 100 days) Miconazole (diet supplementation, 1000 and 2000 ppm for 100 days)	Anethole trithione and miconazole inhibited mammary carcinogenesis PEITC promoted mammary carcinogenesis No effects were observed for NAC (Lubet *et al.*, 1997)
Operculina turpethum	Gavage (100 mg/kg b.w. for 45 days)	Decreased tumor weight (Anbuselvam *et al.*, 2007)
Paclitaxel *Eruca sativa* seeds	Pacliatxel encapsulated liposome (intravenous, 20 mg/kg/week) and *Eruca sativa* seeds extract (oral, 500 mg/kg/week) for 4 weeks	Reduced inflammation and cell proliferation (Shaban *et al.*, 2016)

Photodynamic therapy	Photodithazine (8 mg/kg, i.p.) and 100 J/cm of light at a fluence rate of 100 mW/cm	Upregulation of apoptotic genes and downregulation of anti-apoptotic genes (Silva *et al.*, 2014)
Plasticizer benzyl butyl phthalate	i.p. or gavage (100 and 500 mg/kg for 7 days)	Inhibited mammary carcinogenesis (Singletary *et al.*, 1997)
Pleurotus ostreatus	Gavage (150, 300 and 600 mg/kg b.w. for 16 weeks)	Inhibited mammary carcinogenesis (Krishnamoorthy and Sankaran, 2016)
Pomegranate	Gavage (0.2 g/kg, 1.0 g/kg or 5.0 g/kg, 3 times/week) for 18 weeks (2 weeks before and 16 weeks after DMBA administration)	Chemopreventive effects (Bishayee *et al.*, 2016)
Pomegranate	Diet supplementation (0.2, 1.0 and 5.0 mg/kg) for 18 weeks (2 weeks before and 16 weeks after DMBA administration)	Inhibited mammary carcinogenesis. Inhibited proliferation and promoted the apoptosis (Mandal and Bishayee, 2015a)
RS100642	Intravenous (0.25 mg/kg b.w., once a week for 4 weeks)	Improved survival (Batcioglu *et al.*, 2012)
RU486 CDB-4124	s.c., 10 mg/kg b.w., for 28 days	Inhibited mammary carcinogenesis (Wiehle *et al.*, 2007)
Selenium-enriched Japanese radish sprout	Diet supplementation (8.8 ppm for 13 or 28 weeks)	Inhibited mammary carcinogenesis (Yamanoshita *et al.*, 2007)
Shemamruthaa	Gavage (400 mg/kg b.w., for 14 days)	Inhibited mammary carcinogenesis (Purushothaman *et al.,* 2013)
Simvastatin	Gavage (20 and 40 mg/kg, for 14 days)	Reduced tumor growth (Rennó *et al.*, 2015)
Soy milk	Oral for 20 weeks	Promoted mammary carcinogenesis (Qin *et al.*, 2007)
Taurine	Drinking water (3%) for 16 weeks	Inhibited mammary carcinogenesis (He *et al.*, 2016)
Taurine	Gavage (100 mg/kg) for 5 weeks	Efficient as chemotherapeutic agent (Vanitha *et al.*, 2015)
Tamoxifen Quercetin	Tamoxifen (3 mg/kg, gavage for 3 days) Tamoxifen + Quercetin (3 mg/kg + 6 mg/kg, gavage for 3 days)	Inhibited mammary tumors angiogenesis (Jain *et al.*, 2013)

	Tangeretin (4′,5,6,7,8-pentamethoxyflavone)	Gavage (50 ng/kg, pre-treatment for 30 days or post-treated for 30 days)	Efficient as chemotherapeutic agent (Periyasamy *et al.*, 2015)
	Trianthema portulacastrum	Diet supplementation (50, 100 and 200 mg/kg) for 18 weeks (2 weeks before and 16 weeks after DMBA administration)	Reduced inflammation and suppressed mammary carcinogenesis (Mandal and Bishayee, 2015b)
	Trianthema portulacastrum	Diet supplementation (50, 100 and 200 mg/kg body weight, for 18 weeks)	Inhibited mammary carcinogenesis (Mandal and Bishayee, 2015c)
	Tualang Honey	Oral (0.2, 1.0 or 2.0 g/kg b.w. for 150 days)	Inhibited mammary carcinogenesis (Kadir *et al.*, 2013)
	Vanadium Fish oil	Vanadium (drinking water, 0.5 ppm for 6 weeks) Fish oil (gavage, 0.5 mL/day for 6 weeks) Vanadium + Fish oil (drinking water, 0.5 ppm + gavage, 0.5 mL/day, for 6 weeks)	Vanadium and fish oil alone were effective on mammary tumors inhibition, the combination exhibited higher effectiveness (Manna *et al.*, 2011)
	Wheat bran fiber	Diet supplementation (5, 9.6 and 17.6% for 13 weeks)	Inhibited mammary carcinogenesis (Zile *et al.*, 1998)
	Zearalenone Genistein	Zearalenone and Genistein (s.c., 20 µg for 5 days)	Inhibited mammary carcinogenesis (Hilakivi-Clarke *et al.*, 1999)
	Zinc Resveratrol Genistein	Zinc (231 mg/kg diet, gavage for 40 days) Zinc + Resveratrol (231 mg/kg diet + 0.2 mg/kg b.w., gavage for 40 days) Zinc + Genistein (231 mg/kg diet + 0.2 mg/kg b.w., gavage for 40 days)	The combination of Zinc + Resveratrol increased tumor number (Bobrowska-Korczak *et al.*, 2012)
♀ Wistar-Furth rats	Limonene	Diet supplementation (0, 2.5, 5.0, 7.5 and 10% for 11 weeks)	Regression of mammary tumors above 7.5% (Haag *et al.*, 1992)
♀ Wistar rats	BAY 12-9566N	Diet supplementation (240 mg/kg/day for 52-64 weeks)	Inhibited mammary carcinogenesis (Iatropoulos *et al.*, 2008)
	Celecoxib n-3 polyunsaturated fatty acids (PUFA)	Gavage of celecoxib (20 mg/kg) in combination with PUFA (0.5 mL) for 7 days	Chemopreventive effect in mammary carcinogenesis (Negi *et al.*, 2016)

	Crateva adansonii DC	Gavage (75 and 300 mg/kg b.w., once by day, for 13 weeks)	Inhibited mammary carcinogenesis, mainly at a dose of 75 mg/kg b.w. (Zingue *et al.*, 2016)
	Lycopene Genistein	Lycopene (20 mg/kg b.w., gavage, thrice a week for 20 weeks) Genistein (2 mg/kg b.w., gavage, thrice a week for 20 weeks) Lycopene + Genistein (20 mg/kg b.w. + 2 mg/kg b.w., gavage, thrice a week)	Inhibition of mammary carcinogenesis (Sahin *et al.*, 2011)
	Melissa officinalis	Gavage (100 mg/kg b.w. for 4 weeks after tumors development)	Inhibited tumor growth (Saraydin *et al.*, 2012)
	Organoselenium compounds	i.p. (25 µmol/kg, each two days for four weeks)	Inhibited mammary carcinogenesis (Ozdemir *et al.*, 2006)
	Vincristine Myricetin	Vincristine (i.p., 500µg/kg, 1 administration/week, for 4 weeks) Myricetin (gavage, 50, 100 and 200 mg/kg, every day, 16 weeks)	Each drugs, independently, inhibited mammary carcinogenesis (Jayakumar *et al.*, 2014)
♀ Zucker rats	Casein Soy protein + isoflavones	Casein (diet supplementation, 200 g/kg/diet for 7 weeks) Soy protein + isoflavones (diet supplementation, 202 g/kg/diet + 3.24 mg/g protein for 7 weeks)	Casein inhibited mammary carcinogenesis (Hakkak *et al.*, 2011)
	Dehydroepiandrosterone	Diet supplementation (0.6% for 15 weeks)	Inhibited mammary carcinogenesis (Hakkak *et al.*, 2010)
♀ Donryu rats	Isoflavone aglycones	Diet supplementation (0.2% for 2 weeks, 4 weeks or 40 weeks)	Promoted mammary carcinogenesis (Kakehashi *et al.*, 2012)
♀ Holtzman rats	*Piper aduncum*	Oral (capsules, 50, 150 and 300 mg/kg/body weight)	Decreased mammary carcinogenesis and lymph node metastasis (Arroyo-Acevedo *et al.*, 2015)
♀ albino rats	*Nigella sativa* Thymoquinone	Gavage (1, 5 and 10 mg/kg, 3 times/week, for 4 months)	Inhibited mammary carcinogenesis (Linjawi *et al.*, 2015)
♀ rats	Apigenin	Diet supplementation (0.02, 0.1 and 0.5% for 56 days)	Promoted mammary carcinogenesis (Mafuvadze *et al.*, 2013)

1.1.2. MNU-induced mammary tumors

♀ Lewis rat	Anastrazole Docetaxel HE3235 + Docetaxel 17α-ethynyl-5α-androstane-3α, 17β-diol (HE3235) Tamoxifen	i.p. (2.5 mg/day for 4 weeks) i.p. (1.5 mg, once a week for 4 weeks) i.p. (6.6 mg/day + 1.5 mg, once a week for 4 weeks) i.p. (4-6.6 mg/day for 4 weeks) s.c. (0.25 mg, once a week for 4 weeks)	All compounds decreased incidence and number of mammary tumors; the high dose of HE3235 in combination with docetaxel was the most efficient treatment (Ahelm *et al.*, 2011)
♀ Wistar rat	Carboxy ethyl germanium sesquioxide (Ge-132)	i.p. (1500 mg/kg/day for 34 weeks)	Reduced tumors' growth (Vinodhini and Sudha, 2013)
	Green tea extract	Diet supplementation (30 mg for 9 weeks)	Tumor multiplicity was lower in animals that received green tea extract (Kale *et al.*, 2010)
♀ Ludwig/Wistar/ Olac rat	Pamidronate	s.c. (0.4 mg/kg/b.w. for 4 weeks)	Reduced tumor volume (Colston *et al.*, 2003)
♀ rat	Potato (*Solanum tuberosum* L.)	Diet supplementation (5-50% for 5 weeks)	Reduced cancer incidence (Thompson *et al.*, 2009)
♀ Sprague-Dawley rat	Amphetamine-regulated transcript peptide (CART)	Intracerebroventricular (1 µg/rat/day, for 5 days) Intracerebroventricular (5 µl (1:500)/rat/day, for 5 days)	Reversed cancer cachexia (Nakhate *et al.*, 2010)
	Anastrazole	Diet supplementation (0.05-0.5 mg/kg for 15 weeks)	High concentration reduced mammary tumors incidence and number of tumors *per* animal (Kubatka *et al.*, 2008a)
	Carboplatin Methrotrexate Paclitaxel	Intraductal (6 mg/rat, single administration) Intraductal (4-10 mg/rat, single administration) Intraductal (60 mg/rat, single administration)	Carboplatin was the most efficient agent in the inhibition of mammary carcinogenesis (Stearns *et al.*, 2011)
	Celecoxib	Diet supplementation (1500 ppm, 7-24 weeks)	Suppressed mammary carcinogenesis (Badawi *et al.*, 2004; Lu *et al.*, 1997)

13-cis retinoic acid (13cRA) CpG oligodeoxynucleotides (CpG-ODN) 13-cis retinoic acid (13cRA) + CpG oligodeoxynucleotides	Gavage (1 mg/kg, 3 times a week for 15 weeks) Intraductal (CpG-ODN motifs, 2 administrations) Gavage (1 mg/kg, 3 times a week, 15 weeks + 2 administrations)	CpG-ODN reduced the number of mammary tumors (Liska *et al.*, 2003)
Curcumin	Intraductal (168 µg encapsulated drug/teatment, 2-3 administrations) Gavage (200 mg/kg/b.w., 2-3 adminsitrations)	Reduced the incidence of mammary tumors (Chun *et al.*, 2012)
Doxorubicin (DOX) DOX + Iodine (I2)	i.p. (4-16 mg/kg, 1 day) i.p. (4-16 mg/kg, 1 day) +Drinking water (0.05% for 7 days)	I_2 may be used as adjuvant of doxorubicin in cancer therapy (Alfaro *et al.*, 2013)
Exemestane	Diet supplementation (1-10 mg/kg for 13 weeks)	Administration in premenopausal animals induced mammary carcinogenesis (Kubatka *et al.*, 2008a)
Fluorouracil	i.v. (12 mg/rat, 4 administrations) Intraductal (12 mg/rat, 4 administrations)	Intraductal administration inhibited mammary carcinogenesis (Stearns *et al.*, 2011)
Flurbiprofen	Diet supplementation (31.25-62.5 mg/kg for 26 weeks)	Inhibited mammary carcinogenesis (Mccormick and Moon, 1983)
Garlic powder S -all1cysteine (SAC) Diallyl disulfide (DADS)	Diet supplementation (20 g/kg for 27 weeks) Diet supplementation (57 nmol/kg for 27 weeks) Diet supplementation (57 µmol/kg for 27 weeks)	Inhibited mammary carcinogenesis (Schaffer *et al.*, 1996)
Genistein	s.c. (12.5 mg/day for 3 days)	Promoted mammary carcinogenesis (Yang *et al.*, 2000)
High fat, low fiber diet + phytic acid	Diet supplementation (2% phytic acid from 9-30 weeks)	Phytic acid contributed to the reduction of mammary tumors incidence (Shivapurkar *et al.*, 1996)
1α-Hydroxyvitamin D_5	Diet supplementation (25-50 µg/kg for 18 weeks)	Inhibited mammary carcinogenesis (Mehta *et al.*, 2000)
Keoxifene	s.c. (20-500 µg for 13 weeks)	Inhibited mammary carcinogenesis (Gottardis and Jordan, 1987)

Ketotifen	Drinking water (1 mg/kg for 18 weeks)	Inhibited mammary tumor development (Faustino-Rocha *et al.*, 2014)
Lapatinib	Gavage (25-75 mg/kg/b.w. for 21 weeks)	High dose inhibited mammary carcinogenesis (Li *et al.*, 2011)
Letrozole	Diet supplementation (1-10 mg/kg for 18 weeks)	Inhibited mammary carcinogenesis (Kubatka *et al.*, 2008b)
Lysine, arginine, proline, ascorbic acid and green tea extract	Diet supplementation (0.5% for 24 weeks)	Inhibited mammary carcinogenesis (Roomi *et al.*, 2005)
Mango (*Mangifera indica* L.)	Drinking water (0.02-0.06 g/ml for 2 or 23 weeks)	Did not inhibit mammary carcinogenesis (Garcia-Solis *et al.*, 2008)
2-methoxyestradiol	s.c. (1-5 mg/kg/day for 4 weeks)	Did not inhibit mammary carcinogenesis (Lippert *et al.*, 2003)
Paclitaxel	Intraductal (10-25 mg/kg, for 8 weeks) i.p. (25 mg/kg for 8 weeks)	Local administration may reduce mammary carcinogenesis (Okugawa *et al.*, 2005)
Potassium iodide (KI) Iodine (I_2) Thyroxine (T4)	KI and I_2 (0.05% in drinking water from 3-18 weeks) T4 (3 µg/ml in drinking water from 3-18 weeks)	Long-term I_2 treatment inhibited mammary carcinogenesis (Garcia-Solis *et al.*, 2005)
Raloxifene	Diet supplementation (20-60 mg/kg for 19 weeks)	Inhibited mammary carcinogenesis (Anzano *et al.*, 1996)
Keoxifen (9-cis-retinoic acid + raloxifene)	Diet supplementation (60 mg/kg + 20-60 mg/kg)	
Resveratrol (trans-3,4',5-trihydroxystilbene)	s.c. (10-100 mg/kg/day for 5 days)	Prepubertal treatment promoted mammary carcinogenesis (Sato *et al.*, 2003)
Retinoid 9cUAB30 + Tamoxifen	Diet supplementation (150 mg/kg + 0.4 mg/kg for 21 weeks)	Combination of both agents inhibited mammary carcinogenesis (Grubbs *et al.*, 2003)
Tamoxifen	s.c. (6.25-500 µg for 8 weeks) i.p. (1 mg/kg for 8 weeks)	Inhibited mammary carcinogenesis (Gottardis and Jordan, 1987; Martin *et al.*, 1996)
Targretin	Gavage (6.7-60 mg/kg/day for 17 weeks) Diet supplementation (92-275 mg/kg for 17 weeks)	Inhibited mammary carcinogenesis (Lubet *et al.*, 2005)

1.1.3. Irradiation-induced mammary tumors

♀ Sprague-Dawley rats	Estriol Estradiol	s.c. (638 µ/month for 6 months)	Delayed mammary tumors development (Lemon *et al.*, 1989)

1.1.4. Hormone-induced mammary tumors

♀ August Copenhagen Irish rats (estrogen-induced)	Resveratrol	s.c. implant (50 mg for 8 months)	Inhibited mammary carcinogenesis (Singh *et al.*, 2014)
♀ ACI rats (estrogen-induced)	Dietary restriction	Diet restriction (40% less energy)	Inhibited mammary carcinogenesis (Harvell *et al.*, 2002)
	Tocopherol	Diet supplementation (0.3% for 14 days)	Inhibited mammary carcinogenesis (Das Gupta *et al.*, 2015)
	Vitamin C Butylated hydroxyanisole	Vitamin C (1% drinking water, for 15 days) Butylated hydroxyanisole (diet supplementation, 0.7% for 120 days)	Vitamin C and Butylated hydroxyanisole inhibited mammary carcinogenesis (Singh *et al.*, 2012)
♀ ACI rats (17β-estradiol and testosterone)	Phanobarbital	Drinking water (0.05% for 6, 12 and 28 weeks)	Inhibited mammary carcinogenesis (Mesia-Vela *et al.*, 2006)
	Tamoxifen	Subpanicular implant (40 mg for 6 months)	Inhibited mammary carcinogenesis (Li *et al.*, 2002)
		Subpanicular implant (40 mg for 4 and 7 months)	Inhibited mammary carcinogenesis (Montano *et al.*, 2007)
♀ ACI.COP-Ept2 rats (17β-estradiol and testosterone)	Tamoxifen	s.c. implant (for 5 months)	Inhibited mammary carcinogenesis (Ruhlen *et al.*, 2009)

1.1.5. Xenograft model of mammary cancer

Tumor cells inoculation (T47-D and BT474) in Sprague-Dawley rats 24-48 hours after the implantation of a pellet of 17β-estradiol (1.7 mg, 60 days timed release)	Medroxyprogesterone acetate 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1)	Medroxyprogesterone acetate pellets + YC-1 (10 mg/60-day release + YC (i.p., 600 µg)	Inhibited mammary carcinogenesis (Carroll *et al.*, 2013)

1.1.6. Syngeneic model of mammary cancer

Tumor cells inoculation (MT-450) in Wistar Furth rats	VEGF-C or VEGFR-3 antibodies	Intradermal (5 times/week for 4 weeks)	Inhibited lung metastasis (Quagliata *et al.*, 2014)
	Delphinidin	Gavage (1.18×10^{-5} mol for 28 days)	Promoted tumor growth and metastasis (Thiele *et al.*, 2013)
Tumor cells injection (i.v.., MADB106) in Fischer 344 rats	CpG oligodeoxynucleotides (CpG-C ODN)	i.p. (330 µg/kg)	Reduced lung retention (Goldfarb *et al.*, 2009)
	Interleukin-12 (4×1.5 µg/kg)	s.c. (0.5µg/rat/day for 8 days)	Reduced lung retention (Avraham *et al.*, 2010)
Tumor cells inoculation (Mat B III) in Fischer 344 rats	Antiangiogenic Urokinase-derived Peptide (Å6) Tamoxifen Rat umbilical cord matrix stem cells (rUCMS)	Å6 (i.p., 75 mg/kg/day for 17 days) TAM (i.p., 3 mg/kg/day for 17 days) Å6 + Tamoxifen (i.p., 75 mg/kg/day + 3 mg/kg/day for 17 days) Intratumoral or i.v. (2 administrations)	The Å6 enhanced the antitumor effects of Tamoxifen (Guo *et al.*, 2002) Attenuated mammary cancer growth (Ganta *et al.*, 2009)

	Tamoxifen 4-iodo benzo[b]thiophene-2-carboxamidine (B-428)	Tamoxifen (i.p., 3 mg/kg for 2 weeks) B-428 (i.p., 0.005 ml/h for 2 weeks) Tamoxifen + B-428 (i.p., 3 mg/kg + 0.005 ml/h for 2 weeks)	The combination inhibited mammary carcinogenesis (Xing *et al.*, 1997)
Tumor cells inoculation (Mat B III-uPAR) in Fischer rats	ruPAR IgG	s.c. (50-100 µg/day for 7 days	Inhibited mammary carcinogenesis (Rabbani and Gladu, 2002)
Tumor cells inoculation (R3230) in Fischer 344 rats	Recombinant bone morphogenetic protein-2 (rBMP-2)	i.p. (10, 50 and 100 µg, single injection)	Dose-dependent calcifications were produced (Liu *et al.*, 2010)
Tumor cells (SST-2) inoculation in SHR female rats	Doxorubicin Mito-Tempol (Mito-T) Dexrazoxane	Doxorubicin (i.v.., 10 mg/kg) Mito-T (i.p. , 5 and 25 mg/kg) Dexrazoxane (i.p. 50 mg/kg) Mito-T + Dexrazoxane (i.p. , 5 and 25 mg/kg + 50 mg/kg)	Doxorubicin inhibited mammary carcinogenesis Mito-T and Dexrazoxane inhibited mammary carcinogenesis and ameliorated doxorubicin-induced cardiomyopathy (Dickey *et al.*, 2013)
Tumor cells injection (s.c. or i.v., c-SST-2) in SHR rats	Malotilate	Gavage (150 mg/kg b.w.for 7 days)	Lung metastases were inhibited (Nagayasu *et al.*, 1998)
Tumor cells (Walker 256) inoculation in Sprague-Dawley rats	Sunitinib malate Fingolimod	Sunitinib malate (gavage, 30 mg/kg for 5 or 7 days) Fingolimod (gavage, 5 mg/kg for 5 or 7 days) Sunitinib malate + Fingolimod (gavage, 30 mg/kg + 5 mg/kg for 5 or 7 days)	The drugs combination inhibited mammary carcinogenesis (Mousseau *et al.*, 2012)
Tumor cells inoculation (BN472) in Brown-Norway	NVP-BEZ235	NVP-BEZ235 (gavage, 5 mL/kg for 6 days)	Inhibited mammary carcinogenesis (Schnell *et al.*, 2008)

1.1.7. Genetically engineered model of mammary cancer

MMTV-c-erbB-2 transgenic rat	-	Cystic expansions, sclerosing adenosis, and ductal hyperplasia were developed (Davies *et al.*, 1999)	
MMTV-TGFα transgenic rat	-	Severe hyperplastic lumps, hyperplasia, papillary ductal adenoma, lactating adenoma, ductal carcinoma *in situ* and carcinoma were observed (Davies *et al.*, 1999)	
Transgenic rats carrying human c-Ha-ras proto-oncogenes	*N*-methyl-*N*-nitrosourea (MNU)	i.v. (50 mg/kg at 50 days of age)	All MNU-exposed animals developed mammary tumors (Asamoto *et al.*, 2000)
c-Ha-ras transgenic rats non-transgenic rats	Purple corn color	Diet supplementation (5% for 8 weeks for transgenic animals and 22 weeks for non-transgenic ones)	Inhibited mammary carcinogenesis (Fukamachi *et al.*, 2008)
Transgenic rats carrying human c-Ha-ras proto-oncogenes + *N*-methyl-*N*-nitrosourea (MNU)	Isoflavones	Diet supplementation (0.25% for 20 and 56 days)	Inhibited mammary carcinogenesis (Matsuoka *et al.*, 2003)
Transgenic rats for *neu* proto-oncogene	Androgen 5α-dihydrotestosterone	s.c. implant (6 months)	Both females and males developed mammary carcinomas (Watson *et al.*, 2002)
WKAH and F344 strains carrying the human T-lymphotropic virus type I	-	Spontaneously developed mammary tumors at 5 months of age (Yamada *et al.*, 1995)	
p53 knockout rats	Diethylnitrosamine	i.p. (20 mg/kg b.w., for 5 weeks)	Decreased survival time and latency period (Yan *et al.*, 2012)
Brca2 knockout rats	-	Underdeveloped mammary glands, cataract formation and short lifespan (Cotroneo *et al.*, 2007b)	

b.w. body weight, **i.p.** intraperitoneal injection, **i.v.** intravenous injection, **s.c.** subcutaneous injection

CHAPTER 2

PROGNOSTIC FACTORS IN MNU AND DMBA-INDUCED MAMMARY TUMORS IN FEMALE RATS

THE CONTENT OF THIS CHAPTER WAS PUBLISHED IN:

Antonieta Alvarado, Ana Lopes, Ana I. Faustino-Rocha, António Cabrita, Rita Ferreira, Paula A. Oliveira and Bruno Colaço. (2017). *Pathology- Research and Practice*. 213(5):441-446. (IF 1.388; Q2). (Full paper).

2. PROGNOSTIC FACTORS IN MNU AND DMBA-INDUCED MAMMARY TUMORS IN FEMALE RATS

2.1. Introduction

The MNU and DMBA are the two most widely used chemical carcinogens for the induction of mammary tumor development in female rats (Imaoka *et al.*, 2014). A single administration of MNU or DMBA induces tumor development in female rats within eight to ten weeks after the administration (Ariazi *et al.*, 2005; Sharma *et al.*, 2011; Lopes *et al.*, 2014; Alvarado *et al.*, 2016; Faustino-Rocha *et al.*, 2016d). Although both carcinogenic agents induce mammary tumor development through DNA alkylation, they are different in terms of their metabolism. MNU does not require metabolic activation, being classified as a direct-acting alkylating agent (Lu *et al.*, 1998; Faustino-Rocha *et al.*, 2015a). Unlike MNU, DMBA is an indirect carcinogen that requires prior metabolic activation by liver cytochrome P450 enzymes. In this way, the carcinogenic activity of DMBA is slower when compared with MNU, which results in a longer latency period for DMBA-induced mammary tumors (Budan *et al.*, 2008). Both carcinogens modify the expression of several micro RNAs (miRNAs) in a short period of time, such as miRNA-21, miRNA-34a and miRNA-155, supporting their role in initial process of chemical carcinogenesis. The effect of MNU on miRNAs is dominant when compared with DMBA due to the direct acting effect of this carcinogen in contrast with DMBA (O'Day Elizabeth and Lal Ashish, 2010; Juhasz *et al.*, 2013).

Estrogen and progesterone are steroid hormones that play an important role in sexual differentiation and fertility (Kariagina *et al.*, 2008; Obr and Edwards, 2012). They act by binding to specific nuclear receptors commonly colocalized within the same cell: ER (isoforms α and β) and PR (isoforms A and B) (Lannigan, 2003; Hanstein *et al.*, 2004). Similarly to that observed in woman and mouse mammary gland, the rat PR is regulated by ER that sustains its high expression. These receptors are considered prognostic factors for mammary cancer. The simultaneous expression of both receptors (ER^+/PR^+) is suggestive of less aggressiveness of mammary tumors and better response to hormone therapy, when compared with those mammary tumors that only express one of these receptors (ER^-/PR^+, ER^+/PR^-) or none of them (ER^-/PR^-). Approximately 70% of human mammary tumors express both hormone receptors (Lange and Yee, 2008).

Beyond the hormone receptors, the Ki-67 is involved in the cellular proliferation and despite the fact its role in breast cancer management is uncertain, nowadays it is considered in conjunction with the hormone receptors as an important prognostic marker in breast cancer (Yerushalmi *et al.*, 2010; Dowsett *et al.*, 2011).

Although the histological characteristics of MNU and DMBA-induced mammary tumors have been well-described, there are no previous studies comparing the immunoexpression of the prognostic factors (ERα, PR and Ki-67) between them. So, this study aimed to evaluate the immunoexpression of these prognostic factors in MNU and DMBA-induced rat mammary tumors, in order to know the model that best suits to woman breast cancer.

2.2. Materials and methods

2.2.1 Animals and mammary tumors

Forty-four chemically-induced mammary tumors developed in Sprague-Dawley female rats (28 MNU-induced mammary tumors in 11 animals and 16 DMBA-induced mammary tumors in 12 animals) were used. Briefly, female rats were obtained from Harlan Interfauna Inc. (Barcelona, Spain) and maintained in polycarbonate cages (1500U Eurostandard Type IV S, Tecniplast, Buguggiate, Italy) with corncob for bedding (Mucedola, Italy), at a temperature of 23±2ºC and a humidity of 50±10%, with light/dark cycle (12h:12h). Water and food (Standard diet 4RF21®, Mucedola, Italy) were supplied *ad libitum*. MNU or DMBA were administered at the 50th day of age. Animals from MNU group received a single intraperitoneal injection (50 mg/kg body weight) of the carcinogenic agent MNU (ISOPAC®, lot 100 M1436V, Sigma Chemical Co., Madrid, Spain), dissolved in 0.9% saline solution to a concentration of 11 mg/ml. Animals from DMBA group received one administration of DMBA (65 mg/Kg body weight) diluted in virgin olive oil by gavage (a maximum of one milliliter *per* animal). All the biosecurity standards specified for the studies using animal models were followed (European Directive 2010/63/EU and National Decree-Law 113/2013), and the experimental protocols were approved by *Direção Geral de Alimentação e Veterinária* (Approval nº 008961 for MNU project and Approval nº 004543/2011 for DMBA project).

2.2.2 Animals' sacrifice and necropsy

MNU and DMBA-treated animals were sacrificed at the 35th week and 27th week after the carcinogen administration, respectively, by exsanguination by cardiac puncture under anesthesia (ketamine and xylazine), as indicated by the Federation of European Laboratory Animal Science Associations (Forbes *et al.*, 2007). At necropsy, all animals were externally examined and scalped, the skin was carefully observed under a light to detect mammary tumors. The tumors were collected and immersed in 10% phosphate buffered-formaldehyde during 24 hours.

2.2.3. Histopathology and immunohistochemistry:

Fixated tumors were cut, processed, embedded in paraffin and 2 µm-thick trimmed sections were stained with H&E for histopathological evaluation by two independent pathologists (A. Alvarado and A. Lopes). The mammary tumors were classified and categorized considering the predominant histological patterns according to Russo and Russo (Russo and Russo, 2000). Two µm-thick sections were also obtained for immunohistochemistry, the Novolink^{TM} Polymer Detection System (Leica Biosystems, Newcastle, UK) was used according to the instructions provided by manufacturer. The primary antibodies for ERα (clone 6F11, obtained from Novocastra, Newcastle, UK) and PR (clone SP2, obtained from Abcam, Cambridge, UK) were incubated at a dilution of 1:50 for one and half hour at room temperature or 16 hours (overnight) at 4ºC, respectively. The primary antibody for Ki-67 (clone MIB-5, obtained from Dako, Glostrup, Denmark) was incubated for 16 hours (overnight) at 4˚C at a dilution of 1:50. The antigen retrieval was performed with thermic treatment by microwave using 3 cycles of 5 minutes each one for ERα and PR, and 4 cycles for Ki-67 in citrate buffer solution. The tissues were counterstained with hematoxylin. Normal rat mammary tissue incubated with or without the primary antibody was used as positive and negative control, respectively.

The immunoexpression of ERα, PR and Ki-67 was evaluated as the percentage of nuclei-stained cells in at least 1,000 neoplastic cells, independently of invasiveness of the tumor, using a 40× objective for high power fields (HPF). A tumor was considered positive for ERα and PR if >1% of tumor cell nuclei were immunoreactive (Hammond *et al.*, 2010). The Ki-67 immunoexpression was denoted as proliferation index (Ki-67 PI). Mitotic activity was denoted as MAI, evaluated in 10 HPF of the most proliferative areas of the tumor, *i.e.*,

tumor periphery excluding necrosis and apoptosis, using a 40× objective, 0.45mm in diameter (LEICA DM500 diagnostic microscope, Leica®, Wetzlar, Germany).

2.2.4. Statistical analysis

Descriptive statistics, such as mean, standard deviation (S.D.), proportion in base to percentage and Chi-test were used to express the presence, classification and comparison of mammary histopathological patterns between experimental groups. ANOVA and Tukey-Kramer honestly significant difference (HSD) tests were used to compare the carcinoma patterns between groups. T-test was used to compare the factors observed between groups. The statistical analysis was performed using the JMP starter 5.0.1. program (SAS Institute Inc., Cary, NC, USA). *p*-values lower than 0.05 were considered statistically significant.

2.3. Results

2.3.1. Histopathological analysis

At histopathological analysis, each mammary tumor exhibited more than one histological pattern, independently of the carcinogen agent used. Taking into account the predominant histological patterns in each tumor, a total of 37 and 25 mammary lesions were identified in MNU and DMBA groups, respectively. Carcinomas were the lesions most frequently induced by both carcinogens: 33 carcinomas MNU-induced and 23 carcinomas DMBA-induced. Papillary carcinoma was the pattern most commonly observed in both groups (Figure 2.1). The number of lesions for each histological pattern was not statistically different between groups ($p>0.05$).

2.3.2. Immunohistochemical analysis

Only the carcinomas developed in both groups were studied because the benign (fibroadenoma, observed in DMBA group) and premalignant (intraductal proliferation, observed in MNU group) lesions were only identified in one of the experimental groups (Figure 2.1, Table 2.1).

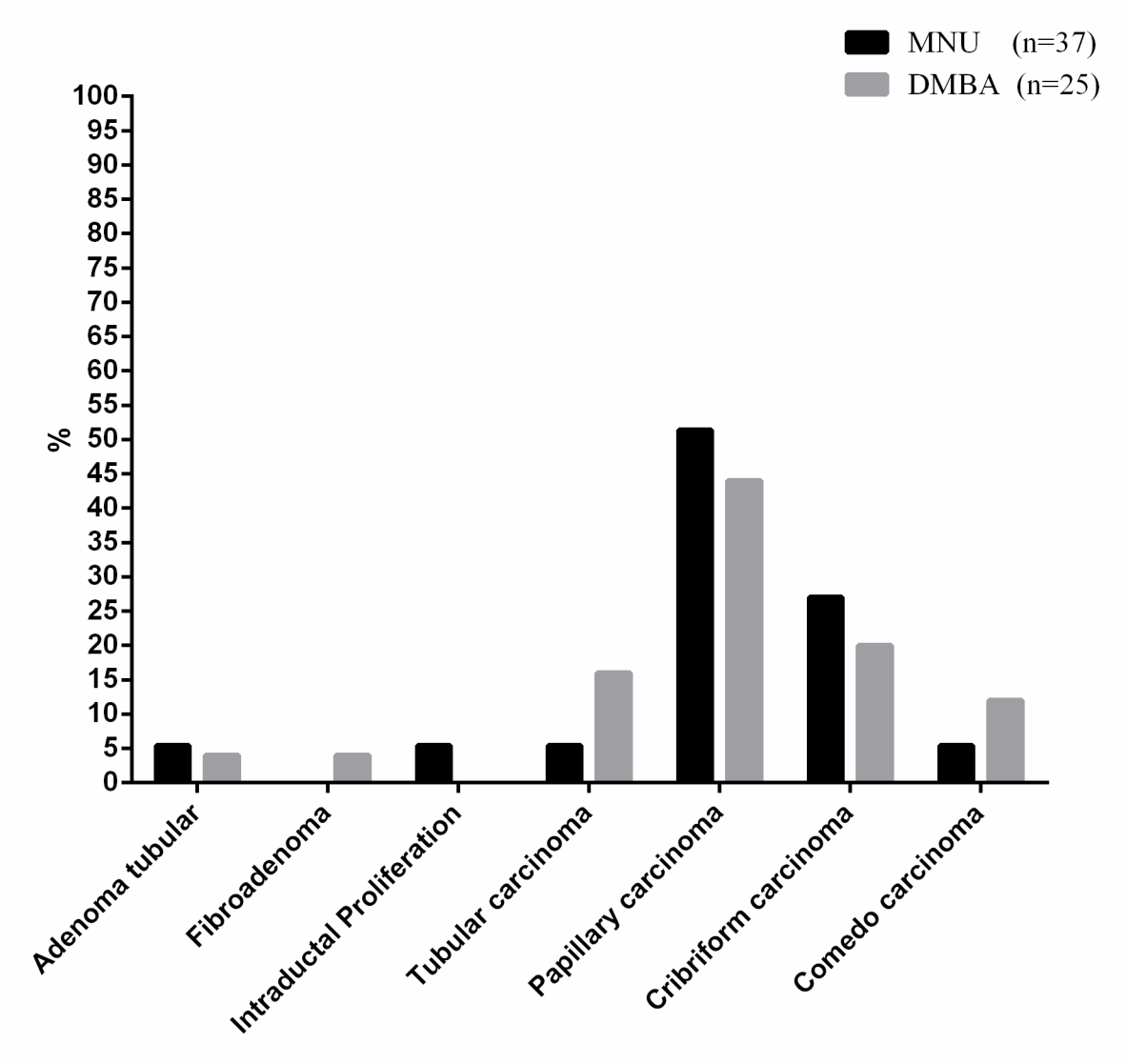

Figure 2.1. Percentage of histological patterns observed in MNU and DMBA-induced mammary tumors.

Table 2.1. Immunoexpression of ERα and PR, Ki-67 PI and MAI for each MNU or DMBA-induced mammary carcinoma (mean±S.D).

Histological pattern	MNU					DMBA				
	n	ERα(%)	PR(%)	Ki-67 PI	MAI	n	ERα(%)	PR(%)	Ki-67 PI	MAI
Tubular carcinoma	2	57.00±19.80	44.10±3.25	1.05±1.48	0.70±0.42	4	35.35±14.50	13.23±9.45	3.54±1.45	0.05±0.06
Papillary carcinoma	19	47.08±9.77	46.27±10.17*	3.86±4.55	1.05±1.08	11	37.29±7.69	18.26±10.48	3.81±0.84	0.43±0.35
Cribriform carcinoma	10	54.97±11.57	33.64±8.62	11.88±13.04	2.04±1.33‡	5	44.10±12.18	33.68±10.97	4.41±1.40	0.34±0.35
Comedo carcinoma	2	51.75±36.98	16.10±16.12	2.65±2.62	5.35±0.64 **	3	48.47±10.92	24.53±12.82	4.84±1.09	0.97±.025

*Statistically different from MNU-induced cribriform and comedo carcinomas, and DMBA-induced tubular, papillary and comedo carcinomas ($p<0.05$). **Statistically different from all MNU and DMBA-induced carcinomas ($p<0.05$). ‡ Statistically different from DMBA-induced tubular, papillary and cribriform carcinomas ($p<0.05$).

All MNU and DMBA-induced mammary carcinomas were ERα and PR positive (ER^+/PR^+) with punctual nuclei immunolabeling (Figure 2.2 and 2.3). The ERα immunoexpression was higher when compared with PR immunoexpression. ERα and PR immunoexpresion was higher in all MNU-induced mammary carcinomas when compared with DMBA-induced carcinomas, with the exception of PR immunoexpression in cribriform and comedo carcinomas. Although some numerical differences were observed, statistically significant differences in the Ki-67 PI between MNU and DMBA-induced mammary lesions were not found ($p>0.05$). MAI was higher in all MNU-induced carcinomas when compared with the DMBA-induced ones, being the highest value for comedo carcinoma ($p<0.05$ for MNU-induced comedo carcinoma).

When carcinomas from both groups were compared independently of the histological pattern, it was observed that the tumors' weight, ERα, PR, Ki-67 PI and MAI were higher in MNU-induced mammary carcinomas when compared with the DMBA-induced carcinomas. Statistically significant differences were observed for ERα, PR immunoexpression, and MAI ($p<0.05$) (Table 2.2).

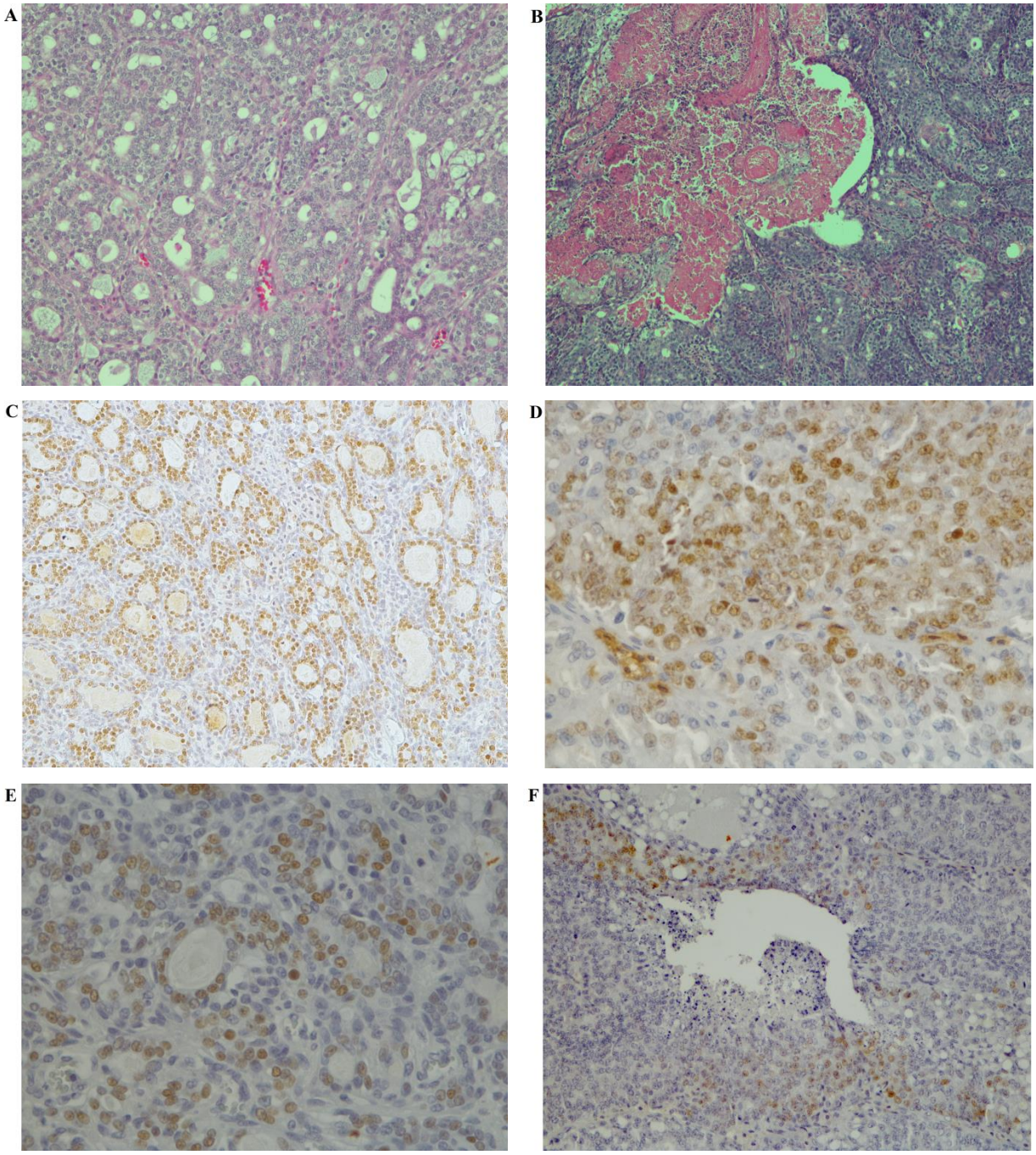

Figure 2.2. **A** MNU cribriform carcinoma (H&E 200X magnification) and **B** DMBA comedo carcinoma, (H&E 100X magnification). **C** ERα immunoexpression in MNU cribriform carcinoma (200X magnification) and **D** DMBA comedo carcinoma (400X magnification). **E** PR immunoexpression in MNU cribriform carcinoma (400X magnification) and **F** DMBA comedo carcinoma (200X magnification).

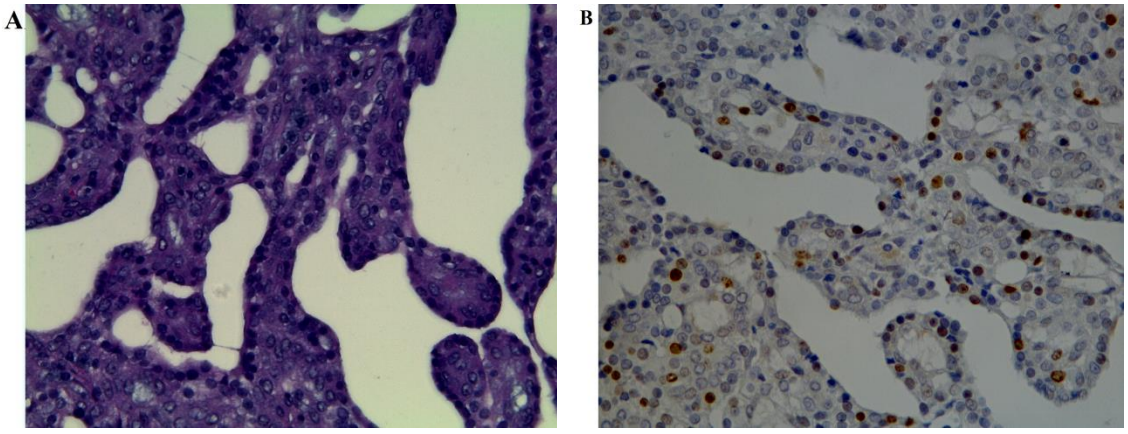

Figure 2.3. **A** MNU papillary carcinoma (H&E 400X magnification). **B** Ki-67 immunoexpression in the same carcinoma (400X magnification).

Table 2.2. Immunoexpression of ERα and PR, Ki-67 PI and MAI for MNU and DMBA-induced carcinomas, independently of the histological pattern. Mean±S.D.

Group		Factors				
	n	Tumor weight (g)	ERα(%)	PR(%)	Ki-67 PI	MAI
DMBA	23	1.06±1.97	39.89±10.70	21.56±12.31	3.99±1.09	0.41±0.39
MNU	33	5.47±10.78	50.35±12.74*	40.48±12.65*	6.01±8.69	1.56±1.52*

*Statistically different from DMBA group ($p<0.05$).

2.4. Discussion

MNU and DMBA-induced mammary tumors in female rats have been extensively used for the study of mammary carcinogenesis. This study intended to assess the biological behavior of these tumors, determining the hormone receptors immunoexpression (ERα and PR) and tumor proliferation (Ki-67 PI and MAI).

Similarly to previous works with MNU and DMBA induced mammary tumors, concluding that they are almost indistinguishable (Shirai *et al.*, 1997; Russo and Russo, 2000; Kang *et al.*, 2004; Russo, 2015), we verified that animals MNU and DMBA-exposed

developed similar histopathological mammary lesions. Benign lesions could be observed in one or both groups, while the premalignant lesion (intraductal proliferation) was only observed in MNU group. The malignant mammary lesions, more specifically the papillary carcinoma and cribriform carcinoma, were the lesions most frequently developed in both experimental groups, in accordance with that previously reported by Russo and Russo (Russo and Russo, 2000).

The MNU and DMBA mammary tumors in rats are mainly hormone-dependent with an expression of both hormone receptors ERα and PR (Thordarson *et al.*, 2001; Russo, 2015). All MNU and DMBA-induced mammary carcinomas evaluated in the present study were positive for both hormone receptors (ER^+/PR^+), with a higher immunoexpression of ERα when compared with PR, as observed in human mammary tumors. These receptors play an important role in mammary cancer etiology, promoting tumor cells proliferation (Kariagina *et al.*, 2013; Boopalan *et al.*, 2015). Despite this, not all positive cells for PR are proliferative and the negative cells surrounded by these positive cells may proliferate suggesting that the proliferation induced by PR may occur through paracrine system (Obr and Edwards, 2012). The expression of both ERα and PR is indicator of good prognosis for breast cancer patients with evident survival benefit effect. The PRs are only expressed in the epithelial luminal cells of the mammary gland, while the ERsα are also expressed in myoepithelial or stromal cells. This explains the higher expression of ERα when compared with PR in double positive mammary tumors (ER^+/PR^+) (Morgan *et al.*, 2011; Bae *et al.*, 2015; Vici *et al.*, 2015; Ren *et al.*, 2016). Despite that the histological patterns were similar between MNU and DMBA-exposed groups, some differences were observed in ERα and PR immunoexpression and proliferation indexes (Ki-67 PI and MAI). Almost all MNU-induced mammary lesions exhibited higher ERα and PR, Ki-67 PI and MAI when compared with the same histological patterns DMBA-induced.

The ERα and PR immunoexpression in DMBA-induced carcinomas was similar to that reported by Russo (2015) that observed a ERα immunoexpression ranging from 15.1 to 57% and a PR immunoexpression ranging from 11.1 to 61.1% in 59 DMBA-induced carcinomas in rats. The results concerning to the ERα were similar to those observed by Kang *et al*. (Kang *et al.*, 2004), who worked with mammary tumors induced by both carcinogens in Sprague-Dawley rats, using a similar dose of MNU (50 mg/kg) and a dose of 10 mg/rat of DMBA, in an assay lasting 26 weeks. However, the results related to tumor proliferation were

distinct. While we observed a higher proliferation in MNU-induced mammary carcinomas when compared with the DMBA-induced carcinomas, those authors described a similar proliferation between both models. These different results may be related with the different methodology employed to assess tumor proliferation, while the Ki-67 PI and MAI were evaluated in the present study, Kang *et al*. (2004) evaluated the cell nuclear antigen marker (PCNA). The observations with this marker cannot be compared with ours because it is a less specific marker when compared with Ki-67, detecting not only cellular proliferation but also cellular repair. The higher proliferation of MNU-induced mammary carcinomas in our study suggests that these tumors are more aggressive when compared with DMBA-induced ones and probably more able to metastasize to distant organs. The higher tumor weight in MNU-induced mammary carcinomas is related with higher proliferation observed in this group (higher Ki-67 PI and MAI) when compared with DMBA group.

Although it is evident that the dose, route of administration and metabolism (direct or indirect agent) of each carcinogenic compound may influence the time in which the carcinogenesis is initiated (latency period), once initiated, the progression of MNU and DMBA-induced mammary tumors is similar leading to the development of similar histological patterns. Despite the fact that previous studies demonstrated several morphologic, molecular and histopathological similarities between MNU and DMBA-induced mammary lesions, any difference observed between these tumors is really important in order to help the researchers to choose the model that best suits their investigations in future studies on mammary carcinogenesis. In terms of prognostic markers, we believe that this is a reason strong enough to perform further studies comparing these two models of mammary carcinogens.

2.5. Conclusion

Both MNU and DMBA-induced mammary tumors were hormone-dependent (ERα and PR immunoexpression). A higher Ki-67 PI and MAI in MNU-induced mammary carcinomas were suggestive of a higher aggressiveness and worse prognostic of these carcinomas when compared with the DMBA-induced ones. In this way, the use of the rat model of MNU-induced mammary tumors is advised in experimental protocols aiming to study more aggressive mammary tumors.

CHAPTER 3

PROGNOSTIC FACTORS IN AN EXERCISED MODEL OF CHEMICALLY-INDUCED MAMMARY CANCER

THE CONTENT OF THIS CHAPTER WAS PUBLISHED IN:

Alvarado, A.; Faustino-Rocha, A.I.; Ferreira, R.; Mendes, R.; Duarte, J.A.; Pires, M.J.; Colaço, B. and Oliveira, P.A. (2016). Prognostic Factors in an Exercised Model of Chemically-induced Mammary Cancer. *Anticancer Research,* 36(5): 2181-2188. (IF 1.895; Q3). (Full paper).

3. PROGNOSTIC FACTORS IN AN EXERCISED MODEL OF CHEMICALLY-INDUCED MAMMARY CANCER

3.1. Introduction

Breast cancer affects approximately one out of ten women in the world and it has been demonstrated that chemically-induced mammary tumors in female rats histopathologically resemble those developed by humans (Liska *et al.*, 2000). When exposed to the carcinogen agent MNU, female Sprague-Dawley rats develop a high number of mammary tumors (Gal *et al.*, 2011; Faustino-Rocha *et al.*, 2013b, 2015). Additionally, the mammary tumors induced by this chemical carcinogen behave as hormone-dependent and they are characterized by the expression of ERs (Russo and Russo, 1998; Thordarson *et al.*, 2001; Rudmann *et al.*, 2012; Soares-Maia *et al.*, 2013).

ERs are nuclei receptors that regulate cell growth, differentiation and homeostasis. Until now, two ERs isoforms are known: ERα and ERβ. The steroid hormone estradiol, which is necessary for sexual differentiation, fertility and development, acts by binding with these two isoforms (Lannigan, 2003; Hanstein *et al.*, 2004). The ERα expression is a well-established marker of cell proliferation and its high expression is considered a good prognostic and predictive marker related with increase of survival and delay of tumor recurrence after hormone therapy in human mammary cancer (Kinsel *et al.*, 1989; Hammond *et al.*, 2010).

Another important marker of cellular proliferation in mammary cancer is the protein Ki-67, which is involved in cellular proliferation and expressed in different phases of the cell cycle, such as G1, S, G2 and M, but not in the resting phase G0. Furthermore, the expression of Ki-67 is associated with a ribosomal pathway for RNA transcription (Scholzen and Gerdes, 2000; Yerushalmi *et al.*, 2010; Mrklic *et al.*, 2013). Although Ki-67 is not included as a routine biological marker, it is recommended as a prognostic and predictive marker in human mammary cancer (Yerushalmi *et al.*, 2010).

Researchers who conducted studies in this field using the female Sprague-Dawley rat model of mammary cancer have observed positive effects of physical exercise on these tumors, including the rate of tumors *per* animal and the type of tumors in terms of their histopathological grade (Thompson, 1994, 1997; Whittal-Strange *et al.*, 1998; Thompson *et*

al., 2010). This work aimed to evaluate the effects of lifelong exercise training on the immunoexpression of ERα, Ki-67 and the MAI in MNU-induced mammary tumors in female Sprague-Dawley rats.

3.2. Materials and Methods

3.2.1. Animals and experimental design

All the biosecurity standards specified for the studies using animal models were followed (European Directive 2010/63/EU and National Decree-Law 113/2013), and the experimental protocol was approved by *Direção Geral de Alimentação e Veterinária* (Approval nº 008961). Thirty outbred female Sprague-Dawley rats (*Rattus norvegicus*) with five weeks of age (Harlan Interfauna Inc., Barcelona, Spain) were maintained in polycarbonate cages (1500U Eurostandard Type IV S, Tecniplast, Buguggiate, Italy) with corncob for bedding (Mucedola, Italy), at a temperature of 23±2ºC and a humidity of 50±10%, with a controlled light/dark cycle (12h:12h). Water and food (Diet Standard 4RF21®, Mucedola, Italy) were supplied *ad libitum*.

At 50th day of age, all animals received an intraperitoneal injection (50 mg/kg body weight) of the carcinogen agent MNU (ISOPAC®, lot 100 M1436V; Sigma Chemical Co., Madrid, Spain), dissolved in 0.9% saline solution to a concentration of 11 mg/ml. Animals were randomly divided into two experimental groups as follows: sedentary (n=15) and exercised (n=15). The day of the carcinogen administration was defined as the first day of the experiment.

A Treadmill Control® LE 8710 (Panlab, Harvard Apparatus, Massachusetts; USA) was used for the exercise training. At the day after the carcinogen administration, animals from exercised group started an acclimatization period to the treadmill for five days with a progressive increase from 20 to 60 min of exercise *per* day with a constant speed of 20 m/min. Since the following week, animals were subjected to the exercise training during 35 weeks (60 min/day, at a speed of 20 m/min, 5 days a week with a rest of 2 days). Both mammary gland chains of all animals were weekly palpated.

3.2.2. Sacrifice and necropsy of animals.

In the 35th week of the experimentation, all animals were sacrificed by exsanguination by cardiac puncture under deep anesthesia (ketamine and xylazine), as indicated by the Federation of European Laboratory Animal Science Associations (Forbes *et al.*, 2007). All animals were scalped and the skin was carefully observed under a light to detect mammary tumors; all mammary tumors were excised and immediately fixed in 10% phosphate buffered-formaldehyde during 24 hours.

3.2.3. Histopathology and immunohistochemistry.

The fixed tissues were cut, processed, embedded in paraffin and 2-µm-thick cut sections were stained with H&E for histopathological evaluation by three independent pathologists. The tumors were histologically classified and categorized according to their histogenesis and biological behavior, according to Russo and Russo (Russo and Russo, 2000), taking into consideration only the concordance type of lesion or lesions with a high proportion in each tumor section. For immunohistochemistry, the Novolink™ Polymer Detection System (Leica®, Newcastle, UK) was used according to the instructions provided by the manufacturer. The sections were incubated with the primary antibody for ERα (clone 6F11, mouse monoclonal anti-human; Leica®) at a dilution of 1:50 for one and half hour at room temperature and with the primary antibody for Ki-67 (clone MIB-5, mouse monoclonal anti-rat; DAKO®, Glostrup, Denmark) at a dilution of 1:50, overnight at 4ºC. The antigen retrieval was performed with thermic treatment by microwave using 3 cycles for ERα and 4 cycles for Ki-67 of 5 minutes each one in citrate buffer solution. The tissues were counterstained with hematoxylin.

The H-score method was used for assessment of the ERα immunoexpression in a total of 1,000 neoplastic cells, as previously described by Kinsel *et al*. (1989). The evaluation of each histological pattern was recorded as percentage of positively stained target cells in each of four intensity categories which were denoted as follows: 0 (no staining), 1+ (weak but detectable above control), 2+ (distinct) and 3+ (strong with minimal light transmission through stained nuclei). The score was obtained adding the percentage of cells stained at each intensity multiplied by the weighted intensity of staining, producing a score range from 0 to 300, as follows:

H-Score = (0 × % at 0) + (1 × % at 1+) + (2 ×% at 2+) + (3 × % at 3+)

An H-score of more than 1 was considered as positive. Normal mammary tissue with and without the primary antibody was used as positive and negative control, respectively.

The Ki-67 was scored as the percentage of nuclei-stained cells in a total of 1,000 neoplastic cells, independently of invasiveness of the tumor, using a 40× objective for HPF and denoted as Ki-67 PI. The tumor tissue without the primary antibody was used as negative control and the hair follicles present in the histological cut that looked a correct intensity of nuclear staining were used as positive control.

The MAI was evaluated in 10 HPF of the most proliferative areas of the tumor, *i.e.* tumor periphery excluding necrosis and apoptosis, using a 40× objective, 0.45mm diameter (LEICA DM500 diagnostic microscope; Leica®). The MAI was scored taking into account the mitotic figures *per* microscopic field and denoted as: low (0-6), intermediate (7-14) or high (≥15) (Lehr *et al.*, 2013).

ER H-score, Ki-67 PI and MAI were evaluated by two independent pathologists.

3.2.4. Statistical analysis.

Descriptive statistics, such as mean, S.D. and proportion in base to percentage were used to express the presence and classification of mammary tumors in both experimental groups. For the comparison between groups, the ANOVA and Tukey-Kramer HSD tests for all pairs were used . The statistical analysis was performed using the JMP starter 5.0.1. program (SAS Institute Inc, North Caroline, USA). *p* values lower than 0.05 were considered statistically significant.

3.3. Results

3.3.1. General findings

Eight animals died during the experiment: four animals from the sedentary group and four animals from the exercised one. Additionally, one animal from the exercised group did not adapt to the exercise training and was excluded from the experiment. At the end of the experiment, the sedentary group had 11 animals and the exercised group had 10 animals. All

animals from both groups, exercised and sedentary, developed mammary tumors (100% of incidence).

3.3.2. Histopathological evaluation of mammary tumors.

Considering the predominant histological patterns in each mammary tumor, a total of 32 mammary lesions (all neoplasms) in the exercised group and 37 mammary lesions (neoplasms and preneoplastic lesions) in the sedentary group ware identified (Figures 3.1A and 3.1B). Papillary carcinoma was the histological pattern most frequently identified in both groups, followed by cribriform carcinoma pattern.

The grade of malignancy of mammary tumors was higher in sedentary group than in exercised one; the majority of the mammary neoplasms identified in both groups were classified as epithelial malignant lesions ($p>0.05$) (Figure 3.1B). Additionally, the animals from sedentary group developed two preneoplastic lesions (intraductal proliferation (IDP)) and two comedo carcinomas that were not found in exercised group (Figure 3.1A and 3.2A).

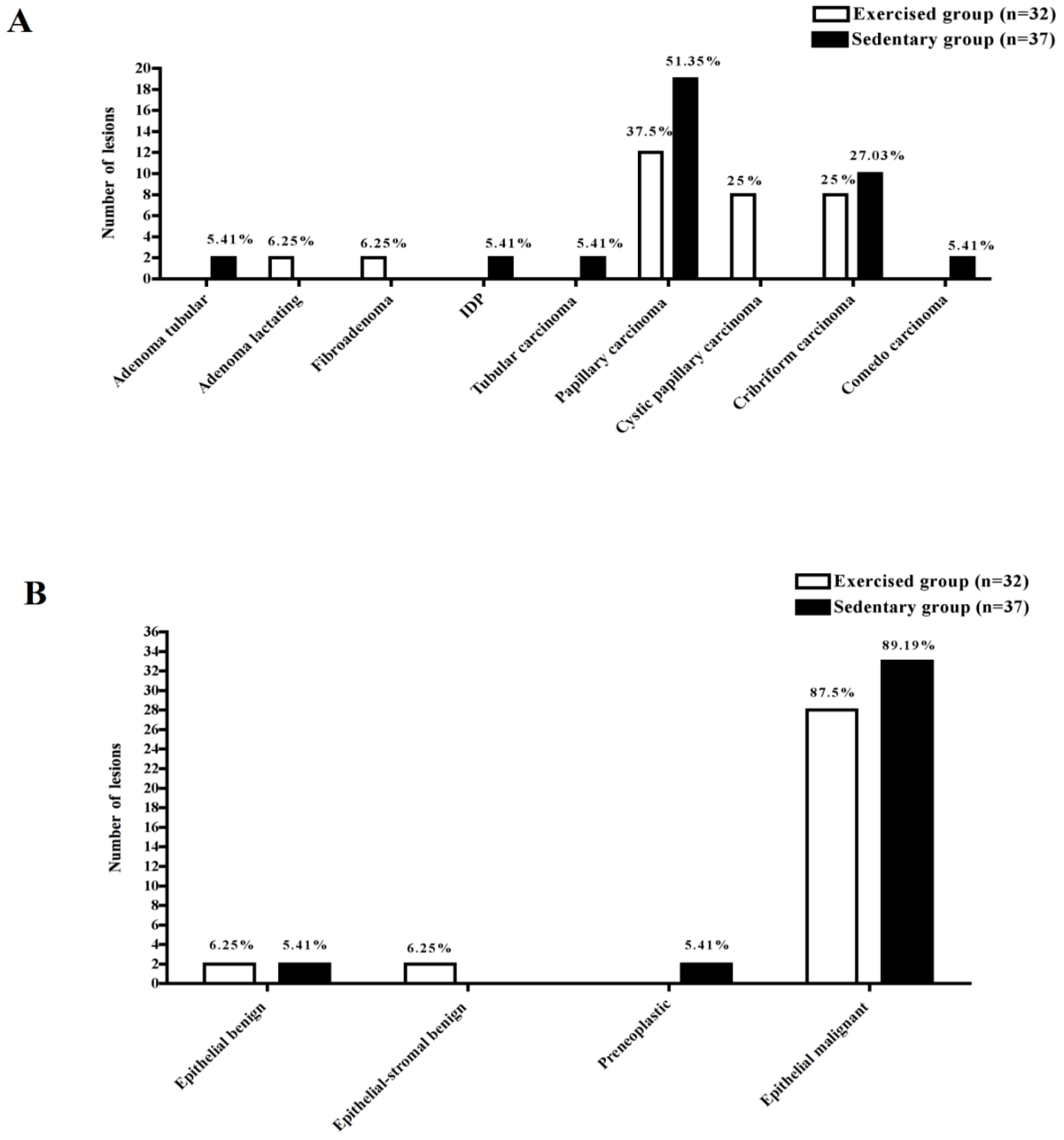

Figure 3.1. Histological patterns of mammary tumors. (**A**) Number and percentage of each histological pattern and (**B**) histological lesions classified according to their histogenesis in both groups, exercised and sedentary. IDP, intraductal proliferation.

3.3.3. Immunohistochemical evaluation of mammary tumors.

All mammary lesions from both groups were ERα-positive with an H-score ≥20. Concerning the ERα H-score in each histological pattern, the papillary pattern in exercised group exhibited the highest mean and fibroadenoma the lowest mean, with these values being statistically different (p<0.05) (Table 3.1). However, the differences of Ki-67 PI among histological patterns did not reach the level of statistical significance (p>0.05). In general, the MAI was low (<5). The comedo carcinoma in sedentary group was the histological pattern with the highest MAI (5.35±0.64), followed by cribriform carcinoma pattern in the same group; the differences between these two histological patterns were considered statistically significant (p<0.05) (Table I). Statistically significant differences were also found between the comedo carcinoma and the remaining histological patterns from both groups, exercised and sedentary (p<0.05) (Table 3.1).

Table 3.1. ERα H-score, Ki-67 PI and MAI of each histological pattern of rat mammary tumors developed by animals from exercised and sedentary groups. Mean ± S.D.

Histological pattern	ERα H-score		Ki-67 PI		MAI	
	Exercised	Sedentary	Exercised	Sedentary	Exercised	Sedentary
Adenoma tubular	-	112.25±52.68	-	0.65±0.35	-	0.28±0.04
Adenoma lactating	112.50±99.70	-	0.40±0.57	-	0.20±0.00	-
Fibroadenoma	34.00±12.72 *	-	0.10±0.14	-	0.35±0.35	-
IDP	-	86.92±94.02	-	1.85±2.62	-	0.55±0.49
Tubular carcinoma	-	162.35±54.94	-	1.05±1.48	-	0.70±0.42
Papillary carcinoma	168.61±45.82	129.10±32.05	5.02±5.75	3.86±4.55	1.08±0.58	1.05±1.08
Cystic papillary carcinoma	129.85±49.69	-	1.78±1.40	-	0.34±0.15*	-
Cribriform carcinoma	148.88±30.22	146.60±28.24	7.08±5.88	11.88±13.04	1.73±0.99	2.04±1.33
Comedo carcinoma	-	105.65±110.80	-	2.65±2.62	-	5.35±0.64‡

ERα H-score: *Fibroadenoma was statistically different from papillary carcinoma in exercised group; MAI: *Cystic papillary carcinoma from exercised group was statistically different from cribriform and comedo carcinoma from sedentary group; ‡Comedo carcinoma from sedentary group was statistically different from the remaining histological patterns from both groups exercised and sedentary.

In both groups, the highest H-score was identified in epithelial malignant neoplasms with a statistically significant difference between this histological type and epithelial-stromal

benign neoplasm from exercised group (p<0.05) (Table 3.2, Figure 3.2B). Statistically significant differences were not found in Ki-67 PI between groups (p>0.05); the epithelial malignant lesions were the histological type with the highest PI in both groups (Table 3.2, Figure 3.2C). Statistically significant differences in MAI were not found among the histological types in both groups (p>0.05) and, similarly to the Ki-67 PI, the epithelial malignant lesions were the histological type with the highest MAI (Table 3.2).

Table 3.2. ERα H-score, Ki-67 PI and MAI in different histological types of rat mammary tumors classified according to their histogenesis and biological behavior in exercised and sedentary groups. Mean ± S.D.

Histological type	ERα H-score		Ki-67 PI		MAI	
	Exercised	Sedentary	Exercised	Sedentary	Exercised	Sedentary
Epithelial benign	112.50±99.70	112.25±52.68	0.40±0.57	0.65±0.35	0.20±0.00	0.28±0.04
Epithelial-stromal benign	34.00±12.73*	-	0.10±0.14	-	0.35±0.35	-
Precancerous (IDP)	-	86.92±94.02	-	1.85±2.62	-	0.55±0.49
Epithelial malignant	151.87±44.76	135.51±37.91	4.68±5.21	6.01±8.69	1.05±0.83	1.56±1.52

ERα H-score: *Epithelial-stromal benign type was statistically different from epithelial malignant in both exercised and sedentary groups.

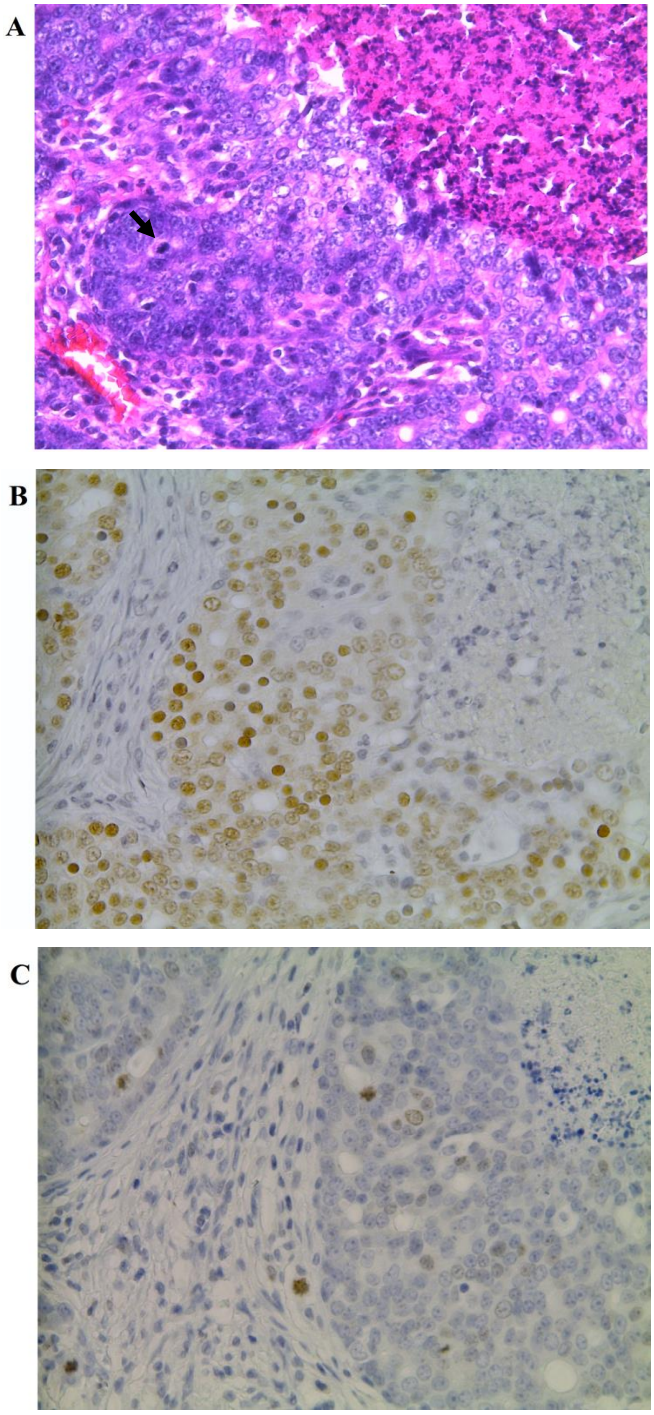

Figure 3.2. Comedo carcinoma pattern. **A,** H&E staining, (atypical mitosis figure with black arrow); **B,** ERα immunoexpression and **C,** Ki-67 immunoexpression in the same carcinoma (400X magnification).

3.3.4. Comparison of ERα H-score, Ki-67 PI and MAI between groups.

Regardless of the histological type or pattern of the mammary lesions, statistically significant differences were not found in the means of ERα H-score, Ki-67 PI and MAI between groups (p>0.05). However, the absolute mean value of ERα H-score was higher in the exercised group, while the Ki-67 PI and MAI were higher in the sedentary group (Table 3.3).

Table 3.3. Means' comparison of ERα H-score, Ki-67 PI and MAI between exercised and sedentary groups. Mean ± S.D.

Parameters	Exercised group	Sedentary group	*P*-value
ERα H-score	142.07±54.48	131.63±41.79	0.36
Ki-67 PI	4.13±5.09	5.53±8.34	0.45
MAI	0.96±0.82	1.46±1.49	0.11

3.4. Discussion

The mammary tumors identified in this study were similar to those previously described by Russo and Russo (2000) who further reported that the papillary carcinoma is the most typical and frequent pattern observed in MNU-induced mammary tumors. Concerning the effects of exercise training on chemically-induced mammary tumorigenesis, a beneficial effect was observed, *i.e.* decrease of the number of lesions and their malignancy, which is in accordance with previous reports (Thompson, 1997; Whittal-Strange *et al.*, 1998; Thompson *et al.*, 2010).

It has been proved that the semi-quantitative method to quantify the expression of ERα in breast cancer through immunohistochemical markers is especially powerful to predict the response of women's breast cancer to endocrine therapy. The high expression of ER is an indicator of better response to antiestrogen therapy and good prognostic in breast cancer patients; while its low expression indicates the need to apply chemotherapy in addition to endocrine therapy (Morgan *et al.*, 2011). In these results, despite the fact that the number of

malignant lesions was higher in sedentary animals, the ERα H-score was higher in the exercised group. Away from the fact that the exercise training did not decrease the expression of ERα, as expected, higher expression of ER in epithelial malignant lesions from exercised group suggests that these animals exhibited more differentiated mammary malignant lesions. This is in accordance with the findings of Qiu *et al*. (2005) and Chan *et al*. (2007) who reported that the rats' chemically-induced mammary carcinomas show papillary and cribriform carcinoma patterns with a greater cellular differentiation and higher ERα positive expression. These data are also in agreement with those described by Mccormick *et al*. (1996) and Russo and Russo (1998) who reported that high levels of steroid hormones and their precursors can be inhibitors of chemically-induced carcinogenesis in rats by the induction of the differentiation of the mammary parenchyma.

Ki-67 expression has been recently identified as a predictive and prognostic factor in breast cancer. Its high expression is associated with poor prognosis and can be used to monitor the tumors' response to treatment (Mrklic *et al.*, 2013; Tan *et al.*, 2014; Wishart *et al.*, 2014; Yuan *et al.*, 2015). In a previous study, it was reported that Sprague-Dawley rats have an elevation of Ki-67 expression in line with an altered ribosomal pathway, which could be related to deregulated protein synthesis machinery and the promotion of malignant tumor progression in this strain (Luzhna *et al.*, 2015). In this work, the Ki-67 PI exhibited low values in both groups, exercised and sedentary. According to Yerushalmi *et al.* (2010), Ki-67 PI <14 indicates a low risk of neoplasms' recurrence and a better response to therapy. The lower Ki-67 PI in exercised than in sedentary group is an indicator of better prognosis in exercised animals. These results were different from those observed by Malicka *et al*. (2015) who, in a similar study using an exercised model of chemically-induced mammary cancer in female rats, did not observe any differences in the Ki-67 expression. However, these authors did not present the values obtained for the Ki-67 immunoexpression. Westerlind *et al*. (2002) evaluated the expression of PCNA in exercised and sedentary animals under a protocol of chemical carcinogenesis. They observed that the expression of PCNA was higher in exercised animals. These results cannot be compared with ours because PCNA is a less specificity marker detecting not only cellular proliferation but also cellular reparation. Additionally, these different results may be due to the shorter period of exercise training employed in the studies (12 weeks in the aforementioned study and 35 weeks in the present work).

Mitotic count is primordial in cancer study and made by histopathological evaluation (Elston and Ellis, 1991). In the last years, this proliferation index was considered to be a strong prognostic factor in breast cancer and employed as a routine component of histopatological studies with clinical applicability (Klintman *et al.*, 2013). Despite the limitations for their estimation, mainly due to the variability of scoring and the standardization of immunohistochemical diagnostic (Bertucci *et al.*, 2013), nowadays, mitotic counts in conjunction with Ki-67 PI are important predictors for mammary cancer. In this study, a low MAI it was obtained inclusively in the most aggressive histological pattern, the comedo carcinoma. Despite the fact that the comedo carcinoma is a malignant neoplasia, highly cellular with a solid and expansive growth, the MNU-induced comedo carcinomas of the present study exhibited low proliferation (MAI was <6 and PI<14).

These results denoted that the MNU-induced mammary tumors have, in general, a low rate of proliferation similar to that reported in human mammary cancer where the ER-positive mammary tumors showed in general a lower rate of proliferation (lower KI-67 PI and MAI) and better prognosis than the ER-negative ones (Klintman *et al.*, 2013). In relation to the effect of the exercise, a positive effect was observed, since both proliferation indexes were lower in exercise than in sedentary group.

3.5. Conclusion

In this study, the comparison between ERα H-score, Ki-67 PI and MAI was not statistical significant but, taking into account their mean values, interesting results were observed: (i) ERα H-score was higher in exercised group and (ii) Ki-67 PI and MAI were higher in sedentary group denoting the positive effects of exercise training in the development of mammary cancer in rats, thus suggesting a better response to hormonal therapy on mammary tumorigenesis.

CHAPTER 4

EFFECTS OF EXERCISE TRAINING ON BREAST CANCER METASTIZATION IN A RAT MODEL

THE CONTENT OF THIS CHAPTER WAS PUBLISHED IN:

Alvarado, A.; Gil Da Costa, R.M.; Faustino-Rocha, A.I.; Ferreira, R.; Lopes, C.; Oliveira, P.A. and Colaço B. (2016). Effects of Exercise Training on Breast Cancer Metastization in a Rat Model**.** *International Journal of Experimental Pathology*, 98(1): 40-46. (IF 2.125; Q2). (Full paper).

Alvarado, A., Faustino-Rocha, A.; Gil Da Costa, R.; Lopes, C.; Ferreira, R.; Colaço, B. and Oliveira P.A. LUNG METASTASIS IN EXERCISED MODEL OF MNU-INDUCED MAMMARY CANCER IN RATS. 2015. I Congreso Ibérico de Ciencias del Animal de Laboratorio. VIII Congreso SECAL. III Congresso SPCAL. Poster.

Alvarado, A.; Faustino-Rocha, A.; Gil Da Costa, R.M.; Lopes, C.; Ferreira, R.; Oliveira, P.A. and Colaço, B. A POSSIBLE ANTITUMORAL EFFECT OF EXERCISE-TRAINING ON LESIONS INDUCED BY 1-METHYL-1- NITROSOUREA IN A RAT MODEL. 14th European Congress of Toxicologic Pathology. 20th – 23rd of September 2016, Barcelona, Catalonia, Spain. Poster-Abstrac, that conferred by the International Federation of Societies of Toxicologic Pathologists the **Trainee Award 2016**.

4. EFFECTS OF EXERCISE TRAINING ON BREAST CANCER METASTIZATION IN A RAT MODEL

4.1. Introduction

Breast cancer is one of the main causes of death in women worldwide, especially due to its high rate of metastasis development in several organs, most commonly the lung, bone, liver and brain (Valastyan and Weinberg, 2011). Lung metastases are the most frequently encountered: 10 to 24 % of women with breast cancer were reported to developed pulmonary metastases (Kayser *et al.*, 1998; Cummings *et al.*, 2014; Wu *et al.*, 2016). Metastatic disease is a great challenge to oncologists, due to its destructive and often deadly development, but also because metastases show a highly heterogeneous and difficult to treat cell population.

Physical activity and exercise training have been appointed as beneficial factors from the standpoint of cancer control and prevention. However, the complex relation between these factors and the biological behaviour of cancer is complex and remains poorly understood (Westerlind, 2003). Exercise training has been suggested to play a protective role against various cancers (Wolff and Toborek, 2013), such as lung (Paceli *et al.*, 2012) and colon (Aoi *et al.*, 2010) cancer. Experimental and epidemiological studies suggest that the prevention of hormone-dependent cancers, such as breast and prostate cancer, may also benefit from moderate exercise training (Gago-Dominguez *et al.*, 2007; Mccullough *et al.*, 2014; Tai *et al.*, 2016)

Our team and others have shown that exercise training is able to reduce the incidence of breast neoplasms in rats, using rat models induced by administering chemical carcinogens (Malicka *et al.*, 2015; Faustino-Rocha *et al.*, 2016b). Such models are highly reliable for comparative studies with human cancer (Faustino-Rocha *et al.*, 2015c). Using a rat model of mammary cancer induced by MNU, our team characterized the immunoexpression of oestrogen receptors in the tumors developed (Soares-Maia *et al.*, 2013), established accurate and non-invasive methods for monitoring tumor vascularization and development (Faustino-Rocha *et al.*, 2013a, 2013c, 2016c, 2016e), and demonstrated the anti-cachexia effects of exercise training on muscle tissues (Padrao *et al.*, 2015). However, despite extensive work done on experimental breast cancer models by our team and others, there remains a lack of experimental data concerning the effects of exercise training on the metastatic spread of breast cancer. Taking into consideration that metastatic disease is incurable and treatment with

chemotherapy can slow progression but ultimately the cancer develops resistance mechanisms and spreads to vital organs eventually leading to death, this study reports the effects of exercise training on the development of breast cancer metastases, as well as a characterization of those metastatic lesions.

4.2. Materials and Methods

4.2.1. Animals and Experimental Design

All the biosecurity standards specified for the studies using animal models were followed (European Directive 2010/63/EU and National Decree-Law 113/2013), and the experimental protocol was approved by *Direção Geral de Alimentação e Veterinária* (Approval nº 008961). Fifty female Sprague-Dawley rats (*Rattus norvegicus*) (Harlan Interfauna Inc., Barcelona, Spain) were maintained in polycarbonate cages (1500U Eurostandard Type IV S, Tecniplast, Buguggiate, Italy) with corncob for bedding (Mucedola, Italy), at a temperature of 23±2ºC and a humidity of 50±10%, with light/dark cycles (12h:12h). Water and food (Diet Standard 4RF21®, Mucedola, Italy) were supplied *ad libitum*. Animals were randomly divided into four groups as follows: sedentary MNU (n=15), exercised MNU (n=15), sedentary control (n=10) and exercised control (n=10). At the 50th day of age (defined as experimental day 1), all animals from MNU groups received an intraperitoneal injection (50 mg/kg body weight) of the carcinogenic agent MNU (ISOPAC®, lot 100 M1436V, Sigma Chemical Co., Madrid, Spain), dissolved in 0.9% saline solution to a concentration of 11 mg/ml. In the same day, animals from control groups received an intraperitoneal injection of 0.9% saline solution.

4.2.2. Exercise training

A Treadmill Control® LE 8710 (Panlab, Harvard Apparatus, USA) was used for exercise training. Starting at experimental day 2, animals from exercised groups underwent an acclimatization period to the treadmill for five days, with a progressive increase of 20 to 60 min of exercise *per* day at 20 m/min. Starting on experimental day 9, animals were subjected to exercise training (60 min/day with a speed of 20 m/min, 5 days a week) until perform 35 weeks of moderate exercise training.

4.2.3. Sacrifice and necropsy of animals

All animals were sacrificed in the 35th week of the experimentation, under anaesthesia (ketamine and xylazine), by exsanguination by cardiac puncture as indicated by the Federation of European Laboratory Animal Science Associations (Forbes *et al.*, 2007). At necropsy, all animals were externally examined and weighed, scalped and the skin was carefully observed under a light to detect mammary tumors. Accurate body weight was obtained by the subtraction of tumor weight to body weight. The mammary tumors and the thoracic and abdominal organs were weighted and immediately fixated in 10% phosphate buffered-formaldehyde during 24 hours.

4.2.4. Histopathology and Immunohistochemistry

The fixated tissues were trimmed, processed, embedded in paraffin and 2 µm-thick sections were stained with H&E for histopathological evaluation by three independent pathologists (A. Alvarado, R.M. Gil da Costa, C. Lopes). The mammary tumors were classified and categorized considering the predominant histological patterns in each mammary tumor according to Russo and Russo (Russo and Russo, 2000). Immunohistochemistry was performed on 2 µm-thick sections, using the Novolink Polymer Detection System (Leica Biosystems, Newcastle, UK) according to the instructions provided by the manufacturer. Both primary antibodies for ER α (clone 6F11, Novocastra, Newcastle, UK) and PR (clone SP2, Abcam, Cambridge, UK) were diluted in phosphate buffered saline (PBS) at 1:50, and incubated for 1.5 hours at room temperature and 16 hours (overnight) at 4ºC, respectively. The immunoexpression of ERα and PR was evaluated in primary tumors (in each histological pattern) and their metastases, counting the number of immunostained cells (cells with punctual nuclear labelling) in at least 500 neoplastic cells. Results were expressed as the percentage of immunopositive neoplastic cells. Normal rat mammary tissues incubated with and without the primary antibody were used as positive and negative controls, respectively.

4.2.5. Statistical Analysis

Descriptive statistics such as mean, S.D. and proportion in base to percentage were used to express the number of mammary tumors and metastases, and lung weight. The number of mammary tumors and lesions was compared using the Chi-square test. For the comparison between groups, the ANOVA and Tukey-Kramer HSD tests for all pairs were

used. The association between ERα and PR immunoexpression was assessed using linear Pearson correlation. The statistical analysis was performed using the JMP starter 5.0.1. program (SAS Institute Inc., Cary, NC, USA). The Odds Ratio (OR) was used to analyze the risk of development of metastases in exercised MNU and sedentary MNU groups with the online program MedCalc statistical software (MedCalc Software bvba, Ostend, Belgium). *P* values lower than 0.05 were considered statistically significant.

4.3. Results

4.3.1. General findings

Nine animals died during the experiment: four animals from each MNU group and one animal from the sedentary control group. One MNU-exposed animal did not adapt to the exercise protocol and was excluded. The final groups were: sedentary MNU (n=11), exercised MNU (n=10), sedentary control (n=9), exercised control (n=10).

4.3.2. Evaluation of mammary tumors

Control animals did not develop mammary tumors, while all MNU-exposed animals developed mammary tumors (100% of incidence). The number of palpable masses and mean number of masses *per* animal were higher in the sedentary when compared with the exercised group. In both groups, the majority of mammary tumors were palpated in thoracic mammary glands (Table 4.1).

Table 4.1. Number of palpable mammary tumors, anatomic location and the mean number of mammary masses *per* animal (mean±S.D.) in MNU groups.

MNU Groups	Number of mammary tumors	Number of mammary tumors (%)		Mean number of tumors *per* animal
		Thoracic mammary glands	Inguinal mammary glands	
Sedentary	28	23 (82.14)*	5 (17.86)	2.55±1.44
Exercised	23	18 (78.26)*	5 (21.74)	2.30±1.42

* Statistically different from the number of mammary tumors in inguinal mammary glands ($P<0.05$).

Histologically, mammary tumors were heterogeneous, showing multiple different histological patterns (Figure 4.1A). Thus, the number of histological lesions was higher than the number of palpable masses. A total of 32 and 37 mammary lesions were identified in the exercised and sedentary groups, respectively ($p>0.05$). Most of these were characterized as malignant epithelial neoplasms, with 28 and 33 malignant epithelial lesions identified in the exercised and sedentary MNU groups, respectively.

4.3.3. Evaluation of organs and metastasis

Control groups showed no relevant lesions. One animal from the MNU sedentary group showed pulmonary nodules measuring 2.0 mm in diameter. The absolute and relative lung weight from sedentary MNU group was slightly higher when compared with the remaining groups ($P>0.05$) (Table 4.2).

Table 4.2. Mean accurate final absolute and relative lung weight in all experimental groups. Mean±S.D.

Groups	N	Absolute lung weight (g)	Relative lung weight (%)
Sedentary MNU	11	1.699±0.111	0.0061±0.0006
Exercised MNU	10	1.618±0.128	0.0058±0.0004
Sedentary control	9	1.656±0.164	0.0056±0.0004
Exercised control	10	1.650±0.182	0.0056±0.0006

(P>0.05)

Multifocal to extensive acute and severe mononuclear interstitial pneumonia, non-suppurative hepatitis, lymphoid hyperplasia of the spleen and thymus were observed in both MNU groups.

On light microscopy, two lung metastasis were observed in two animals from the sedentary MNU group while no metastasis were observed in the exercised MNU group (OR *P-value*>0.05). The metastasis exhibited a poorly-differentiated solid pattern, similar to those commonly observed on mammary carcinomas, and multifocally adopted an alveolar conformation (Figure 4.2A).

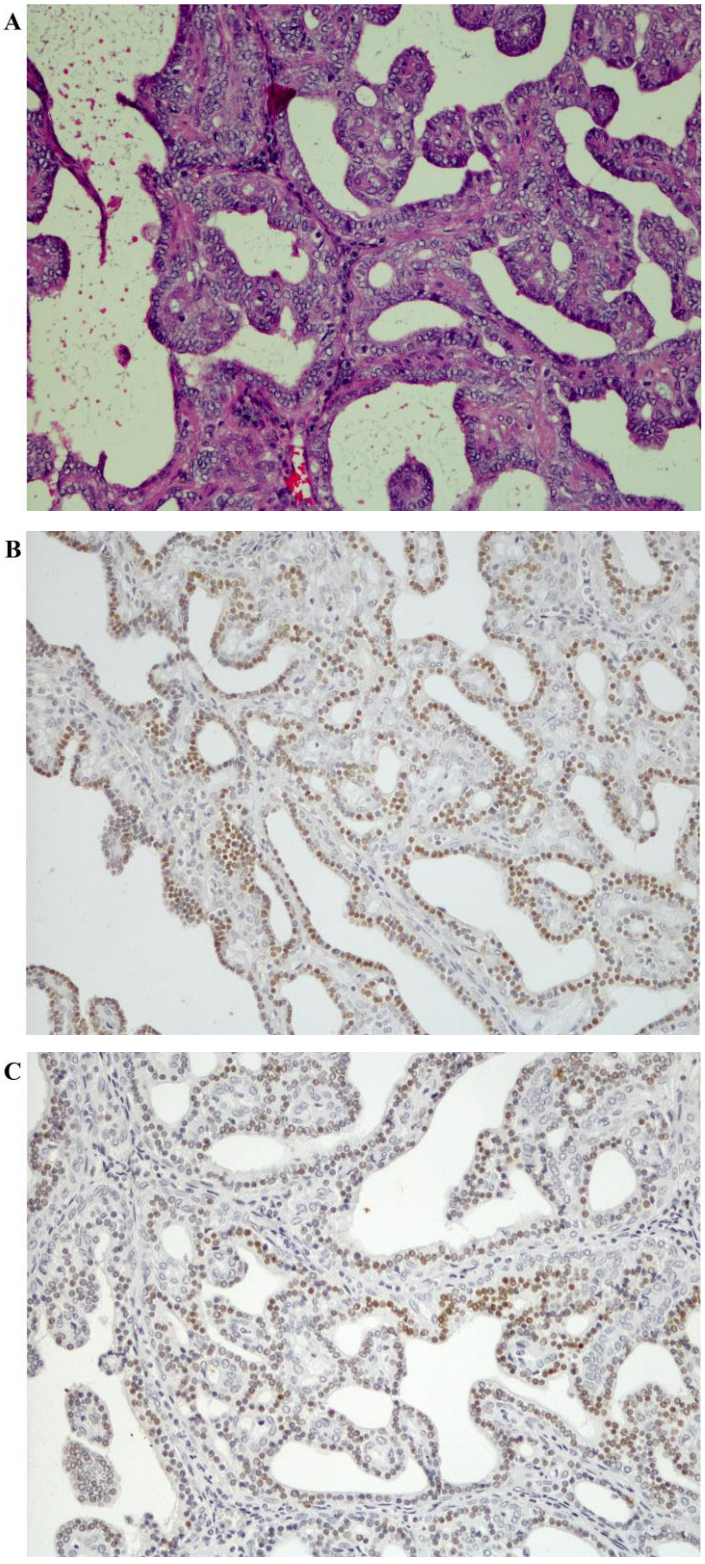

Figure 4.1. Papillary carcinoma. **A** This type of lesion shows an epithelial growth forming epithelial papillae with a light fibrovascular core (H&E, 200X magnification). **B** The same lesion shows the positive cell immunoexpression for ERα and **C** for PR (200X magnification).

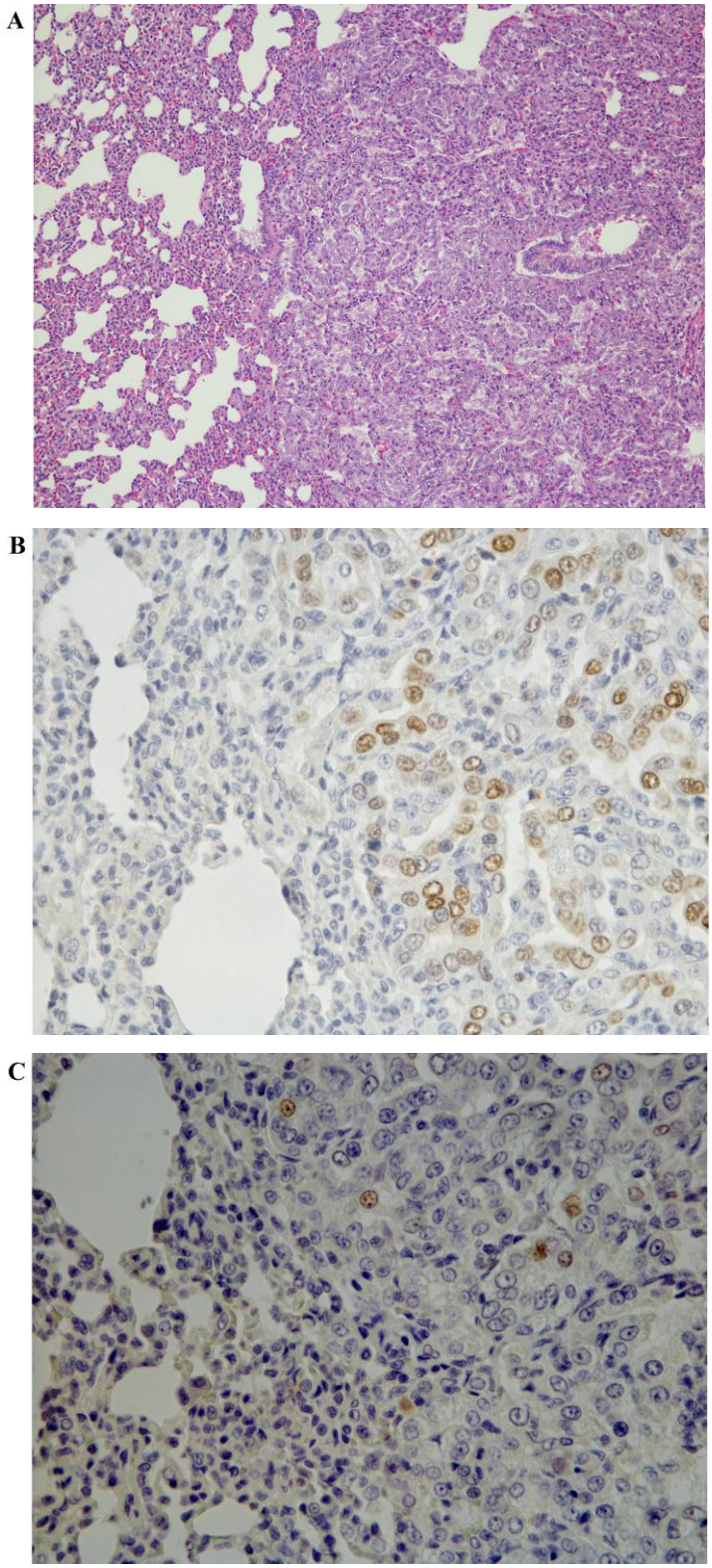

Figure 4.2. Lung metastasis of mammary carcinoma. **A** The metastatic cells growth with a solid pattern (H&E, 200X magnification). **B** The cells show a positive immunoexpression for ERα and **B** PR (400X magnification).

4.3.4. Immunohistochemical evaluation of mammary tumors and metastasis

All mammary neoplasms from both exercised and sedentary groups showed intense ERα and PR immunoexpression (Figure 4.1B and C, Table 4.3). The immunoexpression of ERα and PR was positively correlated (0.389, P=0.001) in both MNU groups. Lung metastasis also showed intense immunostaining for both steroid receptors (ER^+/PR^+) (Figure 4.2B and C, Table 4.3), but showed lower percentage of immunoexpression when compared with primary neoplasms (P<0.05).

Table 4.3. Mammary tumors and lung metastasis in exercised and sedentary MNU groups: histopathological type, ER α and PR immunoexpression. Mean±S.D.

Mammary and pulmonary lesions	Exercised MNU			Sedentary MNU		
	n	**ERα (%)**	**PR (%)**	***n***	**ERα (%)**	**PR (%)**
Epithelial benign	2	46.50±20.51	4.85±1.20‡	2	39.45±18.03	38.20±21.21
Epithelial-stromal benign	2	13.95±7.14*	18.00±25.46	-	-	-
Precancerous (IDP)	-	-	-	2	32.96±32.16	19.85±22.13
Epithelial malignant	28	52.65±14.66	38.91±16.91	33	50.35±12.74	40.48±12.65
Total	32	49.85±17.12	35.47±18.99	37	48.82±14.25	39.25±13.81
Lung metastasis	0	-	-	2	17.46±20.74**	2.50±0.71**

IDP: Intraductal proliferation

* Statistically different from epithelial malignant in both exercised and sedentary groups (p<0.05). ‡ Statistically different from epithelial malignant in both exercised and sedentary groups (p<0.05). ** Statistically different from total ERα and PR on mammary neoplasm from exercised and sedentary groups (p<0.05).

4.4. Discussion

The effects of exercise training on cancer development and treatment are of great interest (Wolff and Toborek, 2013). Recent findings in male mice suggest that exercise training is able to reduce the spread of lung carcinoma metastatic cells in brain that is the principal organ metastasized (Wolff *et al.*, 2014, 2015). The present study addressed the effects of long-term exercise training on the expression of hormone receptors and metastatic dissemination of breast cancer in a rat model. Additionally, in accordance with previous findings (Thompson, 1994; Whittal-Strange *et al.*, 1998; Thompson *et al.*, 2010) in this study was observed that the exercise training reduced the number of palpable masses (28 in

sedentary *versus* 23 in exercised group), histological lesions (37 in sedentary *versus* 32 in exercised group) and malignant lesions (33 in sedentary *versus* 28 in exercised group).

The key finding in this study was the absence of pulmonary metastasis in exercised rats, although the small number of cases does not allow our analysis to reach statistical significance. While the occurrence of metastasis in this model was reported in early studies (Mccormick *et al.*, 1981; Russo and Russo, 2000; Perse *et al.*, 2009), a more detailed study to understand their development in exercised animals was never performed. Although two lung metastases were observed in sedentary animals, only one of them was detected macroscopically (2.0 mm in diameter). Since each animal developed more than one mammary tumor with distinct histological patterns, it was not possible to identify the specific mammary lesion which originated each metastasis.

Chemically-induced mammary tumors in rats are often hormone-dependent, expressing hormone receptors for both oestrogen and progesterone, which play an important role in breast cancer (Kariagina *et al.*, 2013). Positive expression of ERα and PR is widely regarded as indicating a favourable prognostic and response to hormonal therapy (McGuire and Clark, 1986; Lanari *et al.*, 2009; Mohammed *et al.*, 2015; Bae *et al.*, 2015; Vici *et al.*, 2015).

McTiernan *et al.* (2004) and Wolff and Toborek (2013), suggest that long-term exercise training may decrease serum estrogens levels, theoretically decreasing the risk of breast cancer development and metastatic disease. However, our previous findings (Faustino-Rocha *et al.*, 2016d) are not in accordance with those results, and we observed that exercise training causes a significant increase in serum oestrogen levels. To us, this suggests that the anti-metastatic effects of exercise training was hormone-independent. In order to check this possibility, we analyse the expression of hormone receptors in the primary tumors. We recently reported in this model that the high expression of ERα is correlated with differentiated phenotype (Alvarado *et al.*, 2016). Our present findings confirm that mammary lesions induced by MNU strongly express ERα and PR. However, metastatic cells may show significant genotypic and phenotypic differences regarding their primary neoplasm, reflecting local adaptations or the selection of the more metastatic-prone cellular clones (Grewal *et al.*, 2010; Cummings *et al.*, 2014). Recently, Cummings *et al.* (2014) reported a loss of ERα and PR immunoexpression in lung metastasis of human breast cancer, with a consequent

resistance to hormone therapy and increased mortality rates. Accordingly, we also examined by immunohistochemistry ERα and PR expression in metastatic lesions to make sure that metastases maintained the same hormone-sensitive phenotype of their primary lesions. We observed that metastasis maintain the same general pattern of ERα and PR expression observed in primary mammary tumors (with higher immunoexpression for ERα than PR), even if the mean immunoexpression for both receptors was lower.

Recent studies provide another possible explanation for the anti-metastatic effects of exercise training, based on its ability to modulate blood flow and the permeability of blood vessels, making it more difficult for cancer cells to cross vascular walls and establish metastases (Wolff *et al.*, 2014, 2015). These studies point out that exercise training protects blood vessels in the brain against extravasation by injected Lewis lung carcinoma cells in mice. This effect was proposed to be mediated by proteins involved in endothelial tight junctions such as occludin, ZO-1 and claudin-5 (Wolff *et al.*, 2015). The authors proposed that exercised training helped maintaining a near-normal vascular status even in the presence of cancer cells. Some results recently published by our team point in the same direction. Using the same experimental protocol described in the present paper, we showed that mammary tumors from exercised animals demonstrate enhanced vascularization, compared with sedentary animals (Faustino-Rocha *et al.*, 2016e). In view of these findings, it is interesting to speculate that the reduced metastization now observed may be due to a healthier intratumoral vasculature, allowing less cancer cell intravasation. The detailed molecular characterization of the vascular component in these tumors will be the object of a further study.

4.5. Conclusion

Our results support the hypothesis that long-term exercise training may decrease the risk of metastatic disease dissemination in woman breast cancer patients. Two mammary cancer metastasis were found and characterized in the lungs of sedentary animals, closely resembling those observed in cancer patients. This occurred in the presence of enhanced oestrogen levels, with hormone-sensitive primary and metastatic lesions, indicating that the effect was hormone-independent. Since only two lung metastasis of small dimensions were found in this experiment, no studies were performed on the metastasis microenvironment. So,

additional studies are warranted in order to address the potential protective vascular changes involved in the anti-metastatic effects of exercised training on mammary cancer.

GENERAL DISCUSSION

Since the breast cancer is the main cause of cancer death in woman, the last 70 years have been dedicated to develop animal models able to develop mammary cancer similar to that found in woman (Murray *et al.*, 2009). The *in vivo* rat model is internationally recognized as the best model to study the women mammary cancer (Russo, 2015). This study intended to improve a histopathologic and immunohistochemical description of the mammary tumors induced by MNU and DMBA, with a special focus on the expression of the prognostic factors expressed in woman breast cancer and to establish a comparison between both models. This thesis also aimed to evaluate the effects of long-term moderate exercise training on the development and metastization of MNU-induced mammary tumors, emphasizing the modulation of prognostic factors by the practice of exercise. The mammary lesions developed by animals exposed to MNU or DMBA were histopathologically similar, being the papillary and cribriform carcinomas the most frequent histological patterns identified. These results were in accordance with those described by other authors over the last years (Shirai *et al.*, 1997; Russo and Russo, 2000; Kang *et al.*, 2004; Qiu *et al.*, 2005; Chan *et al.*, 2007). However, Ariazi *et al.* (2005) reported that MNU was more carcinogenic to immature F344 female rat mammary gland than DMBA in the same strain, inducing 0.92 carcinomas and 0.23 carcinomas *per* rat respectively, when both carcinogens were administered in a prepubertal period of mammary gland development.

Taken into consideration that both carcinogens act by different pathways, it was possibly to hypothesize that mammary carcinomas chemically-induced by these carcinogens had some differences related to the prognostic factors and malignancy. The epithelial cells from female rat mammary carcinomas induced by MNU and DMBA have a similar differentiation profile. However, the DNA lesions are different due to the distinct mechanism of each carcinogenic agent (Sharma *et al.*, 2011). MNU induces a point mutation in codon 12 of the Ha-Ras-1 gene (Zarbl *et al.*, 1985) while DMBA is primarily activated by liver cytochrome P450, generating metabolites able to change the DNA (Russo *et al.*, 1982). Independently of the distinct mechanisms for tumor induction, mammary neoplasms induced by both chemical carcinogens are hormone dependent (Russo and Russo, 1996b; Thordarson *et al.*, 2001). Similarly to that reported by Thordarson *et al.* (2001) the mammary neoplasms induced by both carcinogens in our study were positive for both hormone receptors, ERα and PR. In humans, ER expression is strongly associated with PR expression and the status of their immunoexpression is considered the most important factor for the establishment of

hormone therapy in breast carcinomas (Hammond *et al.*, 2010). The expression of high levels of both hormone receptors (ERα and PR) in all mammary tumors identified in our experience indicates a good response to hormone therapy and consequently a favorable prognostic. Additionally to the expression of hormone receptors, the interest on the assessment of proliferating cells has lead to the establishment of Ki-67 as a good proliferation marker to predict the course of therapy (Purdie *et al.*, 2014). In our experiment, the Ki-67 immunoexpression was slightly higher in MNU-induced carcinomas when compared with the DMBA-induced ones. All data together indicate that although the MNU- and DMBA-induced carcinomas are similar in terms of their histopathology, the prognostic factors are suggestive of a higher aggressiveness of MNU-induced carcinomas and consequently a worse response to hormone therapy and a worse prognosis. So, the choice of the rat model of mammary carcinogenesis should be based on the purpose of the research, and the model of MNU-induced mammary tumors is advised in experimental protocols aiming to study more aggressive mammary tumors within the group of double-positive mammary tumors (ER^+/PR^+).

Although some studies indicate that the physical activity and exercise reduce the risk of cancer development and positively influence the physical condition and mental status of the humans to cope to the clinical treatment, the role of exercise on cancer remains unclear (Thompson, 1994, 1997; Courneya and Friedenreich, 2007; Thompson *et al.*, 2010). In order to contribute for understanding the role of exercise on mammary carcinogenesis, we evaluated the effects of long-term moderate exercise training in a model of MNU-induced mammary cancer in female Sprague-Dawley rats. After 35 weeks of exercise training, mammary tumors from sedentary and exercised animals were analyzed. Exercised group developed less tumours *per* animal when compared with the sedentary group. Similarly, a lower number of mammary lesions was observed in exercised group. The prognostic markers ERα, Ki-67 PI and MAI were studied in mammary lesions from both exercised and sedentary animals. The combination of the proliferation factors Ki-67 PI and MAI is crucial to determine the risk and medical treatment in human patients with ER positive breast cancer (Klintman *et al.*, 2013). Mammary lesions from MNU exercised group exhibited higher ERα H-score, lower Ki-67 PI and MAI, and higher differentiation when compared with mammary lesions from MNU sedentary group. Once the mammary tumors MNU-induced in female rats resemble those developed by woman in terms of their histopathology and hormone dependency, these results

may be transposed for women. Since the lower immunoexpression of Ki-67 and high MAI, and higher immunoexpression of ERα are related with better prognosis and longer survival post-treatment (Kinsel *et al.*, 1989; Bertucci *et al.*, 2013), our results suggest beneficial effects of exercise training on mammary carcinogenesis.

In cancer, metastasis constitute a "ticking time bombs" because the metastatic cells can be as micrometastasis, migrate to other organs and die or remains dormant, later activate and grow in different organs, establishing a metastatic disease and become refractory to the treatment (Bailey-Downs *et al.*, 2014). The immunoexpression of the primary tumor is essential in human breast cancer, and the principal complexity between the primary tumor and its metastasis is the immunophenotype heterogeneity and genomic profile. The ERs and PRs immunoexpression in breast cancer can vary from the primary tumor to its metastasis, indicating a difficult response to hormone therapy (Cummings *et al.*, 2014). ERs isoforms and their proteins can vary, associated with variations for the endocrine therapy response and the consequent metastatic disease (Thomas and Gustafsson, 2015). In this work, the histopathological analysis showed two lung metastasis from mammary tumors in MNU sedentary animals and no metastasis were found in MNU exercised animals. Based on this, the immunoexpression of hormone receptors was analysed in primary mammary carcinomas and in their metastasis. First, the fact that it was not observed any metastasis from MNU exercised group, suggested that exercise training had a higher positive effect, taking into consideration the important fact of reduction in the number of tumors. Second, in the sedentary MNU group were observed two lung metastasis, in which the hormone expression was reduced when was compared with the primary mammary carcinomas. The loss of immunoexpression in both hormone receptors (ERα/PR) suggest a probable resistance to hormone therapy, similarly to human breast cancer (Cummings *et al.*, 2014), constituting an indicator of more aggressiveness than the primary tumor cells. These results propose an immune independency between the primary tumors and their metastasis. Another important observation from this study was previously reported by our team (Faustino-Rocha *et al.*, 2016e), indicating the enhanced vascularisation in the tumors developed from MNU exercised group. On the microenvironment generated around the neoplasm, the neovasculature is described as tortuous, leaky, heterogeneous and branching irregularly with the absence of extensive pericyte coverage that probably facility the intravasation and neoplastic cells dissemination into the blood circulation (Carmeliet and Jain, 2011; Valastyan and Weinberg,

2011). So, the fact that the exercise training enhanced the vasculature and decrease the numbers of tumor, the lesions *per* tumors and metastasis, suggest definitely the positive effect of the exercise training over mammary cancer development.

To our knowledge, this is the first study addressing the effect of such a long time period of exercised-training in the development of mammary tumor. This study has some limitations, for instance, although it was the longer exercised-training study in mammary cancer to our knowledge, the duration of the experimental protocol was too short to allow the development of more metastasis. One of the reasons why the period was not extended was due to the fact that the size and the number of tumors were compromising the welfare of the animals, and thus, a human endpoint had to be applied. Although it would be interesting to have an increment of metastasis in order to analyze the microenvironment surrounding the mestastatic cells, specifically inflammatory cells and blood vessels, this is also difficult to achieve due to the compromising of animal welfare. For future studies, the immunoexpression of the proto-oncogene ErBb2 could be analyzed, since it is also an important prognostic factor in woman breast cancer and essential to establish the immunotherapy in the positive tumors expressions, this would be of major interest.

FINAL CONCLUSIONS

Breast cancer is the most common cancer in woman, representing the first cancer cause of death among women in the world. The *in vivo* rat models of mammary cancer conduce to study many different stages of breast cancer, since they lead to development similar tumors. Chemical-induced rat models can be used as well-established models of breast cancer in women because their histopathology resemblance. The behavior of MNU and DMBA-induce mammary tumors related with the prognostic factors, as well as the effects of exercise training at different stages of carcinogenesis has been studied in this work, which led to the following conclusions:

- Comparing MNU and DMBA-induced mammary tumors, mammary carcinomas derived from the induction by MNU have a more aggressive behavior with worse therapeutic prognostic;

- Rat mammary tumors derived from animals subjected to long-term exercise training are less proliferative and more differentiated, suggesting that the long-term exercise training induces a better therapeutic prognostic;

- Metastases from mammary carcinomas were hormone-independent from their primary tumors, showing loss of expressivity of ERα and PR, suggesting a worse therapeutic prognostic;

- The long-term exercise training decreases the risk of lung metastases.

REFERENCES

Ahelm, C.N.; Frincke, J.M.; White, S.K.; Reading, C.L.; Trauger, R.J. and Lakshmanaswamy, R. (2011). 17α-ethynyl-5α-androstane-3α, 17β-diol treatment of MNU-induced mammary cancer in rats. *International Journal of Breast Cancer,* 2011: 1–9.

Al-Dhaheri, W.S.; Hassouna, I.; Al-Salam, S. and Karam, S.M. (2008). Characterization of Breast Cancer Progression in the Rat. *Recent Advances in Clinical Oncology*, 1138: 121–131.

Alfaro, Y.; Delgado, G.; Cárabez, A.; Anguiano, B. and Aceves, C. (2013). Iodine and doxorubicin, a good combination for mammary cancer treatment: antineoplastic adjuvancy, chemoresistance inhibition, and cardioprotection. *Molecular Cancer,* 12(45): 1–11.

Al-Saeedi, F.J. (2014). Study of the cytotoxicity of asiaticoside on rats and tumour cells. *BMC Cancer,* 14: 220.

Alshafie, G.A.; Walker, J.R.; Curley, R.W.; Clagett-Dame, M.; Highland, M.A.; Nieves, N.J.; Stonerock, L.A. and Abou-Issa, H. (2005). Inhibition of mammary tumor growth by a novel nontoxic retinoid: chemotherapeutic evaluation of a C-linked analog of 4-HPR-glucuronide. *Anticancer Research,* 25(3c): 2391–2398.

Alvarado, A.; Faustino-Rocha, A.I.; Ferreira, R.; Mendes, R.; Duarte, J.A.; Pires, M.J.; Colaço, B. and Oliveira, P.A. (2016). Prognostic Factors in an exercised model of chemically-induced mammary cancer. *Anticancer Research,* 36(5): 2181–2188.

Anbuselvam, C.; Vijayavel, K. and Balasubramanian, M.P. (2007). Protective effect of Operculina turpethum against 7,12-dimethyl benz(a)anthracene induced oxidative stress with reference to breast cancer in experimental rats. *Chemico-Biological Interactions,* 168(3): 229–236.

Anzano, M.A.; Peer, C.W.; Smith, J.M.; Mullen, L.T.; Shrader, M.W.; Logsdon, D.L.; Driver, C.L.; Brown, C.C.; Roberts, A.B. and Sporn, M.B. (1996). Chemoprevention of mammary carcinogenesis in the rat: combined use of raloxifene and 9-cis-retinoic acid. *Journal of the National Cancer Institute*, 88(2): 123–125.

Aoi, W.; Naito, Y.; Takagi, T.; Kokura, S.; Mizushima, K.; Takanami, Y.; Kawai, Y.; Tanimura, Y.; Hung, L.P.; Koyama, R.; Ichikawa, H. and Yoshikawa, T. (2010). Regular exercise reduces colon tumorigenesis associated with suppression of iNOS. *Biochemical and Biophysical Research Communications*, 399(1): 14-19.

Arcos, J. (1995). Chemical induction of cancer: modulation and combination effects. Arcos J, Argus M, Woo Y-F (eds). Birkhäuser: Boston. , pp.1-674.

Ariazi, J.L.; Haag, J.D.; Lindstrom, M.J. and Gould, M.N. (2005). Mammary glands of sexually immature rats are more susceptible than those of mature rats to the carcinogenic, lethal, and mutagenic effects of N-nitroso-N-methylurea. *Molecular Carcinogenesis*, 43(3): 155-164.

Arpino, G.; Milano, M. and De, P.S. (2015). Features of aggressive breast cancer. *Breast*, 24(5): 594-600.

Arroyo-Acevedo, J.; Chávez-Asmat, R.J.; Anampa-Guzmán, A.; Donaires, R. and Ráez-

Gonzáles, J. (2015). Protective effect of piper aduncum capsule on DMBA-induced breast cancer in rats. *Breast Cancer: Basic and Clinical Research,* 9: 41–48.

Asamoto, M.; Ochiya, T.; Toriyama-Baba, H.; Ota, T.; Sekiya, T.; Terada, M. and Tsuda, H. (2000). Transgenic rats carrying human c-Ha-ras proto-oncogenes are highly susceptible to N-methyl-N-nitrosourea mammary carcinogenesis. *Carcinogenesis,* 21(2): 243–249.

Avraham, R.; Benish, M.; Inbar, S.; Bartal, I.; Rosenne, E. and Ben-Eliyahu, S. (2010). Synergism between immunostimulation and prevention of surgery-induced immune suppression: an approach to reduce post-operative tumor progression. *Brain, Behavior, and Immunity*, 24(6): 952–958.

Avril, N.; Menzel, M.; Dose, J.; Schelling, M.; Weber, W.; Jänicke, F.; Nathrath, W. and Schwaiger, M. (2001). Glucose metabolism of breast cancer assessed by 18F-FDG PET: histologic and immunohistochemical tissue analysis. *Journal of Nuclear Nedicine,* 42(1): 9–16.

Badawi, A.F.; Eldeen, M.B.; Liu, Y.Y.; Ross, E.A. and Badr, M.Z. (2004). Inhibition of rat mammary gland carcinogenesis by simultaneous targeting of cyclooxygenase-2 and peroxisome proliferator-activated receptor gamma. *Cancer Research,* 64(3): 1181–1189.

Badyal, D.K. and Desai, C. (2014). Animal use in pharmacology education and research: the changing scenario. *Indian Journal of Pharmacology*, 46(3): 257–265.

Bae, S.Y.; Kim, S.; Lee, J.H.; Lee, H.C.; Lee, S.K.; Kil, W.H.; Kim, S.W.; Lee, J.E. and Nam, S.J. (2015). Poor prognosis of single hormone receptor- positive breast cancer: similar outcome as triple-negative breast cancer. *BMC Cancer*, 15(138): 1-9.

Bailey-Downs, L.C.; Thorpe, J.E.; Disch, B.C.; Bastian, A.; Hauser, P.J.; Farasyn, T.; Berry, W.L.; Hurst, R.E. and Ihnat, M.A. (2014). Development and characterization of a preclinical model of breast cancer lung micrometastatic to macrometastatic progression. *Plos One*, 9(5): e98624.

Barros, A.C.; Muranaka, E.N.; Mori, L.J.; Pelizon, H.T.; Iriya, K.; Giocondo, G. and Pinotti, J.A. (2004). Induction of Experimental Mammary Carcinogenesis in Rats with 7,12-Dimethylbenz(a)Anthracene. *Revista do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo,* 59(5): 257–261.

Bartella, L.; Thakur, S.B.; Morris, E.A.; Dershaw, D.D.; Huang, W.; Chough, E.; Cruz, M.C. and Liberman, L. (2007). Enhancing nonmass lesions in the breast: evaluation with proton (1H) MR spectroscopy. *Radiology,* 245(1): 80–87.

Bartstra, R.W.; Bentvelzen, P.A.; Zoetelief, J.; Mulder, A.H.; Broerse, J.J. and Van Bekkum, D.W. (1998). Induction of mammary tumors in rats by single-dose gamma irradiation at different ages. *Radiation Research,* 150(4): 442.

Bastos, J.; Barros, H. and Lunet, N. (2007). [Breast cancer mortality trend in Portugal (1955-2002)]. *Acta Médica Portuguesa*, 20(2): 139-144.

Batcioglu, K.; Uyumlu, A.B.; Satilmis, B.; Yildirim, B.; Yucel, N.; Demirtas, H.; Onkal, R.; Guzel, R.M. and Djamgoz, M.B. (2012). Oxidative stress in the in vivo DMBA rat model of breast cancer: suppression by a voltage-gated sodium channel inhibitor (RS100642).

Basic & Clinical Pharmacology & Toxicology, 111(2): 137–141.

Benakanakere, I.; Besch-Williford, C.; Carroll, C.E. and Hyder, S.M. (2010). Synthetic progestins differentially promote or prevent 7,12-dimethylbenz(a)anthracene-induced mammary tumors in sprague-dawley rats. *Cancer Prevention Research,* 3(9): 1157–1167.

Bertucci, F.; Finetti, P.; Roche, H.; Le Doussal, J.M.; Marisa, L.; Martin, A.L.; Lacroix-Triki, M.; Blanc-Fournier, C.; Jacquemier, J.; Peyro-Saint-Paul, H.; Viens, P.; Sotiriou, C.; Birnbaum, D. and Penault-Llorca, F. (2013). Comparison of the prognostic value of genomic grade index, Ki67 expression and mitotic activity index in early node-positive breast cancer patients. *Annals of Oncology*, 24(3): 625-632.

Bhowmik, A.; Das, N.; Pal, U.; Mandal, M.; Bhattacharya, S.; Sarkar, M.; Jaisankar, P.; Maiti, N.C. and Ghosh, M.K. (2013). 2,2'-diphenyl-3,3'-diindolylmethane: a potent compound induces apoptosis in breast cancer cells by inhibiting EGFR pathway. *Plos One,* 8(3): e59798.

Białek, A.; Tokarz, A. and Zagrodzki, P. (2016). Conjugated linoleic acids (cla) decrease the breast cancer risk in dmba-treated rats. *Acta Poloniae Pharmaceutica,* 73(2): 315–327.

Bishayee, A.; Mandal, A.; Bhattacharyya, P. and Bhatia, D. (2016). Pomegranate exerts chemoprevention of experimentally induced mammary tumorigenesis by suppression of cell proliferation and induction of apoptosis. *Nutrition and Cancer,* 68(1): 120–130.

Bobrowska-Korczak, B.; Skrajnowska, D. and Tokarz, A. (2012). The effect of dietary zinc-- and polyphenols intake on DMBA-induced mammary tumorigenesis in rats. *Journal of Biomedical Science,* 19: 43.

Boopalan, T.; Arumugam, A.; Parada, J.; Saltzstein, E. and Lakshmanaswamy, R. (2015). Receptor activator for nuclear factor-kappaB ligand signaling promotes progesterone-mediated estrogen-induced mammary carcinogenesis. *Cancer Science*, 106(1): 25-33.

Boyland, E. and Sydnor, K. (1962). The induction of mammary cancer in rats . *British Journal of Cancer,* 16(4): 731–739.

Budan, F.; Varjas, T.; Nowrasteh, G.; Varga, Z.; Boncz, I.; Cseh, J.; Prantner, I.; Antal, T.; Pazsit, E.; Gobel, G.; Bauer, M.; Gracza, T.; Perjesi, P.; Ember, I. and Gyongyi, Z. (2008). Early modification of c-myc, Ha-ras and p53 expressions by N-methyl-N-nitrosourea. *In Vivo*, 22(6): 793-797.

Budde, M.D.; Gold, E.; Jordan, E.K.; Smith-Brown, M. and Frank, J.A. (2012). Phase contrast MRI is an early marker of micrometastatic breast cancer development in the rat brain. *NMR in Biomedicine,* 25(5): 726–736.

Cardiff, R.D. (2007). Epilog: comparative medicine, one medicine and genomic pathology. *Breast Disesases,* 28: 107–110.

Carmeliet, P. and Jain, R.K. (2011). Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. *Nature Reviews Drug Discovery*, 10(6): 417-427.

Carroll, C.E.; Liang, Y.; Benakanakere, I.; Besch-Williford, C. and Hyder, S.M. (2013). The anticancer agent YC-1 suppresses progestin-stimulated VEGF in breast cancer cells and arrests breast tumor development. *International Journal of Oncology,* 42(1): 179–187.

Castillo, M.; Arbelaez, A.; Smith, J.K. and Fisher, L.L. (2000). Diffusion-weighted MR imaging offers no advantage over routine noncontrast MR imaging in the detection of vertebral metastases. *AJNR. American Journal of Neuroradiology,* 21(5): 948–953.

Chan, M.M.; Lu, X.; Merchant, F.M.; Iglehart, J.D. and Miron, P.L. (2007). Serial transplantation of NMU-induced rat mammary tumors: A model of human breast cancer progression. *International Journal of Cancer*, 121(3): 474-485.

Chandra, M.; Riley, M.G. and Johnson, D.E. (1992). Spontaneous neoplasms in aged Sprague-Dawley Rats. *Archives of Toxicology,* 66(7): 496–502.

Chun, Y.S.; Bisht, S.; Chenna, V.; Pramanik, D.; Yoshida, T.; Hong, S.M.; De Wilde, R.F.; Zhang, Z.; Huso, D.L.; Zhao, M.; Rudek, M.A.; Stearns, V.; Maitra, A. and Sukumar, S. (2012). *Carcinogenesis*, 33(11): 2242–2249.

Clarke, R. (1996). Animal models of breast cancer: their diversity and role in biomedical research. *Breast Cancer Research and Treatment*, 39(1): 1–6.

Colditz, G.A. and Frazier, A.L. (1995). Models of breast-cancer show that risk is set by events of early-life - prevention efforts must shift focus. *Cancer Epidemiology Biomarkers & Prevention,* 4(5): 567–571.

Colston, K.W.; Pirianov, G.; Bramm, E.; Hamberg, J. and Binderup, L. (2003). Effects of Seocalcitol (EB1089) on nitrosomethyl urea-induced rat mammary tumors. *Breast Cancer Research and Treatment*, 80(3): 303–311.

Conn, P.M. (2013). Animal models for the study of human disease. *Animal Models for the Study of Human Disease*, Conn P.M. (ed). Elsevier Science & Technology. , pp.1-1057.

Corsetti, V.; Ferrari, A.; Ghirardi, M.; Bergonzini, R.; Bellarosa, S.; Angelini, O.; Bani, C. and Ciatto, S. (2006). Role of ultrasonography in detecting mammographically occult breast carcinoma in women with dense breasts. *La Radiologia Medica,* 111(3): 440–448.

Cortés-García, J.; Aguilera-Méndez, A.; Higareda-Mendoza, A.; Beltrán-Peña, E. and Pardo-Galván, M. (2009). Inducción de cáncer en rata wistar (*Rattus norvegicus*) mediante el uso de dimetil benzo(a)antraceno (DMBA). *EDEMM,* 1(1): 11–17.

Cos, S.; González, A.; Güezmes, A.; Mediavilla, M.D.; Martínez-Campa, C.; Alonso-González, C. and Sánchez-Barceló, E.J. (2006). Melatonin inhibits the growth of DMBA-induced mammary tumors by decreasing the local biosynthesis of estrogens through the modulation of aromatase activity. *International Journal of Cancer,* 118(2): 274–278.

Cotroneo, M.S.; Haag, J.D.; Zan, Y.; Lopez, C.C.; Thuwajit, P.; Petukhova, G.V.; Camerini-Otero, R.D.; Gendron-Fitzpatrick, A.; Griep, A.E.; Murphy, C.J.; Dubielzig, R.R. and Gould, M.N. (2007). Characterizing a rat BRCA2 knockout model. *Oncogene*, 26(11): 1626–1635.

Courneya, K.S. and Friedenreich, C.M. (2007). Physical activity and cancer control. *Seminars in Oncology Nursing*, 23(4): 242-252.

Cummings, M.C.; Simpson, P.T.; Reid, L.E.; Jayanthan, J.; Skerman, J.; Song, S.; Reed, A.E.; Kutasovic, J.R.; Morey, A.L.; Marquart, L.; O'Rourke, P. and Lakhani, S.R. (2014).

Metastatic progression of breast cancer: insights from 50 years of autopsies. *Journal of Pathology*, 232(1): 23-31.

Curado, M.P. (2011). Breast cancer in the world: incidence and mortality. *Salud Pública de México*, 53(5): 372-384.

Currier, N.; Solomon, S.E.; Demicco, E.G.; Chang, D.L.; Farago, M.; Ying, H.Q.; Dominguez, I.; Sonenshein, G.E.; Cardiff, R.D; Xiao, Z.X.; Sherr, D.H. and Seldin, DC. (2005). Oncogenic signaling pathways activated in DMBA-induced mouse mammary tumors. *Toxicologic Pathology*, 33(6): 726–737.

Dai, X.; Xiang, L.; Li, T. and Bai, Z. (2016). Cancer Hallmarks, Biomarkers and Breast Cancer Molecular Subtypes. *Journal of Cancer*, 7(10): 1281-1294.

Das Gupta, S.; So, J.Y.; Wall, B.; Wahler, J.; Smolarek, A.K.; Sae-Tan, S.; Soewono, K.Y.; Yu, H.; Lee, M.-J.; Thomas, P.E.; Yang, C.S. and Suh, N. (2015). Tocopherols inhibit oxidative and nitrosative stress in estrogen-induced early mammary hyperplasia in ACI rats. *Molecular Carcinogenesis,* 54(9): 916–25.

Davies, B.R.; Platt-Higgins, A.M.; Schmidt, G. and Rudland, P.S. (1999). Development of hyperplasias, preneoplasias, and mammary tumors in MMTV-c-erbB-2 and MMTV-TGFalpha transgenic rats. *The American Journal of Pathology*, 155(1): 303–314.

Deepalakshmi, K. and Mirunalini, S. (2013). Modulatory effect of Ganoderma lucidum on expression of xenobiotic enzymes, oxidant-antioxidant and hormonal status in 7,12-dimethylbenz(a)anthracene-induced mammary carcinoma in rats. *Pharmacognosy Magazine,* 9(34): 167–175.

Dickey, J.S.; Gonzalez, Y.; Aryal, B.; Mog, S.; Nakamura, A.J.; Redon, C.E.; Baxa, U.; Rosen, E.; Cheng, G.; Zielonka, J.; Parekh, P.; Mason, K.P.; Joseph, J.; Kalyanaraman, B.; Bonner, W.; Herman, E.; Shacter, E. and Rao, V.A. (2013). Mito-tempol and dexrazoxane exhibit cardioprotective and chemotherapeutic effects through specific protein oxidation and autophagy in a syngeneic breast tumor preclinical model. *Plos One*, 8(8): e70575.

Diwan, B.A.; Kasprzak, K.S. and Anderson, L.M. (1997). Promotion of dimethylbenz[a]anthracene-initiated mammary carcinogenesis by iron in female Sprague-Dawley rats. *Carcinogenesis,* 18(9): 1757–1762.

Doctores, A.; Martínez, J.; Merchán, J.; Sala, L.; Renedo, G.; Fernández-Pascual, J. and Bullón, A. (1974). Carcinogenesis y Mitrosoamidas. *Patología,* 7: 225–230.

Dowsett, M.; Nielsen, T.O.; A'Hern, R.; Bartlett, J.; Coombes, R.C.; Cuzick, J.; Ellis, M.; Henry, N.L.; Hugh, J.C.; Lively, T.; McShane, L.; Paik, S.; Penault-Llorca, F.; Prudkin, L.; Regan, M.; Salter, J.; Sotiriou, C.; Smith, I.E.; Viale, G.; Zujewski, J.A. and Hayes, D.F. (2011). Assessment of Ki67 in breast cancer: recommendations from the International Ki67 in Breast Cancer working group. *Journal of the National Cancer Institute*, 103(22): 1656-1664.

Doyle, A.; McGarry, M.P.; Lee, N.A. and Lee, J.J. (2012). The construction of transgenic and gene knockout/knockin mouse models of human disease. *Transgenic Research,* 21(2): 327–349.

Eickmeyer, S.M.; Gamble, G.L.; Shahpar, S. and Do, K.D. (2012). The role and efficacy of exercise in persons with cancer. *The Journal of Injury, Function, and Rehabilitation,* 4(11): 874–881.

Elston, C.W. and Ellis, I.O. (1991). Pathological Prognostic Factors in Breast-Cancer .1. the Value of Histological Grade in Breast-Cancer - Experience from A Large Study with Long-Term Follow-Up. *Histopathology*, 19(5): 403-410.

Ericsson, A.C.; Crim, M.J. and Franklin, C.L. (2013). A brief history of animal modeling. *Missouri Medicine,* 110(3): 201–205.

European Union Commission. (2010). Of mice and men - are mice relevant models for human disease? Outcomes of the European Commisiion worshop 'Are mice relevant models for human disease?' London, UK. , pp.1-10.

European Union Comission. (2013). Seventh report on the statistics on the number of animals used for experimental and other scientific purposes in the member states of the European Union. , pp.1-14.

Fagundes, D.J. and Taha, M.O. (2004). Modelo animal de doença: critérios de escolha e espécies de animais de uso corrente. *Acta Cirúrgica Brasileira,* 19(1): 59–65.

Fahey, J.W.; Zhang, Y. and Talalay, P. (1997). Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proceedings of the National Academy of Sciences of the United States of America,* 94(19): 10367–10372.

Faustino-Rocha, A.; Gama, A.; Oliveira, P.; Alvarado, A.; Ferreira, R. and Ginja, M. (2016a). A spontaneous high-grade carcinoma in seven-week-old female rat. (Epub ahead of print).

Faustino-Rocha, A.; Gama, A.; Oliveira, P.; Ferreira, R. and Ginja, M. (2016b). Spontaneous mammary tumor in a 7-week-old female rat. In: *XXI Meeting of the Portuguese Society of Animal Pathology,* 22–23.

Faustino-Rocha, A.I.; Gama, A.; Oliveira, P.A.; Alvarado, A.; Fidalgo-Gonçalves, L.; Ferreira, R. and Ginja, M. (2016c). Ultrasonography as the gold standard for *in vivo* volumetric determination of chemically-induced mammary tumors. *In vivo,* 30(4): 465–472.

Faustino-Rocha, A.; Gama, A.; Oliveira, P.A.; Alvarado, A.; Neuparth, M.J.; Ferreira, R. and Ginja, M. (2016d). Effects of lifelong exercise training on mammary tumorigenesis induced by MNU in female Sprague-Dawley rats. *Clinical and Experimental Medicine*, DOI: 10.1007/s10238-016-0419-0.

Faustino-Rocha, A.; Oliveira, P.A.; Pinho-Oliveira, J.; Teixeira-Guedes, C.; Soares-Maia, R.; Gil da Costa, R.; Colaço, B.; Pires, M.J.; Colaço, J.; Ferreira, R. and Ginja, M. (2013a). Estimation of rat mammary tumor volume using caliper and ultrasonography measurements. *Lab Animal,* 42(6): 217–224.

Faustino-Rocha, A.; Silva, A.; Gabriel, J.; Gil da Costa, R.M.; Moutinho, M.; Oliveira, P.; Gama, A.; Ferreira, R. and Ginja, M. (2016e). Long-term exercise training as a modulator of mammary cancer vascularization. *Biomedicine & Pharmacotherapy,* 81: 273–280.

Faustino-Rocha, A.I.; Ferreira, R.; Oliveira, P.A.; Gama, A. and Ginja, M. (2015a). N-methyl-N-nitrosourea as a mammary carcinogenic agent. *Tumor Biology,* 36(12): 9095–9117.

Faustino-Rocha, A.I.; Gama, A.; Oliveira, P.A.; Alvarado, A.; Vala, H.; Ferreira, R. and Ginja, M. (2015b). Expression of estrogen receptors- alpha and beta in chemically induced mammary tumours. *Virchows Archives,* 467: S51–S51.

Faustino-Rocha, A.I.; Oliveira, P.A.; Duarte, J.A.; Ferreira, R. and Ginja, M. (2013b). Ultrasonographic evaluation of gastrocnemius muscle in a rat model of N-methyl-N-nitrosourea-induced mammary mumor. *In Vivo*, 27(6): 803–807.

Faustino-Rocha, A.I.; Pinto, C.; Gama, A. and Oliveira, P.A. (2014). Effects of ketotifen on mammary tumors volume and weight. *Anticancer Research*, 34(10): 5902.

Faustino-Rocha, A.I.; Silva, A.; Gabriel, J.; Teixeira-Guedes, C.; Lopes, C.; Gil da Costa, R.; Gama, A.; Ferreira, R.; Oliveira, P. and Ginja, M. (2013c). Ultrasonographic, thermographic and histologic evaluation of MNU-induced mammary tumors in female Sprague-Dawley rats. *Biomedicine & Pharmacotherapy*, 67(8): 771–776.

Fedrowitz, M.; Kamino, K. and Löscher, W. (2004). Significant differences in the effects of magnetic field exposure on 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis in two substrains of Sprague-Dawley rats. *Cancer research* 64(1): 243–251.

Feig, S.A.; Shaber, G.S.; Schwartz, G.F.; Patchefsky, A.; Libshitz, H.I.; Edeiken, J.; Nerlinger, R.; Curley, R.F. and Wallace, J.D. (1977). Thermography, mammography, and clinical examination in breast cancer screening. Review of 16,000 studies. *Radiology,* 122(1): 123–127.

Ferlay, J.; Soerjomataram, I.; Dikshit, R.; Eser, S.; Mathers, C.; Rebelo, M.; Parkin, D.M.; Forman, D. and Bray, F. (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *International Journal of Cancer*, 136(5): E359-E386.

Forabosco, F.; Löhmus, M.; Rydhmer, L. and Sundström, L.F. (2013). Genetically modified farm animals and fish in agriculture: a review. *Livestock Science,* 153(1): 1–9.

Forbes, D.; Blom, H.; Kostomitsopoulos, N.; Moore, G. and Perretta, G. (2007). Felasa Euroguide on the accommodation and care of animals used for experimental and other scientific purposes Royal Society of Medicine, London.

Fred, H.L. (2004). Drawbacks and limitations of computed tomography: views from a medical educator. *Texas Heart Institute Journal*, 31(4): 345–348.

Freudenstein, J.; Dasenbrock, C. and Nisslein, T. (2002). Lack of promotion of estrogen-dependent mammary gland tumors in vivo by an isopropanolic Cimicifuga racemosa extract. *Cancer Research,* 62(12): 3448–3452.

Fukamachi, K.; Imada, T.; Ohshima, Y.; Xu, J. and Tsuda, H. (2008). Purple corn color suppresses Ras protein level and inhibits 7,12-dimethylbenz[a]anthracene-induced mammary carcinogenesis in the rat. *Cancer Science,* 99(9): 1841–1846.

Gago-Dominguez, M.; Jiang, X.J. and Castelao, J.E. (2007). Lipid peroxidation and the protective effect of physical exercise on breast cancer. *Medical Hypotheses*, 68(5): 1138-1143.

Gal, A.; Baba, A.; Miclaus, V.; Bouari, C.; Taulescu, M.; Bolfa, P.; Borza, G. and Catoi, C. (2011). Comparative aspects regarding MNU-induced mammary carcinogenesis in immature Sprague-Dowley and Whistar rats. *Bulletin UASVM,* 68(1): 159–163.

Ganta, C.; Chiyo, D.; Ayuzawa, R.; Rachakatla, R.; Pyle, M.; Andrews, G.; Weiss, M.; Tamura, M. and Troyer, D. (2009). Rat umbilical cord stem cells completely abolish rat mammary carcinomas with no evidence of metastasis or recurrence 100 days post-tumor cell inoculation. *Cancer Research*, 69(5): 1815–1820.

Garcia-Solis, P.; Alfaro, Y.; Anguiano, B.; Delgado, G.; Guzman, R.C.; Nandi, S.; Diaz-Munoz, M.; Vazquez-Martinez, O. and Aceves, C. (2005). Inhibition of N-methyl-N-nitrosourea-induced mammary carcinogenesis by molecular iodine (I-2) but not by iodide (I-) treatment evidence that I-2 prevents cancer promotion. *Molecular and Cellular Endocrinology*, 236(1-2): 49–57.

Garcia-Solis, P.; Yahia, E.M. and Aceves, C. (2008). Study of the effect of 'Ataulfo' mango (Mangifera indica L.) intake on mammary carcinogenesis and antioxidant capacity in plasma of N-methyl-N-nitrosourea (MNU)-treated rats. *Food Chemistry*, 111(2): 309–315.

Gershon-Cohen, J. and Haberman, J.D. (1964). Thermography. *Radiology,* 82: 280–285.

Goldfarb, Y.; Benish, M.; Rosenne, E.; Melamed, R.; Levi, B.; Glasner, A. and Ben-Eliyahu, S. (2009). CpG-C oligodeoxynucleotides limit the deleterious effects of beta-adrenoceptor stimulation on NK cytotoxicity and metastatic dissemination. *Journal of Immunotherapy,* 32(3): 280–291.

Gordon, P.B. and Goldenberg, S.L. (1995). Malignant breast masses detected only by ultrasound. A retrospective review. *Cancer,* 76(4): 626–630.

Gottardis, M.M. and Jordan, C. (1987). Antitumor actions of keoxifene and tamoxifen in the N-nitrosomethylurea-induced rat mammary carcinoma model. *Cancer Research,* 47: 4020–4024.

Grewal, J.; Zhou, H.D.; Factor, R. and Kesari, S. (2010). Isolated Loss of Hormonal Receptors in Leptomeningeal Metastasis From Estrogen Receptor- and Progesterone Receptor-Positive Lobular Breast Cancer. *Journal of Clinical Oncology*, 28(13): E200-E202.

Grubbs, C.J.; Hill, D.L.; Bland, K.I.; Beenken, S.W.; Lin, T.H.; Eto, I.; Atigadda, V.R.; Vines, K.K.; Brouillette, W.J. and Muccio, D.D. (2003). 9cUAB30, an RXR specific retinoid, and/or tarnoxifen in the prevention of methylnitrosourea-induced mammary cancers. *Cancer Letters*, 201(1): 17–24.

Guo, Y.; Mazar, A.P.; Lebrun, J.J. and Rabbani, S.A. (2002). An antiangiogenic urokinase-derived peptide combined with tamoxifen decreases tumor growth and metastasis in a syngeneic model of breast cancer. *Cancer Research,* 62(16): 4678–4684.

Haag, J.D.; Lindstrom, M.J. and Gould, M.N. (1992). Limonene-induced regression of

mammary carcinomas. *Cancer Research,* 52(14): 4021–4026.

Hakkak, R.; Shaaf, S.; Jo, C.H.; Macleod, S. and Korourian, S. (2011). Effects of high-isoflavone soy diet vs. casein protein diet and obesity on DMBA-induced mammary tumor development. *Oncology Letters,* 2(1): 29–36.

Hakkak, R.; Shaaf, S.; Jo, C.H.; MacLeod, S. and Korourian, S. (2010). Dehydroepiandrosterone intake protects against 7,12-dimethylbenz(a)anthracene-induced mammary tumor development in the obese Zucker rat model. *Oncology Reports,* 24(2): 357–362.

Hammond, M.E.H.; Hayes, D.F.; Dowsett, M.; Allred, D.C.; Hagerty, K.L.; Badve, S.; Fitzgibbons, P.L.; Francis, G.; Goldstein, N.S.; Hayes, M.; Hicks, D.G.; Lester, S.; Love, R.; Mangu, P.B.; McShane, L.; Miller, K.; Osborne, C.K.; Paik, S.; Perlmutter, J.; Rhodes, A.; Sasano, H.; Schwartz, J.N.; Sweep, F.C.G.; Taube, S.; Torlakovic, E.E.; Valenstein, P.; Viale, G.; Visscher, D.; Wheeler, T.; Williams, R.B.; Wittliff, J.L. and Wolff, A.C. (2010). American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Immunohistochemical Testing of Estrogen and Progesterone Receptors in Breast Cancer. *Journal of Clinical Oncology*, 28(16): 2784-2795.

Hanstein, B.; Djahansouzi, S.; Dall, P.; Beckmann, M.W. and Bender, H.G. (2004). Insights into the molecular biology of the estrogen receptor define novel therapeutic targets for breast cancer. *European Journal of Endocrinology*, 150(3): 243-255.

Harms, S.E. (1998). Breast magnetic resonance imaging. *Seminars in ultrasound, CT, and MR.* 19(1): 104–120.

Haroon, S.; Hashmi, A.A.; Khurshid, A.; Kanpurwala, M.A.; Mujtuba, S.; Malik, B. and Faridi, N. (2013). Ki67 index in breast cancer: correlation with other prognostic markers and potential in pakistani patients. *Asian Pacific Journal of Cancer Prevention*, 14(7): 4353-4358.

Harvell, D.M.; Strecker, T.E.; Xie, B.; Pennington, K.L.; McComb, R.D. and Shull, J.D. (2002). Dietary energy restriction inhibits estrogen-induced mammary, but not pituitary, tumorigenesis in the ACI rat. *Carcinogenesis,* 23(1): 161–169.

He, Y.U.; Li, Q.Q. and Guo, S.C. (2016). Taurine attenuates Dimethylbenz[a]anthracene-induced breast tumorigenesis in rats: A plasma metabolomic study. *Anticancer Research*, 36(2): 533–543.

Hilakivi-Clarke, L.; Onojafe, I.; Raygada, M.; Cho, E.; Skaar, T.; Russo, I. and Clarke, R. (1999). Prepubertal exposure to zearalenone or genistein reduces mammary tumorigenesis. *British Journal of Cancer,* 80(11): 1682–1688.

Hinck, L. and Silberstein, G.B. (2005). Key stages in mammary gland development - the mammary end bud as a motile organ. *Breast Cancer Research*, 7(6): 245–251.

Hoenerhoff, M.J.; Shibata, M.A.; Bode, A. and Green, J.E. (2011). Pathologic progression of mammary carcinomas in a C3(1)/SV40 T/t-antigen transgenic rat model of human triple-negative and Her2-positive breast cancer. *Transgenic Research*, 20(2): 247–259.

Holtzman, S.; Stone, J.P. and Shellabarger, C.J. (1982). Radiation-induced mammary carcinogenesis in virgin, pregnant, lactating, and post-lactating rats. *Cancer Research,* 42(1): 50–53.

Howell, J.S. (1963). Studies on chemically induced breast tumours in the rat. *Acta - Unio Internationalis Contra Cancrum,* 19: 762–764.

Hvid, H.; Thorup, I.; Oleksiewicz, M.B.; Sjogren, I. and Jensen, H.E. (2011). An alternative method for preparation of tissue sections from the rat mammary gland. *Experimental and Toxicologic Pathology*, 63(4): 317–324.

Hvid, H.; Thorup, I.; Sjogren, I.; Oleksiewicz, M.B. and Jensen, H.E. (2012). Mammary gland proliferation in female rats: effects of the estrous cycle, pseudo-pregnancy and age. *Experimental and Toxicologic Pathology*, 64(4): 321–332.

Iannaccone, P.M. and Jacob, H.J. (2009). Rats! *Disease models & mechanisms-The Company of Biologists*, 2(5-6): 206–210.

Iatropoulos, M.J; Cerven, D.R; De George, G.; Von Keutz, E. and Williams, G.M. (2008). Reduction by dietary matrix metalloproteinase inhibitor BAY 12-9566N of neoplastic development induced by diethylnitrosamine, N-nitrosodimethylamine, or 7,12-dimethylbenz(a)anthracene in rats. *Drug and Chemical Toxicology,* 31(3): 305–316.

Ikezaki, S.; Takagi, M. and Tamura, K. (2011). Natural occurrence of neoplastic lesions in young Sprague-Dawley rats. *Journal of Toxicologic Pathology*, 24(1): 37–40.

Imaoka, T.; Nishimura, M.; Doi, K.; Tani, S.; Ishikawa, K.; Yamashita, S.; Ushijima, T.; Imai, T. and Shimada, Y. (2014). Molecular characterization of cancer reveals interactions between ionizing radiation and chemicals on rat mammary carcinogenesis. *International Journal of Cancer*, 134(7): 1529-1538.

Ip, C.; Thompson, H.J.; Zhu, Z. and Ganther, H.E. (2000). *In vitro* and *in vivo* studies of methylseleninic acid: evidence that a monomethylated selenium metabolite is critical for cancer chemoprevention. *Cancer Research*, 60(11): 2882–2886.

Ito, M.; Yang, D.J.; Mawlawi, O.; Mendez, R.; Oh, C.S.; Azhdarinia, A.; Greenwell, A.C.; Yu, D.F.; Kim, E.E. (2006). PET and planar imaging of tumor hypoxia with labeled metronidazole. *Academic Radiology,* 13(5): 598–609.

Jain, A.K.; Thanki, K. and Jain, S. (2013). Co-encapsulation of tamoxifen and quercetin in polymeric nanoparticles: implications on oral bioavailability, antitumor efficacy, and drug-induced toxicity. *Molecular Pharmaceutics,* 10(9): 3459–3474.

Jang, T.J.; Jung, H.G.; Jung, K.H. and O, M.K. (2002). Chemopreventive effect of celecoxib and expression of cyclooxygenase-1 and cyclooxygenase-2 on chemically-induced rat mammary tumours. *International Journal of Experimental Pathology,* 83(4): 173–182.

Jayakumar, J.K.; Nirmala, P.; Kumar, B.A. and Kumar, A.P. (2014). Evaluation of protective effect of myricetin, a bioflavonoid in dimethyl benzanthracene-induced breast cancer in female Wistar rats. *South Asian Journal of Cancer,* 3(2): 107–111.

Juhasz, K.; Gombos, K.; Szirmai, M.; Gocze, K.; Wolher, V.; Revesz, P.; Magda, I.; Sebestyen, A.; Nemeth, A. and Ember, I. (2013). Very early effect of DMBA and MNU on microRNA expression. *In Vivo*, 27(1): 113-117.

Kadir, E.A.; Sulaiman, S.A.; Yahya, N.K. and Othman, N.H. (2013). Inhibitory effects of Tualang Honey on experimental breast cancer in rats: a preliminary study. *Asian Pacific Journal of Cancer Prevention,* 14(4): 2249–2254.

Kakehashi, A.; Tago, Y.; Yoshida, M.; Sokuza, Y.; Wei, M.; Fukushima, S. and Wanibuchi, H. (2012). Hormonally active doses of isoflavone aglycones promote mammary and endometrial carcinogenesis and alter the molecular tumor environment in Donryu rats. *Toxicological Sciences,* 126(1): 39–51.

Kale, A.; Gawande, S.; Kotwal, S.; Netke, S.; Roomi, M.W.; Ivanov, V.; Niedzwecki, A. and Rath, M. (2010). A combination of green tea extract, specific nutrient mixture and quercetin: an effective intervention treatment for the regression of N-methyl-N-nitrosourea (MNU)-induced mammary tumors in Wistar rats. *Oncology Letters*, 1(2): 313–317.

Kalender, W.A.; Beister, M.; Boone, J.M.; Kolditz, D.; Vollmar, S.V. and Weigel, M.C. (2012). High-resolution spiral CT of the breast at very low dose: concept and feasibility considerations. *European Radiology*, 22(1): 1–8.

Kang, J.S.; Jung, N.J.; Kim, S.; Kim, D.J.; Jang, D.D. and Yang, K.H. (2004). Downregulation of estrogen receptor alpha and beta expression in carcinogen-induced mammary gland tumors of rats. *Experimental Oncology*, 26(1): 31-35.

Kaplan, S.S. (2001). Clinical utility of bilateral whole-breast US in the evaluation of women with dense breast tissue. *Radiology,* 221(3): 641–649.

Karathanasis, E.; Chan, L.; Balusu, S.R.; D'Orsi, C.J.; Annapragada, A.V.; Sechopoulos, I. and Bellamkonda, R.V. (2008). Multifunctional nanocarriers for mammographic quantification of tumor dosing and prognosis of breast cancer therapy. *Biomaterials,* 29(36): 4815–4822.

Karathanasis, E.; Suryanarayanan, S.; Balusu, S.R.; McNeeley, K.; Sechopoulos, I.; Karellas, A.; Annapragada, A.V. and Bellamkonda, R.V. (2009). Imaging nanoprobe for prediction of outcome of nanoparticle chemotherapy by using mammography. *Radiology*, 250(2): 398–406.

Karellas, A. and Vedantham, S. (2008). Breast cancer imaging: a perspective for the next decade. *Medical Physics,* 35(11): 4878–4897.

Kariagina, A.; Aupperlee, M.D. and Haslam, S.Z. (2008). Progesterone receptor isoform functions in normal breast development and breast cancer. *Critical Reviews in Eukaryotic Gene Expression*, 18(1): 11-33.

Kariagina, A.; Xie, J.; Langohr, I.M.; Opreanu, R.C.; Basson, M.D. and Haslam, S.Z. (2013). Progesterone stimulates proliferation and promotes cytoplasmic localization of the cell cycle inhibitor p27 in steroid receptor positive breast cancers. *Hormones & Cancer*, 4(6): 381-390.

Kaspareit, J. and Rittinghausen, S. (1999). Spontaneous neoplastic lesions in Harlan Sprague-

Dawley rats. *Experimental and Toxicologic Pathology*, 51(1): 105–107.

Kayashita, J.; Shimaoka, I.; Nakajoh, M.; Kishida, N. and Kato, N. (1999). Consumption of a buckwheat protein extract retards 7,12-dimethylbenz[alpha]anthracene-induced mammary carcinogenesis in rats. *Bioscience, Biotechnology, and Biochemistry,* 63(10): 1837–1839.

Kayser, K.; Biechele, U.; Kayser, G.; Dienemann, H.; Andre, S.; Bovin, N.V. and Gabius, H.J. (1998). Pulmonary metastases of breast carcinomas: Ligandohistochemical, nuclear, and structural analysis of primary and metastatic tumors with emphasis on period of occurrence of metastases and survival. *Journal of Surgical Oncology*, 69(3): 137-146.

Khanna, C. and Hunter, K. (2005). Modeling metastasis *in vivo*. *Carcinogenesis,* 26(3): 513–523.

Kim, H.; Hall, P.; Smith, M.; Kirk, M.; Prasain, J.K.; Barnes, S. and Grubbs, C. (2004a). Chemoprevention by grape seed extract and genistein in carcinogen-induced mammary cancer in rats is diet dependent. *The Journal of Nutrition,* 134(12 Suppl): 3445S–3452S.

Kim, J.B.; O'Hare, M.J. and Stein, R. (2004b). Models of breast cancer: is merging human and animal models the future? *Breast Cancer Research*, 6(1): 22–30.

Kinsel, L.B.; Szabo, E.; Greene, G.L.; Konrath, J.; Leight, G.S. and Mccarty, K.S. (1989). Immunocytochemical Analysis of Estrogen-Receptors As A Predictor of Prognosis in Breast-Cancer Patients - Comparison with Quantitative Biochemical Methods. *Cancer Research*, 49(4): 1052-1056.

Kirubha, S.P.; Anburajan, M.; Venkataraman, B.; Akila, R.; Sharath, D. and Raj, B. (2012). Evaluation of mammary cancer in 7,12-dimethylbenz(a)anthracene-induced Wistar rats by asymmetrical temperature distribution analysis using thermography: a comparison with serum CEA levels and histopathology. *Journal of Biomedicine & Biotechnology*, 2012: 786417.

Klintman, M.; Strand, C.; Ahlin, C.; Beglerbegovic, S.; Fjallskog, M.L.; Grabau, D.; Gudlaugsson, E.; Janssen, E.A.M.; Lovgren, K.; Skaland, I.; Bendahl, P.O.; Malmstrom, P.; Baak, J.P.A. and Ferno, M. (2013). The Prognostic Value of Mitotic Activity Index (MAI), Phosphohistone H3 (PPH3), Cyclin B1, Cyclin A, and Ki67, Alone and in Combinations, in Node-Negative Premenopausal Breast Cancer. *Plos One*, 8(12): e81902.

Koh, D.M. and Collins, D.J. (2007). Diffusion-weighted MRI in the body: applications and challenges in oncology. *American Journal of Roentgenology*, 188(6): 1622–1635.

Kolb, T.M.; Lichy, J. and Newhouse, J.H. (1998). Occult cancer in women with dense breasts: detection with screening US-diagnostic yield and tumor characteristics. *Radiology,* 207(1): 191–199.

Kolb, T.M.; Lichy, J. and Newhouse, J.H. (2002). Comparison of the performance of screening mammography, physical examination, and breast US and evaluation of factors that influence them: an analysis of 27,825 patient evaluations. *Radiology,* 225(1): 165–175.

Krishnamoorthy, D. and Sankaran, M. (2016). Modulatory effect of Pleurotus ostreatus on

oxidant/antioxidant status in 7, 12-dimethylbenz (a) anthracene induced mammary carcinoma in experimental rats - a dose-response study. *Journal of Cancer Research and Therapeutics,* 12(1): 386–394.

Kristoffersen, M.; Aspelin, P.; Perbeck, L. and Boné, B. (2002). Value of MR imaging in clinical evaluation of breast lesions. *Acta Radiologica,* 43(3): 275–281.

Kubatka, P.; Sadlonova, V.; Kajo, K.; Machalekova, K.; Ostatnikova, D.; Nosalova, G. and Fetisovova, Z. (2008a). Neoplastic effects of exemestane in premenopausal breast cancer model. *Neoplasma,* 55(6): 538–543.

Kubatka, P.; Sadlonova, V.; Kajo, K.; Nosalova, G. and Fetisovova, Z. (2008b). Preventive effects of letrozole in the model of premenopausal mammary carcinogenesis. *Neoplasma,* 55(1): 42–46.

Kubota, K.; Hisa, N.; Ogawa, Y. and Yoshida, S. (2002). Evaluation of tissue harmonic imaging for breast tumors and axillary lymph nodes. *Oncology Reports,* 9(6): 1335–1338.

Lamartiniere, C.A.; Jenkins, S.; Betancourt, A.M.; Wang, J. and Russo, J. (2011). Exposure to the endocrine disruptor bisphenol A alters susceptibility for mammary cancer. *Hormone Molecular Biology and Clinical Investigation,* 5(2): 45–52.

Lanari, C.; Lamb, C.A.; Fabris, V.T.; Helguero, L.A.; Soldati, R.; Bottino, M.C.; Giulianelli, S.; Cerliani, J.P.; Wargon, V. and Molinolo, A. (2009). The MPA mouse breast cancer model: evidence for a role of progesterone receptors in breast cancer. *Endocrine-Related Cancer*, 16(2): 333-350.

Lange, C.A. and Yee, D. (2008). Progesterone and breast cancer. *Womens Health (Lond)*, 4(2): 151-162.

Lannigan, D.A. (2003). Estrogen receptor phosphorylation. *Steroids*, 68(1): 1-9.

Lehr, H.A.; Rochat, C.; Schaper, C.; Nobile, A.; Shanouda, S.; Vijgen, S.; Gauthier, A.; Obermann, E.; Leuba, S.; Schmidt, M.; Ruegg, C.C.; Delaloye, J.F.; Simiantonaki, N. and Schaefer, S.C. (2013). Mitotic figure counts are significantly overestimated in resection specimens of invasive breast carcinomas. *Modern Pathology*, 26(3): 336-342.

Lemon, H.M.; Kumar, P.F.; Peterson, C.; Rodriguez-Sierra, J.F. and Abbo, K.M. (1989). Inhibition of radiogenic mammary carcinoma in rats by estriol or tamoxifen. *Cancer,* 63(9): 1685–1692.

Li, J.X.; Cho, Y.Y.; Langfald, A.; Carper, A.; Lubet, R.A.; Grubbs, C.J.; Ericson, M.E. and Bode, A.M. (2011). Lapatinib, a preventive/therapeutic agent against mammary cancer, suppresses RTK-mediated signaling through multiple signaling pathways. *Cancer Prevention Research,* 4(8): 1190–1197.

Li, S.A.; Weroha, S.J.; Tawfik, O. and Li, J.J. (2002). Prevention of solely estrogen-induced mammary tumors in female aci rats by tamoxifen: evidence for estrogen receptor mediation. *The Journal of Endocrinology,* 175(2): 297–305.

Lin, Y.; Collier, A.C.; Liu, W.; Berry, M.J. and Panee, J. (2008). The inhibitory effect of bamboo extract on the development of 7,12-dimethylbenz[a]anthracene (DMBA)-induced breast cancer. *Phytotherapy Research,* 22(11): 1440–1445.

Lindfors, K.K.; Boone, J.M.; Newell, M.S. and D'Orsi, C.J. (2010). Dedicated breast computed tomography: the optimal cross-sectional imaging solution?. *Radiologic Clinics of North America*, 48(5): 1043–1054.

Linjawi, S.A.; Khalil, W.K.; Hassanane, M.M. and Ahmed, E.S. (2015). Evaluation of the protective effect of Nigella sativa extract and its primary active component thymoquinone against DMBA-induced breast cancer in female rats. *Archives of Medical Science ,* 11(1): 220–229.

Lippert, T.H.; Adlercreutz, H.; Berger, M.R.; Seeger, H.; Elger, W. and Mueck, A.O. (2003). Effect of 2-methoxyestradiol on the growth of methyl-nitroso-urea (MNU)-induced rat mammary carcinoma. *Journal of Steroid Biochemistry and Molecular Biology*, 84(1): 51–56.

Liska, J.; Galbavy, S.; Macejova, D.; Zlatos, J. and Brtko, J. (2000). Histopathology of mammary tumours in female rats treated with 1-Methyl-1-Nitrosourea. *Endocrine Regulations,* 34: 91–96.

Liska, J.; Macejova, D.; Galbavy, S.; Baranova, M.; Zlatos, J.; Stvrtina, S.; Mostbock, S.; Weiss, R.; Scheiblhofer, S.; Thalhamer, J. and Brtko, J. (2003). Treatment of 1-methyl-1-nitrosourea-induced mammary tumours with immunostimulatory CpG motifs and 13-cis retinoic acid in female rats: histopathological study. *Experimental and Toxicologic Pathology,* 55(2-3): 173–179.

Liu, F.; Bloch, N.; Bhushan, K.R.; De Grand, A.M.; Tanaka, E.; Solazzo, S.; Mertyna, P.M.; Goldberg, N.; Frangioni, J.V. and Lenkinski, R.E. (2008a). Humoral bone morphogenetic protein 2 is sufficient for inducing breast cancer microcalcification. *Molecular Imaging,* 7(4): 175–186.

Liu, F.; Misra, P.; Lunsford, E.P.; Vannah, J.T.; Liu, Y.; Lenkinski, R.E. and Frangioni, J. V. (2010). A dose- and time-controllable syngeneic animal model of breast cancer microcalcification. *Breast Cancer Research and Treatment*, 122(1): 87–94.

Liu, J.R.; Dong, H.W.; Chen, B.Q.; Zhao, P. and Liu, R.H. (2009). Fresh apples suppress mammary carcinogenesis and proliferative activity and induce apoptosis in mammary tumors of the Sprague-Dawley rat. *Journal of Agricultural and Food Chemistry,* 57(1): 297–304.

Liu, J.R.; Sun, X.R.; Dong, H.W.; Sun, C.H.; Sun, W.G.; Chen, B.Q.; Song, Y.Q. and Yang, B.F. (2008b). β-Ionone suppresses mammary carcinogenesis, proliferative activity and induces apoptosis in the mammary gland of the Sprague-Dawley rat. *International Journal of Cancer*, 122(12): 2689–2698.

Lopes, A.C.; Cova, T.F.; Pais, A.A.; Pereira, J.L.; Colaço, B. and Cabrita, A.M. (2014). Improving discrimination in the grading of rat mammary tumors using two-dimensional mapping of histopathological observations. *Experimental and Toxicologic Pathology,* 66(1): 73–80.

Lu, J.X.; Jiang, C.; Mitrenga, T.; Cutter, G. and Thompson, H.J. (1998). Pathogenetic characterization of 1-methyl-1-nitrosourea-induced mammary carcinomas in the rat. *Carcinogenesis,* 19(1): 223–227.

Lu, J.X.; Pei, H.Y.; Kaeck, M. and Thompson, H.J. (1997). Gene expression changes associated with chemically induced rat mammary carcinogenesis. *Molecular Carcinogenesis,* 20(2): 204–215.

Lubet, R.A.; Christov, K.; Nunez, N.P.; Hursting, S.D.; Steele, V.E.; Juliana, M.M.; Eto, I. and Grubbs, C.J. (2005). Efficacy of Targretin on methylnitrosourea-induced mammary cancers: prevention and therapy dose-response curves and effects on proliferation and apoptosis. *Carcinogenesis*, 26(2): 441–448.

Lubet, R.A.; Heckman, B.M.; De Flora, S.L.; Steele, V.E.; Crowell, J.A.; Juliana, M.M. and Grubbs, C.J. (2011). Effects of 5,6-benzoflavone, indole-3-carbinol (I3C) and diindolylmethane (DIM) on chemically-induced mammary carcinogenesis: is DIM a substitute for I3C? *Oncology Reports,* 26(3): 731–736.

Lubet, R.A.; Steele, V.E.; Eto, I.; Juliana, M.M.; Kelloff, G.J. and Grubbs, C.J. (1997). Chemopreventive efficacy of anethole trithione, N-acetyl-L-cysteine, miconazole and phenethylisothiocyanate in the DMBA-induced rat mammary cancer model. *International Journal of Cancer,* 72(1): 95–101.

Lucas, J.N.; Rudmann, D.G.; Credille, K.M.; Irizarry, A.R.; Peter, A. and Snyder, P.W. (2007). The rat mammary gland: Morphologic changes as an indicator of systemic hormonal perturbations induced by xenobiotics. *Toxicologic Pathology,* 35(2): 199–207.

Luzhna, L.; Kutanzi, K. and Kovalchuk, O. (2015). Gene expression and epigenetic profiles of mammary gland tissue: Insight into the differential predisposition of four rat strains to mammary gland cancer. *Mutation Research-Genetic Toxicology and Environmental Mutagenesis,* 779: 39–56.

Lyng, H.; Olsen, D.R.; Southon, T.E. and Rofstad, E.K. (1993). 31P-nuclear magnetic resonance spectroscopy in vivo of six human melanoma xenograft lines: tumour bioenergetic status and blood supply. *British Journal of Cancer*, 68(6): 1061–1070.

Ma, D.; Zhang, Y.; Yang, T.; Xue, Y. and Wang, P. (2014). Isoflavone intake inhibits the development of 7,12-dimethylbenz(a)anthracene(DMBA)-induced mammary tumors in normal and ovariectomized rats. *Journal of Clinical Biochemistry and Nutrition,* 54(1): 31–38.

Ma, D.F.; Katoh, R.; Zhou, H. and Wang, P.Y. (2007). Promoting effects of milk on the development of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in rats. *Acta Histochemica et Cytochemica,* 40(2): 61–67.

Maeda, K.; Ohkura, S. and Tsukamura, H. (2000). Physiology of reproduction. In *The Laboratory Rat*, Krinke G (ed). Academic Press: UK: 145–176.

Mafuvadze, B.; Cook, M.; Xu, Z.; Besch-Williford, C.L. and Hyder, S.M. (2013). Effects of dietary apigenin on tumor latency, incidence and multiplicity in a medroxyprogesterone acetate-accelerated 7,12-dimethylbenz(a)anthracene-induced breast cancer model. *Nutrition and Cancer,* 65(8): 1184–1191.

Malicka, I.; Siewierska, K.; Pula, B.; Kobierzycki, C.; Haus, D.; Cegielski, M.; Dziegiel, P.; Podhorska-Okolow, M. and Wozniewski, M. (2015). The effect of physical training on

the N-methyl-N-nitrosourea induced mammary carcinogenesis of Sprague–Dawley rats. *Experimental Biology and Medicine*, 240(11): 1408-1415.

Mandal, A.; Bhatia, D. and Bishayee, A. (2013). Simultaneous disruption of estrogen receptor and Wnt/β-catenin signaling is involved in methyl amooranin-mediated chemoprevention of mammary gland carcinogenesis in rats. *Molecular and Cellular Biochemistry*, 384(1-2): 239–250.

Mandal, A. and Bishayee, A. (2015a). Mechanism of breast cancer preventive action of pomegranate: disruption of estrogen receptor and Wnt/β-Catenin signaling pathways. *Molecules.* 20(12): 22315–22328.

Mandal, A. and Bishayee, A. (2015b). Trianthema portulacastrum Linn. displays anti-inflammatory responses during chemically induced rat mammary tumorigenesis through simultaneous and differential regulation of NF-κB and Nrf2 signaling pathways. *International Journal of Molecular Sciences,* 16(2): 2426–2445.

Manivannan, S. and Nelson, C.M. (2012). Dynamics of branched tissue assembly. *Stem Cell Research and Therapeutics,.* 3(1757-6512): 42.

Manna, S.; Das, S.; Chatterjee, M.; Janarthan, M. and Chatterjee, M. (2011). Combined supplementation of vanadium and fish oil suppresses tumor growth, cell proliferation and induces apoptosis in DMBA-induced rat mammary carcinogenesis. *Journal of Cellular Biochemistry,* 112(9): 2327–2339.

Manshadi, S.; Ishiguro, L.; Sohn, K.J.; Medline, A.; Renlund, R.; Croxford, R. and Kim, Y.I. (2014). Folic acid supplementation promotes mammary tumor progression in a rat model. *Plos One,* 9(1): e84635.

Marchesi, V. (2013). Breast cancer: stable breast cancer xenograft models. *Nature Reviews Clinical Oncology,* 10(8): 426–426.

Martin, G.; Melito, G.; Rivera, E.; Levin, E.; Davio, C.; Cricco, G.; Andrade, N.; Caro, R. and Bergoc, R. (1996). Effect of tamoxifen on intraperitoneal N-nitroso-N-methylurea induced tumors. *Cancer Letters,* 100(1-2): 227–234.

Matsuoka, Y.; Fukamachi, K.; Hamaguchi, T.; Toriyama-Baba, H.; Kawaguchi, H.; Kusunoki, M.; Yoshida, H. and Tsuda, H. (2003). Rapid emergence of mammary preneoplastic and malignant lesions in human c-Ha-ras proto-oncogene transgenic rats: possible application for screening of chemopreventive agents. *Toxicologic Pathology,* 31(6): 632–637.

Mccormick, D.L.; Adamowski, C.B.; Fiks, A. and Moon, R.C. (1981). Lifetime Dose-Response Relationships for Mammary-Tumor Induction by A Single Administration of N-Methyl-N-Nitrosourea. *Cancer Research*, 41(5): 1690-1694.

Mccormick, D.L. and Moon, R.C. (1983). Inhibition of mammary carcinogenesis by Flurbiprofen, a non-steroidal anti-inflammatory agent. *British Journal of Cancer,* 48(6): 859–861.

Mccormick, D.L.; Rao, K.V.N.; Johnson, W.D.; BowmanGram, T.A.; Steele, V.E.; Lubet, R.A. and Kelloff, G.J. (1996). Exceptional chemopreventive activity of low-dose dehydroepiandrosterone in the rat mammary gland. *Cancer Research*, 56(8): 1724-1726.

Mccullough, D.J.; Stabley, J.N.; Siemann, D.W. and Behnke, B.J. (2014). Modulation of Blood Flow, Hypoxia, and Vascular Function in Orthotopic Prostate Tumors During Exercise. *Jnci-Journal of the National Cancer Institute*, 106(4): 1-8.

Mceuen, C.S. (1938). Occurrence of cancer in rats treated with oestrone. *The American Journal of Cancer,* 34: 184–195.

Mcgregor, D.H.; Land, C.E.; Choi, K.; Tokuoka, S.; Liu, P.I.; Wakabayashi, T. and Beebe, G.W. (1977). Breast-cancer incidence among atomic bomb survivors, Hiroshima and Nagasaki. *Journal of the National Cancer Institute*. 59(3): 799–811.

Mcguire, W. and Clark, G. (1986). Role of Progesterone Receptors In Breast Cancer. *A Cancer Journal For Clinicians*, 36(5): 302-309.

Mcmartin, D.N.; Sahota, P.S.; Gunson, D.E.; Hsu, H.H. and Spaet, R.H. (1992). Neoplasms and Related proliferative lesions in control Sprague-Dawley rats from carcinogenicity studies - historical data and diagnostic considerations. *Toxicologic Pathology,* 20(2): 212–225.

Mctiernan, A.; Tworoger, S.S.; Ulrich, C.M.; Yasui, Y.; Irwin, M.L.; Rajan, K.B.; Sorensen, B.; Rudolph, R.E.; Bowen, D.; Stanczyk, F.Z.; Potter, J.D. and Schwartz, R.S. (2004). Effect of exercise on serum estrogens in postmenopausal women: A 12-month randomized clinical trial. *Cancer Research*, 64(8): 2923-2928.

Medina, D. (2007). Chemical carcinogenesis of rat and mouse mammary glands. *Breast Disease,* 28: 63–68.

Mehta, R.; Hawthorne, M.; Uselding, L.; Albinescu, D.; Moriarty, R.; Christov, K. and Mehta, R. (2000). Prevention of N-methyl-N-nitrosourea-induced mammary carcinogenesis in rats by 1 alpha-hydroxyvitamin D-5. *Journal of the National Cancer Institute*, 92(22): 1836–1840.

Meisamy, S.; Bolan, P.J.; Baker, E.H.; Pollema, M.G.; Le, C.T.; Kelcz, F.; Lechner, M.C.; Luikens, B.A.; Carlson, R.A.; Brandt, K.R.; Amrami, K.K.; Nelson, M.T.; Everson, L.I.; Emory, T.H.; Tuttle, T.M.; Yee, D. and Garwood, M. (2005). Adding in vivo quantitative 1H MR spectroscopy to improve diagnostic accuracy of breast MR imaging: preliminary results of observer performance study at 4.0 T. *Radiology,* 236(2): 465–475.

Merlo, D.F.; Rossi, L.; Pellegrino, C.; Ceppi, M.; Cardellino, U.; Capurro, C.; Ratto, A.; Sambucco, P.L.; Sestito, V.; Tanara, G. and Bocchini, V. (2008). Cancer incidence in pet dogs: findings of the Animal Tumor Registry of Genoa, Italy. *Journal of Veterinary Internal Medicine*, 22(4): 976-984.

Mesia-Vela, S.; Sanchez, R.I.; Reuhl, K.R.; Conney, A.H. and Kauffman, F.C. (2006). Phenobarbital treatment inhibits the formation of estradiol-dependent mammary tumors in the August-Copenhagen Irish rat. *The Journal of Pharmacology and Experimental Therapeutics,* 317(2): 590–597.

Mohammed, H.; Russell, I.A.; Stark, R.; Rueda, O.M.; Hickey, T.E.; Tarulli, G.A.; Serandour, A.A.; Birrell, S.N.; Bruna, A.; Saadi, A.; Menon, S.; Hadfield, J.; Pugh, M.; Raj, G.V.; Brown, G.D.; D'Santos, C.; Robinson, J.L.; Silva, G.; Launchbury, R.; Perou,

C.M.; Stingl, J.; Caldas, C.; Tilley, W.D. and Carroll, J.S. (2015). Progesterone receptor modulates ERalpha action in breast cancer. *Nature*, 523(7560): 313-317.

Montano, M.M.; Chaplin, L.J.; Deng, H.; Mesia-Vela, S.; Gaikwad, N.; Zahid, M. and Rogan, E. (2007). Protective roles of quinone reductase and tamoxifen against estrogen-induced mammary tumorigenesis. *Oncogene,* 26(24): 3587–3590.

Morgan, D.A.; Refalo, N.A. and Cheung, K.L. (2011). Strength of ER-positivity in relation to survival in ER-positive breast cancer treated by adjuvant tamoxifen as sole systemic therapy. *Breast*, 20(3): 215-219.

Mori, M.; Rao, S.K.; Popper, H.H.; Cagle, P.T. and Fraire, A.E. (2001). Atypical adenomatous hyperplasia of the lung: A probable forerunner in the development of adenocarcinoma of the lung. *Modern Pathology*, 14(2): 72-84.

Morris, E.A. (2002). Breast cancer imaging with MRI. *Radiologic Clinics of North America,* 40(3): 443–466.

Moss, H.A.; Britton, P.D.; Flower, C.D.; Freeman, A.H.; Lomas, D.J. and Warren, R.M. (1999). How reliable is modern breast imaging in differentiating benign from malignant breast lesions in the symptomatic population? *Clinical Radiology,* 54(10): 676–682.

Mousseau, Y.; Mollard, S.; Faucher-Durand, K.; Richard, L.; Nizou, A.; Cook-Moreau, J.; Baaj, Y.; Qiu, H.; Plainard, X.; Fourcade, L.; Funalot, B. and Sturtz, F.G. (2012). Fingolimod potentiates the effects of sunitinib malate in a rat breast cancer model. *Breast Cancer Research and Treatment,* 134(1): 31–40.

Mrklic, I.; Capkun, V.; Pogorelic, Z. and Tomic, S. (2013). Prognostic value of Ki-67 proliferating index in triple negative breast carcinomas. *Pathology Research and Practice*, 209(5): 296-301.

Mullins, L.J.; Brooker, G. and Mullins, J.J. (2002). Transgenesis in the rat. In *Transgenesis techniques: principles and protocols*, Clarke AR (ed). Humana Press: Cardiff, 255–270.

Mundhe, N.A.; Kumar, P.; Ahmed, S.; Jamdade, V.; Mundhe, S. and Lahkar, M. (2015). Nordihydroguaiaretic acid ameliorates cisplatin induced nephrotoxicity and potentiates its anti-tumor activity in DMBA induced breast cancer in female Sprague–Dawley rats. *International Immunopharmacology,* 28(1): 634–642.

Murata, N.; Fujimori, S.; Ichihara, Y.; Sato, Y.; Yamaji, T.; Tsuboi, H.; Uchida, M.; Suzuki, H.; Yamada, M.; Oikawa, T.; Nemoto, H.; Nobuhiro, J.; Choshi, T. and Hibino, S. (2006). Synthesis and anti-tumor activity of a fluorinated analog of medroxyprogesterone acetate (MPA), 9α-Fluoromedroxyprogesterone Acetate (FMPA). *Chemical & Pharmaceutical Bulletin,* 54(11): 1567–1570.

Murray, T.J.; Ucci, A.A.; Maffini, M.V.; Sonnenschein, C. and Soto, A.M. (2009). Histological analysis of low dose NMU effects in the rat mammary gland. *BMC Cancer*, 9: 267.

Nagayasu, H.; Hamada, J.; Kawano, T.; Konaka, S.; Nakata, D.; Shibata, T.; Arisue, M.; Hosokawa, M.; Takeichi, N. and Moriuchi, T. (1998). Inhibitory effects of malotilate on invasion and metastasis of rat mammary carcinoma cells by modifying the functions of

vascular endothelial cells. *British Journal of Cancer,* 77(9): 1371–1377.

Nakhate, K.T.; Kokare, D.M.; Singru, P.S.; Taksande, A.G.; Kotwal, S.D. and Subhedar, N.K. (2010). Hypothalamic cocaine- and amphetamine-regulated transcript peptide is reduced and fails to modulate feeding behavior in rats with chemically-induced mammary carcinogenesis. *Pharmacology Biochemistry and Behavior*, 97(2): 340–349.

Nandhakumar, R.; Salini, K. and Devaraj, S. (2012). Morin augments anticarcinogenic and antiproliferative efficacy against 7,12-dimethylbenz(a)-anthracene induced experimental mammary carcinogenesis. *Molecular and Cellular Biochemistry,* 364(1-2): 79–92.

Nandi, S.; Guzman, R.C. and Yang, J. (1995). Hormones and mammary carcinogenesis in mice, rats, and humans - a unifying hypothesis. *Proceedings of the National Academy of Sciences of the United States of America*, 92(9): 3650–3657.

Negi, A.K.; Bhatnagar, A. and Agnihotri, N. (2016). Fish oil augments celecoxib mediated alteration in apoptotic pathway in the initiation phase of 7,12-dimethylbenz(α)anthracene-induced mammary carcinogenesis. *Biomedicine & Pharmacotherapy,* 79: 9–16.

Nielsen, T.S.; Khan, G.; Davis, J.; Michels, K.B. and Hilakivi-Clarke, L. (2011). Prepubertal exposure to cow's milk reduces susceptibility to carcinogen-induced mammary tumorigenesis in rats. *International Journal of Cancer,* 128(1): 12–20.

Nishikawa, R.M.; Mawdsley, G.E.; Fenster, A. and Yaffe, M.J. (1987). Scanned-projection digital mammography. *Medical Physics,* 14(5): 717–727.

Noble, R.L. and Cutts, J.H. (1959). Mammary Tumors of the rat - review. *Cancer Research,* 19(11): 1125–1139.

Noguchi, M.; Minami, M.; Yagasaki, R.; Kinoshita, K.; Earashi, M.; Kitagawa, H.; Taniya, T. and Miyazaki, I. (1997). Chemoprevention of DMBA-induced mammary carcinogenesis in rats by low-dose EPA and DHA. *British Journal of Cancer,* 75(3): 348–353.

O'Day, E. and Lal, A. (2010). MicroRNAs and their target gene networks in breast cancer. *Breast Cancer Research*, 12(201): 1-10.

Obr, A.E. and Edwards, D.P. (2012). The biology of progesterone receptor in the normal mammary gland and in breast cancer. *Molecular and Cellular Endocrinology*, 357(1-2): 4-17.

Oishi, Y.; Yoshizawa, K.; Suzuki, J.; Makino, N.; Hase, K.; Yamauchi, K. and Tsubura, A. (1995). Spontaneously occurring mammary adenocarcinoma in a l0-wk-old female rat. *Toxicologic Pathology,* 23(6): 696–700.

Okugawa, H.; Yamamoto, D.; Uemura, Y.; Sakaida, N.; Tanano, A.; Tanaka, K. and Kamiyama, Y. (2005). Effect of perductal paclitaxel exposure on the development of MNU-induced mammary carcinoma in female S-D rats. *Breast Cancer Research and Treatment*, 91(1): 29–34.

Oliveira, P.A. (2016). Chemical carcinogens. In *Oxford Textbook of Oncology*, Kerr D, Heller D, Van de Velde C, Baumann M (eds). Oxford University Press: New York. pp.142-149

Oliveira, P.A; Colaço, A.; Chaves, R.; Guedes-Pinto, H.; De-La-Cruz P. and Lopes, C.

(2007). Chemical carcinogenesis. *Anais da Academia Brasileira de Ciências,* 79(4): 593–616.

Oliveira, P.A.; Colaco, A.; De la Cruz P. and Lopes, C. (2006). Experimental bladder carcinogenesis-rodent models. *Experimental Oncology,* 28(1): 2–11.

Orel, S.G. and Schnall, M.D. (2001). MR imaging of the breast for the detection, diagnosis, and staging of breast cancer. *Radiology,* 220(1): 13–30.

Ouhtit, A.; Ismail, M.F.; Othman, A.; Fernando, A.; Abdraboh, M.E.; El-Kott, A.F.; Azab, Y.A.; Abdeen, S.H.; Gaur, R.L.; Gupta, I.; Shanmuganathan, S.; Al-Farsi, Y.M.; Al-Riyami, H. and Raj, M.H. (2014). Chemoprevention of rat mammary carcinogenesis by spirulina. *The American Journal of Pathology,* 184(1): 296–303.

Ozdemir, I.; Talas, Z.; Gul, M.; Ates, B.; Gok, Y.; Esrefoglu, M. and Yilmaz, I. (2006). Inhibition of DMBA induced rat mammary duct damage by novel synthetic organoselenium compounds. *Experimental Animals / Japanese Association for Laboratory Animal Science,* 55(5): 449–455.

Paceli, R.B.; Cal, R.N.; dos Santos, C.H.F.; Cordeiro, J.A.; Neiva, C.M.; Nagamine, K.K. and Cury, P.M. (2012). The influence of physical activity in the progression of experimental lung cancer in mice. *Pathology Research and Practice*, 208(7): 377-381.

Padrao, A.I.; Moreira-Goncalves, D.; Oliveira, P.A.; Teixeira, C.; Faustino-Rocha, A.I.; Helguero, L.; Vitorino, R.; Santos, L.L.; Arnado, F.; Duarte, J.A. and Ferreira, R. (2015). Endurance training prevents TWEAK but not myostatin-mediated cardiac remodelling in cancer cachexia. *Archives of Biochemistry and Biophysics*, 567: 13-21.

Parkin, D.M.; Bray, F.; Ferlay, J. and Pisani, P. (2005). Global cancer statistics, 2002. *CA: A Cancer Journal for Clinicians.*, 55(2): 74-108.

Partridge, S.C. and McDonald, E.S. (2013). Diffusion weighted magnetic resonance imaging of the breast: protocol optimization, interpretation, and clinical applications. *Magnetic Resonance Imaging Clinics of North America,* 21(3): 601–624.

Pedersen, L.; Idorn, M.; Olofsson, G.H.; Lauenborg, B.; Nookaew, I.; Hansen, R.H.; Johannesen, H.H.; Becker, J.C.; Pedersen, K.S.; Dethlefsen, C.; Nielsen, J.; Gehl, J.; Pedersen, B.K.; Thor, S.P. and Hojman, P. (2016). Voluntary Running Suppresses Tumor Growth through Epinephrine- and IL-6-Dependent NK Cell Mobilization and Redistribution. *Cell Metabolism*, 23(3): 554-562.

Periyasamy, K.; Baskaran, K.; Ilakkia, A.; Vanitha, K.; Selvaraj, S. and Sakthisekaran, D. (2015). Antitumor efficacy of tangeretin by targeting the oxidative stress mediated on 7,12-dimethylbenz(a) anthracene-induced proliferative breast cancer in Sprague-Dawley rats. *Cancer Chemotherapy and Pharmacology,* 75(2): 263–272.

Perletti, G.; Concari, P.; Giardini, R.; Marras, E.; Piccinini, F.; Folkman, J. and Chen, L. (2000). Antitumor activity of endostatin against carcinogen-induced rat primary mammary tumors. *Cancer Research,* 60(7): 1793–1796.

Perse, M.; Cerar, A.; Injac, R. and Strukelj, B. (2009). N-methylnitrosourea Induced Breast Cancer in Rat, the Histopathology of the Resulting Tumours and its Drawbacks as a Model. *Pathology & Oncology Research*, 15(1): 115-121.

Polyak, K. (2011). Heterogeneity in breast cancer. *The Journal of Clinical Investigation*, 121(10): 3786-3788.

Pugalendhi, P.; Manoharan, S.; Suresh, K. and Baskaran, N. (2011). Genistein and daidzein, in combination, protect cellular integrity during 7,12-dimethylbenz[a]anthracene (DMBA) induced mammary carcinogenesis in Sprague-Dawley rats. *African Journal of Traditional, Complementary, and Alternative Medicines,* 8(2): 91–97.

Purdie, C.A.; Quinlan, P.; Jordan, L.B.; Ashfield, A.; Ogston, S.; Dewar, J.A. and Thompson, A.M. (2014). Progesterone receptor expression is an independent prognostic variable in early breast cancer: a population-based study. *British Journal of Cancer*, 110(3): 565-572.

Purushothaman, A.; Nandhakumar, E. and Sachdanandam, P. (2013). Phytochemical analysis and anticancer capacity of Shemamruthaa, a herbal formulation against DMBA- induced mammary carcinoma in rats. *Asian Pacific Journal of Tropical Medicine*, 6(12): 925–933.

Qin, L.Q.; Xu, J.Y.; Tezuka, H.; Wang, P.Y. and Hoshi, K. (2007). Commercial soy milk enhances the development of 7,12-dimethylbenz(a)anthracene-induced mammary tumors in rats. *In vivo,* 21(4): 667–671.

Qin, L.-Q.; Xu, J.-Y.; Wang, P.-Y.; Ganmaa, D.; Li, J.; Wang, J.; Kaneko, T.; Hoshi, K.; Shirai, T. and Sato, A. (2004). Low-fat milk promotes the development of 7,12-dimethylbenz(A)anthracene (DMBA)-induced mammary tumors in rats. *International Journal of Cancer,* 110(4): 491–496.

Qiu, C.P.; Shan, L.; Yu, M.S. and Snyderwine, E.G. (2005). Steroid hormone receptor expression and proliferation in rat mammary gland carcinomas induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. *Carcinogenesis*, 26(4): 763-769.

Quagliata, L.; Klusmeier, S.; Cremers, N.; Pytowski, B.; Harvey, A.; Pettis, R.J.; Thiele, W. and Sleeman, J.P. (2014). Inhibition of VEGFR-3 activation in tumor-draining lymph nodes suppresses the outgrowth of lymph node metastases in the MT-450 syngeneic rat breast cancer model. *Clinical & Experimental metastasis,* 31(3): 351–365.

Rabbani, S.A. and Gladu, J. (2002). Urokinase receptor antibody can reduce tumor volume and detect the presence of occult tumor metastases *in vivo*. *Cancer Research*, 62(8): 2390–2397.

Radisky, D.C.; Hirai, Y. and Bissell, M.J. (2003). Delivering the message: epimorphin and mammary epithelial morphogenesis. *Trends in Cell Biology,* 13(0962-8924): 426–434.

Rajakumar, T.; Pugalendhi, P. and Thilagavathi, S. (2015). Dose response chemopreventive potential of allyl isothiocyanate against 7,12-dimethylbenz(a)anthracene induced mammary carcinogenesis in female Sprague-Dawley rats. *Chemico-Biological Interactions,* 231: 35–43.

Ravoori, S.; Vadhanam, M.V.; Sahoo, S.; Srinivasan, C. and Gupta, R.C. (2007). Mammary tumor induction in ACI rats exposed to low levels of 17 beta-estradiol. *International Journal of Oncology*, 31(1): 113–120.

Ren, Z.; Li, Y.; Shen, T.; Hameed, O.; Siegal, G.P. and Wei, S. (2016). Prognostic factors in advanced breast cancer: Race and receptor status are significant after development of metastasis. *Pathology Research and Practice*, 212(1): 24-30.

Rennó, A.L.; Alves-Júnior, M.J.; Rocha, R.M.; De Souza, P.C.; De Souza, V.B.; Jampietro, J.; Vassallo, J.; Hyslop, S.; Anhê, G.F.; De Moraes, N.G.; Soares, F.A. and Schenka, A.A. (2015). Decreased expression of stem cell markers by simvastatin in 7,12-dimethylbenz(a)anthracene (DMBA)-induced breast cancer. *Toxicologic Pathology,* 43(3): 400–410.

Roomi, M.W.; Roomi, N.W.; Ivanov, V.; Kalinovsky, T.; Niedzwiecki, A. and Rath, M. (2005). Modulation of N-methyl-N-nitrosourea induced mammary tumors in Sprague-Dawley rats by combination of lysine, proline, arginine, ascorbic acid and green tea extract. *Breast Cancer Research*, 7(3): R291–R295.

Roy, P.; George, J.; Srivastava, S.; Tyagi, S. and Shukla, Y. (2011). Inhibitory effects of tea polyphenols by targeting cyclooxygenase-2 through regulation of nuclear factor kappa B, Akt and p53 in rat mammary tumors. *Investigational New Drugs,* 29(2): 225–231.

Rudmann, D.; Cardiff, R.; Chouinard, L.; Goodman, D.; Kuttler, K.; Marxfeld, H.; Molinolo, A.; Treumann, S. and Yoshizawa, K. (2012). Proliferative and nonproliferative lesions of the rat and mouse mammary, zymbal's, preputial, and clitoral glands. *Toxicologic Pathology*, 40(6): 7S–39S.

Ruhlen, R.L.; Willbrand, D.M.; Besch-Williford, C.L.; Ma, L.; Shull, J.D. and Sauter, E.R. (2009). Tamoxifen induces regression of estradiol-induced mammary cancer in the ACI.COP-Ept2 rat model. *Breast Cancer Research and Treatment,* 117(3): 517–524.

Rundle, A. (2005). Molecular epidemiology of physical activity and cancer. *Cancer epidemiology, biomarkers & prevention*, 14(1): 227-236.

Russo, I.H. and Russo, J. (1998). Role of hormones in mammary cancer initiation and progression. *Journal of Mammary Gland Biology and Neoplasia*, 3(1): 49–61.

Russo, I.H. and Russo, J. (1996a). Mammary gland neoplasia in long-term rodent studies. *Environmental Health Perspectives*, 104(9): 938-967.

Russo, J. (2015). Significance of rat mammary tumors for human risk assessment. *Toxicologic Pathology*, 43(2): 145-170.

Russo, J. and Russo, I.H. (1996b). Experimentally induced mammary tumors in rats. *Breast Cancer Research and Treatment,* 39(1): 7–20.

Russo, J. and Russo, I.H. (2000). Atlas and histologic classification of tumors of the rat mammary gland. *Journal of Mammary Gland Biology and Neoplasia*, 5(2): 187–200.

Russo, J.; Tay, L. and Russo, I. (1982). Differentiation of the mammary gland and susceptibility to carcinogenesis. *Breast Cancer Research and Treatment,* 2: 5–73.

Saarinen, N.M.; Huovinen, R.; Wärri, A.; Mäkelä, S.I.; Valentín-Blasini, L.; Sjöholm, R.; Ammälä, J.; Lehtilä, R.; Eckerman, C.; Collan, Y.U. and Santti, R.S. (2002). Enterolactone inhibits the growth of 7,12-dimethylbenz(a)anthracene-induced mammary carcinomas in the rat. *Molecular Cancer Therapeutics,* 1(10): 869–876.

Sahin, K.; Tuzcu, M.; Sahin, N.; Akdemir, F.; Ozercan, I.; Bayraktar, S. and Kucuk, O. (2011). Inhibitory effects of combination of lycopene and genistein on 7,12- dimethyl benz(a)anthracene-induced breast cancer in rats. *Nutrition and Cancer,* 63(8): 1279–1286.

Salas, Y.; Marquez, A.; Diaz, D. and Romero, L. (2015). Epidemiological Study of Mammary Tumors in Female Dogs Diagnosed during the Period 2002-2012: A Growing Animal Health Problem. *Plos One*, 10(5): e0127381.

Samy, R.P.; Rajendran, P.; Li, F.; Anandi, N.M.; Stiles, B.G.; Ignacimuthu, S.; Sethi, G. and Chow, V.T.K. (2012). Identification of a novel Calotropis procera protein that can suppress tumor growth in breast cancer through the suppression of NF-κB pathway. *Plos One,* 7(12): e48514.

Sano, D. and Myers, J.N. (2009). Xenograft models of head and neck cancers. *Head & neck Oncology*, 1: 32.

Saraydin, S.U.; Tuncer, E.; Tepe, B.; Karadayi, S.; Özer, H.; Şen, M.; Karadayi, K.; Inan, D.; Elagöz, Ş.; Polat, Z.; Duman, M. and Turan, M. (2012). Antitumoral effects of Melissa officinalis on breast cancer in vitro and in vivo. *Asian Pacific Journal of Cancer Prevention,* 13(6): 2765–2770.

Sato, M.; Pei, R.J.; Yuri, T.; Danbara, N.; Nakane, Y. and Tsubura, A. (2003). Prepubertal resveratrol exposure accelerates N-methyl-N-nitrosourea-induced mammary carcinoma in female Sprague-Dawley rats. *Cancer Letters,* 202(2): 137–145.

Schaffer, E.M.; Liu, J.Z.; Green, J.; Dangler, C.A. and Milner, J.A. (1996). Garlic and associated allyl sulfur components inhibit N-methyl-N-nitrosourea induced rat mammary carcinogenesis. *Cancer Letters*, 102(1-2): 199–204.

Schnell, C.R.; Stauffer, F.; Allegrini, P.R.; O'Reilly, T.; McSheehy, P.M.; Dartois, C.; Stumm, M.; Cozens, R.; Littlewood-Evans, A.; García-Echeverría, C. and Maira, S.M. (2008). Effects of the dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235 on the tumor vasculature: implications for clinical imaging. *Cancer Research,* 68(16): 6598–6607.

Scholzen, T. and Gerdes, J. (2000). The Ki-67 protein: From the known and the unknown. *Journal of Cellular Physiology*, 182(3): 311-322.

Schuurman, H.J.; Hougen, H.P. and Van Loveren, H. (1992). The rnu (Rowett Nude) and rnuN (nznu, New Zealand Nude) Rat: An Update. *ILAR Journal,* 34: 1–2.

Sengupta, P. (2013). The Laboratory Rat: Relating Its Age With Human's. *International Journal of Preventive Medicine*, 4(6): 624–630.

Shaban, N.; Abdel-Rahman, S.; Haggag, A.; Awad, D.; Bassiouny, A. and Talaat, I. (2016). Combination between Taxol-encapsulated liposomes and eruca sativa seed extract

suppresses mammary tumors in female rats induced by 7,12 Dimethylbenz(α)anthracene. *Asian Pacific Journal of Cancer Prevention,* 17(1): 117–123.

Sharma, D.; Smits, B.M.; Eichelberg, M.R.; Meilahn, A.L.; Muelbl, M.J.; Haag, J.D. and Gould, M.N. (2011). Quantification of epithelial cell differentiation in mammary glands and carcinomas from DMBA- and MNU-Exposed Rats. *Plos One*, 6(10): e26145.

Shellabarger, C.J.; Stone, J.P. and Holtzman, S. (1983). Effect of interval between neutron radiation and diethylstilbestrol on mammary carcinogenesis in female acl rats. *Environmental Health Perspectives,* 50(APR): 227–232.

Shivapurkar, N.; Tang, Z.C.; Frost, A. and Alabaster, O. (1996). A rapid dual organ rat carcinogenesis bioassay for evaluating the chemoprevention of breast and colon cancer. *Cancer Letters*, 100(1-2): 169–179.

Shirai, K.; Uemura, Y.; Fukumoto, M.; Tsukamoto, T.; Pascual, R.; Nandi, S. and Tsubura, A. (1997). Synergistic effect of MNU and DMBA in mammary carcinogenesis and H-ras activation in female Sprague-Dawley rats. *Cancer Letters*, 120(1): 87-93.

Shull, J.D.; Spady, T.J.; Snyder, M.C.; Johansson, S.L. and Pennington, K.L. (1997). Ovary-intact, but not ovariectomized female ACI rats treated with 17 beta estradiol rapidly develop mammary carcinoma. *Carcinogenesis*, 18(8): 1595–1601.

Siddiqui, I.A.; Sanna, V.; Ahmad, N.; Sechi, M. and Mukhtar, H. (2015). Resveratrol nanoformulation for cancer prevention and therapy. *Annals of the New York Academy of Sciences,* 1348(1749-6632): 20–31.

Silva, J.C.; Ferreira-Strixino, J.; Fontana, L.C.; Paula, L.M.; Raniero, L.; Martin, A.A. and Canevari, R.A. (2014). Apoptosis-associated genes related to photodynamic therapy in breast carcinomas. *Lasers in Medical Science,* 29(4): 1429–1436.

Singh, B.; Bhat, N.K. and Bhat, H.K. (2012). Induction of NAD(P)H-quinone oxidoreductase 1 by antioxidants in female ACI rats is associated with decrease in oxidative DNA damage and inhibition of estrogen-induced breast cancer. *Carcinogenesis,* 33(1): 156–163.

Singh, B.; Shoulson, R.; Chatterjee, A.; Ronghe, A.; Bhat, N.K.; Dim, D.C. and Bhat, H.K. (2014). Resveratrol inhibits estrogen-induced breast carcinogenesis through induction of NRF2-mediated protective pathways. *Carcinogenesis,* 35(8): 1872–1880.

Singletary, K.; MacDonald, C.; Iovinelli, M.; Fisher, C. and Wallig, M. (1998). Effect of the beta-diketones diferuloylmethane (curcumin) and dibenzoylmethane on rat mammary DNA adducts and tumors induced by 7,12-dimethylbenz[a]anthracene. *Carcinogenesis,* 19(6): 1039–1043.

Singletary, K.; MacDonald, C. and Wallig, M. (1997). The plasticizer benzyl butyl phthalate (BBP) inhibits 7,12-dimethylbenz[a]anthracene (DMBA)-induced rat mammary DNA adduct formation and tumorigenesis. *Carcinogenesis,* 18(8): 1669–1673.

Skrajnowska, D.; Bobrowska-Korczak, B.; Tokarz, A.; Bialek, S.; Jezierska, E. and Makowska, J. (2013). Copper and resveratrol attenuates serum catalase, glutathione peroxidase, and element values in rats with DMBA-induced mammary carcinogenesis. *Biological Trace Element Research,* 156(1-3): 271–278.

Smits, B.M.; Cotroneo, M.S.; Haag, J.D. and Gould, M.N. (2007). Genetically engineered rat models for breast cancer. *Breast Disease,* 28: 53–61.

Soares-Maia, R.; Faustino-Rocha, A.I.; Teixeira-Guedes, C.I.; Pinho-Oliveira, J.; Talhada, D.; Rema, A.; Faria, F.; Ginja, M.; Ferreira, R.; da Costa, R.M.G.; Oliveira, P.A. and Lopes, C. (2013). MNU-Induced Rat Mammary Carcinomas: Immunohistology and Estrogen Receptor Expression. *Journal of Environmental Pathology Toxicology and Oncology*, 32(2): 157-163.

Son, W.C.; Bell, D.; Taylor, I. and Mowat, V. (2010). Profile of early occurring spontaneous tumors in Han Wistar Rats. *Toxicologic Pathology*, 38(2): 292–296.

Song, H.T.; Jordan, E.K.; Lewis, B.K.; Liu, W.; Ganjei, J.; Klaunberg, B.; Despres, D.; Palmieri, D. and Frank, J.A. (2009). Rat model of metastatic breast cancer monitored by MRI at 3 tesla and bioluminescence imaging with histological correlation. *Journal of Translational Medicine,* 7(1): 88.

Song, L.L.; Kosmeder, J.W.; Lee, S.K.; Gerhäuser, C.; Lantvit, D.; Moon, R.C.; Moriarty, R.M. and Pezzuto, J.M. (1999). Cancer chemopreventive activity mediated by 4'-bromoflavone, a potent inducer of phase II detoxification enzymes. *Cancer Research,* 59(3): 578–585.

Soriano, O.; Delgado, G.; Anguiano, B.; Petrosyan, P.; Molina-Servín, E.D.; Gonsebatt, M.E. and Aceves, C. (2011). Antineoplastic effect of iodine and iodide in dimethylbenz[a]anthracene-induced mammary tumors: association between lactoperoxidase and estrogen-adduct production. *Endocrine-Related Cancer,* 18(4): 529–539.

Srivastava, P.; Russo, J. and Russo, I.H. (1997). Chorionic gonadotropin inhibits rat mammary carcinogenesis through activation of programmed cell death. *Carcinogenesis,* 18(9): 1799–808.

Stearns, V.; Mori, T.; Jacobs, L.K.; Khouri, N.F.; Gabrielson, E.; Yoshida, T.; Kominsky, S.L.; Huso, D.L.; Jeter, S.; Powers, P.; Tarpinian, K.; Brown, R.J.; Lange, J.R.; Rudek, M.A.; Zhang, Z.; Tsangaris, T.N. and Sukumar, S. (2011). Preclinical and clinical evaluation of intraductally administered agents in early breast cancer. *Science Translational Medicine*, 3(106): 106-108.

Steele, V.E. and Lubet, R.A. (2010). The use of animal models for cancer chemoprevention drug development. *Seminars in Oncology*, 37(4): 327–338.

Sternlicht, M.D. (2006). Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis. *Breast Cancer Research,* 8(1465): 201.

Stubbs, M.; Coombes, R.C.; Griffiths, J.R.; Maxwell, R.J.; Rodrigues, L.M. and Gusterson, B.A. (1990). 31P-NMR spectroscopy and histological studies of the response of rat mammary tumours to endocrine therapy. *British Journal of Cancer,* 61(2): 258–262.

Stubbs, M.; Rodrigues, L.M. and Griffiths, JR. (1989). Growth studies of subcutaneous rat tumours: comparison of 31P-NMR spectroscopy, acid extracts and histology. *British Journal of Cancer,* 60(5): 701–707.

Sukumar, S.; Notario, V.; Martinzanca, D. and Barbacid, M. (1983). Induction of mammary

carcinomas in rats by nitroso-methylurea involves malignant activation of H-Ras-1 locus by single point mutations. *Nature,* 306(5944): 658–661.

Szaefer, H.; Krajka-Kuźniak, V.; Ignatowicz, E.; Adamska, T.; Markowski, J. and Baer-Dubowska, W. (2014). The effect of cloudy apple juice on hepatic and mammary gland phase I and II enzymes induced by DMBA in female Sprague-Dawley rats. *Drug and Chemical Toxicology,* 37(4): 472–479.

Tabaczar, S.; Domeradzka, K.; Czepas, J.; Piasecka-Zelga, J.; Stetkiewicz, J.; Gwoździński, K. and Koceva-Chyła, A. (2015). Anti-tumor potential of nitroxyl derivative Pirolin in the DMBA-induced rat mammary carcinoma model: a comparison with quercetin. *Pharmacological Reports,* 67(3): 527–534.

Tai, S.Y.; Hsieh, H.M.; Huang, S.P. and Wu, M.T. (2016). Hair dye use, regular exercise, and the risk and prognosis of prostate cancer: multicenter case-control and case-only studies. *BMC Cancer*, 16(242): 1-12.

Tan, Q.X.; Qin, Q.H.; Yang, W.P.; Mo, Q.G. and Wei, C.Y. (2014). Prognostic value of Ki67 expression in HR-negative breast cancer before and after neoadjuvant chemotherapy. *International Journal of Clinical and Experimental Pathology*, 7(10): 6862-6870.

Tepsuwan, A.; Kupradinun, P. and Kusamran, W.R. (2002). Chemopreventive potential of neem flowers on carcinogen-induced rat mammary and liver carcinogenesis. *Asian Pacific Journal of Cancer Prevention,* 3(3): 231–238.

Thiele, W.; Rothley, M.; Teller, N.; Jung, N.; Bulat, B.; Plaumann, D.; Vanderheiden, S.; Schmaus, A.; Cremers, N.; Göppert, B.; Dimmler, A.; Eschbach, V.; Quagliata, L.; Thaler, S.; Marko, D.; Bräse, S. and Sleeman, J.P. (2013). Delphinidin is a novel inhibitor of lymphangiogenesis but promotes mammary tumor growth and metastasis formation in syngeneic experimental rats. *Carcinogenesis,* 34(12): 2804–2813.

Thomas, C. and Gustafsson, J.A. (2015). Estrogen receptor mutations and functional consequences for breast cancer. *Trends in Endocrinology & Metabolism*, 26(9): 467-476.

Thompson, H.J. (1994). Effect of Exercise Intensity and Duration on the Induction of Mammary Carcinogenesis. *Cancer Research*, 54(7): S1960-S1963.

Thompson, H.J. (1997). Effects of physical activity and exercise on experimentally-induced mammary carcinogenesis. *Breast Cancer Research and Treatment*, 46(2-3): 135-141.

Thompson, H.J. and Singh, M. (2000). Rat models of premalignant breast disease. *Journal of Mammary Gland Biology and Neoplasia*, 5(4): 409–420.

Thompson, H.J.; Wolfe, P.; McTiernan, A.; Jiang, W.Q. and Zhu, Z.J. (2010). Wheel Running-Induced Changes in Plasma Biomarkers and Carcinogenic Response in the 1-Methyl-1-Nitrosourea-Induced Rat Model for Breast Cancer. *Cancer Prevention Research*, 3(11): 1484-1492.

Thompson, M.D.; Thompson, H.J.; McGinley, J.N.; Neil, E.S.; Rush, D.K. and Holm, D.G.; Stushnoff, C. (2009). Functional food characteristics of potato cultivars (Solanum tuberosum L.): phytochemical composition and inhibition of 1-methyl-1-nitrosourea induced breast cancer in rats. *Journal of Food Composition and Analysis,* 22(6): 571–576.

Thordarson, G.; Lee, A.V.; McCarty, M.; Van Horn, K.; Chu, O.; Chou, Y.C.; Yang, J.; Guzman, R.C.; Nandi, S. and Talamantes, F. (2001). Growth and characterization of N-methyl-N-nitrosourea-induced mammary tumors in intact and ovariectomized rats. *Carcinogenesis,* 22(12): 2039–2048.

Turan, V.K.; Sanchez, R.I.; Li, J.J.; Li, S.A.; Reuhl, K.R.; Thomas, P.E.; Conney, A.H.; Gallo, M.A.; Kauffman, F.C. and Mesia-Vela, S. (2004). The effects of steroidal estrogens in ACI rat mammary carcinogenesis: 17 beta-estradiol, 2-hydroxyestradiol, 4-hydroxyestradiol, 16 alpha-hydroxyestradiol, and 4-hydroxyestrone. *Journal of Endocrinology,* 183(1): 91–99.

Ueda, M.; Imai, T.; Takizawa, T.; Onodera, H.; Mitsumori, K.; Matsui, T. and Hirose, M. (2005). Possible enhancing effects of atrazine on growth of 7,12-dimethylbenz(a) anthracene-induced mammary tumors in ovariectomized Sprague-Dawley rats. *Cancer Science,* 96(1): 19–25.

Valastyan, S. and Weinberg, R.A. (2011). Tumor Metastasis: Molecular Insights and Evolving Paradigms. *Cell*, 147(2): 275-292.

Van der Gulden, W.J.I.; Beynen, A.C. and Hau, J. (1999). Modelos Animales. In *Principios de la Ciencia del Animals de Laboratorio*, Van Zutphen L.F.M., Baumans V., Beynen A.C. (eds). Elsevier: Granada: 211–219.

Vandamme, T.F. (2014). Use of rodents as models of human diseases. *Journal of Pharmacy & Bioallied Sciences,* 6(1): 2–9.

Vanitha, M.K.; Priya, K.D.; Baskaran, K.; Periyasamy, K.; Saravanan, D.; Venkateswari, R.; Mani, B.R.; Ilakkia, A.; Selvaraj, S.; Menaka, R.; Geetha, M.; Rashanthy, N.; Anandakumar, P. and Sakthisekaran, D. (2015). Taurine regulates mitochondrial function during 7,12-Dimethyl Benz[a]anthracene induced experimental mammary carcinogenesis. *Journal of Pharmacopuncture,* 18(3): 68–74.

Vargo-Gogola, T. and Rosen, J.M. (2007). Modelling breast cancer: one size does not fit all. *Nature Reviews Cancer,* 7(9): 659–672.

Veena, K.; Shanthi, P. and Sachdanandam, P. (2006). Anticancer effect of Kalpaamruthaa on mammary carcinoma in rats with reference to glycoprotein components, lysosomal and marker enzymes. *Biological & Pharmaceutical Bulletin,* 29(3): 565–569.

Vici, P.; Pizzuti, L.; Natoli, C.; Gamucci, T.; Di, L.L.; Barba, M.; Sergi, D.; Botti, C.; Michelotti, A.; Moscetti, L.; Mariani, L.; Izzo, F.; D'Onofrio, L.; Sperduti, I.; Conti, F.; Rossi, V.; Cassano, A.; Maugeri-Sacca, M.; Mottolese, M. and Marchetti, P. (2015). Triple positive breast cancer: a distinct subtype? *Cancer Prevention Research*, 41(2): 69-76.

Vinodhini, J. and Sudha, S. (2013). Effect of bis-carboxy ethyl germanium sesquoxide on N-nitroso-N-methylurea-induced rat mammary carcinoma. *Asian Journal of Pharmaceutical and Clinical Research,* 6(2): 242–244.

Vrdoljak, E.; Bodoky, G.; Jassem, J.; Popescu, R.A.; Mardiak, J.; Pirker, R.; Cufer, T.; Beslija, S.; Eniu, A.; Todorovic, V.; Kubackova, K.; Kurteva, G.; Tomasevic, Z.; Sallaku, A.; Smichkoska, S.; Bajic, Z. and Sikic, B.I. (2016). Cancer Control in Central and

Eastern Europe: Current Situation and Recommendations for Improvement. *Oncologist*, 21: 1-8.

Wagner, K.U. (2004). Models of breast cancer: quo vadis, animal modeling?. *Breast Cancer Research,* 6(1): 31–38.

Watson, P.A.; Kim, K.; Chen, K.S. and Gould, M.N. (2002). Androgen-dependent mammary carcinogenesis in rats transgenic for the Neu proto-oncogene. *Cancer Cell,* 2(1): 67–79.

Westerlind, K.C. (2003). Physical activity and cancer prevention-mechanisms. *Medicine and Science in Sports and Exercise*, 35(11): 1834-1840.

Westerlind, K.C.; McCarty, H.L.; Gibson, K.J. and Strange, R. (2002). Effect of exercise on the rat mammary gland: implications for carcinogenesis. *Acta Physiologica Scandinavica*, 175(2): 147-156.

Whittal-Strange, K.S.; Chadau, S. and Parkhouse, W.S. (1998). Exercise during puberty and NMU induced mammary tumorigenesis in rats. *Breast Cancer Research and Treatment*, 47(1): 1-8.

Wiberg, M.K.; Aspelin, P. ; Perbeck L. ; Boné, B. (2002). Value of MR imaging in clinical evaluation of breast lesions. *Acta Radiologica,* 43 (3): 275-281.

Wiehle, R.D.; Christov, K. and Mehta, R. (2007). Anti-progestins suppress the growth of established tumors induced by 7,12-dimethylbenz(a)anthracene: comparison between RU486 and a new 21-substituted-19-nor-progestin. *Oncology Reports,* 18(1): 167–174.

Wishart, G.C.; Rakha, E.; Green, A.; Ellis, I.; Ali, H.R.; Provenzano, E.; Blows, F.M.; Caldas, C. and Pharoah, P.D.P. (2014). Inclusion of KI67 significantly improves performance of the PREDICT prognostication and prediction model for early breast cancer. *BMC Cancer*, 14(908): 1-6.

Wolff, G.; Balke, J.E.; Andras, I.E.; Park, M. and Toborek, M. (2014). Exercise Modulates Redox-Sensitive Small GTPase Activity in the Brain Microvasculature in a Model of Brain Metastasis Formation. *Plos One*, 9(5): e97033.

Wolff, G.; Davidson, S.J.; Wrobel, J.K. and Toborek, M. (2015). Exercise maintains blood-brain barrier integrity during early stages of brain metastasis formation. *Biochemical and Biophysical Research Communications*, 463(4): 811-817.

Wolff, G. and Toborek, M. (2013). Targeting the therapeutic effects of exercise on redox-sensitive mechanisms in the vascular endothelium during tumor progression. *International Union of Biochemistry and Molecular Biology*, 65(7): 565-571.

Woodhams, R.; Ramadan, S.; Stanwell, P.; Sakamoto, S.; Hata, H.; Ozaki, M.; Kan, S. and Inoue, Y. (2011). Diffusion-weighted imaging of the breast: principles and clinical applications. *Radiographics,* 31(4): 1059–1084.

World Health organization (WHO). (2015). Cancer - Fact sheet nº 297. *World Health Organization*. http://www.who.int/mediacentre/factsheets/fs297/en/. Updated February 2017.

Wu, I.; Wang, H.; Huso, D. and Wahl, R.L. (2015). Optimal definition of biological tumor volume using positron emission tomography in an animal model. *European Journal of Nuclear Medicine and Molecular Imaging research*, 5(1): 58.

Wu, S.G.; Sun, J.Y.; Yang, L.C.; Tang, L.Y.; Wang, X.; Chen, X.T.; Liu, G.H.; Lin, H.X.; Lin, Q. and He, Z.Y. (2016). Patterns of distant metastasis in Chinese women according to breast cancer subtypes. *Oncotarget*, 7(2): 47975-47984.

Xing, R.H.; Mazar, A.; Henkin, J. and Rabbani, S.A. (1997). Prevention of breast cancer growth, invasion, and metastasis by antiestrogen tamoxifen alone or in combination with urokinase inhibitor B-428. *Cancer Research,* 57(16): 3585–3593.

Yamada, S.; Ikeda, H.; Yamazaki, H.; Shikishima, H.; Kikuchi, K.; Wakisaka, A.; Kasai, N.; Shimotohno, K. and Yoshiki, T. (1995). Cytokine-producing mammary carcinomas in transgenic rats carrying the pX gene of human T-lymphotropic virus type I. *Cancer Research,* 55(12): 2524–2527.

Yamanoshita, O.; Ichihara, S.; Hama, H.; Ichihara, G.; Chiba, M.; Kamijima, M.; Takeda, I. and Nakajima, T. (2007). Chemopreventive effect of selenium-enriched Japanese radish sprout against breast cancer induced by 7,12-dimethylbenz[a]anthracene in rats. *The Tohoku Journal of Experimental Medicine,* 212(2): 191–198.

Yan, H.X.; Wu, H.P.; Ashton, C.; Tong, C. and Ying, Q.L. (2012). Rats deficient for p53 are susceptible to spontaneous and carcinogen-induced tumorigenesis. *Carcinogenesis,* 33(10): 2001–2005.

Yang, D.; Kuang, L.R.; Cherif, A.; Tansey, W.; Li, C.; Lin, W.; Liu, C.W.; Kim, E. and Wallace, S. (1993). Synthesis of [^{18} F]Fluoroalanine and [^{18} F]Fluorotamoxifen for Imaging Breast Tumors. *Journal of Drug Targeting,* 1(3): 259–267.

Yang, J.H.; Nakagawa, H.; Tsuta, K. and Tsubura, A. (2000). Influence of perinatal genistein exposure on the development of MNU-induced mammary carcinoma in female Sprague-Dawley rats. *Cancer Letters*, 149(1-2): 171–179.

Yang, N.; Huang, B.; Tsinkalovsky, O.; Brekka, N.; Zhu, H.Y.; Leiss, L.; Enger, O.; Li, X.G. and Wang, J. (2014). A novel GFP nude rat model to investigate tumor-stroma interactions. *Cancer Cell International*, 14: 541.

Yerushalmi, R.; Woods, R.; Ravdin, P.M.; Hayes, M.M. and Gelmon, K.A. (2010). Ki67 in breast cancer: prognostic and predictive potential. *Lancet Oncology*, 11(2): 174-183.

Yoshikawa, T.; Kawaguchi, H.; Umekita, Y.; Souda, M.; Gejima, K.; Kawashima, H.; Nagata, R. and Yoshida, H. (2008). Effects of neonatally administered low-dose diethylstilbestrol on the induction of mammary carcinomas and dysplasias induced by 7,12-dimethylbenz [a] anthracene in female rats. *In vivo*, 22(2): 207–213.

Yuan, J.P.; Wang, L.W.; Qu, A.P.; Chen, J.M.; Xiang, Q.M.; Chen, C.; Sun, S.R.; Pang, D.W.; Liu, J. and Li, Y. (2015). Quantum Dots-Based Quantitative and In Situ Multiple Imaging on Ki67 and Cytokeratin to Improve Ki67 Assessment in Breast Cancer. *Plos One*, 10(4): e0122734.

Zan, Y.H.; Haag, J.D.; Chen, K.S.; Shepel, L.A.; Wigington, D.; Wang, Y.R.; Hu, R.; Lopez-Guajardo, C.C.; Brose, H.L.; Porter, K.I.; Leonard, R.A.; Hitt, A.A.; Schommer, S.L.;

Elegbede, A.F. and Gould, M.N. (2003). Production of knockout rats using ENU mutagenesis and a yeast-based screening assay. *Nature Biotechnology*. 21(6): 645–651.

Zarbl, H.; Sukumar, S.; Arthur, A.; Martin-Zanca, D. and Barbacid, M. (1985). Direct mutagenesis of Ha-ras-1 oncogenes by N-nitroso-N-methylurea during initiation of mammary carcinogenesis in rats. *Nature*, 315: 382-385.

Zhai, G.; Grubbs, C.J.; Stockard, C.R.; Umphrey, H.R.; Beasley, T.M.; Kim, H.; Briest, S.; Stearns, V.; Henriksen, K.; Rasmussen, B.; Lykkesfeldt, A.; Moller, S.; Ejlertsen, B.; Buzdar, A.; Miller, W.; Anderson, T.; Dixon, J.; Saunders, P. and Rose, D. (2013). Diffusion weighted imaging evaluated the early therapy effect of tamoxifen in an MNU-induced mammary cancer rat model. *Plos One,* 8(5): e64445.

Zile, M.H.; Welsch, C.W. and Welsch, M.A. (1998). Effect of wheat bran fiber on the development of mammary tumors in female intact and ovariectomized rats treated with 7,12-dimethylbenz(a)anthracene and in mice with spontaneously developing mammary tumors. *International Journal of Cancer,* 75(3): 439–443.

Zingue, S.; Cisilotto, J.; Tueche, A.B.; Bishayee, A.; Mefegue, F.A.; Sandjo, L.P.; Magne, C.B.; Winter, E.; Michel, T.; Ndinteh, D.T.; Awounfack, C.F.; Silihe, K.K.; Tanekou, T.T.; Creczynski-Pasa, T.B. and Njamen, D. (2016). Crateva adansonii DC, an African ethnomedicinal plant, exerts cytotoxicity in vitro and prevents experimental mammary tumorigenesis *in vivo*. *Journal of Ethnopharmacology,* 190: 183–199.