Universidade do Algarve

Departamento de Ciências Biomédicas e Medicina

Androgen receptor expression in Triple-negative Breast Cancer

Orientadora: Doutora Sofia Braga, Hospital CUF Descobertas Lisboa

Discente: Filipa Nascimento Rodrigues

Dissertação de Mestrado de Ciências Biomédicas da Universidade do Algarve

Faro, Outubro de 2014

Androgen receptor expression in Triple-negative Breast Cancer

Declaração de autoria de trabalho:

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Filipa Nascimento Rodrigues

Index

"A natureza é frequentemente escondida, algumas vezes dominada, mas raramente extinta."

Francis Bacon

Acknowledgments

Os meus agradecimentos começam por se dirigir à Dra. Sofia Braga pela oportunidade que me apresentou de desenvolver a dissertação em Lisboa. De seguida, agradeço à Dra. Paula Borralho por toda atenção e ajuda que me facultou durante o ano para a realização da tese de mestrado. Um obrigado dirigido à Técnica Catarina Talina pois sem a sua ajuda não conseguiria realizar o trabalho experimental. No seguimento um obrigado ao Dr. Francisco Tortosa, que se mostrou disposto ajudar na aquisição dos anticorpos necessários na elaboração do trabalho laboratorial. A toda a equipa do Hospital CUF Descobertas, por me ter acolhido da melhor forma, eu dirijo o meu sincero obrigado.

De seguida os meus agradecimentos vão para os meus pais, pilar e força constante de vida. Sem eles, realmente não conseguiria chegar onde estou.

Agradeço também, ao meu namorado Fernando, que se manteve sempre pronto apoiar-me sem nunca me deixar desistir.

Ao meu melhor amigo, eu deixo os mais sinceros agradecimentos por toda a caminhada que fizemos e que mesmo longe, não há nada que separe uma verdadeira amizade.

Os meus restantes agradecimentos vão para todos aqueles que direta ou indiretamente possibilitaram que eu conseguisse chegar aqui, que se mostraram prontos ajudar-me, que me incentivaram e acreditaram ate ao fim que seria capaz de alcançar mais uma etapa da minha vida.

A mudança não transforma só uma pessoa num melhor profissional, a mudança transforma uma pessoa num melhor ser humano.

Bem-hajam!

Abbreviations

% - Percentage

ºC - Degrees Celsius

µL - Microliter

µm - Micrometer

AR - Androgen receptor

BC - Breast Cancer

BRCA1 - *breast cancer 1, Gene*

BRCA 2 - *breast cancer 2, Gene*

cm- Centimeter

CK- Cytokeratin

CK5/6- Cytokeratin 5/6

DAB- 3,3′-Diaminobenzidine tetrahydrochloride hydrate

DFS- Disease Free Survival

DNA - Deoxyribonucleic acid

e-cadherin- *Epithelial caderin*

EGFR- *Epidermal growth factor receptor*

ER - Estrogen receptor

g- Gram

GE- Gene Expression

HE- Hematoxylin & Eosin

HER2 – *Human epithelial receptor 2*

HCD- Hospital CUF Descobertas

IC 95%- Confidence interval of 95%

IHC - Immunohistochemical

µm - Micrometer

min – Minutes

mL- Milliliter

WHO- World Health Organization

OS- Overall Survival

PR - Progesterone receptor

PHH3- Phosphohystone H3

p53 - *Tumor suppressor p53*

p-cadherin- *Placental* cadherin

TIL- Tumor Infiltrating Lymphocytes

TMA - *TissueMicroarray*

TNBC – Triple-negative Breast Cancer

Abstract

Worldwide, 3 million the women are diagnosed with breast cancer which is the most common malignancy in female gender. Breast Cancer is a heterogeneous disease and clinically it is evaluated using three markers: the estrogen receptor (ER), the progesterone receptor (PR) and the epidermal growth factor receptor type 2 (HER2).

The tumors that do not express any of these receptors are called triple-negative breast cancers (TNBC). TNBC represent approximately 20% of BC.

These tumors constitute an important clinical challenge, as they do not respond to endocrine treatment and to anti HER2 directed therapy. As a group they harbor an aggressive clinical phenotype with early development of visceral metastases and a poor long-term prognosis. The only systemic treatment for TNBC is chemotherapy. Numerous experiments and trials have been done to try to understand what is targetable in TNBC and if so, if the clinical trials achieve their endpoints. Today, the trend in clinical practice is individualized treatment based on molecular biology markers of tumor and patient.

Lehmann *et. al*. attempted to characterize TNBC and put forward a microarray signature that divides these tumors in 6 groups, according to differential gene expression profiles: basal-like 1 (BL1); basal-like 2 (BL2); immunomodulatory (IM); mesenchymal (M); mesenchymal stem–like (MSL) and luminal androgen receptor (LAR).

Androgen receptor is member of the steroid hormone receptor family, expressed in more than 70% of breast cancers and has been implicated in breast cancer pathogenesis. The role of AR is of particular interest in patients with TNBC.

In this project, immunohistochemistry was used to characterize the AR expression in a consecutive cohort of TNBC cases from Hospital CUF Lisboa. The immunohistochemical panels of markers used to characterize this subgroup of breast cancer, along with AR, were the CK 5/6, p-cadherin, PHH3 and EGFR in an attempt to further characterize TNBC subtypes.

Of all the TNBC cases, 56% (28/50) were CK5/6, 0; 22% (11/50) were CK5/6, 1+; 12% (6/50) were CK5/6, 2+; and 10% (5/50) were CK5/6, 3+. In 96% (48/50) the expression of p-cadherin was positive while only 4% (2/50) was negative.

Regarding EGFR, 38% (19/50) were EGFR, 0; 26% (13/50) were EGFR, 1+; 24% (12/50) were EGFR, 2+; and 12% (6/50) were EGFR, 3+. The expression of AR was considered positive in 62% of cases of TNBC.

We have known from the literature that AR status is a significant independent prognostic factor, although we cannot show this in this sample. In this study was not possible to implement the Lehmann classification because it was not possible to separate the different TNBC by Immunohistochemical staining into 6 different categories of the Lehmann classification.

The functional role of AR in breast cancer remains unclear, further exploration of this area could expand the repertoire of potential treatments for patients with AR+ TNBC.

Key words: Breast Cancer, Triple-negative Breast Cancer, prognostic markers, Immunohistochemistry, Androgen Receptor, Therapeutic Target.

Resumo

O cancro da mama assume uma grande relevância na sociedade, a sua incidência na Europa ocidental é de 90 novos casos por ano em cada 100.000 habitantes e em Portugal é semelhante. O cancro da mama apresenta-se como uma doença heterogénea, não só clínica e histologicamente, como também no seu perfil de expressão genética.

As modalidades terapêuticas têm evoluído, minimizando as cirurgias mais mutilantes, e a implementação de terapêuticas dirigidas, tanto anti-estrogénicas como anti-HER2. No seguimento deste conceito, a pesquisa de marcadores moleculares com potencial alvo terapêutico, assume um crescente interesse no seio da comunidade científica. Neste contexto temos como órfãos de terapêuticas alvo os tumores da mama triplo-negativo (TMTN), porque não expressam receptores de estrogénio, progesterona e do fator de crescimento epidérmico humano tipo 2.

Lehmann *et. al.* apresentou uma classificação de seis subtipos histológicos para identificar os TMTN, com implicações genéticas, epidemiológicas, terapêuticas e práticas. Esta classificação foi obtida com recurso a expressão genética de milhares de genes, através de "microarrays". Um dos objetivos deste trabalho é adaptar a classificação de Lehmann a uma técnica de utilização corrente nos hospitais que recorre a proteínas (anticorpos) pré-definidas com sistema de alta sensibilidade de deteção, a técnica de imunohistoquímica (IHQ). A classificação de Lehmann divide em 6 subtipos o cancro da mama triplo-negativo (CMTN). "Basal-like" 1 e 2 (BL1 e BL2) correspondem a um grupo de tumores susceptíveis à quimioterapia clássica, porque eles têm mutações nos genes de reparação do ADN, estes são, também, os tumores que respondem bem à quimioterapia neoadjuvante e desenvolvem-se frequentemente em doentes portadores da mutação BRCA1 e BRCA2; em seguida o grupo com evidências genómicas de modulação imunológica, grupo de bom prognóstico; o terceiro grupo em que se divide ao cancro CMTN é o subgrupo classificado como mesenquimal. Estes tumores têm características mesenquimais e expressão de marcadores de células tronco, como a p-caderina. Estes tumores são quimioresistentes e poderão explicar a associação com mau prognóstico. Por fim o subgrupo de CMTN que expressam o

receptor de androgénio, descritos histologicamente, como tumores apócrinos, com tendência a recidiva local e que poderão ser susceptíveis à terapêutica com anti-androgénios.

Apesar de uma década de investigação clínica, continuamos sem ter qualquer modalidade terapêutica alvo para tratar os TMTN, recorremos ainda e só à cirurgia, quimioterapia (QT) e radioterapia (RT). Neste trabalho usámos um método auxiliar de diagnóstico na anatomia patológica que se tem mostrado de extrema importância, na prática clínica, que é o método da imunohistoquímica. O crescente uso desta técnica deve-se à necessidade de diagnósticos precisos para prognóstico e tratamento, principalmente de neoplasias. Vários avanços, nomeadamente um de fundamental importância, a chamada técnica de recuperação antigênica (sistemas de recuperação de epítopos através do calor (radiação ou calor húmido), e o uso de um número crescente de anticorpos disponíveis em tecidos fixados em formaldeído e incluídos em parafina, permitiram uma progressão na técnica. Estratégias utilizadas para obter uma melhor qualidade na marcação de anticorpos em tecidos pré- fixados, são também executadas pela IHQ. A utilização de enzimas específicas que amplificam uma grande quantidade de moléculas propiciadoras de visualização, são uma mais-valia pelo menor dispêndio do anticorpo primário, permitindo o aumento de diluições sem comprometer a intensidade de marcação antigénio-anticorpo.

Neste trabalho será determinado, como objetivo principal a expressão do receptor de androgénio (RA), a partir do método imunohistoquímico. Na clínica o desdobramento de diferentes subtipos de CMTN não pode ser determinada, por esse motivo em conjunto com o RA, foram utilizados quatro anticorpos: citoqueratina 5/6 (CK5/6), o factor de crescimento epidérmico (EGFR), p-caderina e a fosfohistona H3 (PHH3). O nosso objetivo é dividir BL1 e BL2 através da avaliação do EGFR e CK 5/6 e um marcador de proliferação (PHH3); TMTN com fenótipo mesenquimal através da expressão de p-caderina; a infiltração linfocitária tumoral (ILT) permite a descrição de TMTN com fenótipo descrito como imunomodulador e os tumores que expressam receptores de androgénio, também chamados de tumores apócrinos, consequentemente com RA.

O estudo abrange uma amostra de 80 casos de pacientes, previamente selecionados. Cerca de 80 casos de cancro da mama triplo negativo foram

analisados e feita a sua associação, posterior, com características clinico-patológicas. Os casos CMTN foram conservados em blocos de parafina, selecionados consecutivamente no Serviço de Anatomia Patológica do Hospital CUF Descobertas de Lisboa.

Nos casos de CMTN, a expressão de CK5/6 foi em 56% (28/50) de 0, em 22% (11/50) de 1+, em 12% (6/50) de 2+ e em 10% (5/50) de 3+. Em 96% dos casos de CMTN (48/50) a expressão de p-caderina foi positiva enquanto em apenas 4% (2/50) foi negativa. A expressão de EGFR foi em 38% (19/50) dos casos de 0, 26% (13/50) dos casos de 1+, 24% (12/50) dos cacos de 2+ e 12% (6/50) dos casos de 3+. A expressão de RA foi elevada em 62% dos casos de CMTN. O RA é um fator de prognóstico independente significativo, embora não tenha sido possível demostra-lo nesta amostra. Neste estudo não foi possível implementar classificação de Lehmann à clínica porque não era possível separar os diferentes CMTN pelo método imunohistoquímico nos 6 subgrupos desta classificação.

O papel funcional da RA no cancro da mama ainda não está claro. Um tratamento individualizado, tendo por base os novos conhecimentos moleculares, irá permitir a selecção de terapias efectivas com menores efeitos adversos. Todavia, mais estudos e ensaios clínicos são necessários para determinar o seu benefício.

Palavras-chave: cancro de mama, cancro de mama triplo-negativo, marcadores prognósticos, imunohistoquímica, receptor de androgénio, terapêutica dirigida.

1. Introdution

1.1 Female breast

The breast of mammals is important for milk production that ensures the survival of the newborn and the development of this species.

The breast consists of three tissue types: fatty tissue, connective tissue and mammary gland tissue. The glandular tissue is a specialized tissue whose function is to produce milk. The structures related to milk production are the lobules. Lobules are structures organized in 15 to 20 sections. In each lobule there is a secondary structure called lobe, where milk is produced. The structures responsible for directing the milk to the nipple are the ducts. The dark area of skin around the nipple is called the areola (figure 1) [1].

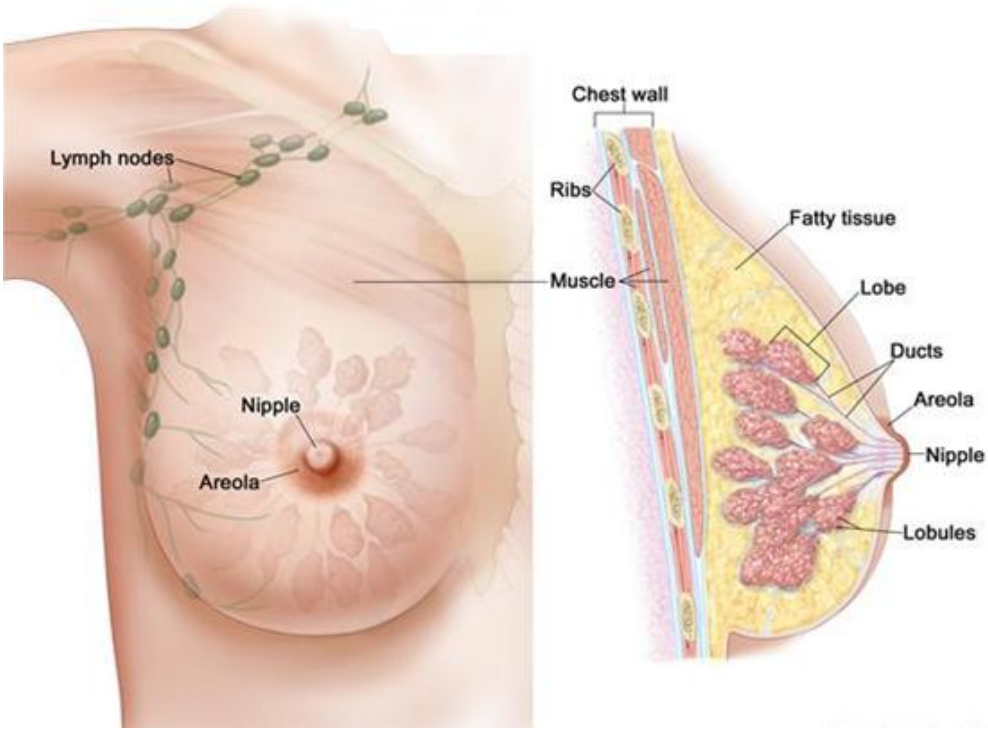

Figure 1. Illustration of female breasts. The main components are the lobules and ducts, conductors and producers of breast milk, respectively; fat tissue, connective tissues and lymphatic vessels or blood. *Adapted from National Cancer Institute (NCI) http://www.cancer.gov/images/cdr/live/CDR415520-750.jpg*

The rest of the breast is composed of connective tissue (collagen and elastin), adipose tissue (fat) and an aponeurosis called Cooper ligament. The breast consists as well of skin, nerves, blood vessels, lymph vessels and lymph nodes [1].

The ducts are coated with 1 to 2 layers of cylindrical luminal cells. The lobular-alveolar portion is coated with two layers of cuboidal cells with secretory capacity. A layer of myoepithelial cells is located in close contact with the basement membrane of the alveolar epithelium and the smaller ducts (figure 2) [1, 2]. These cells are sensitive to oxytocin, have clear portion of cytoplasm and oval nuclei and a part of dense cytoplasm containing myofibrils. Myoepithelial cells show positivity for immunohistochemical markers of epithelial origin as well as mesenchymal smooth muscle actin, p63, S100 protein and cytokeratins 5, 6, 14 and 17. These cells have contractile capacity and are responsible for the renewal of the luminal epithelium. The basal myoepithelial cells have high levels of type IV collagenase, with probable critical role in the renovation and remodeling of the basement membrane of the glands [3].

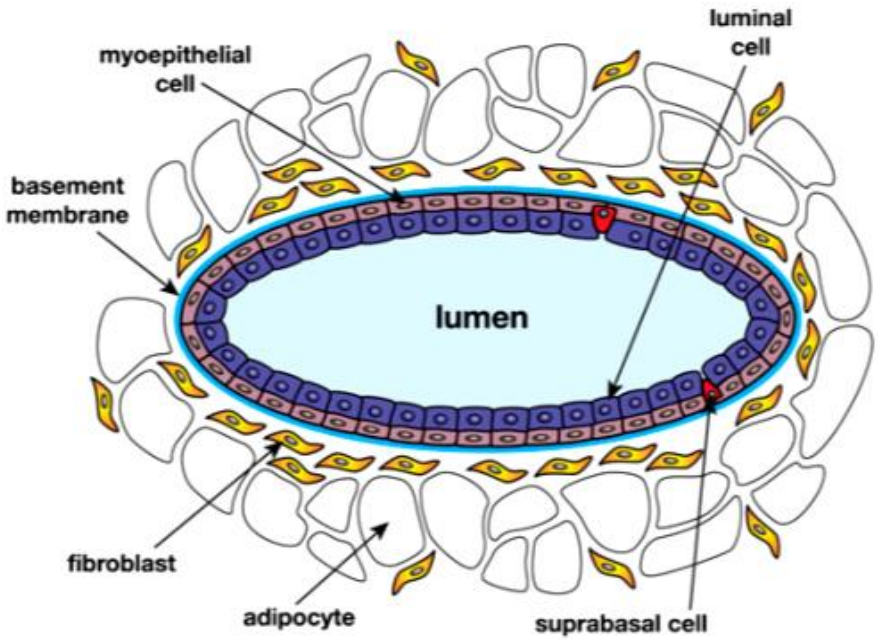

Figure 2A. Schematic representations of a duct. The luminal lineage can be further subdivided into ductal and alveolar luminal cells that line the ducts. In contrast, myoepithelial cells are specialized, contractile cells located at the basal surface of the epithelium adjacent to the basement membrane [2].

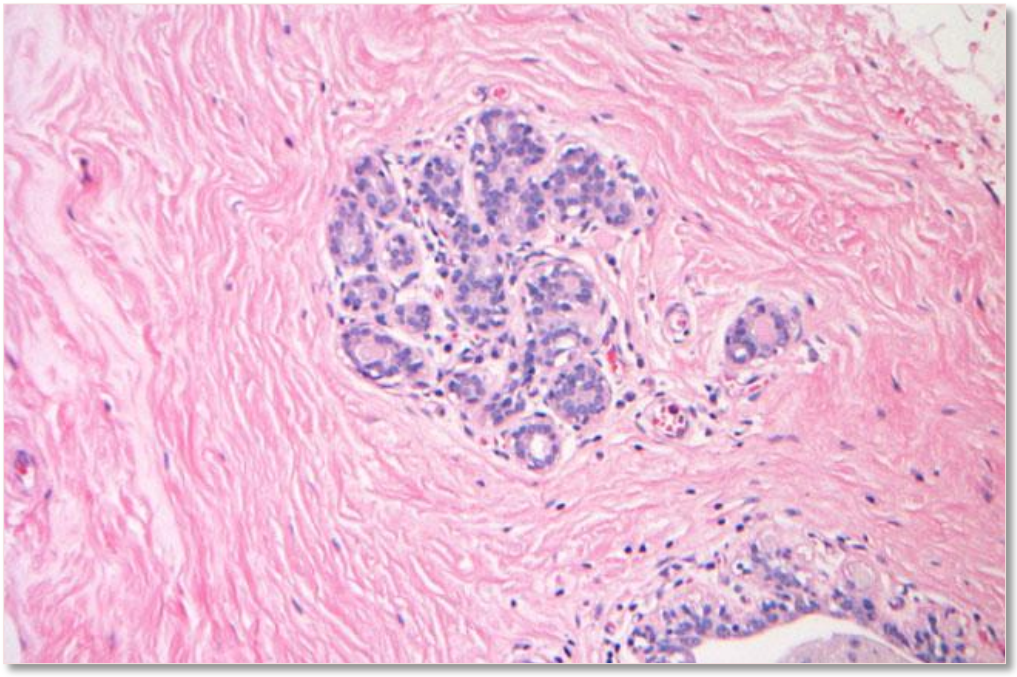

Figure 2B. Histology of normal breast. It is possible to observe the benign lobule, surrounded by myoepithelial cells with an adjacent interlobular duct; H&E stain. *Adapted from Meenakshi Singh, MD © - Department of Pathology, Stony Brook University Medical Center.*

Mammary stem cells (MASCS) have assumed an important interest in organ development and maintenance of tissue homeostasis. These cells give rise to mature epithelium of either the luminal or myoepithelial lineage via a series of lineage-restricted intermediates. The profound expansion of mammary epithelium that occurs during puberty and pregnancy further implicates a stem-like cell with remarkable regenerative capability [2].

Several factors can influence the regulation of normal development and progression of carcinogenesis of the breast. Steroid hormones have a very important role, these produced by the ovaries, pituitary, endocrine pancreas, thyroid, adrenal cortex and adipose tissue. These hormones act through nuclear receptors or cell surface receptors and regulate the transcription of specific sets of genes [4].

In normal breast tissue there are three main groups of cells, namely epithelial, myoepithelial/basal and stromal. Paracrine factors play a role in their interactions, and between the epithelial cells, autocrine mechanisms also play an important role.

In normal conditions, the human body produces various types of hormones responsible, for example, by the growth of several types of cells. One of these hormones is estrogen which is responsible for female development, systematic and organized with cell multiplication. If this cell proliferation stimulated by several factors, including hormonal, occurs in an uncontrolled manner, this can result in the appearance of cancers - particularly breast cancer. With this assumption, one way to inhibit the growth of neoplastic mammary cells (cancer) is to eliminate the production of female hormones, especially estrogen and/or blocking the action of hormones on the cells. Only cells that have receptors for estrogen and/or progesterone in its surface are grow in response to a hormonal stimulus. The hypothalamus is the initial route of estrogen production, which "sends" the pituitary gland signals to order production of several hormones, including the gonadotropic hormones FSH (follicle stimulating hormone) and LH (luteinizing hormone). These hormones will act on the female sexual glands - ovaries – stimulating the production of estrogens. Estrogen may also be produced on a smaller scale, by another gland, the adrenal. Blocking this pathway production, in its different stages, is the main target of endocrine therapy for breast carcinoma. In all women the adipose tissue (fat cells) is a site of estrogen production. When in childbearing age (menstrual cycles), the production of female hormones is made primarily in the ovary, however, after menopause the adrenal gland is responsible for this function, producing hormones which will then be "converted" into estrogens in adipose tissue [1].

Cancer results from an imbalance between of these interactions and the loss of control factors that promote cell survival (figure 3), activation of oncogenes, inactivation or loss of tumor suppressor genes, with participation of hereditary factors and exposure to factors that promote carcinogenesis. Breast carcinomas are characterized by uncontrolled and unregulated proliferation of epithelial cells (figure 4), the disappearance of the myoepithelial cells, changes in the pattern of programmed cell death (apoptosis) and, sometimes, loss of certain epithelial characteristics, known as epithelial-mesenchymal transition (EMT) [5].

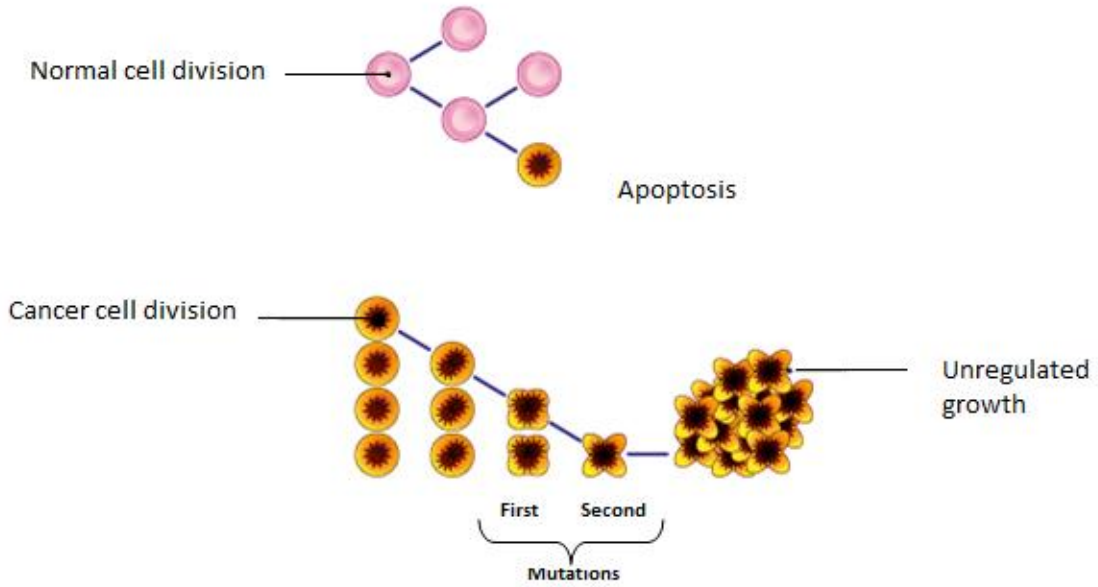

Figure 3. Normal growth requires a balance between the activity of genes that promote and suppress cell proliferation. It also relies on the activities of genes that signal when damaged cells should undergo apoptosis. Cancer cells do not respond to many of the signals that control cellular growth and death. Cancer cells escape programmed cell death (apoptosis). Over time, these cells become increasingly resistant to the controls that maintain normal tissue. As a result, cancer cells divide more rapidly and become less dependent on signals from other cells. In the late stages of cancer, cells break through normal tissue boundaries and metastasize (spread) to other organ sites in the body. *Adapted from oncologiacuf*.

Both normal breast and neoplastic tissues are able to produce similar substances and hormones by its own cells, which act locally (paracrine hormones), and modulate the function of the epithelial, stromal and vascular/endothelial adjacent cells. The tumor cells are capable of producing polypeptides that act as autocrine growth hormone factors regulating gene transcription by acting in its own surface receptors [1]. Therefore, it is important to understand the origin of cancer and the factors behind this so that biological processes can help find targetable drugs for clinical success.

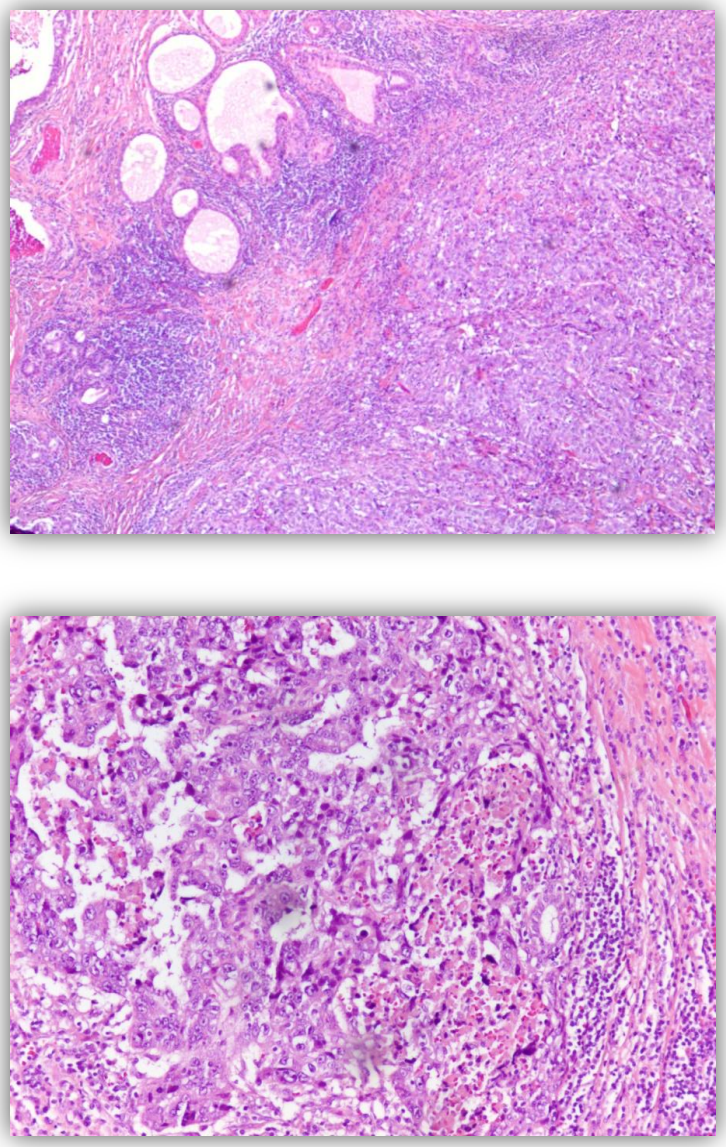

Figure 4. Examples of poorly differentiated breast cancer. The cancer cells change and become specialized. This description of breast can inform the grade of the cancer. As more the cells look like normal cells, the lower the cancer grade and the less normal they look, the higher the grade**.** The histological images of breast cancer were provided by the Anatomic Pathology Laboratory, of Hospital CUF Descobertas.

1.2 What is Breast Cancer?

Breast cancer is an uncontrolled growth of breast cells (figure 5). Cancer occurs as a result of mutations, or abnormal changes, in the genes responsible for regulating the growth of cells and keeping them healthy [6].

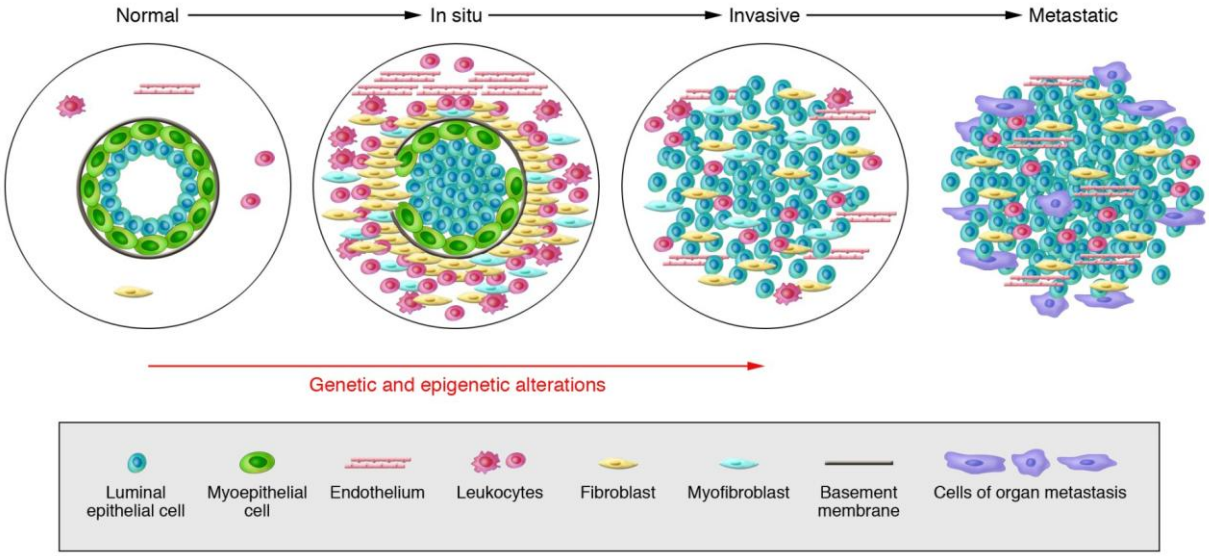

Figure 5. Hypothetical model of breast tumor progression. Schematic view of normal, in situ, invasive, and metastatic carcinoma progression.[7].

When normal mammary cells age or are damaged, they die naturally. When the cells loose this control mechanism and undergo changes in its genome they become cancer cells which do not die when they age or are damaged, and produce new cells that are not necessary in an uncontrolled manner, resulting in the formation of cancer [6, 8]. Another event that occurs is genomic instability (mutations, deletions, chromosomal rearrangements and amplifications), with an overall loss of normal tissue organization and possibly giving rise to metastases. The genomic stability is maintained by several modular components, which have a primary role in the suppression of cancer. The occurrence of inherited mutations in the major pathways of cell cycle control and repair of DNA damage causes an increase in susceptibility to breast cancer in women carrying these mutations

(BRCA1 and 2). On the other hand, mutations of the p53 gene are frequent in sporadic breast carcinomas.

Breast cancer occurs when malignant tumors develop in the breast. These cells can spread by breaking away from the original tumor and entering blood vessels or lymph vessels, which branch into tissues throughout the body. When cancer cells circulate and seed in distant organs and damage tissues and organs, the process is called metastasis. The most common sites of cancer metastasis are (in alphabetical order) the bone, brain, liver, and lung [9].

1.3 Incidence

Breast cancer is a problem for global public health and is the non-cutaneous malignancy more common in females, with increasing incidence in developed countries.

About 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008 worldwide, with 56% of the cases and 64% of the deaths in the developing world [10].

Breast cancer is the second most common cancer in the world and, by far, the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers) [11].

In Portugal BC remains the leading cause of cancer death in women with about 5513 new cases per 100 000 persons per year [12].

More than half of these cases occur in industrialized countries. In the European Union, 367,000 new cases of BC were diagnosed in 2012 (about 28.8 % of cancer in women) [13]. The incidence of BC has increased more in developed countries but there has been an increase in incidence in developing countries, particularly in Africa, Latin America and Asia [14] [15].

Breast cancer is the leading cause of death in women between the ages of 25 and 55 years in developed countries [16].

The factors that contribute to the international variation in incidence rates are largely originated from differences in reproductive and hormonal factors and the availability of early detection services [17, 18].

1.4 Breast Cancer Stages

Clinical staging (designated cTNM or TNM) accordding to the AJCC system (American Joint Committee on Cancer) is based on all information available prior the first definitive treatment and includes the findings on pathological examination, imaging studies, operative findings on physical examination of the breast or other tissue [19, 20]. The extent of tissue examined pathologically for clinical staging includes tumor size (T), involvement of lymph nodes in the homolateral axilla (N) and whether the cancer is invasive or non-invasive. The presence or absence of distant metastases (M) is assessed through imaging [21]. Pathologic staging is based on a pathologist's study of the lymph nodes and tumor tissue removed during surgery. The pathological classification requires the resection and examination of at least the low axillary lymph nodes (level I). If the lymph nodes are negative, but the number ordinarily examined is not met, classify as pN0. Often this is surgery to remove the cancer and nearby lymph nodes, but sometimes surgery may be done to just look at how much cancer is in the body and take out tissue samples. The pathological stage gives the health care team more precise information that can be used to predict treatment response and outcomes (prognosis) [21] (appendix I).The invasive nature of cancer is defined by the rupture of the basal membrane. The term "locally advanced" or "regionally advanced" is used to refer large tumors involving the skin of the breast, underlying chest structures, altering the shape of the breast and lymph nodes that are visible or palpable during clinical examination.

The stage of breast cancer is essential to determine prognosis and therapy. Breast cancer is classified into 4 stages (Table I) [1].

Most breast malignancies arise from epithelial elements and are categorized as carcinomas. The invasive breast carcinomas consist of several histologic subtypes. Breast carcinomas are a diverse group of lesions that differ in microscopic appearance and biologic behavior, although these disorders are often discussed as a single disease. The *in situ* carcinomas of the breast are either ductal (also known as intraductal carcinoma) or lobular. This distinction is primarily based upon the growth pattern and cytologic features of the lesions, rather than their anatomic location within the mammary ductal-lobular system [22].

BC is also classified by its histopathologic characteristics [22] which are presented in table II and figure 6 .

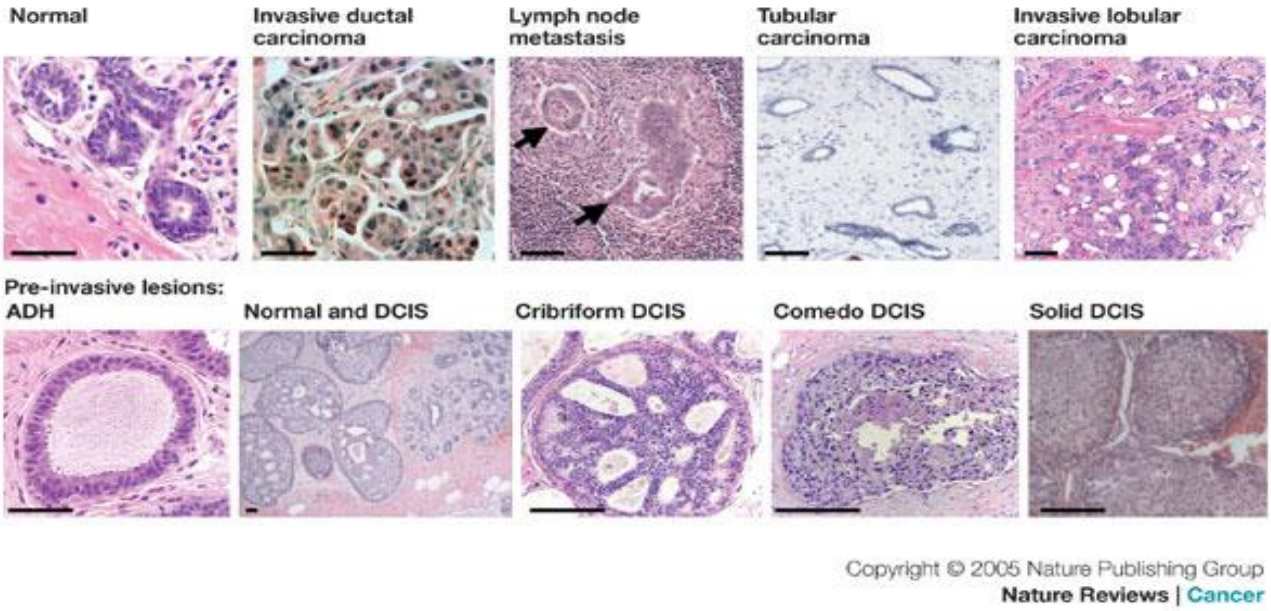

Figure 6. Histological images of different types of breast cancer.

	Table I: The stage of breast cancer.
O	This stage describes noninvasive (“in situ”) breast cancer. Ductal carcinoma in situ (DCIS) is an example of stage 0 cancer. *Adapted from National Cancer Institution*
I	The cancer is completely inside the breast. **IA**: The tumors is 2 cm or smaller and has not spread outside the breast; **IB**: Small areas of breast cancer cells are found in the lymph nodes close to the breast and either. The tumors is 2cm or smaller or no tumors is found in the breast.
II	A stage II breast tumor is larger than a stage I tumor, but the cancer hasn't spread to a distant part of your body. **IIA**: The tumors are larger than 2cm but not larger than 5cm and there is no cancer in the lymph nodes or cancer cells are found in 1 to 3 lymph nodes in the armpit or in the lymph nodes near the breastbone. **IIB**: The tumors is larger than 2cm but not larger than 5cm and small areas of cancer cells are in the lymph nodes or The tumors is larger than 2cm but not larger than 5cm and the cancer has spread to 1 to 3 lymph nodes in the armpit or to the lymph nodes near the breastbone or The tumors is larger than 5cm and has not spread to the lymph nodes.
III	A stage III breast cancer, known as locally or regionally advanced cancer. This cancer may have spread to lymph nodes near your breast, those located under your arm or by your collarbone, but not to more-distant parts of your body. **IIIA**: tumors are either larger than 5 centimeters (2 inches) and have spread to one to three lymph nodes under the arm, or are any size and have spread into multiple lymph nodes. **IIIB:** tumor of any size has spread to tissues near the breast - the skin and chest muscles-and may have spread to lymph nodes within the breast or under the arm. **IIIC:** cancer is a tumor of any size that has spread: to 10 or more lymph nodes under the arm; to lymph nodes above or beneath the collarbone and near the neck; to lymph nodes within the breast itself and under the arm.
IV	The tumors can be any size, the lymph nodes may or may not contain cancer cells and the cancer has spread (metastasized) to other parts of the body such as the bones, lungs, liver or brain.

Adapted from National Cancer Institution (NCI).

TableII. Breast Cancer Types.

Invasive carcinoma of no special type (NST)
Pleomorphic carcinoma
Carcinoma with osteoclast-like stromal giant cells
Carcinoma with choriocarcinomatous features
Carcinoma with melanotic features
Invasive lobular carcinoma
Classic lobular carcinoma
Solid lobular carcinoma
Alveolar lobular carcinoma
Pleomorphic lobular carcinoma
Tubulolobular carcinoma
Mixed lobular carcinoma
Tubular carcinoma
Cribriform carcinoma
Mucinous carcinoma
Carcinoma with medullary features
Medullary carcinoma
Atypical medullary carcinoma
Invasive carcinoma NST with medullary features
Carcinoma with apocrine differentiation
Carcinoma with signet-ring-cell differentiation
Invasive micropapillary carcinoma
Metaplastic carcinoma of no special type
Low-grade adenosquamous carcinoma
Fibromatosis-like metaplastic carcinoma
Squamous cell carcinoma
Spindle cell carcinoma
Metaplastic carcinoma with mesenchymal differentiation
-Chondroid differentiation

-Osseous differentiation

-Other types of mesenchymal differentiation

Mixed metaplastic carcinoma

Myoepithelial carcinoma

Epithelial-myoepithelial tumors

Adenomyoepithelioma with carcinoma

Adenoid cystic carcinoma

Rare types

Carcinoma with neuroendocrine features

Neuroendocrine tumor, well-differentiated

Neuroendocrine carcinoma poorly differentiated (small cell carcinoma)

Carcinoma with neuroendocrine differentiation

Secretory carcinoma

Invasive papillary carcinoma

Acinic cell carcinoma

Mucoepidermoid carcinoma

Polymorphous carcinoma

Oncocytic carcinoma

Lipid-rich carcinoma

Glycogen-rich clear cell carcinoma

Sebaceous carcinoma

Adapted from WHO of Breast Tumors, 4th Edition [22].

1.5 Clinical and histopathological prognostic factors

Breast cancer is the most common cancer in women and its incidence is increasing. Breast cancer is a heterogeneous disease [23-26] which comprises a number of distinct biological entities that are associated with morphological and immunohistochemical outcomes and clinical characteristics [23, 26].

The invasive breast carcinomas were for decades, only classified according to histological grade and hormone receptor expression [23]. The most important histopatological prognostic factors are the expression of ER and PR. These have enabled clinicians to treat BC in the last half a decade with anti-estrogens, significantly reducing BC mortality. Anti-estrogenic therapy is not toxic, well tolerated and cheap. Recently, after the success of clinical trials involving adjuvant trastuzumab, expression of the human epidermal growth factor receptor 2 (HER2) has become an integral part of the pathological workup for patients with breast cancer. HER2-positive breast cancer had worse prognosis compared with HER2-negative tumors [27]. HER2 amplification is widely known to indicate an aggressive tumor behavior and a poor clinical outcome in breast cancer patients [28]. Simultaneously with the development of trastuzumab as a targeted therapy for breast cancer, some results of genome microarray began to be reported [29]. There are several types of clinical breast cancer, defined by amplification of specific markers. The over expression of steroid hormone (like estrogen and progesterone receptors) defines the most abundant type of breast cancer, accounting for about 70%. (ER and PR positive) [24, 30, 31]. Therefore, several studies demonstrate that breast cancers can be divided according to hormone receptor (HR) expression (negative or positive) and/or epithelial cellular origin (basal or luminal), that have clinical implication [32]. Breast cancers can be divided into three main groups: (1) hormone-positive breast tumors; (2) HER2 –positive breast cancer and (3) basal or triple-negative breast tumors (classification summarized in table III). The overlap between basal and triple negative BC is not complete [32]. Generally, there are 20% of classifications in either direction i.e. there are 20% of basal like BC that are not TNBC and similarly there are 20% of TNBC that are not basal-like BC.

The tumors that are HR-positive are luminal A e B. This subtypes originate from inner (“luminal”) cells that line the mammary ducts, and they are dissimilar in their expression of HER2 (luminal A, which is negative and luminal B which is positive). Most carcinomas not related to genetic mutations, are characterized by the luminal A type [25]. The luminal B type is more likely to be lymph node-positive and to have high proliferation. Unlike the luminal A which tends to have a better prognosis, they often diagnosed in young women, the luminal B tumors tend to have a higher tumor grade, poorer prognosis and probably associated to genetic mutations. These phenotypes are associated with elevated gene expression by luminal epithelial cells, of molecules such as cytokeratins (CK) 7,8,18 and 19 [24, 33, 34].

The tumors that are HR-negative cannot be treated with anti-estrogens, are associated with a higher recurrence rate and a decreased overall survival. These tumors are more likely to be poorly differentiated, with higher histological grade [32].

HER2 tumors tend to be HR-negative and lymph node-positive. HER2 has been a major target for the development of the new cancer therapies in the last 20 years. Its greatest value as a predictive marker lies in the prediction of response to therapies that target HER2, such as trastuzumab (Herceptin) and with neoadjuvant anthracycline/taxane-based chemotherapy [35]. The HER2 oncogene amplification and concomitant overexpression of its protein, is currently implicated as an important prognostic biomarker in breast cancer [36].

Basal-like tumors originate in the outer (“basal”) cells that line the mammary ducts. This subtype has a gene expression profile similar to the genes that are identified in normal basal/myoepithelial cells of the mammary ducts and acini. The gold standard for the identification of basal-like carcinomas as a particular class of molecular breast cancer in the clinic is TNBC [33].

Their incidence has been estimated to be between 13% and 25% and they occur more frequently in young women and are associated with hereditary BRCA1-related breast cancers. Their metastatic pattern includes early dissemination to the axillary nodes and to viscera (liver, lung, soft tissue and brain) and less frequently to bone. According to the most recent publications, this phenotype shows positivity for

CK5/6, CK14, receptor for epidermal growth factor (EGFR), p-cadherin, p63 and CK17 proteins that are expressed in the basal/myoepithelial cells [24, 30, 37]. This profile is associated with numerous genetic mutations [30].

The original microarray classification published in the year 2000 [24], defined a fourth group called normal-like tumors that account 6%-10% of all breast cancers. These tumors do not fall into any other categories, usually small, typically have a good prognosis and they are more common in postmenopausal than in premenopausal women. Perou *et. al.* [24] identified these group by increasing the expression of many genes known to be expressed by adipose tissue and other non-epithelial cells. These tumors also showed strong expression of basal epithelial genes and low expression genes to the luminal epithelium. Nevertheless, it is still not clear if this distinction is of clinical value [24, 30].

Estrogen receptor and progesterone receptor are the most studied markers in breast tissue. The results of ER and PR have been scored as ‘‘positive’’ and ‘‘negative’’ although receptor protein concentration (in biochemical assays) and the percentage of cells stained and staining intensity (in Immunohistochemical assays) range widely [26]. Several studies report that the ER/PR status is an independent predictor of outcome in breast cancer tumors. The association of this status with the mortality is observed in tumors ER+/PR-, ER-/PR+, and ER-/PR-tumors compared to women with ER+/PR+ tumors. The prognostic usefulness of the expression of steroids hormone receptors is partially independent from the association in clinical characteristics and demographic characteristics of tumors. The higher relative mortality identified among ER-/PR- patients with small or low-grade tumors, raise the question of whether there may be a beneficial role for adjuvant chemotherapy in this population [38].

The problem of steroid receptor testing inaccuracy and non reproducibility is extremely serious because we are inadequately classifying BC but also because we are depriving women of anti-estrogenic therapy. For the determination of prognosis and therapy of BC some other proteins such as HER2, Ki-67, p53 are sought in addition to hormone receptors[39]. When BC does not express ER, PR and HER2 it will not benefit from the currently available receptor-targeted systemic therapy and these tumors are called TN. This study will be focused on TNBC.

Table III. Subtype (hormone and HER2 receptor status)

Luminal A	(ER+ or PR+ or both, HER2-)
Luminal B	(ER+ or PR+ or both, may be HER2+)
HER2	(ER-, PR-, HER2+)
Basal-like	(ER-, PR-, HER2-)
Triple-negative tumors	(ER- , PR-, HER2-)
Normal breast-like	Tumors that do not fall into any the foregoing categories

One of the most important challenges ahead is to identify specific molecules alterations in tumors and to validate targeted therapies for them. This is sometimes called precision/personalized therapy [24]. With the development of primary and secondary resistance to hormonal treatment, new substances and new patterns of association with blockers of growth factor receptors were created, enhancing the effect of drugs and recovering the responsiveness to endocrine therapy. Some of these substances are aromatase inhibitors (anastrozole, letrozole), antagonist of estrogen and progesterone receptors (fulvestrant), pan-EGFR inhibitors (GW572016), mTOR inhibitors (rapamycin RAD001-inhibit tumor growth central controller and angiogenesis), anti-HER2 (trastuzumab), and drug that blocks cell growth by stopping mitosis (cell division) [40].

1.6 Risk factors

Risk factors for breast cancer are age, race/ethnicity, age of menarche and menopause, multiparity, radiation mantle before Hodgkin lymphoma, oral contraceptive use and body mass index.

Family history of breast cancer, particularly having one or more first degree relatives with breast cancer (although most women with breast cancer do not have a family history of the disease) increases the risk of breast cancer. Inherited mutations in breast cancer susceptibility genes account for approximately 5% to 10% of all cancers of the female breast and an estimated 4% to 40% of all male breast cancers, but are very rare in the general population (much less than 1%). Most of these mutations are located in BRCA1 and BRCA2 genes, although mutations in other known genes have also been identified [18] [31].

Increased risk of hormone receptor–positive tumors was also associated with postmenopausal obesity, which probably increases estrogen exposure via different mechanisms. Obesity is associated with increased aromatization of circulating androgens to estrogens in adipose tissue and reduced levels of sex hormone binding globulin, thereby increasing both total and bioavailable estrogens [31]. A lot of studies summarized the risks of hormone receptor-positive and hormone receptor-negative BC. The majority of know BC risk factors are associated with hormone receptor-positive disease. Risk factors for HR-negative BC are young age, African origin and BRCA1/2 germline mutation [31]. In addition, the effect of hormone-related risk factors on hormone content within the breast is unknown. Although many factors have been shown to contribute to elevated systemic levels of estrogens, a relationship between high serum levels and the development of hormone receptor positive tumors has not been established [31].

Potentially modifiable risk factors that can be minimized include overweight, lack of exercise, smoking cigarettes, eating unhealthy food, alcohol consumption.

Two drugs (tamoxifen and raloxifene), have been approved to reduce breast cancer incidence in high risk women. Raloxifene appears to have a lower risk of certain side effects, such as uterine cancer and blood clots; however, it is only approved for use in postmenopausal women.

Women and society have the empowerment to lower BC risk by making healthiest lifestyle options possible.

1.7 Diagnosis Tests

Several tests can help distinguish a benign (noncancerous) lump from a malignant (cancerous) tumor. Because malignant and benign lumps tend to have different physical features, imaging tests such as mammography (figure 7) and ultrasonography can often rule out cancer. The only way to confirm cancer is to perform a needle aspiration or a biopsy to be analyzed. Before BC screening was implemented, breast cancer detection was limited to diagnosis based on physical examination by the woman herself or her physician. The concept of screening is to detect non palpable lesions.

When breast cancer is found at an early stage, the cure rate is high, the aim of screening is to detect non palpable disease and increase survival. Such cancers may be detectable only in mass population screening endeavors. Mammograph was suggested by Salomon, a German pathologist in 1913, as a modality to detect breast cancer and in 1959, Egan at the MD Anderson Hospital in Houston published results from a series of 1000 cases emphasizing the detection of non palpable lesions. In a practical, cheap and feasible, mammography has been implemented as an important method for early detection of BC, reducing mortality.

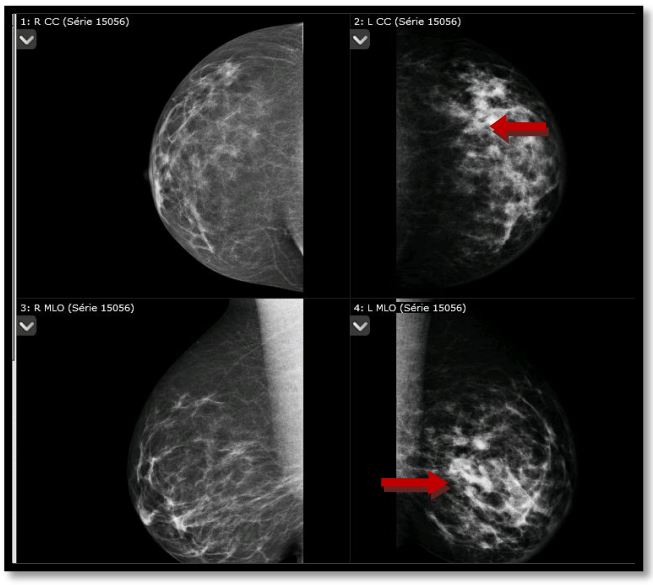

Figure 7. Mammography of locally advanced breast cancer. Mammogram shows dark areas of normal fatty breast tissue and lighter areas are dense breast tissue that includes ducts and lobes. The whitest area is the most dense, indicating a tumor (breast cancer), indicated by a red arrow. This picture was provided by Hospital CUF Descobertas)

Ultrasound testing works by transmitting high-frequency sound waves, inaudible to the human ear, through the breast. The sound waves bounce off surfaces in the breast (tissue, air, fluid) and these "echoes" are recorded and transformed into video or photographic images. Breast ultrasound is a procedure that may be used to determine whether a lump is a cyst (sac containing fluid) or a solid mass which might be cancer (figure 8). If the lump is found to be a cyst, fluid is typically withdrawn from it using a needle and syringe (a process called aspiration). If clear fluid is removed and the mass completely disappears, no further treatment or evaluation is needed.

Ultrasound can also be used to precisely locate the position of a known tumor to help guide the needle during a biopsy or aspiration procedure. Ultrasound helps confirm correct needle placement.

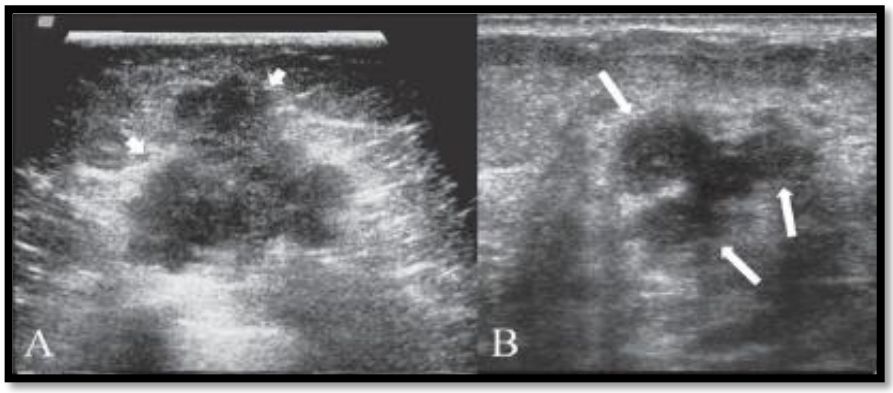

Figure 8. Ultrasound images of a malignant breast lesion. **A.** shows a typical malignant nodule that is taller than wide. **B.** Some of the nodules may reveal a branching pattern, indicated by white arrows [41].

The two main tests used to diagnose breast conditions are mammograms and ultrasounds. Magnetic resonance imaging (MRI) is used in some cases, usually along with one of the other two tests.

A biopsy may be performed when an abnormal finding in the breast is discovered during a mammogram, ultrasound, or physical examination. The method recommended will depend on how large the breast lump or abnormal area where in the breast it is located, how many lumps or abnormal areas such as suspicious calcifications are present. The types of breast biopsies include: ultrasound-guided core biopsy, stereotactic biopsy, open excisional biopsy and sentinel node biopsy.

Imaging studies alone cannot provide a specific diagnosis on which to base treatment decisions. The breast biopsy is a procedure, which confirms whether the tissue is cancerous or precancerous. The table IV summarizes the tests that may be used to diagnose breast cancer or for follow-up testing after the cancer has been diagnosed.

Figure 9. Image of breast biopsy system.

Adpated from *http://www.mammotome.com/images/supplement/BiopsySystem1.png*

Table IV. Tests and diagnostic procedures for breast cancer.	
Imaging tests	Mammography Untrasounds MRI
Surgical tests	Biopsy
Molecular testing of the tumor	ER and PR, HER2; KI-67
Tests and procedures used to stage breast cancer	-Blood tests, such as a complete blood count -Mammogram of the other breast to look for signs of cancer -Breast MRI -Bone scan -Computerized tomography (CT) scan -Positron emission tomography (PET) scan

1.8 Treatment

Treatment involves conservative surgery (surgical removal of the tumor and surrounding tissue) or mastectomy (surgical removal of the breast). The tumor size and breast size grade are the patient characteristics that will be important to choose type of treatment to adopt.

Numerous studies have shown that for early breast cancer (cancer that has not spread to the skin, chest wall, or distant organs), the long-term survival for women treated with breast-conserving surgery plus radiotherapy is similar to those treated with mastectomy. For women undergoing mastectomy, significant advances in reconstruction techniques provide several options for breast reconstruction, including several different options on the timing of the process.

In women with early stage disease, the sentinel lymph node biopsy, a procedure in which only the first lymph nodes to which cancer is likely to spread due to lymph node anatomical drainage system are removed, has a lower chance of long-term side effects and is as effective as a full axillary node dissection, in which numerous nodes are removed, to determine whether the tumor has spread beyond the breast.

Treatment may also involve radiation therapy, chemotherapy (before or after surgery), hormone therapy or targeted therapy.

Tumor cells viable after neoadjuvant chemotherapy (NAC), which is chemotherapy administered before surgery, are a population of cancer cells that is intrinsically resistant to chemotherapy. These tumor cells likely reflect the component of micrometastatic disease, which is responsible for distant metastases, and is unlikely to be sensitive to adjuvant chemotherapy, if so metastases will one day become evident. Specifically, this phenomenon has been observed in patients with TNBC who have residual disease after NAC [40].

2. Triple-negative breast cancer (TNBC)

TNBC has a growing recognition by oncologists, pathologists and geneticists since 2005, when it began to be referred by the term "triple-negative" [42]. TNBC comprises 12 to 17% of BC cases. This terminology reflects a heterogeneous population with a much more complex molecular transcriptome than is suggested by the triple-negative (TN) immunohistochemical (IHC) expression [43]. Furthermore this definition is misleading because it tells us what these cancers are not and not what they are, it is a negative definition.

TNBC is a distinct subtype defined by the lack of immunohistochemical expression of the estrogen receptor (ER) and progesterone receptor (PR), human epidermal receptor growth factor 2 (HER2) amplification. TNBC generally has expression of genes normally found in the basal, myoepithelial cells or normal breast [25, 42-44] .

The frequency of the triple-negative breast cancer increases with age, it comprises approximately 15% to 20% of all breast cancers [25, 45] although TNBC are more common than ER positive BC in younger patients (<50 years) and African American women [42, 44]. TNBC is associated with an advanced stage, increased risk of visceral disease and worse outcome. Several studies have been carried with the purpose of demonstrating these associations [24, 25, 37, 46-48]. These tumors are generally larger in size, are of high histological grade (III) [23], with lymph node involvement at diagnosis, and are biologically more aggressive with worse prognosis [24]. The majority of TNBC are grade III or poorly differentiated, infiltrating ductal carcinoma not otherwise specified (IDC NOS). The few remaining cases are rare histological types like adenoid-cystic, medullary, apocrine, metaplastic or inflammatory BC [23].

The aggressiveness of this subtype of cancer is illustrated by the fact that the peak risk of recurrence is between the first and third year of follow-up in patients diagnosed with TNBC and most deaths occur in the first 5 years after therapy. When compared with patients who have other subtypes of breast cancer [24]. Less than 30% of women with metastatic TNBC survive five years and all die of the

disease, despite having undergone adjuvant chemotherapy and subsequently chemotherapy for advanced disease [24] [49].

Patients with triple negative breast cancer are more likely to develop distant metastases earlier than non-triple negative breast cancer patients, develop brain metastases sooner [28]. The prevalence of genetic mutations among women with TNBC referred for genetic counseling is high and differs significantly by ethnicity/race and age [45].

This molecular subtype of breast cancer is characterized by a profile of gene expression similar to that found in basal/myoepithelial cells. The multiplicity of names reflects an underlying uncertainty about the true nature of this entity.

The breast cancer literature includes a large number of reports on prospective clinical trials examining the effects of endocrine therapy, chemotherapy, targeted therapy combinations, but none has validated a specific systemic therapy for TNBC [42]. Although effective tailored therapies have been developed for patients with HER2 positive or hormone receptor positive, chemotherapy is the only modality of systemic therapy for patients with BC that lack the expression of these three markers [44] [43].

One of the first molecular insights into TNBCs was the finding that they are likely to arise in patients with germline mutations in the BRCA1 gene and have gene expression profiles (GE) similar to those tumors which are not TNBC but arise in germline BRCA1 mutated women [44]. Germline BRCA1 or BRCA 2 mutations among women are the most frequent hereditary breast cancer syndromes and are associated with a 90% lifetime risk of developing BC. Studies have shown the prevalence of BRCA mutations in women with TNBC is high, therefore the finding of a woman with a TNBC and positive family history of BC is a reliable indicator of possible BRCA germline mutation [45]. BRCA1 behaves as an important gene in DNA repair of double strand DNA breaks, similarly contributing to the maintenance and stability of DNA [44]. Among a population of patients with TNBC referred for genetic counseling and genetic testing between 2000 and 2012, the prevalence of BRCA mutation carriers exceeded 30% [45].

2.1 TNBC subtypes

There are several studies that research the molecular profile that distinguishes subtypes of TNBC. Until recently, most studies on TNBC aimed to identify markers that separate TNBC from other BCs, and it is likely that these studies identify molecules that differentiate amongst subtypes of TNBC.

Since 2005, when the widespread use of anti-HER2 therapy started to change HER2 positive BC, TNBC finally became the only subtype of BC for which there is no targeted therapy. This is a very important unmet need in BC. Since then a lot of experiments and clinical trials have been done to try to understand what is targetable in TNBC, and if the clinical trials meet their endpoints, so we might validate usable drugs for daily practice. Besides the therapeutic challenge, TNBC entails a diagnostic challenge and Lehman *et. al.* after several experiments have put forward a convincing classification of six subtypes with histological, genetic, epidemiological, therapeutic, and, therefore, practical implications (appendix II). TNBC subtypes were characterized on the basis of differential GE and gene ontologies and subsequently labeled TNBC as follows: basal-like (basal-like 1 (BL1); basal-like 2 (BL2)); immunomodulatory (IM); mesenchymal (M); mesenchymal stem–like (MSL); luminal androgen receptor (LAR) [44].

The top biological processes present in the BL1 subtype are cell cycle and cell division components and pathways. Elevated DNA damage response (ATR/BRCA) pathways accompany the proliferation pathways in the BL1 subtype. Increased proliferation and cell-cycle checkpoint loss are consistent with the elevated expression of the DNA damage response genes observed. The highly proliferative nature of this subtype is further supported by the finding of high Ki-67 mRNA expression (MKI67). Enrichment of proliferation genes and increased Ki-67 expression in basal-like TNBC tumors suggest that this subtype would preferentially respond to antimitotic agents such as taxanes (paclitaxel or docetaxel). The BL2 subtype displays unique gene ontologies involving growth factor signaling as well as glycolysis and gluconeogenesis. Likewise, the BL2 subtype is uniquely enriched in growth factor receptor expression such as EGFR, MET (proto-oncogene, receptor tyrosine kinase), and EPHA2 (member of the ephrin-A receptor subfamily of

receptor tyrosine kinases). This subtype has features suggestive of basal/myoepithelial origin as demonstrated by higher expression levels of p63 and MME (CD10) [34].

The IM subtype is enriched in immune cell processes. The IM signaling is evidenced by immune signal transduction GE, in addition to immune cell-surface antigens, cytokine signaling, complement cascade, chemokine receptors and ligands, and antigen presentation genes. This subtype of TNBC substantially overlaps with the gene signature for medullary breast cancer, a rare, histologically distinct form of TNBC that despite its high-grade histology is associated with a favorable prognosis.

The M subtype (M and MSL subtypes) displays a variety of unique gene ontologies that are heavily enriched in components and pathways involved in cell motility. The difference between the M and MSL subtypes is that the MSL subtype expresses high levels of proliferation genes. This subtype was associated with a highly dedifferentiated type of breast cancer called metaplastic breast cancer, which is characterized by mesenchymal/sarcomatoid or squamous features is chemoresistant and, therefore, carries severe prognosis.

The LAR group is the most distinct among the TNBC subtypes. This subtype is ER negative, but gene ontologies are heavily enriched in hormonally regulated pathways including steroid synthesis, porphyrin metabolism, and androgen/estrogen metabolism. Others have previously described a breast cancer subgroup expressing AR termed molecular apocrine and this BC has good prognosis.

The identification of different subtypes of TNBC had focused on regulatory molecules, both extracellularly the immune system and at the level of the plasma membrane, a gene set comprised epithelial growth factors. The immune regulation has previously been proposed to play a role in BC and, in particular the ER positive BC. Intracellularly, these gene products bind to the molecules proliferation that control, such as cyclins. The activation of the immune response may preclude a good prognosis; this would be similar to the good prognosis associated with immune activation in melanoma, ovarian and kidney cancer.

P53 expression is a predictor of shorter time to relapse in independent populations diagnosed with TNBC. Similarly, increased expression of EGFR

appears to predict adverse prognosis in TNBC. In the latter report, loss of c-Kit and BRCA1 expression were also reported to be predictive of poor outcome in TNBC.

The cell lines representative of the TNBC subtypes display different sensitivities to a variety of agents, and importantly, these differences can be attributed to distinct expression of cellular components and presence of mutations in key oncogenes and tumor suppressors [44].

In the seventies, the early tests of the presence of AR protein in breast cancer specimens were performed, by the McGuire laboratory [50]. Currently, most of the studies were performed using specific anti-AR antibodies in IHC studies. This study will focus more on the expression of this protein in samples of patients with TNBC.

2.2 Androgen receptor

Steroid hormone receptors are ligand-activated transcription factors that regulate eukaryotic gene expression and affect cellular proliferation and differentiation in target tissues. The AR gene is more than 90 kb long and codes for a protein of 919 amino acids that has three major functional domains, as illustrated in figure 10 [51].

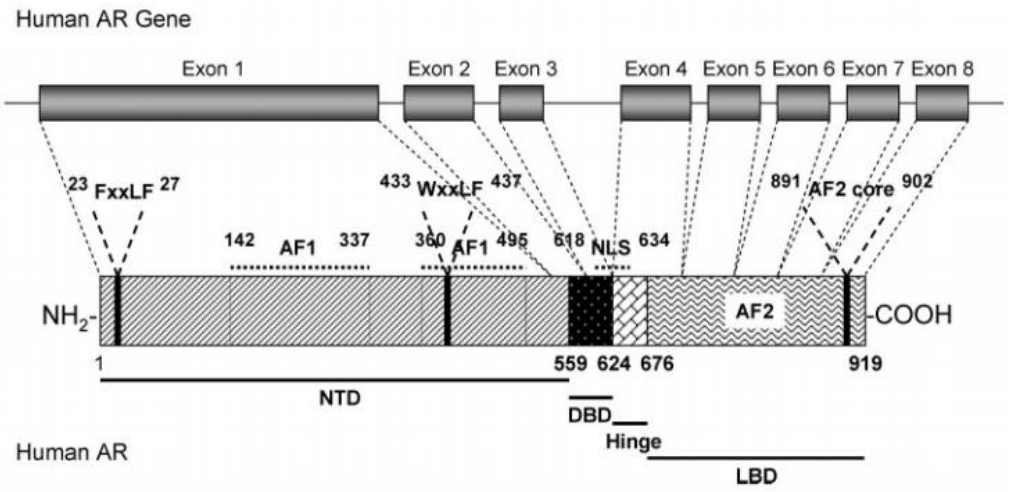

Figure 10. Human androgen receptor gene. The human androgen receptor protein is encoded by 8 exons (1-8). Similarly to other nuclear receptors, the protein consists of several distinct functional domains: the NH2-terminal domain (NTD), the DNA-binding domain (DBD), the hinge region and the ligand binding domain (LBD) [51].

Throughout a woman's life, the production of steroid hormones is prolonged and varied. During the fertile period of a woman’s life, estrogen and progestogens (progesterone and 16-OH progesterone) are secreted by the ovaries. Estrogen is the most important hormone in terms of mitogenic and morphogenic capacity, however it is secreted at low levels, unlike the progestogens that were abundantly secreted. The precursors required for the synthesis of estrogen in the ovaries are androgens (testosterone and androstenedione).

Androgens are also secreted by both the ovaries and adrenal glands and circulate in a similar estradiol concentration during the pre-ovulatory peak and in a higher during the remainder of the menstrual cycle. Androgens are sexual steroids which are dominant throughout a woman's life. After menopause, there is a survival adaptation to an environment with a low concentration of estrogen. This adaptation requires increased capacity of local estrogen synthesis and increased cellular response to these hormones. Together, this change may cause the epithelial cell to have increased levels of aromatase and of receptor coactivators of estrogen. Estrogen drives carcinogenesis because it induces cell survival. The endogenous sex steroids have been implicated in the development of breast cancer with evidence of increased risk among women who had early menarche and decreased in women who experience early natural menopause or had a bilateral ovariectomy at young age.

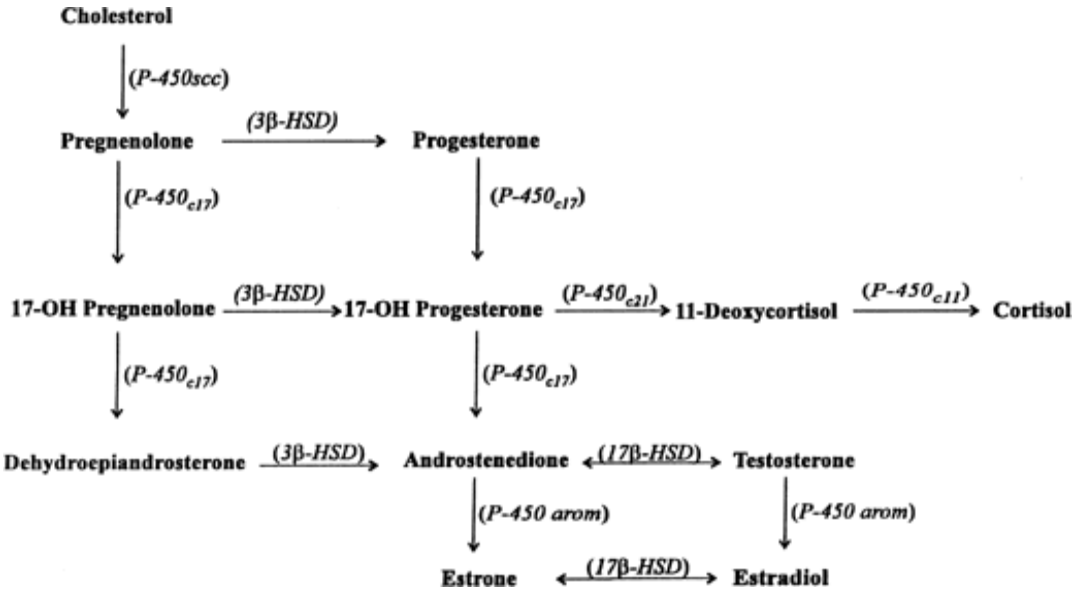

Figure 11. Synthesis of steroid hormones at menopause. Both adrenal and ovarian androgens produce low power, mainly androstenedione and dehydroepiandrosterone sulfate (DHEA-S). Enzymes 17-hydroxysteroid dehydrogenase and aromatase are widespread in peripheral tissues, including brain, liver, adipose tissue and the pathological and normal breast epithelium. Aromatase catalyzes the conversion of androgens to estrogens. Estrogen content of breast cancer cells during menopause is even higher than in tumors in premenopausal women. As the tissue of breast cancer also contains sulfatase activity, DHEA-S also acts as a precursor to estrogen. *(Image adapted from http://www.glowm.com/resources/glowm/cd/graphics/figures/v5/0380/05.gif)*

The androgen receptor is a member of the family of nuclear receptors that are dependent on the binding of transcription factors. The AR has a higher affinity for dihydrotestosterone (DHT) than for testosterone, the two most potent natural androgens.

The process of binding of the hormone results in a conformational change in the molecule that promotes the AR to spill cytosolic heat shock proteins, and translocate to the nucleus. Once bound to a hormone, AR is able to form homodimers that are associated with chaperones and nuclear coactivators and is the active form of the receptor. The probability of accumulating, uncorrected DNA replication errors and mutations, which are the basis of estrogen-related tumorigenesis thus increases. Cells adapted to a low estrogenic environment increase the number of cell cycles per year, these changes include androgen aromatization to produce estradiol which is mitogenic in epithelial breast cells. Hormone-related breast carcinogenesis after menopause possibly is a consequence of subtle changes that allow the epithelial cells to survive in a low estrogenic environment. Older age at diagnosis has previously been reported in patients with AR-positive BC [44].

Besides being able to act as an estrogen precursor, androgen can directly act on epithelial breast cells by activating the androgen receptor which modulates a number of genes at the transcriptional level.

Information on of AR expression in breast cancer is sparse. Studies have focused on the role of androgens and the androgen receptor and they have demonstrated that androgens can lead to proliferation of AR expressing breast cancer cell lines and promote tumor formation in animal models. AR is a marker of good prognosis and this effect appeared independent of coexpression of ER. New drugs targeting AR have been used in cases of BC. Despite an association with good outcome, targeting of breast tumors expressing AR may be beneficial, similar to the effects of pharmacologic targeting of ER (for example, bicalutamide, a non-steroidal anti-androgen).

3. Objective

The main purpose of this study consisted in the evaluation of the expression of androgen receptor in triple-negative breast cancer. The sample was collected at Hospital CUF Descobertas, Lisbon, and consisted of all consecutive TNBC treated and followed in the Institution in the years between 2005 to 2012.

TNBC due to its heterogeneous profile presents a specific problem in detecting targets for systemic therapy.

Thus, in this study, we had a second objective. The relevance of the Lehmann classification of TNBC cannot be ascertained because this classification has not been adapted to IHC. We used, together with the androgen receptor, the following antibodies: CK5/6 (cytokeratin 5/6), p-cadherin, EGFR (epidermal growth factor receptor) and PHH3 (phosphohystone H3) to further separate TNBC.

Gene expression profiles signature derived from the most differentially expressed genes found in the TNBC training set and used to predict which TNBC subtype was best-fit for each of the tumors in the validation set.

In this study, distinct genes ontologies were used to each TNBC subtype. BL1 and BL2 subtypes were determined through the assessment of EGFR and CK5 /6 and a proliferation marker PHH3. The BL1 subtype expressed higher levels of basal cytokeratin expression; enriched in cell cycle, cell division components and pathways and the highly proliferative nature. The BL2 subtype displays unique gene ontologies involving growth factor signaling. Expression of p-cadherin was used for the identification of mesenchymal. This subtype displays a variety of unique gene ontologies that are heavily enriched in components and pathways involved in cell motility; epithelial-mesenchymal transition is associated. The immunomodulatory TNBC subtype will be assess through the evaluation of TIL (tumor infiltration lymphocytic). And for last, the LAR (luminal androgen receptor) subtype was determined by the androgen receptor.

4. Materials and methods

Patients diagnosed with triple-negative breast cancer, between the years 2005-2012, were included in this study from Hospital CUF Descobertas e Hospital Infante Santo.

The initial sample included 80 cases of TNBC. Because of lack of information from the patients and paraffin blocks from the Hospital CUF Infante Santo (patients with an incomplete record and/or patients without tissue available to be studie), the sample includes only patients from Hospital CUF Descobertas and comprises 66 patients with TNBC.

4.1 Clinical-pathological method

Clinicopathological parameters including age, type of surgery, tumor size, histological grade, node involvement, adjuvant treatment, disease free survival (DFS) and Overall survival (OS) were reviewed.

The patients' age categorized in less than 35 years, from 35 to 50 years, from 50 to 65 years and over 65 years.

Ppathological data correspond to the size of the tumor, lymph node involvement and histological grade were also analysed. The size of tumor was categorized as < 2cm, between 2cm and 5cm, >5cm the diameter and/or not determined. For lymph node involvement was described as positive or negative involvement, and/or not determined. The histological grade was classified in 1 (well), 2 (moderate), 3 (poorly) and/or not determined (Scarff-Bloom-Richardson grading system).

Adjuvant treatment refers to the administration of both chemotherapy (CT) and radiotherapy (RT) treatments.

DFS was defined as the interval (mouths) from primary surgery data until the first relapse of disease.

OS was the time, in months, from the date of the primary surgery data to the time of breast cancer related death.

The information from patients was collected from electronic patient records and hospital charts. Before study initiation, Institutional ethics committee approval was obtained.

4.2 Histopathological method

All the material collected had be fixed in 10% formaldehyde included in paraffin (type 6, Richard said AllScientic ®), following the usual methodology. After the inclusion, slices of 2,5 -3 µm thick were performed in a manual Minot microtome, manual (RM2255 leica Microssystems). Then, the sections were placed on slides and held drying in an oven (60 min at 60ºC or overnight at 37ºC). After drying in a row in decreasing alcohol concentration (70%, 96% and absolute alcohol -100%), proceeded to staining with Hematoxylin (Harris) and Eosin (alcoholic), about 40min-1h.

The histopathologic review was made by pathologist with experience in breast cancer at the Pathology Department, Hospital CUF Descobertas.

Figure 12. Minot microtome and cold plate used in the histology laboratory.

4.3 Immunohistochemical method

The blocks for Immunohistochemical analysis of patients with TNBC subtype were obtained from the Pathology files, Hospital CUF Descobertas. After obtaining high quality paraffin blocks, the study comprised only 50 cases. The experimental work was conducted at the Faculty of Medicine, University of Lisbon (FMUL) at the Institute of Pathology, in collaboration with the Technical Catarina Isabel Talina.

After obtaining the blocks of patients with triple-negative breast cancer, 2µm thick slices were made and placed in adherent SuperFrost®Plus slides, which then are putted in the oven to dry for 60 min.

Immunohistochemistry is a set of methodologies that use antibodies as specific reagents that are able to identify and connect with tissue constituents that act as antigens. This connection allows locating and identifying the presence of various substances in cells and tissues by means of color that is associated with the antigen-antibody complexes formed [52].

The possibility of combining a marker with an antibody without causing any damage to the specific connection established between antibody and antigen makes this technology area heavily used in Pathology. This provides the microscopic observation of the location where is the antibody and hence the antigen. Apart from the quality of the antibody, the marking quality also depends on the pre-analytical phase (highlighting tissue fixation and processing), the recovery of antigenic epitopes and sensitivity of the detection system.

In this study, the immunohistochemical method used was the method of indirect polymer. This method applies a primary antibody directed against the desired antigen, and subsequently applies a polymer to which they are coupled "secondary" antibody substances and enables visualization of HRP (Horseradish Peroxidase). It is a quick and easy method, showing a decrease of error factors.

This method enables amplification of weak signals i.e. proteins of low abundance can be visualized. It is however, a very expensive method.

The method of indirect polymer uses a dextran polymer, not containing avidin or biotin, then the markup nonspecific does not occur in various tissues. A solution of 3,3-diaminobenzidine tetrahydrochloride (DAB) was used as chromogen agent.

Dextrans are high molecular weight molecules of approximately 500kDa, with high solubility in water and low toxicity/immunogenicity. These molecules are used as the internal skeleton of the polymer. Rarely bind to poly (1,6-α-D-glucose), which makes them resistant to cleavage by endogenous glycosidases of the cell.

Commercially, these molecules which are presented in the form of a highly flexible polymer agglomerates in solution become very extensible spirals. This compound is readily soluble in water and electrolyte, being colorless, transparent and highly stable solutions. This type of polymer affects large and encompasses approximately 100 molecules of HRP and 20 of the type secondary antibodies goat anti-mouse or goat anti-rabbit, all of these molecules bound directly to the skeleton of the active dextran (figure 14).

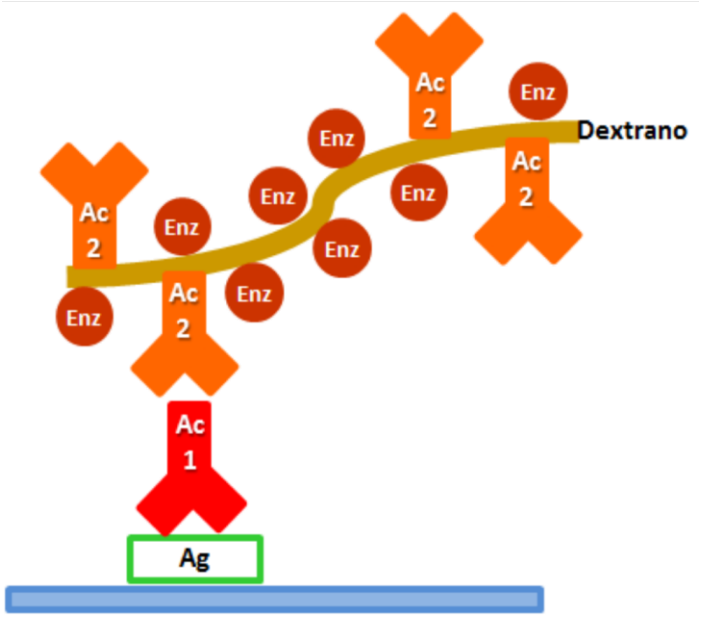

Figure 13. Representation of the polymer dextrano technique.

Adapted from IHC Amadeu BF [52].

Table V shows the different tissues used as positive controls for different antibodies.

Initially several preliminary experiments were performed to optimize the primary antibody (Table VI), for the method of antigen retrieval and incubation time for the clearance technique.

Breast cancer tissue were immunohistochemically stained for cytoqueratin 5/6, androgen receptor, p-cadherin, phosphotistone H3 (PHH3) and epidermal growth factor receptor as previously according to the suppliers data sheet (Abcam, Company- Cambridge Science Park and Cell Marque Corporation-Supplies primary antibodies to Ventana Medical Systems®).

Table V. Controls for CK 5/6, Androgen receptor, P-cadherin, EGFR and PHH3.

Antibodies	**Positive controls**
CK5/6	Mesotheliome
Androgen receptor	Prostate
P-cadherin	Tonsil
PHH3	Tonsil
EGFR	Lymph Node with metastase

Table VI. Log referring to clone, type, cellular location and dilution of the antibodies according to the mark.

Antibody	**Brand**	**Clone**	**Species**	**Cell Location**	**Dilution**
CK5/6	Cell Marque	D5 & 16B4	Mouse monoclonal	Cytoplasmatic	1:200
AR	Cell Marque	SP107	Rabbit monoclonal	Nuclear	1:100
P-caderin	Abcam	Ab140338	Policlonal	Membranar	1:1000
PHH3	Cell Marque	N/A	Policlonal	Nuclear	1:100
EGFR	Abcam	EP38Y	Monoclonal	Membranar	1.100

CK5/6 = cytokeratin 5/6; AR = Androgen receptor; P-caderin; PHH3 = phosphohystone H3; EGFR =epidermal growth factor receptor.

The immunohistochemical analysis followed the following protocol:

Dewaxing: Place slides in the sections marked with study at 60°C for 60min. Allow to dry prior to antigen retrieval for 20 minutes (90°C)

Antigen retrieval: place the rack with the blades filled by antigen retrieval buffer PT module (15ml Buffer Cell Marque Trillogy R 1: 400 in 1485 ml of distilled water) in Electric Pressure camera to immunohistochemistry for 1 hour on high / low pressure; at the end, removed and allowed to cool approximately 37 min in distilled water.

Dehydrate until absolute alcohol (70%, 96%, 100%)

Blocking of endogenous peroxidase: the blades were passe by 4 ml bath of hydrogen peroxide diluted in 200mL methanol for 15 min, rinse in distilled water.

Washes with TBS: two washes with TBS, 5 min each.

Dilution of antibodies that will be used: with the solution (antibody diluents; Diamond) as the blades are in phase antigen retrieval.

Application of the primary antibody (for 60 minutes)

Washes with TBS: two washes with TBS, 5 min each.

Immunoenzymatic labeling method (HRP): 10 minutes applies an amplifier twice, five minutes each; applies then the polymer for 10 minutes twice, five minutes each.

Revelation with DAB ((*3,3'-Diaminobenzidine tetrahydrochloride hydrate*) HRP substrate; Cell Marque chromogens) for 3 minutes solution 50 ml of chromogen more 1000 ml substrate.
Washing with tap water to remove excess

Counter staining with Mayer's Hematoxylin for a few seconds

Wash in warm water

Dehydration of the cuts to the absolute alcohol - xylene.

Clearing and mounting the slides in xylene

5. Statistic Analysis

The statistical analyzes was performed using SPSS for Windows (Version 17). Survival analysis was performed with the Kaplan-Meier method. The Kaplan-Meier estimate is also called as “product limit estimate” and it is the simplest way of computing the survival over time in spite of all the variability associated with cancer patients and cancer follow-up. In clinical trials, the effect of an intervention is assessed by measuring the number of subjects whose survival increased or decreased after that intervention over a period of time. The survival curve provides a measure of mortality of patients with TNBC.

The results of the immunohistochemical analysis of the expression of Ck 5/6 were defined by intensity of staining and percentage of tumor cells. The expression of stained cells was divided into four categories: absence or weak labeling (0); weak staining and less than 1/3 of the cells (1+); moderate intensity staining of 2/3 of cells (2+) and strong staining of 3/3 (3+). The same was applied to the methodology of EGFR protein (score 0-3+). The marking was checked for EGFR membrane labeling. The expression of p-cadherin was staining according to positivity or negativity of the marked tissue. Expression of the androgen receptor was determined by nuclear expression of this protein (≥ 10%).

Proliferation index is calculated the counting of PHH3 by expression of nuclear tumor cells. The percentage of tumors cells expressing the proliferation marker PHH3 determined the growth rate of tumors. The proliferation index was calculated by the total number of PHH3- positive nuclear profiles. Due to poor tissue fixation, some cases were excluded.

Inflammatory infiltration was classified in absent, moderate, slight, intense and not determined.

6. Results

The clinical characteristics of the patients included in this study are presented in table VII. We studied samples of 66 patients with TNBC obtained from Hospital CUF Descobertas, Lisbon, between 2005 and 2012.The clinical chart data were reviewed and analyzed. The majority of patients were middle aged between 50 and 65 years old. The median age was 60 years. About 89% of patients underwent surgery. The majority of these patients also presented a tumor grade II or III. The tumor size had a mean 2,1 cm and a median of 1,8 cm.

TNBC patients had positive axillary nodal involvement was found 74%. In total, 44% of the patients underwent chemotherapy and radiotherapy, 79% underwent chemotherapy.

Subsequently, the median follow-up time for the time diagnosis until the disease free survival (DFS) was 12 months (range, 0-24months). Overall survival for that death population was 3 years (range, 1-6 years). TNBC phenotype was associated with poorer outcome in terms of overall survival and disease-free interval when compared to publish cohorts of BC patients.

The Kaplan-Meier method was used to estimate time-to-event functions. Approximately 75% of patients remain without evidence of disease (Figure 14A). The calculation was made in a range of time in months, we found a median of 12 months and a mean of 11 months to relapse of TNBC. The calculation of OS (Figure 14B) was made in a range of time, in years, with a median of 3 years and a median of 3.28 years from the diagnosis to date of death. DFS and the OS were obtained in a confidence interval of 95%.

Most patients received additional neoadjuvant chemotherapy following FEC (Fluorouracil (5-FU), epirubicin, cyclophosphamide) plus docetaxel.

The most common site of initial relapse was bone. Other sites involved in relapse are lymph nodes, central nervous, system lung and liver.

Table VII. Patient and Tumor Characteristics Associated with TNBC.

	No. of patients (%)	
Patient or tumor Features	***TNBC (N= 66)***	***%***
Age At Diagnosis (y)		
<35	1	2%
35-50	13	20%
50-65	28	42%
>65	24	36%
Surgery		
Positive	59	89%
Negative	1	2%
Not determined	6	9%
Nodal involvement		
Positive	49	74%
Negative	0	0%
Not determined	17	26%
Tumor Grade		
1 (well)	1	2%
2 (moderate)	14	21%
3 (poorly)	45	68%
Not determined	6	9%
Tumor size (cm)		
≤ 2	34	52%
2 < cm ≤ 5	18	27%
≥ 5	4	6%
Not determined	10	15%
Adjuvant treatment		
CT / RT		
Yes	29	44%
No	6	9%
Not determined	31	47%
Disease Free Survival (DFS), months, (Range)	12 (0-24)	
Overall Survive, years, (Range)	3 (1-6)	

Note: Percentage (%) indicates the percentage within the subgroup.

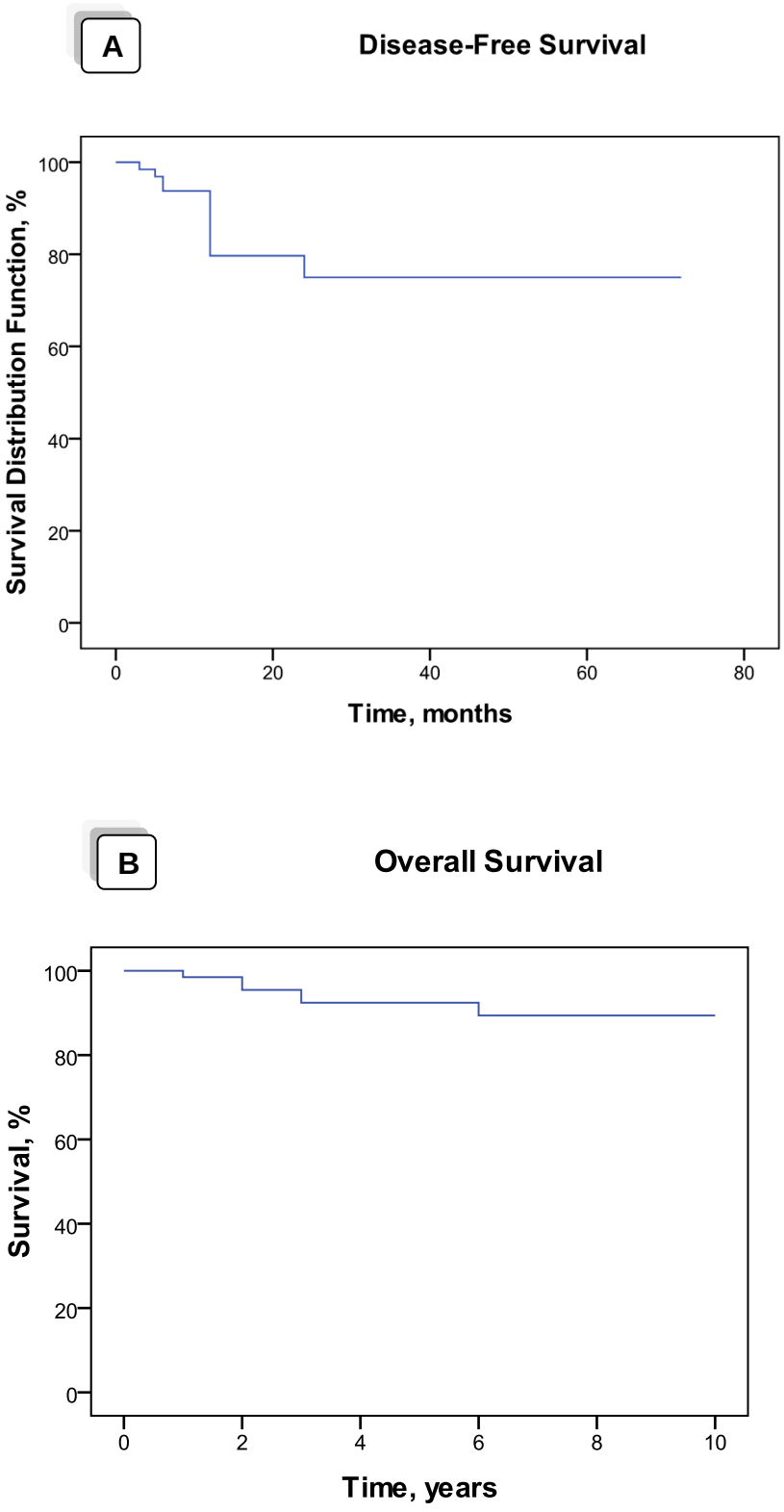

Figure 14. A. Disease-free survival curve for patients with TNBC. Probability of survival estimated using the Kaplan-Meier method. **B.** Overall-survival curve of patients with TNBC. Probability of survival estimated using the Kaplan-Meier method.

After analyzing available clinical data, the tissue samples were reviewed and tested for CK5/6, AR, p-cadherin, EGFR and PHH3 staining. The clinical pathological status of TNBC used CK5/6, AR, p-cadherin, EGFR and PHH3 which are summarized in Table VIII.

Table VIII. Expression of CK5/6, AR, p-cadherin, EGFR and PHH3 in 50 cases of TNBC.

Antibodies	No. of patients (%)
Ck5/6	
0	56% (28/50)
1+	22%(11/50)
2+	12% (6/50)
3+	10%(5/50)
AR	
<10%	62%(31/50)
>10%	38%(19/50)
P-cadherin	
Positive	96%(48/50)
Negative	4%(2/50)
EGFR	
0	38%(19/50)
+1	26%(13/50)
+2	24%(12/50)
+3	12%(6/50)
PHH3	
≥5% positive	80% (40/50)
<5%negative	20%(10/50)

Of all the TNBC cases, 56% (28/50) were CK5/6, 0; 22% (11/50) were CK5/6, 1+; 12% (6/50) were CK5/6, 2+; and 10% (5/50) were CK5/6, 3+. The expression of AR was calculated by upper or lower than 10% of the labeled cells staining. In some cases with higher expression of AR, we attributed scores of 50%, 90% and/or 100%. Thus, there were 62% of cases being lower than 10% and 38% of cases where there was more than 10% AR labeling. In 96% (48/50) the expression of p-cadherin was positive while only 4% (2/50) was negative. The 38% (19/50) were EGFR, 0; 26% (13/50) were EGFR, 1+; 24% (12/50) were EGFR, 2+; and 12% (6/50) were EGFR, 3+.

In this work we assessed proliferation through labeling our samples with the PHH3 antibody. PHH3 is a core histone protein, which together with other histones forms the major protein constituents of the chromatin in eukaryotic cells. Immunohistochemical studies performed with anti-PHH3 antibody have shown that the antibody detects specifically the core protein histone H3 only when is phosphorylated at serine 10 or serine 28. The phosphorylation of histone H3 is a rare event in interphase cells but is a process almost exclusively occurring during mitosis. Studies have also revealed no phosphorylation on the histone H3 during apoptosis. Therefore, PHH3 can serve as a very effective mitotic marker.

A Ki-67 cut-off point of 15% was defined according to the experience of different pathologists as well as national and international recommendations at present [53]. In this study, less than 5% staining with the antibody is considered low for the proliferation index and greater than or equal to 5% is considered high for PHH3 proliferation index. About 80% (40/50) of the sample of TNBC have a high nuclear staining of PHH3 and around 20% (10/50) are considered low.

Inflammatory infiltration (IF) has been considered as a likely prognostic factor in malignant neoplasms, and in this study was classified as absent, slight, moderate, intense and not determined (table IX). This rating was assessed in the samples used for PHH3 staining with the analysis of the antibody PHH3. In 34% (17/50) of TNBC cases IF was classified as absent; in 16% (8/50) of TNBC cases IF was classified as moderate; in 22% (11/50) of TNBC cases IF was classified as slight and in 8% (4/50) TNBC cases IF was classified as intense inflammatory infiltration.

The remaining cases were called “not evaluable” for above mentioned reasons and represent 20% (10/50) of cases TNBC.

Table IX. Lymphocytic infiltrate

absent	17	34%
slight	11	22%
moderate	8	16%
intense	4	8%
Not available	10	20%

In this series of TNBC, basal phenotype was defined by expression of CK5/6, EGFR and PHH3 in tumor cells; mesenchymal phenotype of TNBC was defined by expression of p-cadherin protein; immunomodulatory phenotype of TNBC was defined by the evidence of lymphocytic infiltration and luminal receptor androgen phenotype was defined for the expression of AR. The table X summarizes TNBC subtypes and markers used in this study.

Table X. TNBC subtypes and markers.

Subtype	Markers
Basal -like	CK5/6, EGFR, PHH3
Mesenchymal (M,MSL)	P-cadherin
Immunomodulatory	Lymphocytic infiltration
Luminal androgen receptor	AR

Microphotographs showing the morphology and immunohistochemical results for CK5/6, p-cadherin, EGFR, AR and PHH3 in breast tissue (figures 15-18). Immunohistochemical expression of CK5/6 was observed by cytoplasmatic staining (figure 15), p-cadherin (figure 16A) and EGFR (figure 16B) positive cells were observed by membranar staining and the AR (figure 17) and PHH3 (figure 18) observed by nuclear staining.

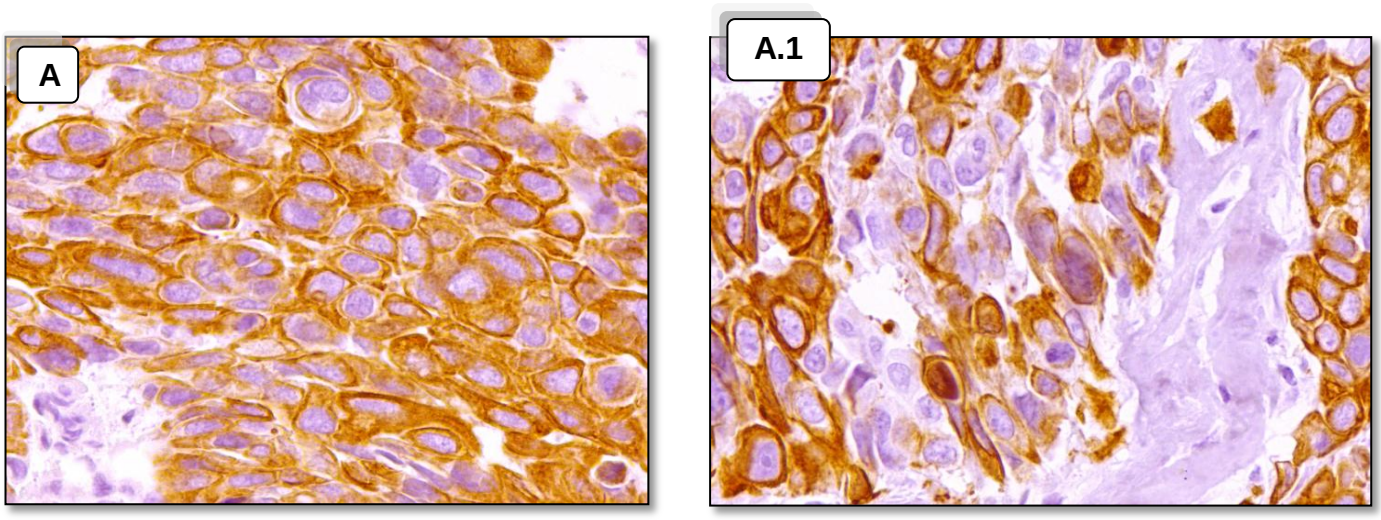

Figure 15. (A) Immunohistochemical preparation of samples from patients with TNBC which shows the cytoplasmic expression of CK5/6 (40x).

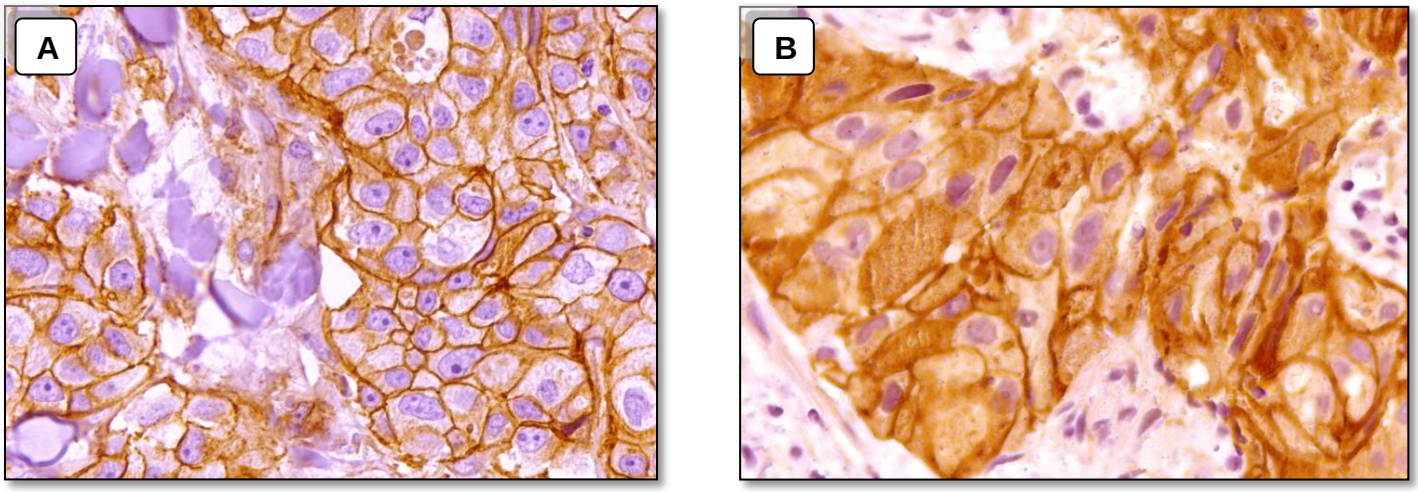

Figure 16. (A) Immunohistochemical preparation of samples from patients with TNBC which shows the p-cadherin expression (40x). **(B)** Immunohistochemical preparation of samples from patients with TNBC, which shows EGFR expression (40x).

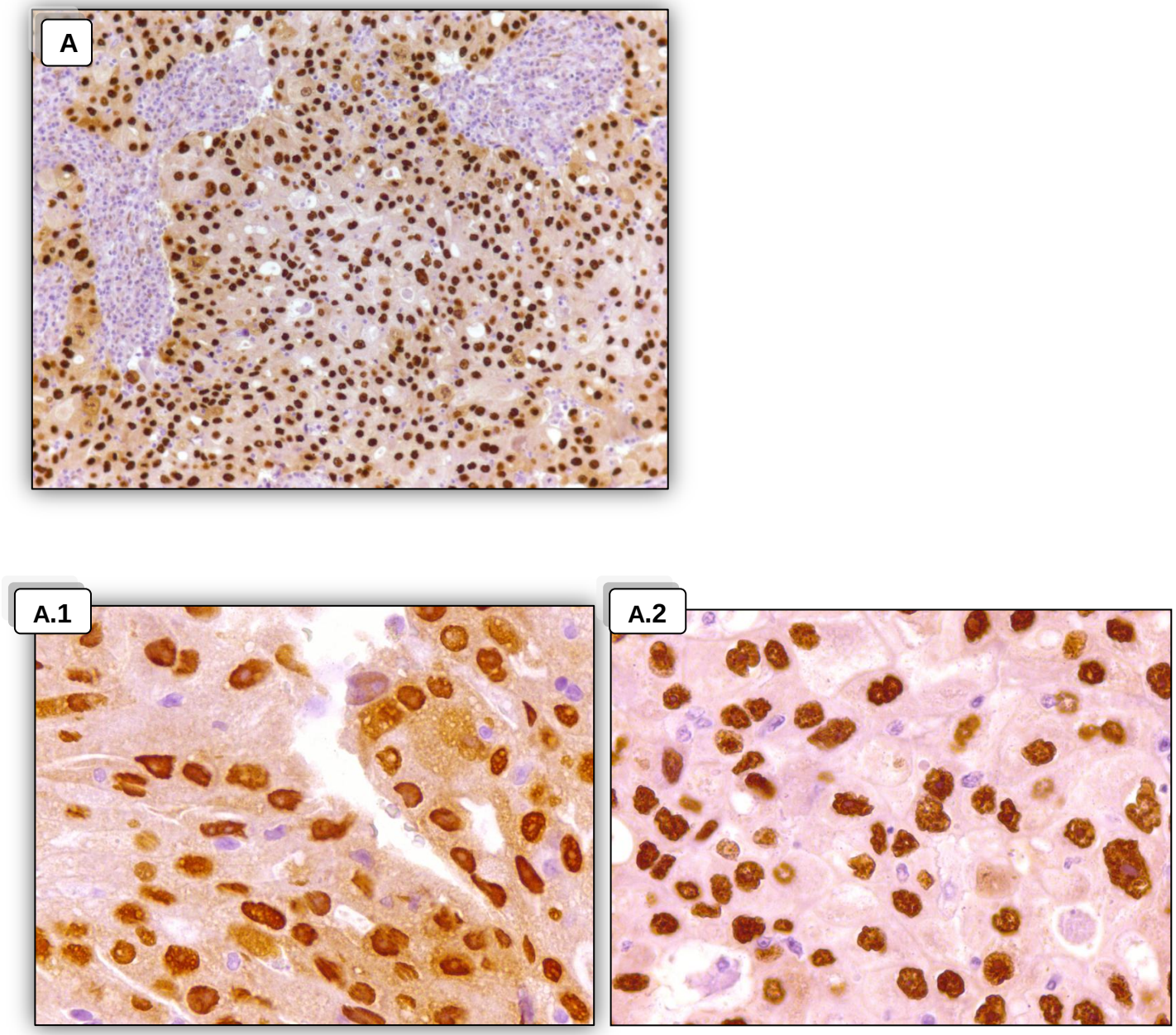

Figure 17. **(A**) Microphotographs showing AR expression (20x);

(A.1, A.2). AR expression (40x); intense nuclear staining.

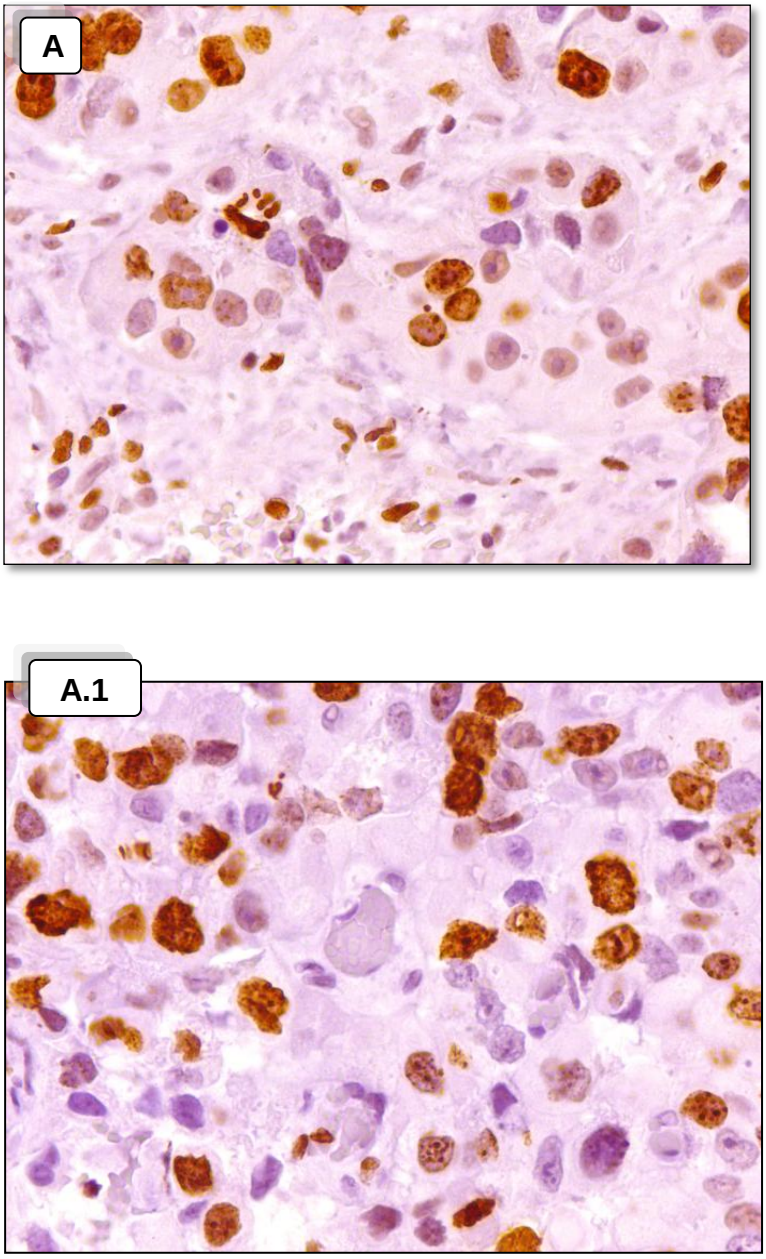

Figure 18. PHH3 nuclear expression (40x). This cell proliferation marker here is positive in about 70% of the nuclei. It is a protein present in all cells which are in cell division and absent in G0 cells that do not divide cycle. PHH3 is a very effective mitotic marker.

7. Discussion

Breast cancer is a heterogeneous disease. Currently, breast cancer, relies on traditional prognostic factors including nodal status, tumor histological grade and primary tumor size, in addition on expression to ER, PR and HER2. The molecular identification has allowed the classification of subtypes of BC, however it remains difficult to predict response to therapy. Surgery, radiotherapy and chemotherapy individually or in combination, are the only available modalities for patients with TNBC. Patients with TNBC do not benefit from hormonal or trastuzumab-based therapies, because of the loss of target receptors such as ER, PR and HER2 [32].

TNBC tumors were mainly of high histological grade, high mitotic index and were found more frequently in old age women [32]. The most common histological types were carcinomas of no specific type, metaplastic and apocrine carcinomas, the majority of these tumors were grade III. 80%-90% of TNBC truly have the basal molecular phenotype, which is known to be associated with worse OS [25].

This study shows the expression of five antibodies in cases of TNBC. These results attempted to apply to IHC the Lehamnn classification of TNBC subtypes performed in with genomics.

The expression profile of cytokeratin intermediate filaments is used in clinical practice and has been applied for the differentiation of metastatic tumors of unknown origin and currently is being used with interest to mark the cells of the mammary gland [54]. Cytokeratins have an important role in the cell motility, cell membrane support, forming pathways for the transport of organelles and other elements in the cytosol, under constant rearrangements that can produce cell movements [55] [56]. Cytokeratins type II (CK5 and 6) and type I (CK14) stain basal and / or myoepithelial cells, while antibodies to cytokeratins type II (CK7 and 8) and type I (CK18 and 19) are associated with the luminal/glandular epithelial cells of the breast [54].

The expression profile of cytokeratin CK5/6 and CK14 is interesting for studying BC. These markers have been used in the identification of basal-like TNBC. Since the early work of Sorlie *et. al*. [30], the terminology and definition (morphological

and genomic) of basal-like cancers, remains controversial. Nielsen *et. al.* [37] proposed an immunohistochemical panel of four antibodies (ER, HER2, HER1 and CK5/6), although there is still no exact correlation with the genomic expression profiles determined by DNA microarrays. Recent European studies propose the use basal expression of cytokeratins (CK5/6 and/or CK/14) as the defining element of the phenotype of basal-like BC. To identify the basal TNBC, Rakha *et. al*. recently proposed to employ the criteria [57]: expression of CK5/6 and/or CK/14. In this study, for characterization the basal TNBC we chose CK5/6, EGFR and PHH3 markers.

In this work, the expression of CK5/6 is negative in 56% of the case is positive in 44% of these cases. The expression of EGFR is positive in 62% of cases and the expression of PHH3 is high (80% the cases of TNBC). This finding allows us to suggest that the subtype that is more dominant in the set, the group BL2 because the high percentage of EGFR and proliferation marker.

EGFR is a cell surface protein which, is activated by binding of epidermal growth factor (EGF), through tyrosine-kinase activity. EGFR gene amplification has been described in different tumors, especially in the central nervous system, lung and stomach [58]. Recently, protein expression of EGFR has been described in breast carcinomas especially in metaplastic carcinoma, which although less frequent, is inserted into the differentiation pathway of progenitor/myoepithelial cells of the mammary carcinogenesis [59] [60]. EGFR immunoreactivity in TNBC IDC (triple-negative breast cancer, invasive ductal carcinoma) of the breast emerges as a clinically relevant prognostic parameter. Recently reported studies already indicated that EGFR expression is associated with significantly worse DFS in early breast cancer [61]. A significant proportion of the patients with TNBC IDC might have tumors concomitantly showing intratumoral necrosis [28], as well as EGFR and basal cytokeratin immunoreactivity [46]. This may explain why these closely associated factors are not all independently predicting DFS or OS when taken together in the full regression models [61].

The adherens-type junctions are related in normal development, to the role of cell sorting during embryogenesis and in the maintenance of specific organ and adult tissue architecture. Any disorders involving dysfunction of classical

cadherins/catenins are related to various disease states, including cancer. P-cadherin is transiently expressed in various tissues during development and its permanent expression is limited to adult epithelial tissues, at cell-cell boundaries. The use of particular markers that are expressed only in basal cells (or myoepithelial cells) of the mammary gland have led to a recently developed strategy to improve the diagnosis, prognosis and treatment in these tumors. In various human tumors, p-cadherin expression is altered, but its role in the carcinogenic process remains unclear because it behaves differently depending on the cancer cell model studied [62].

The expression of p-cadherin is well established as an indicator of poor prognosis in human breast cancer [62]. P-cadherin stimulated our interest in studying its role in TNBC and the results were consistent with what was reported in literature. The p-cadherin is over expressed in TNBC, 96% of cases were positive for this marker.

Several studies analyze lymphocyte infiltration in breast cancer and in fact they show that inflammatory cell infiltration of tumors contributes either positively or negatively to tumor invasion, growth, metastasis, and patient outcomes, which creates a conundrum when examining mechanisms of action. TIL (tumors infiltrative lymphocytes) has shown to be predictive of prognosis in breast cancer and it has been taking emphasis. The correlation between intratumoral immune responses and clinical outcomes in TNBC is potentially related to the role of immune cells in the activity of cytotoxic chemotherapeutics. In mice, chemotherapy with anthracyclines requires priming of IFNγ- producing CD8+ T cells [63]. In humans, correlative studies have reported that high intratumoral levels of IFNγ and CD8+ T cells [64], or TILs, are associated with better clinical responses to anthracycline-based chemotherapy [65].

The immunomodulatory subtype of TNBC was determined by the expression of lymphocytic infiltration. This assessment in our work was not technically optimized and was not planned because many of the cases don´t have good tissue preservation. Evaluation of TIL is thus compromised.

More recently, attention has focused in androgens and androgen receptor (AR) as prognostic markers and therapeutic targets in breast cancer [66]. The significance of AR expression is less well characterized when compared with the prognostic significance of ER and PR expression, in breast cancer and their roles in predicting therapy.

The implication of androgens in breast carcinogenesis was tested in animal models and this showed that testosterone in combination with estrogen can induce mammary carcinomas. The mammary gland density values were significantly higher in animals treated with testosterone alone or in combination with 17β-oestradiol than in those treated with 17β-oestradiol alone or in controls. The strong expression of androgen receptor in epithelium increased the amount of perialveolar and interlobular connective tissue and the proliferation rate of fibroblast-like cells in the stroma and had decreased the surrounding adipose tissue. All these changes were blocked by simultaneous implantation of flutamide, indicating that androgens play a crucial role in the process despite the absence of androgen receptors in stromal cells [67].

Several studies have focused on ER-positive tumors and have shown some association between AR status and disease-free survival, as well as response to endocrine therapy, but these findings have not been confirmed in AR positive and ER negative [68] [69, 70]. The ER-negative tumors characteristically have a poorer prognosis and are thought to respond poorly to endocrine therapy [71].

In one of the largest studies of AR status in breast cancer has found a highly significant association between AR status and survival, that remained significant even when taking ER status into account [72]. AR status was a significant prognostic factor for disease-free survival in univariate analysis, in multivariate analysis, only lymph node status, tumor size, and ER status were independent prognostic variables [68].

Curiously, in our study, the expression of androgen receptor is low. The sample reveals 38% positive case for AR expression. This observation shows that AR is not the most frequently expressed marker. Although statistical significance was lacking in our univariate analysis, a larger sample and additional research may help clarify whether AR status is a significant independent prognostic factor. The functional role

of AR in breast cancer remains unclear, further exploration of this area could expand the repertoire of potential treatments for patients with TNBC.

In this sample, IHC identified the subtypes of TNBC. The biologic validation of all the cases studied by conventional immunohistochemical techniques is laborious, expensive and time consuming. Given the high degree human error and difficulties to maintain its performance under identical conditions of temperature, concentration of reactants, even with the automation of some of the steps involved, there are numerous reports of conflicting results in the literature.

Additional markers [24, 32] are being sought to further refine those classifications, especially in patient subgroups such as TNBC, whose response therapy cannot be predicted accurately by conventional parameters. In this study we obtained the expression of the five markers in our work the expression of a single marker cannot be quantified due to very frequently overlap by the others markers. In clinical practice, this is what happens. Lehamnn starting from an analysis of gene expression was able to individualize each marker and accurately classify the subtypes of TNBC. It is important to note, the level of difficulty in clinical practice to get a correct diagnosis, for better targeted therapeutics. TNBC is an unclarified entity, despite all the advances in trying to respond to this subtype of BC, it requires more research for better pathological, clinical and therapeutic clarification.

Clinical trials evaluating the efficacy of certain receptors as targets for new therapeutic drugs have been performed, for example [40]:

1- Anti-EGFR and C-kit [37] the expression of EGFR has been reported in 66% of BC patients with TNBC tumors cells and basal-like tumors cells. Clinical trials evaluating the efficacy of humanized anti-EGFR monoclonal antibodies and EGFR tyrosine kinase inhibitors in TNBC are currently under way;

2- *In vitro* studies have indicated that breast tumor cells from BRCA1 germline mutations are extremely sensitive to a damaging high importance of these agents is based on the BRCA1 pathway and DNA repair dysfunction seen in TNBC, which may confer enhanced sensitivity to agents that damage DNA; Cells null for BRCA1 were shown to be incapable of repairing DNA double strand breaks [73];

3- The agents that inhibit PARP1 have successfully completed phase II and phase III studies have begun [74]. The PARP1 inhibitors effectively impair the ability of cancer cells to repair DNA damage causing the death on these cells;

4- Several genes have been reported to be amplified in TNBC. Many components of the proliferation pathways (Ras, MEK/ ERK) are overexpressed or mutated in cancer cells [40]. This can represent potential targets for TNBC. Genetic aberrations, such as gain of 1q, 3q, 7q, 8q and 10p and loss of 4p, 5q, 17p and 8p, have also been found in tumors arising in BRAC1 mutations carriers [75].

In this study we predicted that TNBC tumors could belong to the mesenquimal subtype by high expression of p-cadherin and/or, a sizable part of the sample could belong to the basal-like subtype, through the expression of CK5/6, EGFR and PHH3. However this observation is inconclusive, given the fact that we obtained a lot of overlap between the classifiers we used. The scientific advances with a view to clinical practice should move in this direction as future goals to better characterize subtypes of TNBC.

8. Conclusion

Since the sequencing of the human genome in 2000, the ability to predict and treat cancer has increased. Genomic date will be generated for all tumors in the future and we will know the genetic abnormalities between normal and tumor tissue, to be able to design effective targeted therapies [76].

In the era of precision medicine, enabled by affordable next generation sequencing oncologists are often faced with patients requiring treatment and reports describing mutations, amplifications or deletions of oncogenes and tumor suppressor genes. The vision for the treatment of TNBC focuses on potential molecular targets and biomarkers that can be designed or analyzed for the prediction of response of TNBC patients to chemotherapy, radiotherapy and/or biologic targeted therapy.

TNBC is characterized by the absence of ER, PR and HER2 expression these are very aggressive tumors with poor prognosis. This aggressiveness is best illustrated by the fact that the peak risk of recurrence is between the first and third years and the majority of deaths occur in the first 5 years following therapy. An ideal scenario would allow hypotheses around resistance pathway activities, optimal target identification and target combination strategies to be studied driven by pharmacodynamic and short term efficacy biomarker endpoints in accessible residual disease tissue and in blood. Trial designs and platform approaches that are in development to address these points [77].

Currently applied regimens are mostly based on a combination of anthracyclines and taxanes which suggests again the importance of further comparative studies evaluating the efficacy with these standard approaches based or other therapies in TNBC[74].

It is apparent that patients with AR positive TNBC tumors are likely to demonstrate limited benefit from the current standard-of-care chemotherapy regimens for TNBC, and then it will be necessary to study with targeted investigational combination treatments [71].

The molecular apocrine breast cancer subset has recently been identified. LAR cells are in part dependent on AR signaling as siRNA-mediated AR knockdown or pharmacological inhibition of AR by bicalutamide (CDX) greatly decreases cell viability and tumor growth- hormonally responsive cancers. CDX as a single agent in metastatic AR positive, ER/PR negative breast cancers (NCT00468715/ TBCRC011) has demonstrated some efficacy with a clinical benefit rate of 19% [78].

Tumors with AR expression are more likely to harbour PIK3CA mutations. The combination of AR antagonism and PI3K inhibition has found an additive or synergistic effect on AR positive TNBC in cell growth. AR is described as a biomarker for the selection of TNBC patients for clinical trials, which would investigate the efficacy of therapeutic combinations that simultaneously target AR and PI3K [79].

TNBC patients when treated with anthracyclines, cyclophosphamide and taxanes had overall pathological complete response (pCR) of 28% and patients with the AR subtype had a poor response with only 10% achieving pCR prior to surgery. Clinically, AR negative patients have a higher likelihood of achieving pCR with neoadjuvant chemotherapy than AR positive patients [80].

The effective inhibition of sex hormones can be a therapeutic target in patients that present AR expression. Seen that oestrogens and androgens act together in mammary carcinogenesis, with estrogen could possibly be the predominant initiator whereas the androgen acts as a great promoter of carcinogenesis of the mammary gland [67].

Paracrine influences of factors secreted by epithelial cells, such as transforming growth factor (TGF) β is an important growth factor involved in epithelial–stromal interaction. The important point concerning TGF-β1 in epithelial–stromal interaction in carcinogenesis is its various effects on tumors host tissue, particularly with respect to angiogenesis, immunosuppression, fibroblast activation and restructuring of the tumors extracellular matrix. Although TGF-β1 is a powerful inhibitor of normal epithelial cell proliferation, its effect on epithelial tumor cells is slight in adenomas and totally lost in carcinomas. T cell stimulation may act through the AR on mammary epithelial cells. Upon stimulation by T cells, the epithelial cells secrete

TGF-β1, which can act on stromal cells to stimulate growth of the stroma. This may explain growth of the stroma on androgen stimulation [67].

Emerging technological advances in proteomics and transcriptomics requiring complex bioinformatics approaches in the future will pose further challenges and exciting opportunities. AR positive TNBC tumors are likely to demonstrate limited benefit from the current standard-of-care chemotherapy regimens for TNBC. The combination therapy would be the first trial in which TNBC patients are divided based on AR expression and, as a result, aligned to a targeted investigational combination treatment.

9. Future perspectives

As future perspectives, it will be necessary to increase the study sample to clarify a few points, namely to be able to generate better statistical, pathological, clinical and therapeutic results.

A major challenge would bring to the routine clinical practice a more technological and accurate approach, as did Lehamnn.

Analysis of a genomic profile could classify the various subtypes of BC and a better treatment would be applied, including hormone therapy. To validate genomic results with IHC, the use of tissue matrix arrangements tissue microarrays (TMA) has greatly facilitated the rapid and reproducible verification of the relevant candidate genes and molecules. IHC is a relatively simple technique, whose validation over conventional techniques is increasingly consolidated, having quickly spread in major global research and clinical centers.

TMA and IHC have the potential to generate predictive and prognostic markers in data sets of large numbers of samples.

References

1. Harris J , L.M., ,Morrow M, et al., ed. , *Diseases of the Breast*: Philadelphia: Lippinoott Williams & Wilkins.
2. Visvader, J.E., *Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis.* Genes Dev, 2009. **23**(22): p. 2563-77.
3. STERNBERG, S.S., *Histology for Pathologists.* Pennsylvania: Lippincott-Raven Publishers. 1991.
4. Stuart J. , N.H.G.e.a., *Molecular Oncology of Breast Cancer.*
5. Yu, M., et al., *Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition.* Science, 2013. **339**(6119): p. 580-4.
6. BreastCancer.org. *http://www.breastcancer.org/symptoms/understand_bc/what_is_bc; Access in September 2013*.
7. Polyak, K., *Breast cancer: origins and evolution.* J Clin Invest, 2007. **117**(11): p. 3155-63.
8. OncologiaCUF. *http://www.oncologiacuf.pt/; Access in January 2014*.
9. National Breast Cancer Foundation. *2003;http://www.nationalbreastcancer.org/what-is-breast-cancer ; Access in January 2014*.
10. Jemal, A., et al., *Global cancer statistics.* CA Cancer J Clin, 2011. **61**(2): p. 69-90.
11. WHO. *International Agency for Research on Cancer (IARC),Epidemiological data on breast cancer in Portugal (2012);http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx?cancer=breast, access in September 2013*
12. WHO and *International Agency for Research on Cancer (IARC),Epidemiological data on breast cancer in Portugal (2012);http://globocan.iarc.fr/Pages/fact_sheets_population.aspx?country=620; access in September 2013*.
13. WHO. *International Agency for Research on Cancer (IARC), Epidemiological data on breast cancer in Europe (2012);http://globocan.iarc.fr/Pages/fact_sheets_population.aspx?country=968 access in September 2013*.
14. Ginsburg, O.M. and R.R. Love, *Breast cancer: a neglected disease for the majority of affected women worldwide.* Breast J, 2011. **17**(3): p. 289-95.
15. Ferlay, J., et al., *Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008.* Int J Cancer, 2010. **127**(12): p. 2893-917.
16. Li, C.I., J.R. Daling, and K.E. Malone, *Incidence of invasive breast cancer by hormone receptor status from 1992 to 1998.* J Clin Oncol, 2003. **21**(1): p. 28-34.
17. *Global Cancer Statistics: CA Cancer J Clin.* American Cancer Society, Inc., 2011.
18. Cancer, S.A., *Cancer Facts & Figures* Atlanta: American Cancer Society, 2013.
19. Singletary, S.E., et al., *Revision of the American Joint Committee on Cancer staging system for breast cancer.* J Clin Oncol, 2002. **20**(17): p. 3628-36.
20. Fleming ID, C.J., Henson DE, et al (eds), *AJCC Cancer Staging Manual (ed 5)*. 1997: Philadelphia, PA, Lippincott-Raven
21. Edge, S.B. and C.C. Compton, *The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM.* Ann Surg Oncol, 2010. **17**(6): p. 1471-4.

22. Sinn, H.P. and H. Kreipe, *A Brief Overview of the WHO Classification of Breast Tumors, 4th Edition, Focusing on Issues and Updates from the 3rd Edition.* Breast Care (Basel), 2013. **8**(2): p. 149-54.
23. Reis-Filho, J.S. and A.N. Tutt, *Triple negative tumours: a critical review.* Histopathology, 2008. **52**(1): p. 108-18.
24. Perou, C.M., et al., *Molecular portraits of human breast tumours.* Nature, 2000. **406**(6797): p. 747-52.
25. Stagg, J. and B. Allard, *Immunotherapeutic approaches in triple-negative breast cancer: latest research and clinical prospects.* Ther Adv Med Oncol, 2013. **5**(3): p. 169-81.
26. Althuis, M.D., et al., *Etiology of hormone receptor-defined breast cancer: a systematic review of the literature.* Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 2004. **13**(10): p. 1558-68.
27. Slamon, D.J., et al., *Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene.* Science, 1987. **235**(4785): p. 177-82.
28. Yonemori, K., et al., *Disruption of the blood brain barrier by brain metastases of triple-negative and basal-type breast cancer but not HER2/neu-positive breast cancer.* Cancer, 2010. **116**(2): p. 302-8.
29. Stanford, J.L. and N.S. Weiss, *Hormone replacement therapy and breast cancer.* Arch Fam Med, 1996. **5**(6): p. 349-50.
30. Sorlie, T., et al., *Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.* Proc Natl Acad Sci U S A, 2001. **98**(19): p. 10869-74.
31. Althuis, M.D., et al., *Etiology of hormone receptor-defined breast cancer: a systematic review of the literature.* Cancer Epidemiol Biomarkers Prev, 2004. **13**(10): p. 1558-68.
32. Rakha, E.A., et al., *Prognostic markers in triple-negative breast cancer.* Cancer, 2007. **109**(1): p. 25-32.
33. Sotiriou, C. and L. Pusztai, *Gene-expression signatures in breast cancer.* N Engl J Med, 2009. **360**(8): p. 790-800.
34. Lehmann, B.D., et al., *Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies.* J Clin Invest, 2011. **121**(7): p. 2750-67.
35. Darb-Esfahani, S., et al., *Identification of biology-based breast cancer types with distinct predictive and prognostic features: role of steroid hormone and HER2 receptor expression in patients treated with neoadjuvant anthracycline/taxane-based chemotherapy.* Breast Cancer Res, 2009. **11**(5): p. R69.
36. Tanner, M., et al., *Chromogenic in situ hybridization: a practical alternative for fluorescence in situ hybridization to detect HER-2/neu oncogene amplification in archival breast cancer samples.* Am J Pathol, 2000. **157**(5): p. 1467-72.
37. Nielsen, T.O., et al., *Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma.* Clin Cancer Res, 2004. **10**(16): p. 5367-74.
38. Dunnwald, L.K., M.A. Rossing, and C.I. Li, *Hormone receptor status, tumor characteristics, and prognosis: a prospective cohort of breast cancer patients.* Breast Cancer Res, 2007. **9**(1): p. R6.
39. Ahn, S.H., et al., *Effect Modification of Hormonal Therapy by p53 Status in Invasive Breast Cancer.* J Breast Cancer, 2013. **16**(4): p. 386-94.
40. Balko, J.M., et al., *Molecular profiling of the residual disease of triple-negative breast cancers after neoadjuvant chemotherapy identifies actionable therapeutic targets.* Cancer Discov, 2014. **4**(2): p. 232-45.
41. Gokhale, S., *Ultrasound characterization of breast masses.* Indian J Radiol Imaging, 2009. **19**(3): p. 242-7.

42. Foulkes, W.D., I.E. Smith, and J.S. Reis-Filho, *Triple-negative breast cancer.* The New England journal of medicine, 2010. **363**(20): p. 1938-48.
43. Gelmon, K., et al., *Targeting triple-negative breast cancer: optimising therapeutic outcomes.* Ann Oncol, 2012. **23**(9): p. 2223-34.
44. Lehmann, B.D., et al., *Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies.* The Journal of clinical investigation, 2011. **121**(7): p. 2750-67.
45. Greenup, R., et al., *Prevalence of BRCA mutations among women with triple-negative breast cancer (TNBC) in a genetic counseling cohort.* Ann Surg Oncol, 2013. **20**(10): p. 3254-8.
46. Carey, L.A., et al., *Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study.* JAMA, 2006. **295**(21): p. 2492-502.
47. Liedtke, C., et al., *Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer.* J Clin Oncol, 2008. **26**(8): p. 1275-81.
48. Dent, R., et al., *Pattern of metastatic spread in triple-negative breast cancer.* Breast Cancer Res Treat, 2009. **115**(2): p. 423-8.
49. Dent, R., et al., *Triple-negative breast cancer: clinical features and patterns of recurrence.* Clin Cancer Res, 2007. **13**(15 Pt 1): p. 4429-34.
50. Allegra, J.C., et al., *Relationship between the progesterone, androgen, and glucocorticoid receptor and response rate to endocrine therapy in metastatic breast cancer.* Cancer Res, 1979. **39**(6 Pt 1): p. 1973-9.
51. Gao, W., C.E. Bohl, and J.T. Dalton, *Chemistry and structural biology of androgen receptor.* Chem Rev, 2005. **105**(9): p. 3352-70.
52. Amadeu, B.F. *Imunocitoquímica;http://amadeuferro.webs.com/documentos/texto%20total%20v2.4a.pdf*. 2013.
53. Inwald, E.C., et al., *Ki-67 is a prognostic parameter in breast cancer patients: results of a large population-based cohort of a cancer registry.* Breast Cancer Res Treat, 2013. **139**(2): p. 539-52.
54. Bocker, W., et al., *Common adult stem cells in the human breast give rise to glandular and myoepithelial cell lineages: a new cell biological concept.* Lab Invest, 2002. **82**(6): p. 737-46.
55. Alberts, B.e.a., *Molecular biology of the cell*, ed. t. ed. 2002, New York: Garland Science,. p.907-982.
56. LODISH, H.e.a., *Molecular cell biology*, ed. t. ed. 2000, New York: : W. H. Freeman

p.795-847.
57. Rakha, E.A., et al., *Breast carcinoma with basal differentiation: a proposal for pathology definition based on basal cytokeratin expression.* Histopathology, 2007. **50**(4): p. 434-8.
58. DiGiovanna, M.P., et al., *Relationship of epidermal growth factor receptor expression to ErbB-2 signaling activity and prognosis in breast cancer patients.* J Clin Oncol, 2005. **23**(6): p. 1152-60.
59. Reis-Filho, J.S., et al., *Novel and classic myoepithelial/stem cell markers in metaplastic carcinomas of the breast.* Appl Immunohistochem Mol Morphol, 2003. **11**(1): p. 1-8.
60. Reis-Filho, J.S., et al., *EGFR amplification and lack of activating mutations in metaplastic breast carcinomas.* J Pathol, 2006. **209**(4): p. 445-53.
61. Viale, G., et al., *Invasive ductal carcinoma of the breast with the "triple-negative" phenotype: prognostic implications of EGFR immunoreactivity.* Breast Cancer Res Treat, 2009. **116**(2): p. 317-28.
62. Paredes, J., et al., *P-cadherin expression in breast cancer: a review.* Breast Cancer Res, 2007. **9**(5): p. 214.

63. Ghiringhelli, F., et al., *Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors.* Nat Med, 2009. **15**(10): p. 1170-8.
64. Mattarollo, S.R., et al., *Pivotal role of innate and adaptive immunity in anthracycline chemotherapy of established tumors.* Cancer Res, 2011. **71**(14): p. 4809-20.
65. Denkert, C., et al., *Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer.* J Clin Oncol, 2010. **28**(1): p. 105-13.
66. Vera-Badillo, F.E., et al., *Androgen receptor expression and outcomes in early breast cancer: a systematic review and meta-analysis.* J Natl Cancer Inst, 2014. **106**(1): p. djt319.
67. Xie, B., S.W. Tsao, and Y.C. Wong, *Sex hormone-induced mammary carcinogenesis in female noble rats: the role of androgens.* Carcinogenesis, 1999. **20**(8): p. 1597-606.
68. Kuenen-Boumeester, V., et al., *Immunohistochemical determination of androgen receptors in relation to oestrogen and progesterone receptors in female breast cancer.* Int J Cancer, 1992. **52**(4): p. 581-4.
69. Soreide, J.A., et al., *Androgen receptors in operable breast cancer: relation to other steroid hormone receptors, correlations to prognostic factors and predictive value for effect of adjuvant tamoxifen treatment.* Eur J Surg Oncol, 1992. **18**(2): p. 112-8.
70. Isola, J.J., *Immunohistochemical demonstration of androgen receptor in breast cancer and its relationship to other prognostic factors.* J Pathol, 1993. **170**(1): p. 31-5.
71. Agoff, S.N., et al., *Androgen receptor expression in estrogen receptor-negative breast cancer. Immunohistochemical, clinical, and prognostic associations.* Am J Clin Pathol, 2003. **120**(5): p. 725-31.
72. Bryan, R.M., et al., *Androgen receptors in breast cancer.* Cancer, 1984. **54**(11): p. 2436-40.
73. Quinn, J.E., et al., *BRCA1 functions as a differential modulator of chemotherapy-induced apoptosis.* Cancer Res, 2003. **63**(19): p. 6221-8.
74. Bouchalova, K., et al., *Triple negative breast cancer--current status and prospective targeted treatment based on HER1 (EGFR), TOP2A and C-MYC gene assessment.* Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub, 2009. **153**(1): p. 13-7.
75. Turner, N., et al., *Integrative molecular profiling of triple negative breast cancers identifies amplicon drivers and potential therapeutic targets.* Oncogene, 2010. **29**(14): p. 2013-23.
76. DeVita, V.T., Jr. and S.A. Rosenberg, *Two hundred years of cancer research.* N Engl J Med, 2012. **366**(23): p. 2207-14.
77. Bauer, K.R., et al., *Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry.* Cancer, 2007. **109**(9): p. 1721-8.
78. Gucalp, A., et al., *Phase II trial of bicalutamide in patients with androgen receptor-positive, estrogen receptor-negative metastatic Breast Cancer.* Clin Cancer Res, 2013. **19**(19): p. 5505-12.
79. Lehmann, B.D., et al., *PIK3CA mutations in androgen receptor-positive triple negative breast cancer confer sensitivity to the combination of PI3K and androgen receptor inhibitors.* Breast Cancer Res, 2014. **16**(4): p. 406.
80. Masuda, H., et al., *Differential response to neoadjuvant chemotherapy among 7 triple-negative breast cancer molecular subtypes.* Clin Cancer Res, 2013. **19**(19): p. 5533-40.

Appendix

Adapted from AJCC Cancer Staging Manual..Breast. 7th ed. New York, NY: Springer, 2010

DEFINITIONS OF TNM

The increasing use of neoadjuvant therapy in breast cancer and the documented prognostic impact of postneoadjuvant extent of disease and response to therapy warrant clear definitions of the use of the "yp" prefix and response to therapy. The use of neoadjuvant therapy does not change the clinical (pretreatment) stage. As per TNM rules, the clinical stage is identified with the prefix "c". In addition, the use of fine needle aspiration and sentinel lymph node biopsy before neoadjuvant therapy is denoted with the subscripts "f" and "sn," respectively. Nodal metastases detected by FNA or core biopsy are classified as macrometastases (N1) regardless of the size of the tumor focus in the final pathologic specimen. For example, if, prior to neoadjuvant systemic therapy, a patient has no palpable nodes but has an ultrasound-guided FNA biopsy of an axillary lymph node that is positive, the patient will be categorized as cN1 (f) for her clinical (pretreatment) staging and would be considered as stage IIA. Likewise, if the patient has a positive axillary sentinel node identified prior to neoadjuvant systemic therapy, the patient will be categorized as cN1 (sn) (Stage IIA).

As per TNM rules, with the absence of pathologic T evaluation (removal of the primary tumor), microscopic evaluation of nodes before neoadjuvant therapy is still classified as clinical "c."

Primary Tumor (T)

The T classification of the primary tumor is the same regardless of whether it is based on clinical or pathologic criteria, or both. Size should be measured to the nearest millimeter. If the tumor size is slightly less than or greater than a cutoff for a given T classification, it is recommended that the size be rounded to the millimeter reading that is closest to the cutoff. For example, a reported size of 1.1 mm is reported as 1 mm, or a size of 2.01 cm is reported as 2.0 cm. Designation should be made with the subscript "c" or "p" modifier to indicate whether the T classification was determined by clinical (physical examination or radiologic) or pathologic measurements, respectively. In general, pathologic determination should take precedence over clinical determination of T size.

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
Tis (DCIS)	Ductal carcinoma in situ
Tis (LCIS)	Lobular carcinoma in situ
Tis (Paget's)	Paget's disease of the nipple NOT associated with invasive carcinoma and/or carcinoma in situ (DCIS and/or LCIS) in the underlying breast parenchyma. Carcinomas in the breast parenchyma associated with Paget's disease are categorized based on the size and characteristics of the parenchymal disease, although the presence of Paget's disease should still be noted
T1	Tumor ≤20 mm in greatest dimension
T1mi	Tumor ≤1 mm in greatest dimension
T1a	Tumor >1 mm but ≤5 mm in greatest dimension
T1b	Tumor >5 mm but ≤10 mm in greatest dimension
T1c	Tumor >10 mm but ≤20 mm in greatest dimension
T2	Tumor >20 mm but ≤50 mm in greatest dimension
T3	Tumor >50 mm in greatest dimension
T4	Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or skin nodules).

Note: Invasion of the dermis alone does not qualify as T4

T4a	Extension to the chest wall, not including only pectoralis muscle adherence/invasion
T4b	Ulceration and/or ipsilateral satellite nodules and/or edema (including peau d'orange) of the skin, which do not meet the criteria for inflammatory carcinoma
T4c	Both T4a and T4b
T4d	Inflammatory carcinoma (see "Rules for Classification")

Posttreatment ypT. Clinical (pretreatment) T will be defined by clinical and radiographic findings, while y pathologic (posttreatment) T will be determined by pathologic size and extension. The ypT will be measured as the largest single focus of invasive tumor, with the modifier "m" indicating multiple foci. The measurement of the largest tumor focus should not include areas of fibrosis within the tumor bed. The inclusion of additional information in the pathology report such as the distance over which tumor foci extend, the number of tumor foci present, or the number of slides/blocks in which tumor appears may assist the clinician in estimating the extent of disease. A comparison of the cellularity in the initial biopsy to that in the posttreatment specimen may also aid in the assessment of response.

Note: If a cancer was designated as inflammatory before neoadjuvant chemotherapy, the patient will be designated to have inflammatory breast cancer throughout, even if the patient has complete resolution of inflammatory findings.

Regional Lymph Nodes (N)

Clinical

NX	Regional lymph nodes cannot be assessed (e.g., previously removed)
N0	No regional lymph node metastases
N1	Metastases to movable ipsilateral level I, II axillary lymph node(s)
N2	Metastases in ipsilateral level I, II axillary lymph nodes that are clinically fixed or matted; or in clinically detected* ipsilateral internal mammary nodes in the *absence* of clinically evident axillary lymph node metastases
N2a	Metastases in ipsilateral level I, II axillary lymph nodes fixed to one another (matted) or to other structures
N2b	Metastases only in clinically detected* ipsilateral internal mammary nodes and in the *absence* of clinically evident level I, II axillary lymph node metastases
N3	Metastases in ipsilateral infraclavicular (level III axillary) lymph node(s) with or without level I, II axillary lymph node involvement; or in clinically detected* ipsilateral internal mammary lymph node(s) with clinically evident level I, II axillary lymph node metastases; or metastases in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement
N3a	Metastases in ipsilateral infraclavicular lymph node(s)
N3b	Metastases in ipsilateral internal mammary lymph node(s) and axillary lymph node(s)
N3c	Metastases in ipsilateral supraclavicular lymph node(s)

**Note: Clinically detected* is defined as detected by imaging studies (excluding lymphoscintigraphy) or by clinical examination and having characteristics highly suspicious for malignancy or a presumed pathologic macrometastasis based on fine needle aspiration biopsy with cytologic examination. Confirmation of clinically detected metastatic disease by fine needle aspiration without excision biopsy is designated with an (f) suffix, for example, cN3a(f). Excisional biopsy of a lymph node or biopsy of a sentinel node, in the absence of assignment of a pT, is classified as a clinical N, for example, cN1. Information regarding the confirmation of the nodal status will be designated in site-specific factors as clinical, fine needle aspiration, core biopsy, or sentinel lymph node biopsy. Pathologic classification (pN) is used for excision or sentinel lymph node biopsy only in conjunction with a pathologic T assignment.

Pathologic (pN)*

pNX	Regional lymph nodes cannot be assessed (e.g., previously removed, or not removed for pathologic study)
pN0	No regional lymph node metastasis identified histologically

Note: Isolated tumor cell clusters (ITC) are defined as small clusters of cells not greater than 0.2 mm, or single tumor cells, or a cluster of fewer than 200 cells in a single histologic cross-section. ITCs may be detected by routine histology or by immunohistochemical (IHC) methods. Nodes containing only ITCs are excluded from the total positive node count for purposes of N classification but should be included in the total number of nodes evaluated.

pN0(i−)	No regional lymph node metastases histologically, negative IHC
pN0(i+)	Malignant cells in regional lymph node(s) no greater than 0.2 mm (detected by H&E or IHC including ITC)
pN0 (mol−)	No regional lymph node metastases histologically, negative molecular findings (RT-PCR)
pN0 (mol+)	Positive molecular findings (RT-PCR),** but no regional lymph node metastases detected by histology or IHC
pN1	Micrometastases; or metastases in 1–3 axillary lymph nodes; and/or in internal mammary nodes with metastases detected by sentinel lymph node biopsy but not clinically detected***
pN1mi	Micrometastases (greater than 0.2 mm and/or more than 200 cells, but none greater than 2.0 mm)
pN1a	Metastases in 1–3 axillary lymph nodes, at least one metastasis greater than 2.0 mm
pN1b	Metastases in internal mammary nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected***
pN1c	Metastases in 1–3 axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected

pN2	Metastases in 4–9 axillary lymph nodes; or in clinically detected**** internal mammary lymph nodes in the *absence* of axillary lymph node metastases
pN2a	Metastases in 4–9 axillary lymph nodes (at least one tumor deposit greater than 2.0 mm)
pN2b	Metastases in clinically detected**** internal mammary lymph nodes in the *absence* of axillary lymph node metastases
pN3	Metastases in ten or more axillary lymph nodes; or in infraclavicular (level III axillary) lymph nodes; or in clinically detected**** ipsilateral internal mammary lymph nodes in the *presence* of one or more positive level I, II axillary lymph nodes; or in more than three axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected***; or in ipsilateral supraclavicular lymph nodes
pN3a	Metastases in ten or more axillary lymph nodes (at least one tumor deposit greater than 2.0 mm); or metastases to the infraclavicular (level III axillary lymph) nodes
pN3b	Metastases in clinically detected**** ipsilateral internal mammary lymph nodes in the *presence* of one or more positive axillary lymph nodes; or in more than three axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected***
pN3c	Metastases in ipsilateral supraclavicular lymph nodes

Notes:

*Classification is based on axillary lymph node dissection with or without sentinel lymph node biopsy. Classification based solely on sentinel lymph node biopsy without subsequent axillary lymph node dissection is designated (sn) for "sentinel node," for example, pN0(sn).

**RT-PCR: reverse transcriptase/polymerase chain reaction.

***"Not clinically detected" is defined as not detected by imaging studies (excluding lymphoscintigraphy) or not detected by clinical examination.

****"Clinically detected" is defined as detected by imaging studies (excluding lymphoscintigraphy) or by clinical examination and having characteristics highly suspicious for malignancy or a presumed pathologic macrometastasis based on fine needle aspiration biopsy with cytologic examination.

Posttreatment ypN

- Post-treatment yp "N" should be evaluated as for clinical (pretreatment) "N" methods above. The modifier "sn" is used only if a sentinel node evaluation was performed after treatment. If no subscript is attached, it is assumed that the axillary nodal evaluation was by axillary node dissection (AND).
- The X classification will be used (ypNX) if no yp posttreatment SN or AND was performed
- N categories are the same as those used for pN.

Distant Metastases (M)

M0	No clinical or radiographic evidence of distant metastases
cM0(i+)	No clinical or radiographic evidence of distant metastases, but deposits of molecularly or microscopically detected tumor cells in circulating blood, bone marrow, or other nonregional nodal tissue that are no larger than 0.2 mm in a patient without symptoms or signs of metastases
M1	Distant detectable metastases as determined by classic clinical and radiographic means and/or histologically proven larger than 0.2 mm

Posttreatment yp M classification. The M category for patients treated with neoadjuvant therapy is the category assigned in the clinical stage, prior to initiation of neoadjuvant therapy. Identification of distant metastases after the start of therapy in cases where pretherapy evaluation showed no metastases is considered progression of disease. If a patient was designated to have detectable distant metastases (M1) before chemotherapy, the patient will be designated as M1 throughout.

ANATOMIC STAGE/PROGNOSTIC GROUPS

Stage 0	Tis	N0	M0
Stage IA	T1*	N0	M0
Stage IB	T0	N1mi	M0
	T1*	N1mi	M0
Stage IIA	T0	N1**	M0
	T1*	N1**	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1*	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

II

Adapted from Lehmann, B.D., *et al*.

Figure 3

GE patterns within TNBC subtypes are reproducible. Heat maps showing the relative GE (log2, –3 to 3) of the top differentially expressed genes (P < 0.05) in each subtype in the training set (left) and the same differentially expressed genes used to predict the best-fit TNBC subtype of the validation set (right). Overlapping gene ontology (GO) terms for top canonical pathways in both the training and validation sets as determined by GSE-A are shown to the right of the heat maps.