Universidade de Aveiro
2019

Departamento de Ciências Médicas

DANIELA FILIPA DUARTE FIGUEIRA

MORPHOLOGICAL AND PROTEIN AGGREGATION CHANGES IN THE AGING MAMMARY GLAND AND BREAST CANCER

ALTERAÇÕES MORFOLÓGICAS E AGREGAÇÃO PROTEICA NO ENVELHECIMENTO DA GLÂNDULA MAMÁRIA E NO CANCRO DA MAMA

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Luisa Helguero, Professora auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro, e a coorientação das Doutora Adelina Gama, Professora auxiliar da Universidade de Trás-os-Montes, e a Dra. Paula Oliveira, Professora associada com agregação da Universidade de Trás-os-Montes

Financial support by FCT within the projects UID/BIM/04501/2013, POCI-01-0145-FEDER-007628 and UID/BIM/04501/2019 granted to the Aveiro Institute for Biomedicine

Financial support by the SR&TD Integrated Programme “pAGE – Protein aggregation Across the Lifespan” (CENTRO-01-0145-FEDER-000003)

Image acquisition was performed in the LiM facility of iBiMED, a node of PPBI (Portuguese Platform of BioImaging): POCI-01-0145-FEDER-022122.

Dedico este trabalho aos meus pais e irmã

o júri

presidente

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agradecimentos

Um especial agradecimento à minha orientadora, prof. Luisa Helguero, pela oportunidade, confiança e incansável apoio, por toda a disponibilidade e orientação ao longo de todo o processo, por todo o conhecimento e experiência transmitidos; muito obrigada!

Quero também agradecer às minhas coorientadoras, prof. Adelina Gama e prof. Paula Oliveira, pela constante disponibilidade e auxílio.

À Inês Direito e à Liliana Monteiro, por todas as vezes que vos tive de interromper com dúvidas ou dificuldades para encontrar ou preparar algum material, ou mesmo pelo próprio trabalho em execução, muito obrigada pela vossa disponibilidade e apoio.

À Mariana Alves, pela paciência em repetir e explicar tudo sobre os microscópios quantas vezes fossem precisas, e por toda a disponibilidade e apoio nestes procedimentos.

Quero também agradecer à minha Técnica Coordenadora do Serviço de Anatomia Patológica do CHBV, Cristina Soares, e aos meus colegas Ana Marques e Eugénio Matos, pelo vosso apoio e dicas valiosas da vossa experiência.

Ao nosso grupo de meninas do Mestrado em Biomedicina Molecular, pelos inúmeros jantares e almoços de partilhas de sucessos e frustrações e mútuo apoio; e em especial, à Carolina Matos, pela incansável e constante motivação, pelas inúmeras mensagens (mais ou menos sérias) e por todo o companheirismo nestes 2 anos.

À minha maior colega de estágio e amiga, Maria Neto, pelo interesse e paciência, insistência na disponibilidade e por todo o apoio ao longo deste tempo.

Ao Luís Pinho, por todos os cafés de *study sessions*, pela paciência e imensurável ajuda na pesquisa bibliográfica e toda a parte mais tecnológica, bem como por todos os conselhos e constante apoio e motivação. Obrigada por cada minuto e por cada palavra, por não me deixares desistir e por fazeres saber que eu era capaz.

Finalmente, à minha família, em particular aos meus pais e irmã, pelo apoio e motivação, por todas as vezes que se preocuparam "como está a correr a tese? Mostra lá esses resultados", mesmo sem entenderem completamente o que estava a ser tratado. Foram vocês que me "levantaram" de todas as vezes que parecia que não tinha forças para continuar e concluir, foi graças a vocês que cheguei até aqui e que tenciono continuar a aprender e ser melhor!

palavras-chave

Envelhecimento, respostas de stress adaptativo, dano proteico, *unfolded protein response,* agressomas, glândula mamária, cancro da mama, proteostase

resumo

O risco de cancro da mama aumenta com a idade. Alterações durante a diferenciação celular mamária aumentam o risco de cancro da mama. O envelhecimento celular está associado ao aumento do dano proteico, o que pode resultar em agregados proteicos com toxicidade intra e extracelular que influenciam a sobrevivência celular. Consequentemente, as células ativam uma série de respostas ao stress para responder a esta toxicidade potencialmente letal. Contudo, com o envelhecimento, as células aparentam ser menos eficientes, uma vez que tem sido descrita a agregação de proteínas em diversos sistemas biológicos com o envelhecimento. Esta agregação proteica está associada com várias doenças humanas que apresentam elevada incidência na população envelhecida e está relacionada com degeneração e morte, tal como a doença de Alzheimer. Existe pouca informação relativamente aos mecanismos utilizados pelas células humanas de diferentes origens embrionárias e estadios de diferenciação relativamente à eliminação destes agregados, bem como de que forma estes influenciam a carcinogénese. Desde que a glândula mamária se desenvolve e diferencia após o nascimento a partir de células estaminais progenitoras, colocámos a hipótese de diferentes tipos celulares reagirem de forma diferente aos agregados proteicos relacionados com o envelhecimento. Assim, alguns tipos de células mamárias podem ser mais suscetíveis a alterações durante a diferenciação. Este trabalho tem dois objetivos: 1) O estudo de marcadores de diferenciação no epitélio mamário ao longo do envelhecimento; e 2) Avaliação dos agregados proteicos observados através de fluorescência nos casos de cancro da mama humanos, para avaliar se existem alterações na proteostase antes e depois da terapia hormonal. Para o objetivo 1, o modelo experimental consistiu em amostras de tecido de ratinhos C57BL/6 com idades compreendidas entre 1 e 29 meses de idade; e amostras humanas de tumores mamários resistentes à terapia hormonal (amostras antes e após o tratamento) para o objetivo 2. Os métodos incluíram avaliação histológica da glândula mamária do ratinho durante o envelhecimento, nomeadamente constituição e arquitetura da glândula e imunohistoquímica para avaliar a expressão de marcadores epiteliais e de diferenciação da glândula mamária (tais como CK5, CK8, ER e PR); e para o objetivo 2, foi necessário a otimização do protocolo com o ProteoStat® para amostras histológicas. O objetivo 1 não foi cumprido, possivelmente devido artefactos de fixação encontrados nas amostras, que impossibilitaram o estudo imunohistoquímico. No objetivo 2, contrariamente ao esperado, foram detetados mais agregados proteicos nas amostras de tumores após a terapia hormonal, sendo necessários mais estudos para discriminar os agregados intra e extracelulares.

keywords

Aging, adaptative stress responses, protein damage, unfolded protein response, aggresomes, mammary gland, breast cancer, proteostasis

abstract

Breast cancer risk increases with aging. Aberrations occurring in the mammary cell differentiation process increase the risk of breast cancer. In general, cell aging is associated with increased protein damage which can result in toxic intra – and extracellular protein aggregates and influence cell survival. Consequently, the cells activate a series of adaptive stress responses to resolve this potentially lethal toxicity. However, with aging cells appear to become less efficient as protein aggregation in different biological systems has been reported to increase with age. Protein aggregation is associated to human diseases which have higher incidence in the aging population and are related to cell degeneration and death, such as Alzheimer's. Little information exists regarding how human cells from different embryonic origin and differentiation stage resolve toxic protein aggregates and how this can influence carcinogenesis. Since the mammary gland develops after birth through differentiation of adult mammary stem and progenitor cells, we hypothesized that the different cell hierarchies found in the mammary tissue deal differently with age-related protein aggregation. This in turn would make certain mammary cell types more prone to suffer alterations during differentiation. This work has two objectives: 1) the study of differentiation markers in the mammary epithelium throughout aging; and 2) the evaluation of the protein aggregates observed by fluorescence in human breast cancer cases to assess the changes in proteostasis before and after hormone therapy. For objective 1, the experimental model consisted of tissue samples from C57BL/6 mice aged 1 to 29 months; and human samples of hormone therapy resistant breast tumors (samples before and after treatment) for objective 2. Methods included histological evaluation of the mouse mammary gland during aging, namely constitution and architecture of the gland and immunohistochemistry to evaluate expression of epithelial markers and mammary gland differentiation (such as CK5, CK8, ER and PR); and for objective 2, it was necessary to optimize the protocol with ProteoStat® for histological samples. Objective 1 was not achieved, possibly due to the fixation artifacts found in the samples, which made the immunohistochemical study impossible. In Objective 2, opposing the expectations, more protein aggregates were detected in tumor samples after hormone therapy, and further studies will be needed to discriminate intra and extracellular aggregates.

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List of Abbreviations

ABC – avidin-biotin complex

AR – androgen receptor

ASR – age standardized rate

ATF6 - activating transcription factor 6

ATG – autophagy-related gene

ATP – adenosine triphosphate

AUT - autophagy

BC – breast cancer

BiP – binding immunoglobulin protein

CEMIP - cell migration-inducing protein

CK - cytokeratin

DAB - diaminobenzidine

DNA – deoxyribonucleic acid

DUB – deubiquitinating enzyme

ECIS - European Cancer Information System

EnR – endoplasmic reticulum

eIF2α – α subunit of eukaryotic initiation factor 2

ER – estrogen receptor

FBS – fetal bovine serum

HDAC6 – histone deacetylase 6

H&E – hematoxylin and eosin

HER2 – human epidermal growth factor receptor 2

HT – hormone therapy

IRE1 – inositol requiring kinase 1

LC3 – light chain 3

LIR – light chain 3 interacting region

mRNA – messenger ribonucleic acid

NBR1 – neighbor of breast cancer 1

NOS – not otherwise specified

PBS – phosphate buffer solution
PERK – double-stranded ribonucleic acid-activated protein kinase-like endoplasmic reticulum kinase
PR – progesterone receptor
RNA – ribonucleic acid
TNM - tumor, lymph nodes involvement and metastasis
Ub – ubiquitin
UBD – ubiquitin binding domain
UP – unfolded protein
UPR – unfolded protein response
UPS – ubiquitin-proteasome system

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Introduction

The theoretical subjects that were taken into consideration in order to develop this thesis will be approached within this section. To achieve the proposed objectives, topics such as the mammary gland (from its development on both mice and humans, and breast cancer in humans), the phenomenon of aging, and proteostasis are the main focus of this work, simultaneously considering the network between these issues.

Mammary glands, also known as breasts in humans, are unique to mammals, with the specific function of synthetizing, secreting and delivering milk to the newborn. This is a very specialized secretory gland that is composed of alveolus, that are grouped into lobules, where the milk is produced, and the ducts transport the milk produced in the alveoli to the nipple. Both males and females have mammary glands, which are made up of adipose and glandular tissue, both being most abundant in women. The mammary gland develops after birth, but it is only during a pregnancy/lactation that the gland reaches a mature developmental state to functionally differentiate in order to produce and secrete milk. This is accomplished via hormonal influences that exert drastic modifications in its micro and macroanatomy, which result in remodeling of the gland into a milk-secretory organ (1,2) that regresses to its pre-pregnancy state once lactation ceases (3). However, when mammary cells stop responding normally to hormonal stimuli, pathologies including cancer can arise.

Breast cancer is known to affect 46.3% women in the world, and the carcinogenic process has also been correlated with aging. There are several factors that support a relationship between cancer and aging, and several features that some cancers exhibit are similar to those seen in aging, such as the role of genomic instability, telomere attrition, epigenetic changes, loss of proteostasis, decreased nutrient sensing and altered metabolism, but also cellular senescence and stem cell function (4). Here we will focus on the loss of proteostasis, its relationship to cancer, aging, and its relationship with the response to hormone-therapy.

1. Mammary gland development

1.1. The human breast

The female breasts are glandular organs that are very sensitive to hormonal changes in the body and adopt cyclic changes in synchrony with the menstrual cycle (1,2,5). Female breasts contain 12-25 lobes, which are further divided into smaller lobules connected via milk ducts. Besides that, the adipose tissue is supplied by a network of nerves, blood vessels, lymph vessels, lymph nodes, and is also composed of fibrous connective tissue and ligaments (1,2,5).

The mammary gland is composed of multiple cell types, under continuous structural and functional changes that comprise embryonic and postnatal growth, as well as continuous cycles of proliferation, differentiation and cellular death throughout the reproductive life (6).

The rudimentary mammary glands of male and female neonates are identical; and as in the fetus, from birth to puberty there are no identifiable morphological differences in the development of the breasts. This development (Figure 1) begins with the mammary crests (ridges) (Figure 1 B). Involution of the mammary crests (except for those at the site of the future breasts) in the fifth week leads to the formation of the primary mammary buds (Figure 1 C), that will develop into lactiferous ducts and their branches (Figure 1 D-E). The formation of these canals is induced by placental sex hormones entering the fetal circulation and this process continues until the late fetal period. By term, 15 to 20 lobes of glandular tissue have formed, each containing a lactiferous duct that opens onto the breast surface through the mammary pit, and the fibrous connective tissue and fat of the mammary glands develop from the surrounding mesenchyme. During the late fetal period, the epidermis at the site of origin of the mammary glands becomes depressed, forming shallow mammary pits (Figure 1 E). The breasts of neonates contain lactiferous ducts but lack the alveoli. The alveoli are arranged in grape-clusters and will be the sites of milk secretion (7–9).

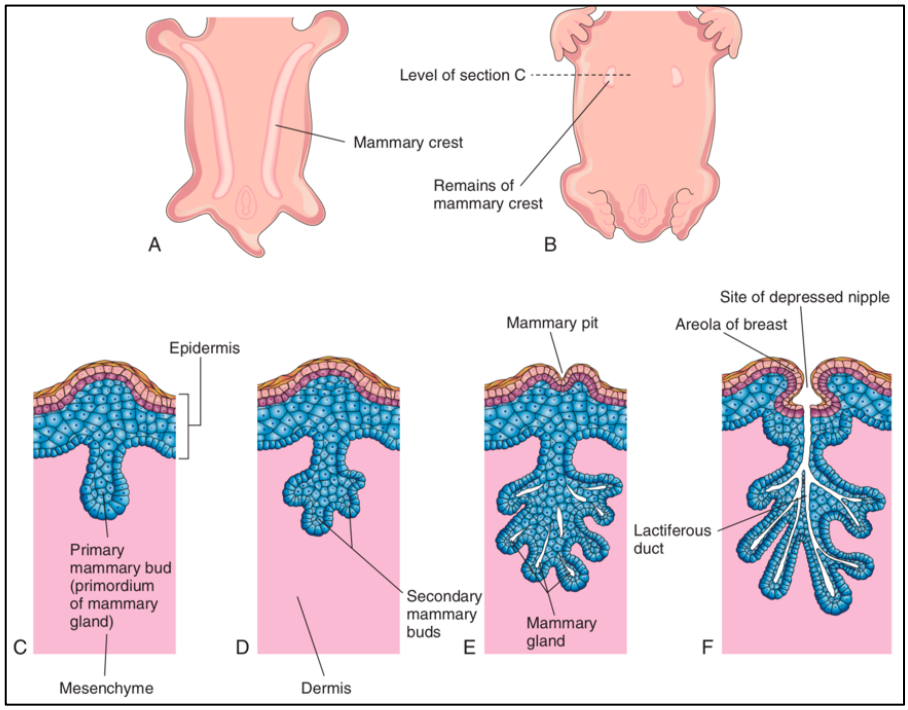


Figure 1: Development of the human mammary gland. **(A)** Ventral view of an embryo at 28-days gestation showing mammary crests. **(B)** Similar view at 6-week gestation showing the remains of the mammary crests. **(C)** Transversal section of a mammary crest at the site of the developing mammary gland. **(D–F)** Similar sections showing successive stages of breast development between the 12th week of gestation and birth.
The development begins with the mammary crests which develop along each side of the ventral surface of the embryo (A). These crests usually disappear, except for the parts at the site of the future breasts (B). Involution of the remaining mammary crests leads to the formation of the primary mammary buds (C), and each will give rise to several secondary mammary buds that will develop into lactiferous ducts and their branches (D-E). By term each lobe contains a lactiferous duct that opens onto the breast surface through the mammary pit, and the fibrous connective tissue and fat of the mammary glands develop from the surrounding mesenchyme. During the late fetal period, the epidermis at the site of origin of the mammary glands becomes depressed, forming shallow mammary pits (E)
Reproduced from *The Developing Human: Clinically Oriented Embryology* (Moore, KL; Persaud, TVN; Torchia, MG. 10tth ed. 2016)

There are wide variations in the degree of glandular development (branching and acinar formation) and in the functional differentiation of the cells lining the ducts and acini of males and females. In girls the breasts enlarge rapidly during puberty due to the proliferation of the mammary epithelium in response to ovarian hormones and the accumulation of the fibrous stroma and fat associated with them, with the full development occurring at approximately 19 years of age. On the other hand, the lactiferous ducts of boys remain rudimentary throughout life (7–9). This dimorphic development of the breast is heavily under the influence of sexual hormones, in particular estrogen (8,9).

It is very difficult to study the human breast during pregnancy and lactation, since most specimens obtained at these stages are from *breast cancer* (BC) surgeries; however, the few specimens examined show similar features to those in rodents (Figure 2), which will be described in the next topic, with an increase in the number of lobules and a loss of the fat. At

weaning, with the end of the suckling stimulus, the breast involutes, and secretory epithelial cells are removed by apoptosis and phagocytosis. At menopause, both the lobules and ducts are reduced in number and the intralobular stroma is replaced by collagen, whereas the glandular epithelium and the interlobular connective tissue regress and are replaced by fat (2,8).

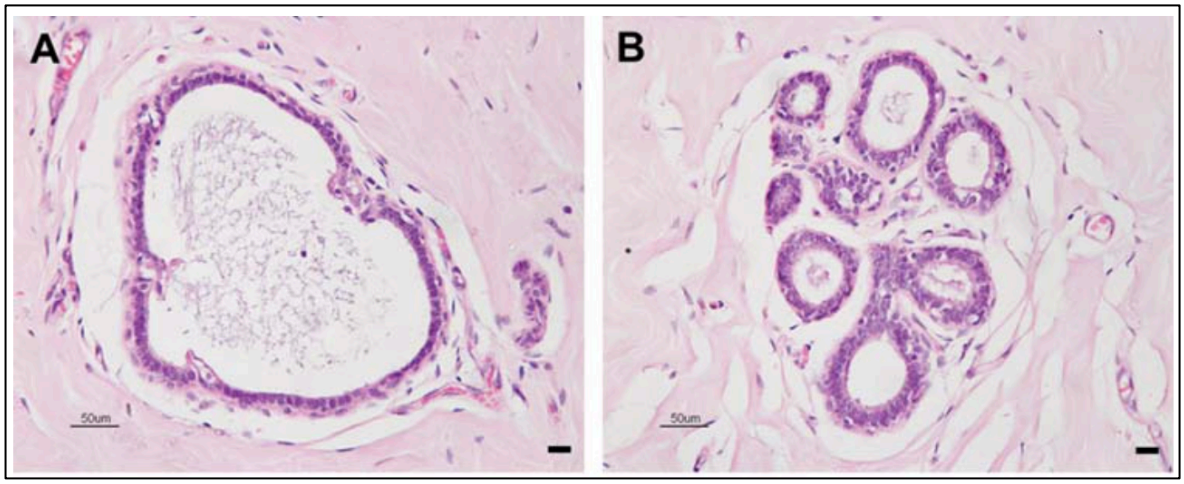


Figure 2: Histology of the human lactating mammary gland. (A) Duct and (B) a group of alveoli, embedded in the surrounding stroma.
Reproduced from *Anatomy of the Human Mammary Gland: Current Status of Knowledge* (Hassiotou, F; Geddes, D. 2012

1.2. The mouse mammary gland

1.2.1. The mouse mammary gland development

There are five pairs of mammary glands in the mouse. These are located below the skin, extending from the cervical (one pair), thoracic (two pairs), abdominal (one pair) to the inguinal (one pair) regions of the animal, along what is termed the mammary line (Figure 3) (10); each fat pad has one to three nipples to which the primary epithelial duct of the gland is connected to allow the release of milk during lactation (6). A gradient of differentiation among the glands is also observed, being the first thoracic gland the least differentiated and the fifth inguinal gland the most differentiated (6,10,11).

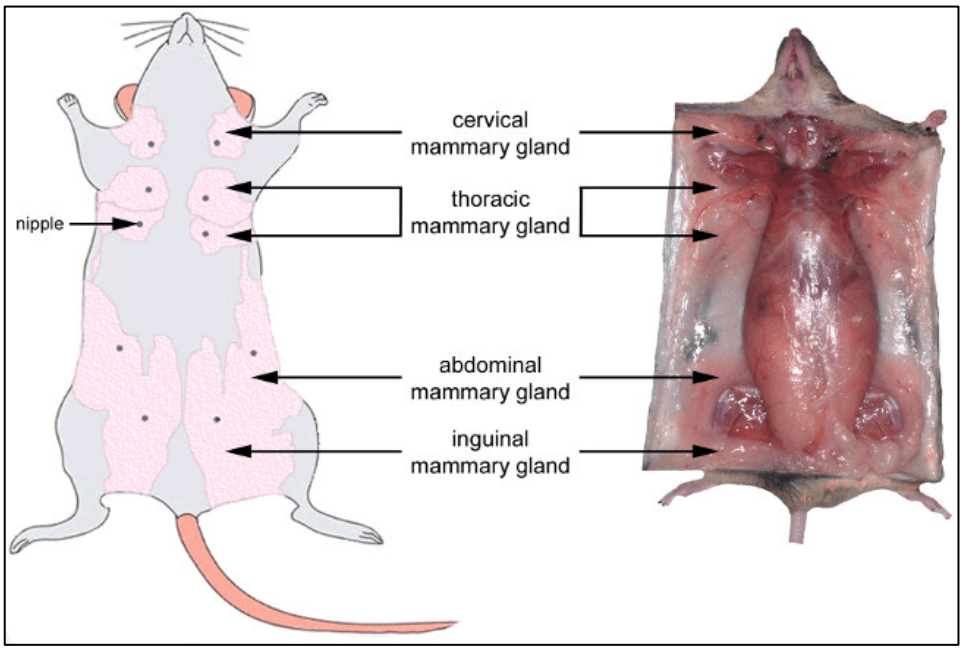


Figure 3: Mammary gland pairs in the mouse.
Five mammary gland pairs: one cervical, two thoracic, one abdominal and one inguinal.
Reproduced from *Indirect immunofluorescence on Frozen Sections of Mouse Mammary Gland* (Honvo-Houéto, N; Truchet, S. 2015)

The majority of the mammary gland development occurs postnatally beginning with the onset of puberty around 4-8 weeks of age (Figure 4). There is an invasion of the mammary fat pad by a primary ductal structure preceding birth (Figure 4 A), and this epithelial structure remains quiescent until approximately 3 weeks of age, when the ovaries begin to secret hormones. At this point, the terminal end buds appear and begin the process of ductal elongation, a process that continues until approximately 10-12 weeks of age, when the mice are considered sexually mature/adult (Figure 4 B). With the onset of the estrous cycle at puberty, the ducts begin to branch and give rise to the alveolar buds (Figure 4 C), which will differentiate completely during pregnancy becoming capable of milk production and secretion at parturition, a process that will continue for about 3 weeks (Figure 4 D). When this period of lactation ends, the gland begins a process of tissue remodeling involving apoptosis of the mammary epithelial cells, known as involution (Figure 4 E), which is irreversible after 2 days After approximately 2 weeks this involution process is complete, the gland regress back to a nearly pre-pregnant state and is ready to initiate another lactation cycle (6,11–13).

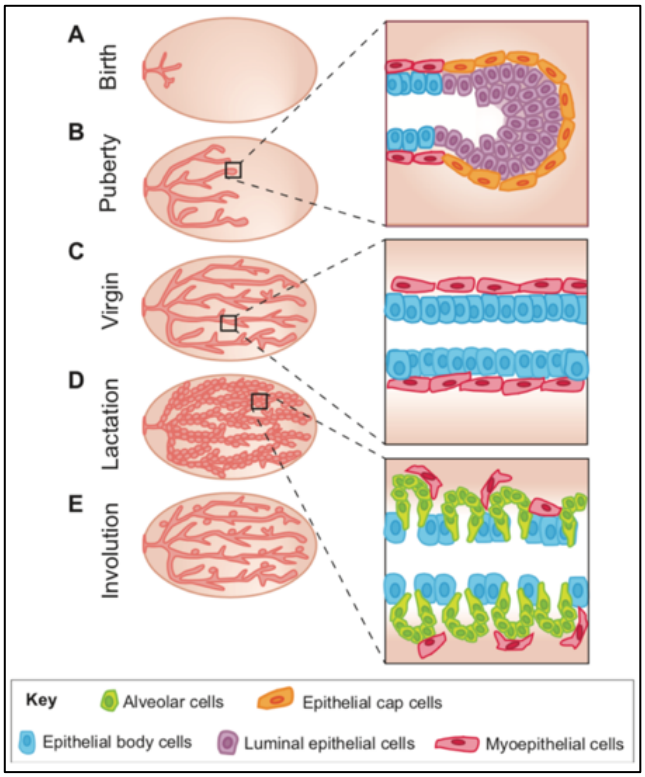


Figure 4: The mammary gland development is multistage and occurs after birth.
Distinct phases with specific features are observed from birth. It begins as a set of epithelial proliferations of ectodermic origin, that go deeper into the underlying mesoderm. At puberty, under the influence of the ovarian cycle, it suffers structural and functional changes, leading to the formation of several branches, forming channels and secretory alveoli. These alveoli reach their maximum expression during lactation and pregnancy.
Reproduced from *Mammary gland development: cell fate specification, stem cells and the microenvironment* (Inman, JL; Robertson, C; Mott, JD and Bissell, MJ. 2002)

Under the influence of the ovarian hormones, the epithelial cells of the ducts suffer some changes after puberty: at the beginning of the cycle the lumen of the gland is not evident; however it becomes more prominent on the later phases, sometimes even containing some eosinophilic secretion (5).

Continuous cycles of proliferation, differentiation and death are possible throughout the reproductive cycle, because of the existence of mammary stem and progenitor cells, capable of sustaining mammary gland morphogenesis in response to ovarian and pregnancy hormonal cues. The epithelium of the mammary gland presents two main lineages, which are the luminal cells and the myoepithelial cells; the first ones surrounding the lumen and the last ones being adjacent to the basement membrane. These lineages are organized and assure the formation of the branching ducts that terminate in secretory alveoli during lactation phases (14), as shown in Figure 5 and can be identified by the expression of specific cell markers.

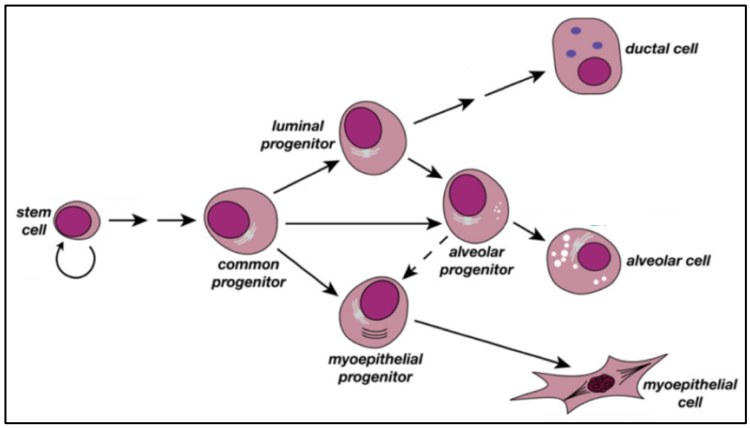


Figure 5: Model of the differentiation hierarchy within mammary epithelium.
From the stem cells arise the common progenitors, which will give rise to the two known progenitor lines: luminal progenitor, which will further originate the ductal and alveolar cells; and myoepithelial progenitor that will further originate the myoepithelial cells. The ductal luminal cells line the ducts and the alveolar luminal cells constitute the alveolar luminal units that arise during pregnancy, while the myoepithelial cells are specialized, contractile cells found at the basal surface of the epithelium.
Adapted from*: Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis* (Visvader, JE. 2009)

1.2.2. Cytokeratins as markers of epithelial and myoepithelial lineages

Keratins make up the largest subgroup of intermediate filament proteins. *Cytokeratins* (CKs) are cytoskeletal proteins, and these filaments that contain keratin-like proteins are characteristic of epithelial cells and represent the most abundant proteins in these cells (15–17). Based on the 2D gel migration, CKs are classified as basic (CK1 to CK8) and acidic (CK9 to CK20), and within each group, they are numbered in order of decreasing size. In a cell, these proteins exist as heterodimers, composed of acidic and basic subunits of a similar size, with certain pairs being expressed jointly, such as CK1/10, CK5/14 and CK8/18 (15,16).

CK5 was recognized as a high molecular weight CK, forming a heterodimer with CK14. It is expressed in the basal cells of stratified epithelium, and thus regarded as a "basal" keratin. This duplet is commonly found in cells that sit adjacent to the basement membrane and in a "basal position" from the ducts to the acini (18). Throughout the mouse mammary gland development, CK5 was found to be restricted to basal cells and was not detected until 10 days of age in basal ductal cells. It was subsequently detected at 42 days in acinar basal cells. Its expression was strongest in interlobular ducts, being absent to weak in acini and terminal buds. Finally, its expression was moderate at puberty, decreased with gestation and was back to moderate during lactation and involution phases (19).

CK8 was recognized as a low molecular weight CK that forms a heterodimer with CK18. This pair is usually expressed in simple epithelial cells, as it forms intermediate filament

proteins that play a key role in maintaining cellular structural integrity, while also functioning in signal transduction and cellular differentiation. CK8 is detected in the early embryonic epithelia (15). The lumen formation in the ducts of the postnatal mammary gland was associated with mild-to-moderate labeling of luminal cells for CK8/CK18 duplet, and this labeling was detected at all stages (early postnatal, puberty, gestation, early and late involution) excluding lactation when it was weak to absent. In the ducts and terminal end buds, the labeling of the pair CK8/18 was stronger, while it was generally weaker in the mammary acini (19).

The nature of CKs expressed by basal and luminal cells is conserved in mice throughout life, although expression levels of each are dependent on location in the mammary tree (19).

1.2.3. Estrogen and progesterone receptors

The expansion of the epithelial cell populations in the mammary ductal tree is regulated by ovarian steroids, particularly estrogen (through the *estrogen receptor* (ER)) and progesterone (through the *progesterone receptor* (PR)) (20–22). Although these hormone receptors are expressed before puberty and the fetus is exposed to maternal and placental hormones, it is generally accepted that the mammary gland develops up to this stage in a hormone-independent manner (21) and only becomes responsive to ovarian hormones when their levels begin to increase at puberty.

In the mammary glands, ER exists as ERα and ERβ, but the second has been shown to be dispensable for estrogen-mediated growth, although it may be important to maintain epithelial differentiation (23,24). Moreover, most antibodies to study ERβ are not reliable (25), which is why most studies regarding estrogen regulation of mammary gland morphogenesis focus on ERα. In mice, ERα is detected by immunohistochemistry in the mammary rudiment of 18-19 days-old embryos, and postnatally, from 1 day-old in both the mammary epithelial cells and in the dense fibrous stroma surrounding the epithelium, with a similar but more intense expression until day 7. In adult female mice, ERα expression is mainly associated with the luminal epithelial cells, not being detected in the fibrous stromal tissue around the periphery of mammary epithelial cells in distal ducts. ERα is also expressed heterogeneously in the adipocytes of the mammary fat pad and in the dense fibrous stroma around the nipple area and the primary mammary ducts. The levels of ERα expression decrease at the onset of pregnancy but is detected again during lactation (22).

Whereas the effects of ER have gathered widespread attention on their role in the mammary gland development and cancer, PR has been given less attention (26).PR is a downstream target gene of ER, which becomes expressed when cells are exposed to estrogen. Historically, PR was considered to be active during pregnancy and responsible for the alveolar cell proliferation leading to expansion of the milk producing units (27–31). However, PR function is also needed for BC growth (32,33) and it has recently been shown to regulate ERα transcriptional activity (27–31). PR expression is low to absent in the primordial epithelial rudiment of the fetus with only about 8% of epithelial cells expressing PR (20,22). In 1-day-old mice, there's a significant decrease in the percentage of PR positive cells in the epithelium, while at day 7 there is a dramatic increase in both percentage of positive epithelial cells and the levels of PR. There is a further increase in the number of PR positive epithelial cells until puberty, where approximately 55% of the epithelial cells are positive for this receptor. In the adult females, PR is only expressed in the luminal epithelial cells of the mammary ducts (22). The different stages as defined by the ER profile are also linked to the stages in breast development: before 3 weeks of age, estrogen does not induce proliferation in the mammary epithelium; however, during pregnancy, estrogen causes a rapid growth and maturation of the mammary gland and the concentration of PR is high. After this period, during lactation, the breast becomes insensitive to proliferative signals and PR-induced effects of estradiol. In the post lactation period, during involution, the plasma levels of estrogen become higher than they were during lactation and PR levels also increase in the breast (34–36).

2. Breast cancer

2.1. General aspects

BC is the most common non-skin malignancy in the world (Figure 6), it represents about one-third of all cancers diagnosed in women; it has the highest mortality rate for women (Figure 7) and second considering both genders (Figure 8). BC incidence increases rapidly after the age of 30 in women, and is rare in women younger than 25 years-old (37–41). According to the American Cancer Society, one in every eight women in the United States will develop BC in her lifetime and it has been predicted that the incidence of this cancer will reach approximately 3.2 million new cases per year by 2050, making it a major public health problem (37–39,42). When it comes to Portugal, the incidence and mortality have also been rising over the past few years, particularly in the North and Central regions (Figure 9). Even though BC

affects mostly women, men can also be diagnosed with BC, although the incidence is very low.

BC initiates when cells in the breast begin to grow out of control, usually forming a tumor that can be seen on an x-ray or felt as a lump, which are the most common signs of this disease. Breast tumors are considered malignant (cancer) when they grow and invade the surrounding tissues or metastasize through the blood or lymph system to distant areas of the body. Most breast lumps are benign and represent abnormal growths that do not spread outside the breast (1,38,43). BC can start from cells located in different parts of the breast, but the majority begins in the ducts (ductal cancers), while some start in the glands (lobular cancers) (1,38,43). There are also other types of cancer that can start in other breast cell types, such as sarcomas and lymphomas but these are not really thought of as BC (1,38,43).

Estrogen plays a critical role in BC development and progression (43). Estrogens bind to ERs which induce the expression of genes responsible for the proliferation of cancer cells. About 60% of premenopausal and 75% of postmenopausal patients present carcinomas that express ER and require estrogen to grow (estrogen-dependent or hormone-dependent). These BC types also express PR in about 70% of the cases (44). Estrogen and progesterone are breast cell mitogens, and the exposure to these hormones throughout a woman´s life has an impact on the risk of developing BC (1,42). The timely exposure according to three moments in a woman's life represents a major impact on BC incidence, which are age at menarche, age at first full-term pregnancy and age at menopause. Moreover, postmenopausal women with high circulating estrogen levels are at a greater risk of BC (1,42).

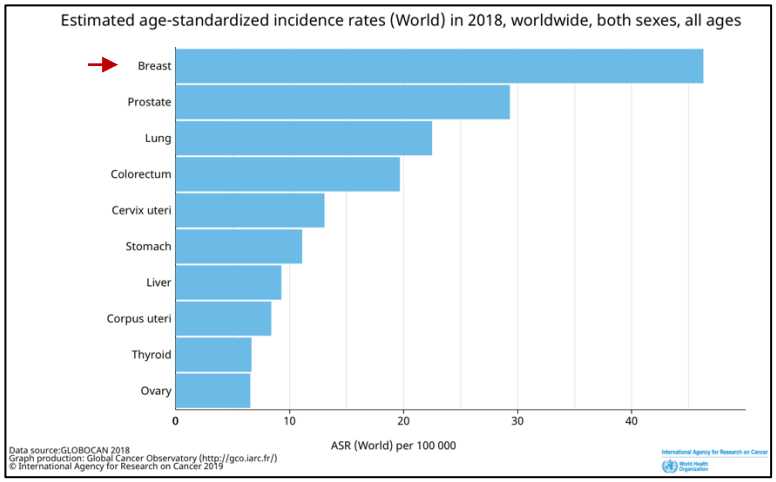


Figure 6: Cancer incidence rates in the world (2018) in both genders.
Breast is the most common non-skin malignancy in the world (red arrow). ASR – *Age Standardized Rate*
Reproduced from *GLOBOCAN* (2018)

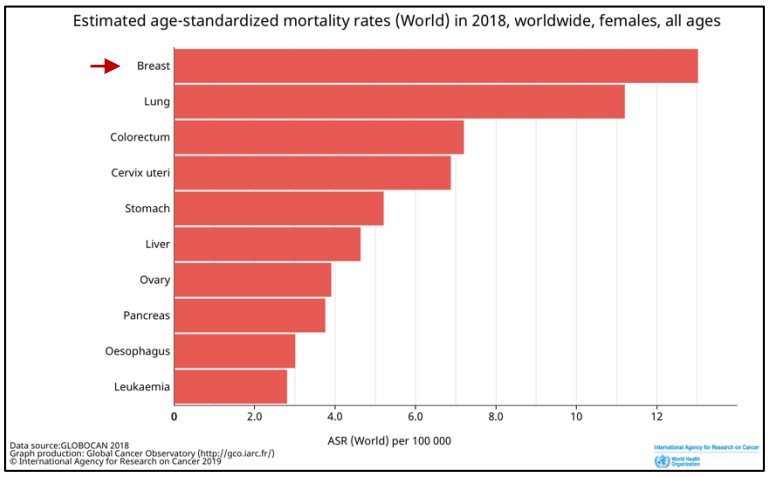


Figure 7: Cancer mortality rates in the world (2018) for females.
Breast as the malignancy with the highest mortality rate for women (red arrow).
Reproduced from *GLOBOCAN* (2018)

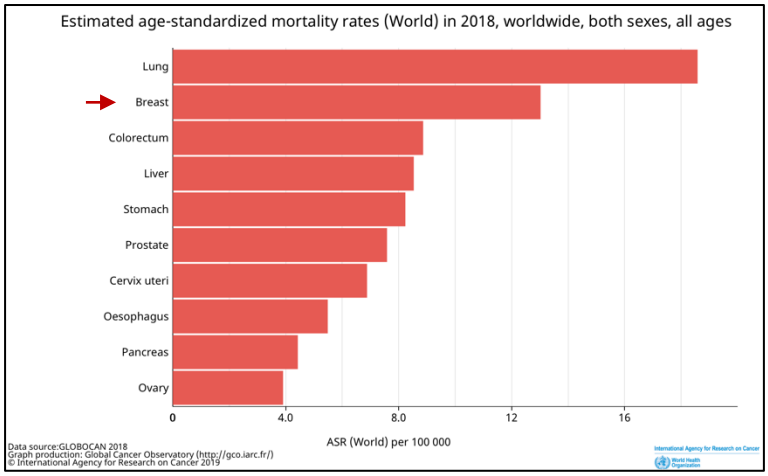


Figure 8: Cancer mortality rates in the world (2018) in both genders.
Breast as the second highest mortality rate considering both genders (red arrow).
Reproduced from *GLOBOCAN* (2018)

Figure 9: Incidence trends by period of BC in Portugal, from 2000 to 2013.
There's a clear rising incidence of BC in females in Portugal over time, particularly in the North and Central regions. These numbers seem more stable in the South,
Reproduced from ECIS – *European Cancer Information System* – Incidence and mortality historical data (2019)

2.2. Subtypes of breast cancer

BC presents marked tumor heterogeneity between patients, with specific subtypes which are associated with differing prognoses (45). Therefore, BC comprises several morphological tumor types, under several classification systems, which can display a distinct clinical behavior (45–47).

Clinically, BC classification is done on the basis of tumor morphological characteristics into different types, considering histological grade (including degree of cellular differentiation, nuclear pleomorphism and mitotic count, which help in further sub-classification) and molecular features (based on the expression of *human epidermal growth factor receptor 2* (HER2) and hormone receptor status, namely ER, and PR expression (38,39,46–48). According to its site and its ability to invade the adjacent tissues, a BC is divided into invasive and non-invasive, with the three most common histological types being a) ductal carcinoma *in situ* (approximately 13%; this type does not involve any metastasis, nevertheless it will become invasive during cancer progression) b) invasive ductal carcinoma (accounting for about 55% of all BCs), and c) invasive lobular carcinoma (about 5% among all BCs) (1,49). Seventy to 80% BCs are described as invasive ductal carcinoma *not otherwise specified* (NOS), while about 25% are defined as "special types" (49). In its histological evaluation, other features are considered to evaluate BC aggressiveness with the TNM system (***T****umor, Lymph* ***N****odes Involvement and* ***M****etastasis*), such as the primary tumor's dimensions and whether or not it

has invaded the chest wall, the number of affected nearby lymph nodes and their position on the nodal system, and identification of distant metastasis which is a sign of cancer spread beyond the site of the primary tumor (45,50).

The molecular subtypes are classified based on their immunohistochemical expression of ER, PR, HER2 and other hormone receptors, according to Table 1. These immunohistochemical markers have been consistently implemented in pathology laboratories with standardized staining and evaluation protocols worldwide (51). Considering the molecular features, different BC molecular subtypes have been described, namely Luminal (divided into Luminal A and B), HER2 positive, Triple negative (Basal-like, Claudin-low, Metaplastic BC and Interferon-rich) and Molecular apocrine cancer (43,46,49,51–53). Along with PR and HER2, ER is one of the most prevalent and important biomarkers for the BC classification, due to its crucial roles in breast carcinogenesis, whose inhibition forms the mainstay of BC endocrine therapy. ER positive tumors include up to 75% of all BC patients, and constitute 65% under 50 years and 80% older than 50 years. ER positive tumors are well-differentiated and less aggressive, being associated to a better outcome after surgical interventions than ER negative ones (47,49,51–54).

The identification of stem and progenitor populations and the consequential evidence of a hierarchical organization in the development of the mammary gland has permitted the characterization of the normal epithelial subtypes, which allowed a better understanding of the cells that are predisposed to oncogenesis, by considering their key molecular regulators. The longevity of several adult stem cells is a strong factor into the prospect of accumulating genetic mutations, and the molecular profiles found on several tumor subtypes might be a reflection of these mutations. With this in mind, a potential relationship between the epithelial hierarchy and the breast tumors subtypes is shown on Figure 10 (55). Particularly, regarding basal-like BC, its origin is not fully understood, this term derives from studies demonstrating tumor gene expression profiles that include some transcripts characteristic of the basal cells of the normal adult human mammary gland and others associated with a subset of normal luminal cells (56).

Age is one of the strongest risk factors for developing BC; however, the factors of aging that mediate these effects on BC are not fully known. It has recently been shown that BC samples from luminal BC exhibit strong positive age acceleration (denoted by epigenetic biomarkers such as *deoxyribonucleic acid* (DNA) methylation levels of 353 cytosine-phosphate-guanines), while basal BCs usually correlate with negative age acceleration (57).

Table 1: Summary of the breast tumor molecular subtypes.
Subtype classification based on the biomarkers' expression, their corresponding grade and clinical outcome.

Subtype	Alias	Biomarker status	Grade *	Outcome
Luminal	*Luminal A*	[ER+|PR+] HER2-**KI67-**	1|2	Good
	Luminal B	[ER+|PR+] HER2-**KI67+** [ER+|PR+] HER2+**KI67+**	2|3	Intermediate |Poor
HER2 positive	*HER2 over-expression*	ER-PR-**HER2+**	2|3	Poor
Triple Negative **	*Basal*	ER-PR-HER2-, **basal marker+**	3	Poor
	Claudin-low	ER-PR-HER2-, EMT marker+, Stem-cell marker+, **claudin-**	3	Poor
	Metaplastic BC	ER-PR-HER2-, EMT marker+, Stem-cell marker+	3	Poor
	Interferon-rich	ER-PR-HER2-, **interferon regulated genes+**	3	Intermediate
Molecular apocrine cancer	*Molecular apocrine cancer*	ER-PR-***androgen receptor*** **(AR) +**	2|3	Poor

* Grade according to the TNM system
** Triple negative types are not mutually exclusive.

Adapted from *Cancer Hallmarks, Biomarkers and Breast Cancer Molecular Subtypes* (Dai, X; Xiang, L; Li, T; Bai, Z. 2016)

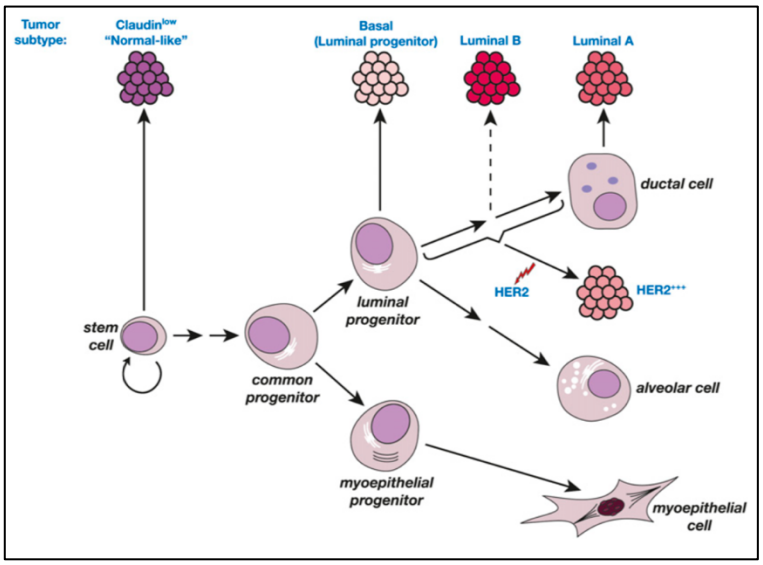


Figure 10: Schematic model of the human breast epithelial hierarchy and potential relationships with breast tumor subtypes.
Reproduced from *Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis (Visvader, JR. 2009)*

3. Aging

Aging is an inevitable process, characterized by an increased mortality rate accompanied by age after maturation, a process often associated with senescence (4,58) which is a state of decreased proliferation potential observed after multiple cell divisions that ultimately leads to cell cycle arrest usually in response to different damaging stimuli (59). Aging is defined as the time-dependent functional decline that affects most living organisms and is revealed through progressive changes in the biochemical composition of tissues, decrease in physiological capacity, reduced ability to respond adaptively to environmental stimuli and increased vulnerability to disease (4,58).

3.1. Common denominators of aging

Some of the common denominators of aging in different organisms have been described as being "*The Hallmarks of Aging*". These hallmarks explain the aging phenotype and evolution (Figure 11). All of these hallmarks have been associated with premature aging diseases or increased susceptibility to age-related deterioration. Since these hallmarks manifest during normal aging, its experimental aggravation accelerates aging and its experimental amelioration retards the normal aging process, hence increasing healthy lifespan (4). However, the processes that retard aging are also activated in cancer cells.

Figure 11: The hallmarks of aging.
The nine hallmarks that contribute to normal aging: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication.
Reproduced from *The Hallmarks of Aging* (López-Otín, C et al. 2013)

Genomic instability is both a source of genetic diversity and natural selection allowing mutations that can be beneficial for evolution, and a strong factor with catastrophic consequences on age-related diseases such as cancer (60). The integrity and stability of DNA are continuously challenged by exogenous physical, chemical and biological agents as well as by endogenous threats, and the mutations arise from inactivation of DNA repair pathways or due to genotoxic stress from cellular processes (60–62).

Telomeres are regions particularly susceptible to age-related deterioration (4,63–66). Telomeres protect the integrity of DNA (63–66), associate with shelter in proteins to prevent recognition by the DNA damage-repair system and regulate the activity of telomerase (64). In the absence of sufficient telomerase activity telomeres shorten with each mitotic cycle resulting in cumulative telomere attrition during aging, and when a critical length is reached, the cell will not further undergo cell divisions and become senescent or otherwise dysfunctional (4,63–66).

Epigenetics can control age-associated degeneration (4,67–70). These epigenetic alterations occur at various levels, during both organismal aging and replicative senescence. This promotes an altered local accessibility to the genetic material, leading to aberrant gene expression, reactivation of transposable elements, and genomic instability (4,67–74).

Proteostasis is defined as the maintenance of the proteome and appears to be highly relevant to both aging and disease. There are multiple mechanisms involved in the maintenance of proteostasis that seem to be indispensable for longevity in several species, including the *unfolded protein response* (UPR), the *ubiquitin (Ub)-proteasome system* (UPS)

and *autophagy* (AUT), which allow the correct folding and assembling of the proteins in well-defined three-dimensional structures (UPR), as well as disposal of misfolded and damaged proteins (UPS and AUT), which is necessary for correct cellular function. Proteostasis is important because when proteins become misfolded or are no longer functionally required, they must be degraded to avoid aggregation and damaging effects (4,75–79).

Nutrient sensing is regulated by several signaling systems, since nutrients are a crucial requirement for biological processes such as reproduction, somatic growth and tissue maintenance; and are, therefore, necessary for life (4,80–83).

Mitochondrial dysfunction has also been linked with aging. As cells and organisms age, the efficacy of the respiratory chain tends to diminish, thus increasing electron leakage and reducing *adenosine triphosphate* (ATP) generation (4,84–92).

Cellular senescence is a permanent state of cell cycle arrest that promotes tissue remodeling. It results from a variety of stresses and has been implicated in the promotion of a variety of age-related diseases (4,59,93–95).

Stem cell exhaustion refers to adult stem cells that are affected by the same aging mechanisms that involve somatic cells, resulting in their impaired regenerative capacity and depletion of stem cell pools in adult tissue (96). It impairs the regenerative capacity of the organs (97) and correlates with accumulation of DNA damage and telomere shortening (4,96–100).

Intercellular communication changes are also involved in the aging process, beyond the cell-autonomous alterations. With this, neurohormonal signaling tends to be deregulated, while inflammatory reactions increase, immunosurveillance against pathogens and premalignant cells declines, and the composition of the peri and extracellular environment changes. These changes in tissues can lead to aging-specific deterioration including carcinogenesis (4,101–106).

3.2. The use of mice as a model to study aging and age-related diseases

The use of humans in research is complicated by ethical issues, environmental and social factors and human´s long natural life span. Although cellular models of human physiology and disease provide valuable mechanistic information, they are limited in that they may not replicate the *in vivo* biology. On the other hand, almost all organisms age, and thus animal models can be useful to study aging (107).

The mouse has become the favorite mammalian model for aging research, especially due to its genetic proximity to humans, the possibilities of genetically manipulating their genomes and the availability of tools, mutants and inbred strains. Besides that, mice have a life expectancy of only a few years, genetic approaches and other strategies for intervening in aging can be tested by examining their effects on life span and aging parameters during a relatively short period. Despite all these advantages, there are some important age-related differences between mice and humans: mice have long telomeres and high telomerase activity in many organs; they can synthesize vitamin C while humans cannot, and the availability of vitamins is of great importance because vitamins can influence certain aging processes; mice do not typically demonstrate age-related diseases seen in humans such as cardiovascular diseases and Alzheimer's disease (107–109). Also, of important notice is the biological phenomenon of menopause: curiously, rodents, rats and mice present some features and endocrine changes found in humans, including decline in follicles, irregular cycling and steroid hormone fluctuations and irregular fertility; occurring approximately at 8 months of age. However, only 25-40% of the animals will naturally model the human menopause, while in the remaining there is a spontaneous transition into a polyfollicular anovulatory state of constant estrus and low levels of progesterone (110).

3.3. Physiologic and pathologic protein aggregation

Protein complexes and their formation are an important part of biological systems. Nonetheless, there are other forms of aggregations that have a negative impact on metabolism and protein turnover, possibly leading to cell death. Even though it is still not clear how this aggregations occur, the irreparable damage to proteins derived from oxidative stress is clearly involved in physiological aging (111).

Most secretory proteins and non-cytosolic domains of transmembrane proteins are co-translationally transported in an unfolded state into the lumen of *endoplasmic reticulum* (EnR), where resident chaperones prevent these nascent proteins from aggregating as they fold into their native conformations (112). This process might fail due to amino acid mis incorporation subsequent from genetic mutations, errors in transcription, *messenger ribonucleic acid* (mRNA) processing or translation; or, alternatively in response to environmental factors such as thermal stress, osmotic and oxidative stress, interference from viral gene products that can interfere with the folding of nascent polypeptides, or unequal

synthesis of subunits of heterooligomeric protein complexes that can lead to the production of unassembled polypeptide chains (113,114).

Many misfolded and unassembled proteins inappropriately expose hydrophobic surfaces that are normally buried in the protein's interior or at the interface with other subunits and that can interact to form aggregates (113). The mechanisms that prevent or regulate protein aggregation tend to decline during aging, so once these aggregates form, they become hard to degrade or unfold, being quite often associated with several aging-related degenerative diseases, including amyloidosis, Alzheimer's and Huntington's diseases. The mechanism by which these aggregates damage the cells is not fully clear yet, and it is not fully understood if these aggregates are a cause or a consequence of the underlying pathology. However, the conformational change and the induced structural instability of these proteins may promote the disease either by gaining toxic activity or by the lack of the biological function of the native protein (113,115,116). One example are the beta-amyloid proteins.

Amyloid diseases are characterized by the deposition of insoluble fibrous amyloid proteins, and although this deposition is a characteristic feature of Alzheimer's disease, it is also common in elderly normal controls (117,118). Several proteins are known to be converted to this aggregated amyloid state, including fibrils associated with systemic and neurodegenerative diseases and cancer, functional amyloid fibrils and denatured proteins (119). However, the mechanism of toxicity of the amyloid aggregates is only partially understood: the soluble oligomers that are formed expose sticky surfaces (hydrophobic amino acid residues and unpaired β-strands), disturbing the phospholipid bilayers and engaging in aberrant interactions with multiple key cellular proteins (116).

3.4. Unfolded protein response

The EnR has elaborated mechanisms to ensure that only properly folded and assembled proteins are released, a process termed as "quality control", which discriminates between folded and misfolded or unfolded polypeptides. When protein folding is inhibited in the EnR, signal transduction pathways that increase the biosynthetic capacity and decrease the biosynthetic burden of the EnR to maintain the homeostasis in this organelle are activated, being these pathways UPR, which is represented on Figure 12. UPR-signaling pathways respond to the nutritional state of a cell and control well-established regulatory gene clusters involved in metabolism and starvation responses. The chaperone machinery recognizes a protein as folded or unfolded (114,120–124). Prolonged interaction of a folding protein with

the chaperone machinery activates three EnR resident transmembrane proteins (EnR stress sensors): *activating transcription factor 6* (ATF6), the *inositol requiring kinase 1* (IRE1), and *double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase* (PERK), which then transduce an *unfolded protein* (UP) signal across the EnR membrane. Under chronic stress Ca^{2+} is then released from the EnR to activate apoptotic-signaling pathways (112,120,121). The *binding immunoglobulin protein* (BiP) is the EnR chaperone, and the master regulator of the activation of the three EnR stress sensors (PERK, ATF6 and IRE1) providing a direct mechanism to sense the folding capacity of the EnR (112,120,121,125–129).

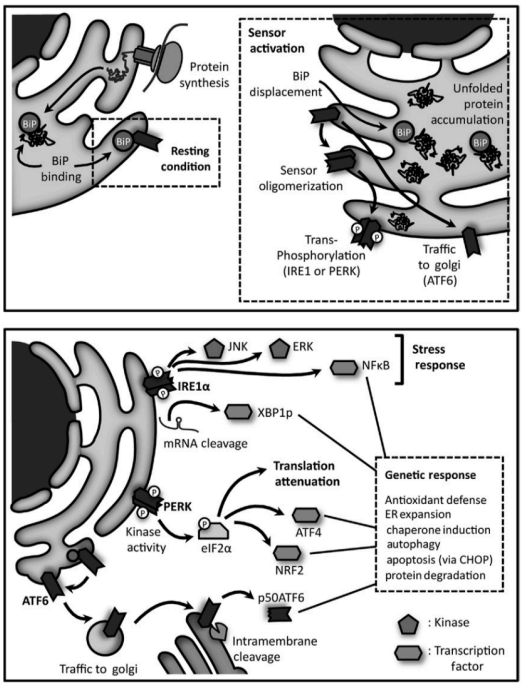


Figure 12: The Unfolded Protein Response.
In resting conditions, the stress sensors IRE1 and PERK and ATF6 interact with BiP/GRP78. Accumulation of misfolded proteins in the EnR lumen separates the chaperone from each sensor. PERK is an EnR transmembrane protein kinase responsible for the phosphorylation of the *α subunit of eukaryotic initiation factor 2* (eIF2α), reducing the formation of translation initiation complexes which in turn leads to reduced recognition of AUG initiation codons and therefore leads to general translational attenuation and reduction of the number of UPs in the EnR. ATF6 is an EnR transmembrane-activating transcription factor which upon EnR stress transits to the Golgi compartment where it is cleaved and the free ATF6 fragment migrates to the nucleus to activate transcription. Finally, IRE1 is an EnR transmembrane glycoprotein with both kinase and RNase activities in the cytoplasmic domains whose RNase activity is activated due to the autophosphorylation of this protein, induced by EnR stress, to splice XBP1 mRNA, the substrate of IRE1α and IRE1β. The signal from downstream effectors of these EnR stress sensors merges in the nucleus to activate the transcription of UPR target genes: transcription factors (XBP1, ATF4, p50ATF6, NF-κB, CHOP) and protein kinases (JNK, AKT). If the cell does not reduce the misfolded protein overload, apoptosis is triggered.
Reproduced from *Endoplasmic Reticulum and the Unfolded Protein Response: Dynamics and Metabolic Integration* (Bravo, R et al. 2013)

3.5. Autophagy and the ubiquitin-proteasome system in the maintenance of proteostasis

Proteolysis in eukaryotic cells is mainly mediated by the UPS and the AUT-lysosome system. Initially, these mechanisms were thought to be independent from one another, with the ubiquitination long been recognized as a key determinator for tagging proteins for proteasomal degradation; however, recent studies suggest that cells operate a single proteolytic network comprising of the UPS and AUT that share notable similarity in many aspects and functionally cooperate with each other to maintain proteostasis (Figure 13).

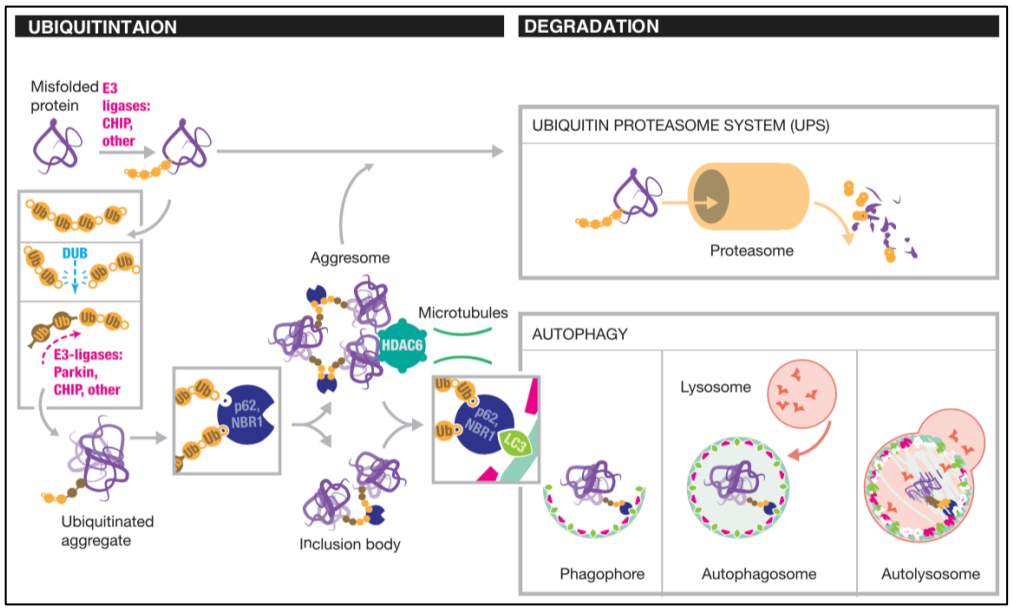


Figure 13: The ubiquitin code links between proteasomal and lysosomal degradation, a model for different degradation routes of a misfolded protein.
A misfolded protein can be degraded by proteasome or lysosomal system. Chaperone-dependent E3-ligase leads to ubiquitination of the misfolded protein, which targets for the proteasome. Under certain conditions i.e., if the capacity of the chaperone-mediated refolding machinery and the UPS are overloaded, protein aggregation occurs, and these aggregates are then targeted for autophagic clearance. Thereby, Ub chains on misfolded proteins can undergo remodeling by combined activity of *deubiquitinating enzyme* (DUB) and E3-ligases. Newly formed Ub chains are then recognized by the *Ub binding domain* (UBD) of p62, *neighbor of breast cancer 1* (NBR1) to form inclusion bodies or by the corresponding UBD of *histone deacetylase 6* (HDAC6), which direct protein aggregates to the aggresome. Aggresomes can be degraded via the proteasome or via autophagy pathway. If degradation occurs via autophagy, targeting of the protein aggregates are determined by the *light chain 3* (LC3) *interacting region* (LIR) motif of p62 and NBR1. Autophagy takes part in distinct steps. Cytosolic components are enclosed by an isolation membrane so-called phagophore, which leads to the formation of the autophagosomes. Thereby, LC3 and other *autophagy-related gene* (ATG) proteins such as the ATG 5/12/16L complex are recruited to autophagosome. Finally, the outer membrane of the autophagosome fuses with the lysosome and the internal material is degraded in the autolysosome.
Reproduced from *Ubiquitination and selective autophagy* (Shaid, S; Brandts, CH; Serve, H; Dikic, I. 2013)

AUT is a general term for the degradation of cytoplasmic components within lysosomes, comprising several steps from the sequestration of the components to be

degraded, their transportation to the lysosomes, degradation and utilization of the degradation products (130–132). Three types of autophagy have been documented and described so far a) macroautophagy, b) microautophagy and c) chaperone-mediated autophagy (Figure 14), which differ in the mechanism by which substrates are delivered to lysosomes, their regulation and their selectivity (133,134).

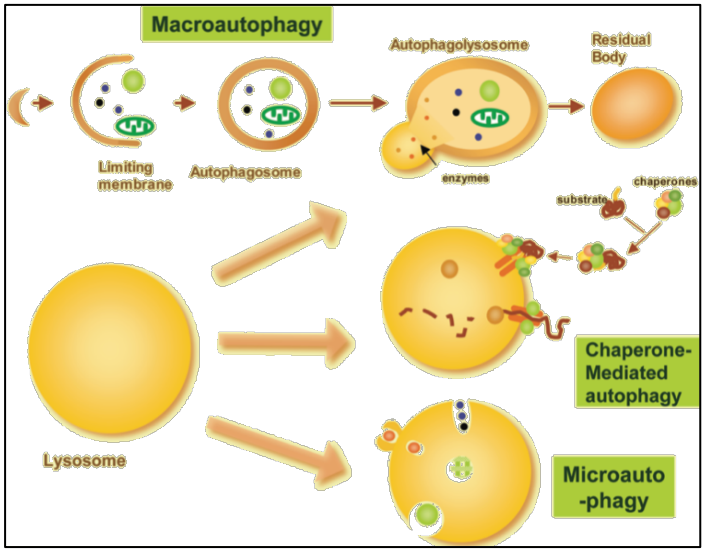


Figure 14: Schematic model of the types of autophagy in mammalian cells.
Internalization of complete regions of cytosol first into autophagosomes that then fuse with lysosomes (macroautophagy), or directly by the lysosomal membrane (microautophagy) contrast with the selective uptake on a molecule-by-molecule basis of cytosolic proteins via chaperone-mediated autophagy.
Reproduced from *Autophagy and Aging: The Importance of Maintaining "Clean" Cells* (Cuervo, AM et al. 2005).

During aging, the efficiency of autophagic degradation declines and intracellular waste products accumulate. Since autophagocytosis has a key role in cellular housekeeping by removing damaged organelles, this decline during aging has also been associated with the regulation of aging and age-related degenerative diseases (131,135,136).

In all tissues, the majority of intracellular proteins are degraded by the UPS; however, extracellular proteins and some cell surface proteins are taken up by endocytosis and degraded within lysosomes, and some cytosolic proteins are degraded in lysosomes after being engulfed in autophagic vacuoles that fuse with lysosomes, through the mechanisms already explained (137).

4. The impact of aging in the mammary gland carcinogenesis

The average lifespan is represented by the age at which half of a given population survives, while the maximum lifespan potential represents the longest-lived members of the population or species. Over time, the average lifespan of humans has increased but the maximum lifespan potential has remained approximately constant. Socioeconomic and nutritional status along with disease have been strongly associated with life expectancy and its variations, and as these causes of early mortality are eliminated (due to public health measures and improved medical care) more individuals reach the maximum lifespan (usually stated to be 90-100 years for humans). Considering this scenario, the maximum lifespan potential appears to be species specific, implying a significant genetic component to the rate of aging (58,138). Many discoveries have suggested a tight link between aging and disease mechanisms, where macromolecular damage and the ability to respond to damage are compromised (76,139,140). More recently, additional commonalities have emerged between aging biology and stress biology; moreover, the current situation of aging research exhibits many parallels with that of cancer research. The time-dependent accumulation of cellular damage is widely considered to be the general cause of aging. Occasionally, cellular damage might provide aberrant advantages to certain cells that can eventually lead to cancer. Therefore, these mechanisms, cancer and aging, can be regarded as two different manifestations of the same underlying process, the accumulation of cellular damage (4,59,141).

Specifically, BC shows a dramatic increase in incidence after menopause, probably associated with the lower protective effect of estrogen. The mammary stem and progenitor cells might also have an impact on this disease. The adult stem cells of the mammary gland are related to processes that involve growth, proliferation, migration, branching, invasion and apoptosis. These processes are present in normal gland development but also occur during the development and progression of malignant diseases, which suggests that these stem cells may be particularly susceptible targets for transformation in BC, due to their relatively long life span and consequent susceptibility to pass on their genetic alterations to their daughter cells along the hierarchical differentiation (142,143).

5. Proteostasis deregulation in BC and its relationship with therapy response

As described on the chapter relative to the UPR, disruption of the EnR homeostasis can cause misfolded proteins to accumulate within the EnR lumen, which normally leads to a transient activation of the UPR to reduce the accumulation of unfolded proteins and restore EnR function or, in case of a prolonged UPR signal, triggers cell death (114,120–122,124,144–147). In the microenvironment of solid tumors, cancer cells are exposed to various stressors that can lead to perturbed protein folding in the EnR and consequent activation of the UPR. Therefore, many tumors display constitutive UPR activation, allowing them to adapt and thrive under stressful conditions. In BC in particular, this increased UPR is believed to contribute to most hallmarks of cancer and therapy resistance (144). Simultaneously, AUT is responsible for the removable of damaged intracellular molecules and organelles, suppressing genomic instability and preventing the accumulation of deleterious alterations in the genome, thus limiting initiation of cancer formation. However, this process might also lead to the AUT-dependent cell death under stressful conditions, revealing its complex role in the context of tumorigenesis and cancer progression (148). Despite its role in inhibition of tumor formation, AUT becomes a necessity for cancer cell survival in an established tumor, and it is activated in response to various stressors. The proliferation of tumors can promote metastasis and lead to nutrient deficiency, one stress factor. Here, AUT tends to induce the metastatic process by maintaining and spreading cell survival and inducing the cells into a dormant state if they cannot establish stable contact with the extracellular matrix in the new environment (148,149).

BiP is a chaperone that has been shown to be upregulated in many human cancers, along with *cell migration-inducing protein* (CEMIP); together driving cancer progression and metastasis CEMIP resides in the EnR and interacts with BiP to induce cell migration, promoting activation of BiP promotor and upregulating BiP transcript and protein levels in BC. This upregulation leads to a reduced apoptosis – by binding directly to the pro-apoptotic protein BIK and blocking its downstream signaling and the consequent release of cytochrome c from the mitochondria -, and enhances autophagic activity under hypoxia, which might support the ability of these cancer cells to survive and proliferate under stressful conditions. However, the mechanisms by which BiP exerts these functions are still poorly understood (150).

The development of the *hormone therapy* (HT) that blocks the estrogen synthesis is a very important treatment for ER positive breast tumors. However, cancer cells might acquire resistance. It was found that the apoptosis induced by the estrogen starvation requires BIK,

the pro apoptotic protein located primarily at the EnR. Since BIK selectively forms a complex with BiP, when BiP is overexpressed in the tumors, the apoptosis induced by this therapy decreases. Therefore, the expression levels of BiP might be a prognostic marker for the response to the HT based on the estrogen starvation (151).

This might be explained by the fact that the higher BiP levels confer a better proteotoxic resolution, which simultaneously leads to a milder UPR activation, favoring the survival responses of the tumor (152). Therefore, HT resistant tumors would be expected to show lower amounts of protein aggregates when in comparison to those non-resistant and this distinction might be useful to predict therapy responsiveness and reduce the exposure to therapeutic agents that are not likely to be beneficial (152).

6. Aims

The mammary gland development and remodeling relies on the differentiation of adult mammary stem and progenitor cells. These cells have a long life and are prone to accumulate genetic and epigenetic alterations that could lead to neoplasia. Since the different BC subtypes correlate with different cell differentiation degrees, it is thought that they could arise from different mammary cell lineages. Therefore, in the first part of this work (Chapter 1) the aim was to investigate if aging targets the different cell types. For this purpose, mammary tissue from C57/Bl6 mice was used to characterize the morphological changes in the mammary glands throughout aging female mice and to evaluate the expression of luminal and basal/myoepithelial cell markers (CK8 and CK5, respectively) and hormone receptors (ER and PR). The second part of this work was based on preliminary data from the lab, where it was showed that in ER+ BC cells, hormone therapy (HT) induces an increase in protein aggregation, which is toxic and leads to cell death, while cells that develop resistance to HT can eliminate protein aggregates more efficiently than sensitive cells. Therefore, in Chapter 2, the levels of protein aggregation were investigated in a cohort of human BC cases before and after HT. Consequently, the specific aims and objectives of this work are:

1. Characterization of the histomorphological changes in mouse mammary glands ages 1-29 months-old using tissue sections stained with H&E and bright field microscopy;
2. Preparation of mouse mammary tissue sections and set up the immunostaining of CK8, CK5, ER, PR proteins in mouse mammary glands ages 1-29 months-old;
3. Quantification of the protein aggregation levels in human breast cancer tissues using ProteoStat®;

4. Evaluation of changes in the percentage of cells expressing CK8, CK5, ER and PR by immunohistochemistry in mouse mammary glands ages 1-29 months;
5. Analysis and quantification of the protein aggregation levels in BC samples using ProteoStat®.

Materials and Methods

1. Evaluation of the mammary gland morphology and epithelial markers throughout aging

1.1. Acquisition of mouse mammary gland samples

A total of 40 mouse mammary gland samples were obtained from C57BL/6 virgin female mice, with ages between 1-29 months-old: 1 month old (n=5); 3 month old (n=2); 6 month old (n=5); 13 month old (n=5); 18 month old (n=6); 24 month old (n=13) and 29 month old (n=4). In addition, 30 uterine samples were also collected from the same animals, even though they were not included in the purpose of this study. The samples were fixed in 10% neutral buffered formalin (Sigma-Aldrich) for 24 hours and post-fixed in 70% ethanol (Sigma-Aldrich). All samples were routinely processed and embedded in paraffin wax. Sections of 3µm thickness were cut using a Thermo Fisher Scientific HM 340E microtome for light microscopy and immunohistochemistry. Due to the amount of tissue, some samples were divided in two or more blocks, but 5 presented poor preservation, 4 were lost during processing, 1 did not present mammary gland (only fat pad) and 5 presented tumors (4 ovary tumors and 1 uterus tumor), so were excluded from this study, while the remaining 32 samples are summarized on Table 2.

Table 2: Description of the mice mammary gland samples used in this study. The table presents details regarding age, mice identification numbers and total number of samples per age group.

Age (months)	Mice identification	Number of samples
1	1	4
	3	
	4	
	5	
6	1	5
	2	
	3	
	4	
	5	
13	X	5
	1	
	3	
	4	
	5	
18	2	4
	3	
	4	
	5	
24	1	11
	3	
	4	
	6	
	7	
	8	
	9	
	10	
	11	
	12	
	13	
29	1	3
	2	
	4	

Note: X – tube of the sample without identification.

1.2. Histological analysis of the mammary gland samples

A section of each sample was stained with *hematoxylin and eosin* (H&E) for quality control of fixation and evaluation of the mammary glands by light microscopy (Nikon Eclipse E600, using the imaging software NIS-Elements D). This protocol was performed by the co-supervisor Prof. Adelina Gama.

The tissue sections were analyzed under a inverted widefield microscope equipped with a CCD color digital camera (Nikon eclipse Ti-U and DS-Fi1c, respectively).

1.3. Immunohistochemistry

The slides containing the tissue sections were deparaffinized in xylene (Sigma, MO, USA) and hydrated through decreasing alcohols (Sigma-Aldrich)) (70%, 80%, 90% and finally 100%), for 5 minutes. Antigen retrieval was performed by microwave treatment in 10mM citrate buffer (2.94g tri-sodium citrate diluted in distilled water up to 1L, adjusting pH=6,0 with 1N Hydrochloridric Acid). After cooling down, slides were washed with *Phosphate Buffered Saline* (PBS) (8g Sodium Chloride, 0.2g Potassium Chloride, 1.44g Sodium Phosphate, dibasic, 0.24g Potassium Phosphate, monobasic, diluted in distilled water up to 1L, adjusting pH=7.4 with 1N Sodium Hidroxide). Endogenous peroxidases were blocked by an incubation with 3% H_2O_2 (diluted in PBS) for 30 minutes. After washing with PBS, protein blocking was performed with 10% *Fetal Bovine Serum* (FBS) (diluted in PBS). Incubation of the primary antibody was performed overnight at 4ºC. The following antibodies were tested on the mice samples: **CK5** [EP1601Y] (ab52635, *abcam*, Cambridge, United Kingdon), **CK8** [EP1628Y] (ab53280, *abcam*, Cambridge, United Kingdom), **ERα** (F-12) (sc-8002, *Santa Cruz Biotechnology*, USA) and **PR** (C19) (sc-538, *Santa Cruz Biotechnology*, USA). After washing with PBS, secondary antibody was incubated for 30 minutes at room temperature and the results were revealed using the Vectastain® Elite® avidin-biotin complex (ABC) Kit (Vector Laboratories, USA) method and color was developed with 3,3' *diaminobenzidine tetrahydrochloride* (DAB) (diluted in PBS, adding 1µL of H_2O_2 30%/mL of solution). Washing with PBS was performed between these two steps. Sections were counterstained with Hematoxylin for 2 minutes, dehydrated, diaphanized and the slides were mounted with mounting medium for evaluation by light microscopy.

To determine the adequate dilution factor for each antibody, several dilutions were tested on different samples (besides the ones included on this study) until the desired results were achieved with no background and clear identification of the positive areas.

When facing troubleshooting issues several conditions were tested: different buffer solutions (prepared from the dissolution of Sodium Chloride, Potassium Chloride, Sodium Phosphate and Potassium Phosphate; or from tablets of PBS diluted in distilled water), different antigen retrieval times and temperatures (from 10 to 30 minutes, on different power modes of the microwave), different incubation times and temperatures (at 4ºC overnight or at

room temperature for 1 hour), different lots of reagents (including the ABC Kit, the *diaminobenzidine* (DAB) solution and the antibodies) and even different clones of primary antibodies (particularly for ER: ER (C-311) (sc-787, *Santa Cruz Biotechnology*) and ER(33) (*Novus Biologicals/GeneTex*)) or other different antibodies that are known to work well in mouse tissues (such as E-cadherin (610181, *BioSciences*); different lots of secondary antibodies) were also tested. These tests, their results and explanations are summarized on the Results section.

1.4. Acquisition of human samples

A total of 23 cases from human breast tumors were obtained from the Instituto Português de Oncologia do Porto through our collaboration with Profs. Rui Henrique and Cármen Jerónimo. The samples were selected by Dr. João Lobo, together with Profs. Carmen Jerónimo and Rui Henrique with the following criteria: samples from patients before HT and metastases appearing after this therapy. Three of the cases were excluded from the study due to the lack of tissue on the slide that prevented any comparison. Acquisition of samples followed all national and European ethical guidelines.

1.5. Detection of protein aggregates

The protein aggregates in the human samples were evaluated through immunofluorescence using the ProteoStat® Aggresome Detection Kit (*Enzo Life Sciences*), which includes a molecular rotor dye that specifically intercalates into the cross-beta spine of quaternary protein structures typically found in misfolded and aggregated proteins, which will inhibit the dye's rotation and lead to a strong fluorescence.

The protocol was applied according to the manufacturer's recommendations, adding deparaffinization and hydration steps in the beginning. The optimal dilution factor was tested, starting with the recommended for the cells and then lower concentrations, defining the final dilution factor at 1:1000. A protocol to lower autofluorescence was also performed with an incubation with Sodium Borohydride (1mg/mL diluted in PBS) for 3 cycles of 10 minutes each before the step with the blocking solution (FBS 10%), but no differences where observed so in the actual samples, this step was overlooked. The remaining steps were taken according to the manufacturer's indications. The tissue slides were observed on The Zeiss Axio Imager Z1 - a motorized upright widefield microscope equipped with a CCD monochromatic digital camera (Axiocam HRm). The amount of fluorescence for the protein aggregates quantification

was performed using Image J in approximately 10 random fields per sample at 63X magnification, by manually selecting the areas of tissue. In the software, the procedure taken for all these samples included defining the "Set Measurements" in order to evaluate the "Area", "Integrated Density" and "Mean Grey Value". Then, the selected areas previously mentioned were measured with the software by addressing to "Analyze" and then "Measure". To these values were subtracted the values calculated for the negative areas. The data obtained from each sample was saved on an Excel file and the average and standard deviation were calculated for each sample. Afterwards, the difference of this average between before and after treatment was calculated for each case.

2. Data analysis

For the mammary gland morphology evaluation, the overall structure (architecture and cell composition) was evaluated; with regard to the immunohistochemical findings, the presence of a brown staining in the epithelial cells was considered positive (cytoplasmic/membranous for CKs and nuclear for hormonal receptors).

Regarding the detection of the protein aggregates, the intensity of fluorescence was evaluated for each tumor before the HT and compared to the fluorescence of the metastasis that occurred after HT.

3. Statistical analysis

The evaluation of the overall structure of the mice mammary gland was qualitative, no statistical tests were used.

For the study of the protein aggregates on the human tumor samples, was applied the Wilcoxon test (non-parametric test for two paired samples) with a significance level of 95% (α=0,05).

Results

1. Histological and immunohistochemical analysis of mouse mammary gland samples throughout aging

1.1. Histological evaluation of mouse mammary gland samples

The mammary gland has a complex architecture and is composed by several cell types, constantly under the influence of hormones and suffering cyclic changes. The morphological changes throughout development of the mammary gland have been described and characterized. The process of aging and cancer development have also started to be linked together, and with the aim to find an association to justify the susceptibility to the development of BC, the morphological changes on the aging mammary gland were analyzed and later correlated to the expression of epithelial differentiation markers.

Mammary gland samples from one-month female mice were characterized by the presence of highly proliferative terminal end buds, which contained an outer layer of cap epithelial cells surrounding multilayered body epithelial cells, with several mitotic figures. The connective tissue proximal to the cap cells of the terminal end buds was absent/reduced, while at its trailing edge the cells were differentiating, and the stroma was forming around the ducts. Sparse ducts were observed surrounded by connective tissue interspersed within an adipose stroma, characterized by a single layer of luminal epithelial cells, surrounded by a single layer of myoepithelial cells. After this time point (3 to 29 months old), the mammary samples showed identical histomorphologies, with an increase in ductal branching, when in comparison to 1 month-old samples. Ductal structures were characterized by an outer layer of myoepithelial cells and an inner layer of luminal epithelial cells, as shown on Figure 15. The mammary gland of the elderly female mice (24 and 29 months old) showed occasional features of mild ductoalveolar hyperplasia characterized by the presence of secretory alveoli and distended ducts containing secretory material. Moreover, at 1 month-old, in some cases, severe features of poor preservation were found, such as lack of nuclear detail and cytoplasmic definition (as seen on Figure 15 H and I) and some residual yellow-brown pigment in the majority of the samples (data not shown due to technical difficulties to obtain a focused picture of it). On the remaining samples were not identified severe features of poor preservation, however, the limits of the cells were hard to perceive, which made impossible to determine the exact proportion of each cell type on each gland; which could also be an indicator of deficient fixation.

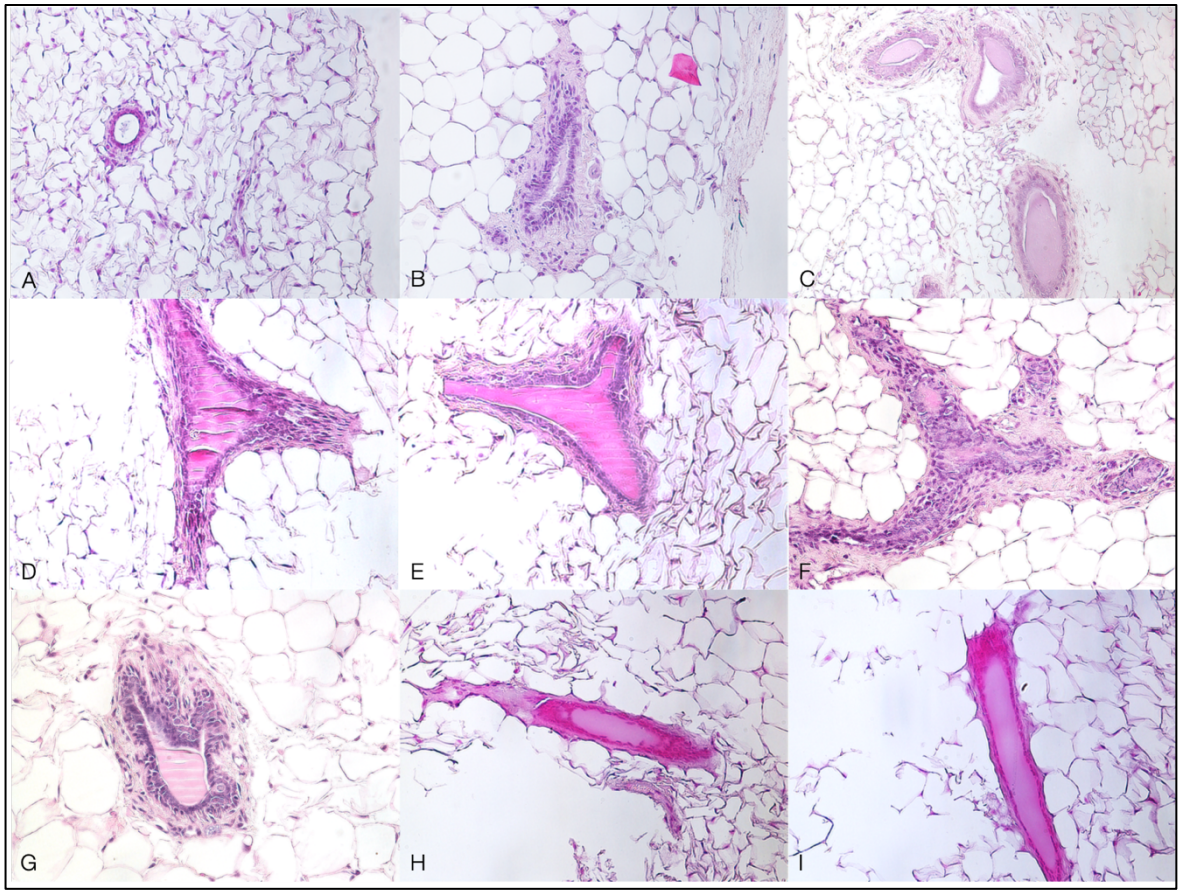


Figure 15: Mammary gland histology throughout aging.
Panel exhibiting the evolution of the mouse mammary gland throughout aging of mice: **(A)** 1-month-old **(B)** 3-months-old **(C)** 6-months-old **(D)** 13-months-old **(E)** 18-months-old **(F)** 24-months-old **(G)** 29-months-old **(H)** and **(I)** mammary gland tissue of a 18-months-old female mouse exhibiting features of a poor cellular preservation, such as lack of nuclear detail with no definition of the cellular limits associated with eosinophilia. The same objective magnification (10x) was used on all images on all images.

1.2. Immunohistochemical analysis of mouse mammary gland samples throughout aging

The mammary epithelium is heterogeneous and presents a series of cell hierarchies, some of which can be identified with specific markers. It has been postulated that the different cell types in the mammary gland originate the different BC subtypes. Given that in post-menopausal women, the most prevalent BC form is the luminal form, which consists of well differentiated epithelial, hormone receptor +, CK8 + cells, we decided to analyze the expression of these markers across the aging mouse mammary glands. For this purpose, we used CK8 and CK5 to differentiate epithelial from basal/myoepithelial cells and ER as well as PR to determine their degree of differentiation.

1.2.1. Cytokeratins 5 and 8

To study the epithelial cell markers in the development of the mouse mammary gland, the antibodies were tested on older histology slides from uterus of Balb/c mouse and mammary tumors to determine the ideal dilution factor and incubation times. During these tests an optimal expression was found with a dilution factor of 1:200 for CK5 and 1:250 for CK8, with an incubation at 4ºC overnight, and the results are shown below (Figure 16 and Figure 17).

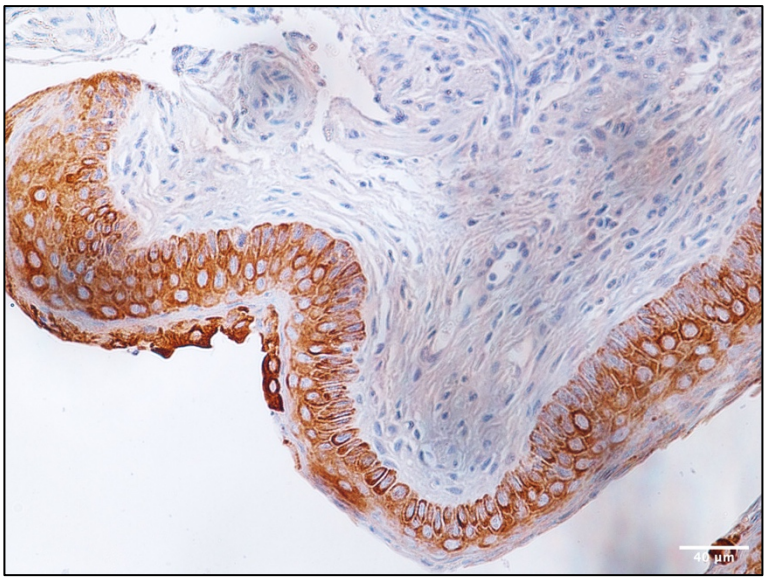


Figure 16: Results of the tests of CK5 on a slide of uterus from a 2-month-old Balb/c mouse. A positive brown cytoplasmic expression was observed in the epithelial cells Counterstain: Hematoxylin. Objective magnification 20x.

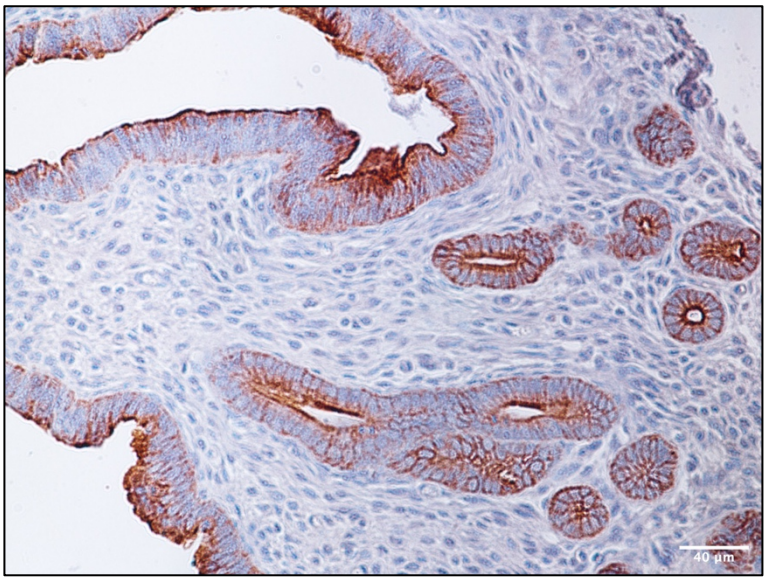


Figure 17: Results of the test of CK8 on a slide of uterus of 2-month-old Balb/c mouse. A positive brown cytoskeleton expression was observed in the epithelial cells, predominantly in the apical epithelium. Counterstain: Hematoxylin. Objective magnification 20x.

When the same protocol was performed on the cases of this study, the results were unexpected. The mammary gland samples showed no CK expression, while on the uterus from the same mice, which were and processed in parallel, showed some expression (Figure 18 and Figure 19), although not uniform.

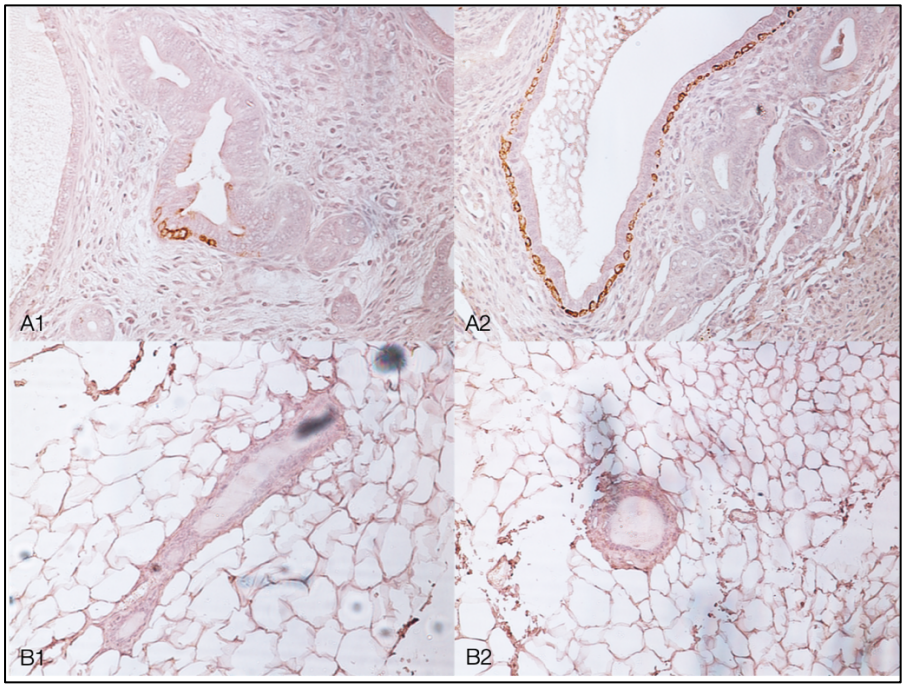


Figure 18: Results of CK5 expression on 13 and 29-months mouse tissue.
(A) (**1** – 13months-old; **2** – 29months-old) Uterus, exhibiting a partial CK5 expression in the basal epithelial cells; **(B)** (**1** – 13months-old; **2** – 29months-old) mammary tissue showing mammary ducts with no CK5 expression. No counterstain was used. Objective magnification 63x.

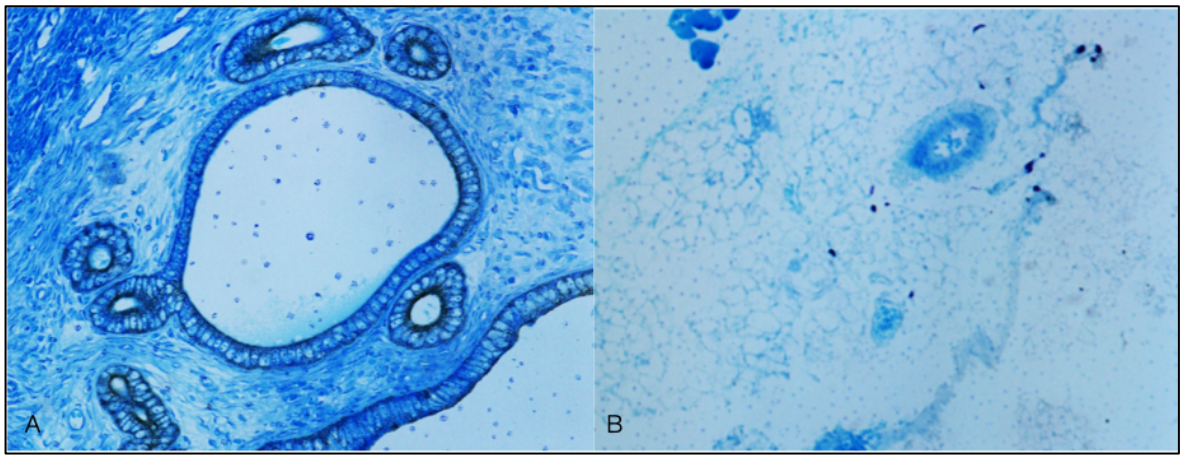


Figure 19: Results of CK8 on 13 months mouse tissue.
A) Uterus, exhibiting a residual CK8 expression on the epithelial cells; **B)** mammary tissue showing mammary ducts with epithelial cells with no CK8 expression. Azur B was used as a counterstain. Objective magnification 63x.

In order to further attempt an optimization for this protocol, the several tests taken, their respective results and possible explanations are summarized on Table 3.

Table 3: Tests performed for the immunohistochemistry optimization, their results and explanations.

Test number	Condition at test	Result	Possible explanation
T1	Testing antibodies that will be used according to the manufacturer's recommendations	No expression	Wrong DAB dilution was used
T2	Repeating previous test with appropriate DAB dilution. Testing three dilution factors for each antibody	No expression	ABC kit expired
T3	Testing expired ABC kit and a new one	No expression	PBS solution not properly prepared
T4	Testing PBS previously used and freshly prepared commercial solution (tablets of PBS diluted in distilled H_2O)	No expression	Primary or secondary antibodies not working
T5	Testing different aliquot of secondary antibody for CKs	Partial expression	-
T6	Repeating T5 with both aliquots of secondary antibody	No expression	Insufficient deparaffinization
T7	New set of deparaffinization with one extra xylene. Testing on CKs	Partial expression	-

Table 3 (continuation)

Test number	Condition at test	Result	Possible explanation
T8	Repeating T7 for the remaining antibodies (ER F10, PR and beta-amyloid)	No expression	Santa Cruz antibodies not working properly; ER antigen not expressed on the tissue at test. Beta-amyloid antibody was never tested before, but PR was working on previous studies
T9	Testing CK5 on the mice samples, one slide per age range	Partial expression on two slides only referring to ages 13 and 29 months, but only on uterus and/or ovary, not mammary gland	Poor preservation of tissues
T10	Repeating CK5 on more samples; testing CK8	No expression for CK5, partial expression for CK8 (uterus and/or ovary)	Poor preservation of tissues
T11	Testing new clones of antibodies such as PR, ER and beta-amyloid	No expression, background only	-
T12	Testing different antibody (BiP) on human prostate and breast tumors, before and after HT	Expression only on prostate samples before HT	-

No results were obtained for the hormonal receptors (ER and PR).

2. Proteostasis in response to hormone-therapy

Previous studies noticed a higher UPR activity in the tumors resistant to HT, therefore, we wanted to test whether we would find less protein aggregates in the metastases found after this treatment when in comparison to the respective primary tumor. The protein aggregates were found both intracellular and within the extracellular matrix of the tumor.

The ProteoStat kit was developed to use on cells in culture and not on tissue sections, so an optimization for this study had to be performed, in order to include a deparaffinization and hydration at the beginning, and to determine the optimal dilution factor of the dye. Optimization of the concentration and incubation conditions was tested on normal mouse mammary tissues. However, very few protein aggregation foci were detected on the normal breast samples, so this protocol was also tested on mouse mammary tumors where more foci were detected (Figure 20).

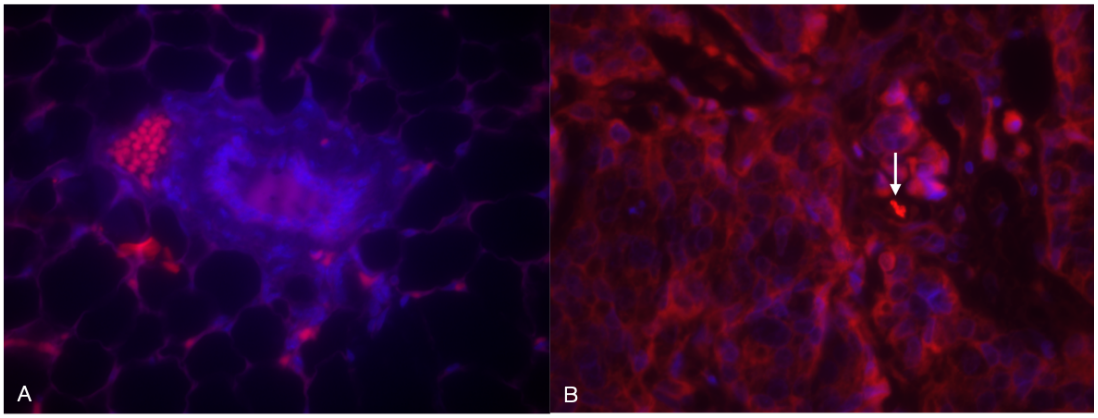
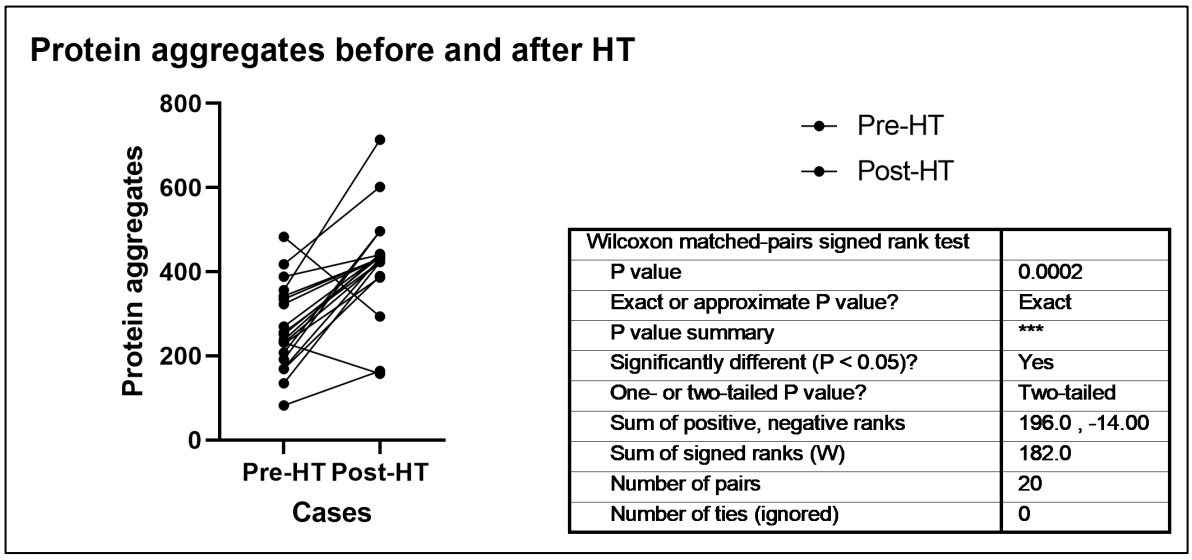


Figure 20: Comparison of the detection of aggresomes through an immunofluorescence probe on human normal breast tissue and human BC tissue.
A) Normal breast tissue, red staining mostly on red cells and adipose tissue; B) Mammary tumor tissue with a more prominent protein aggregate (white arrow). Objective magnification 63x.
Blue: DAPI (nuclear staining); Red: ProteoStat (aggregated proteins).

After the validation of the protocol, it was applied to the cases of human cancers to determine if there were any differences before and after HT. From the 23 pairs of samples, 3 were excluded due to the lack of material on the slide to analyze. The fluorescence levels of ProteoStat (due to the protein aggregates) and the statistical analysis are represented on Figure 21.



Wilcoxon matched-pairs signed rank test	
P value	0.0002
Exact or approximate P value?	Exact
P value summary	***
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of positive, negative ranks	196.0 , -14.00
Sum of signed ranks (W)	182.0
Number of pairs	20
Number of ties (ignored)	0

Figure 21: Protein aggregates found on tumor samples before and after HT.
From the 20 studied samples, 18 showed an increase of the protein aggregates after the HT, with statistically significant results ($p<\alpha=0,05$).

It was observed an increase of the protein aggregates (considering both intracellular and extracellular) on the samples after the HT in 18 of the 20 cases. Thus, there's statistical evidence that the protein aggregates increase on the metastasis of the tumors after HT, with a significance level of 95% ($p<\alpha=0,05$).

However, it should be noted that some of the represented cases had smaller sections of tissue on the slide (sometimes just some scattered cells), and in this scenario there seemed to be an excessive fluorescence signal as can be seen on Figure 22 as an example. The more intense fluorescence on these cases might not reflect the real amount of protein aggregates and may induce to a biased result. This excessive fluorescence was observed on 6 different cases that presented a smaller amount of tissue on the slide, even though all case pairs "before" and "after" HT were simultaneously and equally treated, both during the execution of the protocol and the image acquisition. Therefore, this difference of intensity of the fluorescence must be related to the tissue itself (the way it was preserved, or the little amount of tissue promotes a bigger absorption of the dye) and not to any step of the protocol. However, even excluding these cases from the study, the final statistical analysis still points towards the same direction, but without statistical significance ($p>\alpha=0,05$; data not shown on the table).

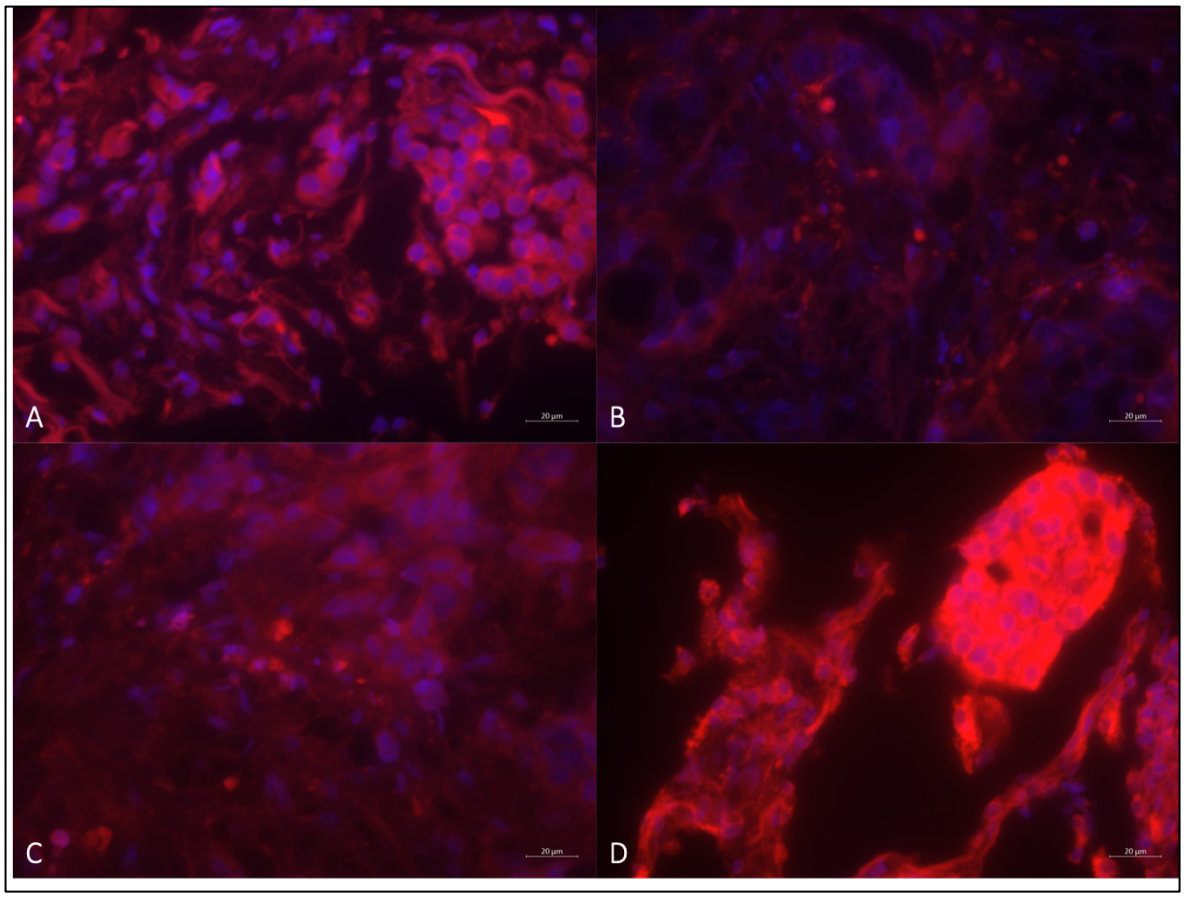


Figure 22: Comparison of the fluorescence signal on two cases (before and after HT).
Figures A and B correspond to one case (before and after HT, respectively) while figures C and D correspond to another case (before and after HT, respectively). While figures A, B and C seem to be qualitatively balanced regarding the fluorescence signal, figure D seems to exhibit an exacerbated fluorescence.

Discussion

1. Histological and immunohistochemical analysis of mouse mammary gland samples throughout

In this work we aimed to correlate the alterations in the mouse mammary gland histomorphology with the expression of epithelial cell markers to attempt to discover a connection with the incidence of ER+ BC in the elderly population.

Generally, there were no major differences in the morphology of the mammary gland of the aging mice, except for the younger ones (1month-old) that presented a simpler structure (no connective tissue proximal to the cap cells of the terminal end bud, less branching of the gland), with more mitotic figures and differentiating cells, and the elderly female mammary glands which exhibited occasional features of mild hyperplasia. In post pubertal females (more than 1-month-old), the histomorphological features of the mammary glands were usually identical, although with a more intensive ductal branching when in comparison to 1-month-old (pre-pubertal) samples; however, the cell components of the mammary structures were identical. This might be related to the fact that mice are considered to be adults when they are around 8 weeks old (approximately 2 months) (153). The lack of qualitative differences between the mammary glands and ducts regarding the type, number and layers of cells and the number of branches through these different ages might be explained by the fact that after 8 weeks old mice are already mature, and that in this study the female mice were nulliparous, The observation of hyperplastic features in the older female mice was described in a previous investigation (154) that studied the morphology of mammary glands of wild-type virgin female FVB/NCr mice. These authors described that older virgin female mice frequently had the appearance of glands from mid-pregnant animals, which was associated with pituitary anomalies.

The immunohistochemical studies for the epithelial cell markers were compromised and no results were obtained on the actual cases. The results observed during the tests of the antibodies and the solutions used for the immunohistochemistry protocol serve as a control for the fact that the method was correctly planned, and the antibodies were reactive for this species. Considering that all samples of the same mouse were stored in the same container with a fixative solution, the absolute lack of expression of the epithelial markers on the mammary tissue, but occasional reactivity in the uterus samples leads into thinking that the source of the problem must rely on the sample itself. As mentioned in the previous section, in

some of the containers, the samples were found to be poorly fixed, which was confirmed on the H&E slides. Since the same protocol was adopted for all the samples, one might consider the hypothesis that maybe all the samples suffered from poor fixation but with less severe effects (not noticeable on the H&E staining) in the majority of them, but severe enough to interfere with the immunohistochemistry protocol. Alternatively, one must consider that the majority of the immunohistochemistry protocols and reagents for this method were optimized for samples fixed in buffered formaldehyde and not alcohol (nor a post-fixation with alcoholic reagents), and the post-fixation in this solution might have compromised the availability of the epitopes to the antibody (155). Several proteins have their protein hydrophilic moieties in contact with water and hydrophobic moieties in closer contact with each other, stabilizing hydrophobic bonding. When this water is removed with the ethanol (used on the fixation solution), these protein hydrophobic bonds are destabilized because the hydrophobic areas are released from the repulsion of water and the protein tertiary structure becomes unfolded. Simultaneously, removal of the water destabilizes hydrogen bonding in hydrophilic areas, resulting in protein denaturation, which in turn will result in inadequate cellular preservation and very likely a shift in the intracellular immunoreactivity (156). Ethanol as a fixative has been shown to be associated with a major loss of proteins (8.3% vs 0% for buffered formalin) (157).

The differences between the mammary tissue and the uterus on the same containers might be explained by the different density of these tissues, since it is known that the diffusion rate of the fixative solution is influenced by this feature (besides temperature, diffusion coefficient, agitation, etc), being more promptly absorbed by the less dense one – the mammary tissue, especially considering that even though some results were seen on uterus slides, there was never an expression throughout all the section (158).

Thus, under these conditions, it was not possible to evaluate the epithelial differentiation in the mammary gland throughout aging and to correlate these with features observed in BC.

2. Proteostasis

The aim was to determine whether there was a difference in the elimination of toxic protein aggregates in HT resistant breast tumors, that could serve as a marker of proteostasis control. In both scenarios, before and after HT, protein aggregates were found. We expected to see a decrease of these aggregates in the HT resistant tumors (the cases that metastasized after HT) when in comparison to the ones before HT, since the former would be expected to demonstrate a better proteotoxic resolution. However, the results present statistical evidence

for the opposite: there seems to be a higher amount of protein aggregates after HT. We hypothesize that maybe the protein aggregates diminish on the intracellular levels, but some are secreted to the extracellular matrix surrounding the tumor cells.

At the same time, some of the cases had no more tissue on the slide (probably due to thinning on the microtome from previous studies with these samples) which were excluded from the study at the beginning, while others presented very little tissue or even just some scattered cells on the slide. The cases that had less tissue to evaluate seemed to exhibit a more intense fluorescence, which might mislead the interpretations of this study, and excluding these cases from the evaluation would leave insufficient samples to guarantee any statistical significance.

Conclusion and Future Perspectives

The samples available for the study of the morphology of the mammary gland throughout aging were not adequate for this purpose given the ages of the mice available, since no major changes are observed nor expected after the age of 3 months regarding the mammary gland general structure. The fixation methods for these samples were not the ideal either for immunohistochemical studies, since the majority of the immunohistochemistry protocols are validated and optimized for formalin fixed tissues only, and the post-fixation with ethanol might have compromised the availability of the protein epitopes. The poor fixation artifacts found in some of the H&E samples also corroborate this, since all of these methods are mostly optimized for formalin fixations when it comes to histology. However, the determination of the exact proportion of each cell type in the mammary gland throughout aging may present valuable information, and in order to better understand the mammary gland development it could be interesting to include more ranges of ages before reaching puberty, and samples from gestation, lactation and involution phases.

Regarding the proteostasis study, further on would be interesting to distinguish the intracellular protein aggregates from the extracellular, and even compare these values within the tumor cells with the normal cells surrounding the tumor to evaluate the behavior of the tumor with its environment. Additionally, a comparison of the aggresomes on breast tumors regarding their type and/or response to therapy might be of interest, to better understand the underlying molecular mechanisms and possible target therapies. Besides this, a correlation between the aggresomes in breast tumors and age of incidence could also be a factor to consider and determine whether the aggresomes are a consequence or related to the cause of the oncologic development. Besides BC, other types of cancer could go under this approach.

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