Chapter

Advances in Molecular and Immunohistochemical Detection of Prognostic and Therapeutic Markers in Breast Cancer

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Abstract

In the last two decades, new discoveries concerning on breast cancer have contributed to important changes on its classification, from purely morphologic to molecular embased, to establish better correlation with clinicopathologic features. The classification in molecular subtypes, based on hormonal receptor and *HER-2* status, have been remarkable not only for its more accurated clinical correlations, but also for its easy applicability in diagnostic routine, better replication of tumor microenvironment through the selection of paraffinized tumor amounts and cost-effectiveness of the detection method, the immunohistochemistry. Hence, this classification may predict the breast cancer prognosis and became an important target for therapy with hormonal and *HER-2* antagonist drugs. Other study models, like cancer-stem cell hypothesis and immunological aspects of human cancer, have brought new emerging ideas regarding on molecular pathways and accurated prognostic preditions. Putative stem-cell markers and *PD-1/PDL-1*, have highlighted among several emerging molecular markers because of the bad cancer prognosis determinated by stem-cell markers expression and for emerging new drugs with selective action to PD-1/PDL-1, with promising results. The therapy of breast cancer have became diverse, target directed and personalized, in order to take in consideration the clinicopathologic cancer aspects, molecular tumor profile and clinical status of the patient.

Keywords: breast cancer, target therapy, molecular markers, prognostic markers, immunomarkers, cancer stem-cell

1. Introduction

Breast cancer is the one of the three most frequent human neoplastic disease worldwide and is the most common female cancer, remaining with considerable impact on general mortality. Worldwide, in the last 10 years, the incidence is growing up, with approximately 2.1 million of new cases per year and estimated mortality of 15%, at about 300.000 per year [1, 2].

Breast cancer remains as an heterogeneous group of disease from the point of view of biological behavior, therapeutic issues and prognostic features, determining different tracks of overall and free of disease survivals [3, 4]. Thus, the

clinicopathologic classification of breast cancer has been challenging over the last years, since the isolated simple morphologic classification of the tumor on histology examination is not necessarily related to the precise biologic behavior of the disease [5, 6].

In this way, especially over the last two decades, important researches revealing novel molecular markers expressed by cancer cells has been published in the literature. The new discoveries have improved the breast cancer classification, which has been progressed from a purely morphologic classification, based on histologic patterns, to a molecular classification, based on expression of oncoproteins and hormonal receptors, detected mainly by immunohistochemical techniques, in paraffin-embedded tumoral specimens [6, 7].

The novel molecular classification of breast cancer seems to exhibit more accurated correlation to the clinicopathological aspects of the tumor, as proliferative index, invasiveness and potential to metastatic spread. Furthermore, some of these molecular markers allowed the development of new drugs with specific actions on populations of cancer cells with specific genes alterations, improving considerably the therapeutic, prognostic and survival issues [7].

Instead of the recent advances on new therapeutic protocols under a new molecular perspective, early breast cancer on clinicopathological classification still remains the single one potencially curative [8]. The management of advanced clinicopathologic stage tumors and some established molecular groups of cancer, especially the 'triple negative' disease, remains with lacks of consensus. Anyway, the molecular markers have just improved the pathophysiology pathways knowledge, with potential future development of promising new drugs for target therapy of breast cancer [8–10] .

2. The molecular subtypes of breast cancer of clinicopathologic importance

In the beginning of the 21st century, breast cancer was classified mainly on histologic basis. The WHO current histologic classification of breast cancer is demonstrated in **Table 1**. Photomicrographies of the most frequent histologic subtypes of invasive breast cancer are represented on **Figure 1**. The hormonal status receptors (estrogen and progesterone) expression by the neoplastic cells was just evaluated by immunohistochemistry on paraffin-embedded specimens of tumor (core needle biopsy or the surgical excision specimen) [6, 7] .

Breast cancer is known for its heterogeneous behavior [3, 4]. The histologic classification has been satisfactory for malignancy determination [6]. Though, the clinical division based on hormonal status was not enough for accurate prediction of the prognosis and of clinical response to the therapy [5]. Thus, until the last decade of 20th century, the clinical treatment of breast cancer was based on unespecific chemotherapy and hormonal therapy with drugs like tamoxifen, a known hormonal receptor antagonist [12].

The hormone positive breast cancer is more "differentiated" than the negative one, as the cancer cells maintain the epithelial original cell feature of hormonal receptor expression and, therefore, the hormonal antagonist drugs are effective against these tumors [8]. On the other hand, the approaching of hormonal negative cancers were variable, since it was forming a kindly heterogeneous group, with different aggressiveness potentials, imprecise therapeutic response and doubtful prognosis [6, 8, 10].

In the first decade of the current century, it was emerged a promising classification of breast cancer, proposing a division of the disease in 3 molecular subtypes:

WHO classification of epithelial breast tumors (5th edition, 2019)

Benign epithelial proliferations and precursors

- Usual ductal hyperplasia
- Columnar cell lesions, including flat epithelial atypia
- Atypical ductal hyperplasia

Adenosis and benign sclerosing lesions

- Sclerosing adenoma
- Apocrine adenoma
- Microglandular adenosis
- Radial scar/complex sclerosing lesion

Adenomas

- Tubular adenoma
- Lactating adenoma
- Duct adenoma

Epithelial-myoepithelial tumors

- Pleomorphic adenoma
- Adenomyoepithelioma NOS
- Adenomyoepithelioma with carcinoma
- Epithelial-myoepithelial carcinoma

Papillary neoplasms

- Intraductal papilloma
- Ductal carcinoma *in situ*, papillary
- Encapsulated papillary carcinoma
- Encapsulated papillary carcinoma with invasion
- Solid papillary carcinoma *in situ*
- Solid papillary carcinoma with invasion
- Intraductal papillary adenocarcinoma with invasion

Non-invasive lobular neoplasia

- Atypical lobular hyperplasia
- Lobular carcinoma *in situ* NOS
  - Classic lobular carcinoma *in situ*
  - Florid lobular carcinoma *in situ*
  - Lobular carcinoma in situ, pleomorphic

Ductal carcinoma *in situ* (DCIS)

- Intraductal carcinoma, non-infiltrating, NOS
  - DCIS of low nuclear grade
  - DCIS of intermediate nuclear grade
  - DCIS of high nuclear grade

Invasive breast carcinoma

- Infiltrating duct carcinoma NOS
- Oncocytic carcinoma
- Lipid-rich carcinoma
- Glycogen-rich carcinoma
- Sebaceous carcinoma
- Lobular carcinoma NOS
- Tubular carcinoma
- Cribriform carcinoma NOS
- Mucinous adenocarcinoma
- Mucinous cystadenocarcinoma NOS
- Invasive micropapillary carcinoma of breast
- Apocrine adenocarcinoma
- Metaplastic carcinoma NOS

Rare and salivary gland-type tumors

- Acinar cell carcinoma
- Adenoid cystic carcinoma
  - Classic adenoid cystic carcinoma
  - Solid-basaloid adenoid cystic carcinoma
  - Adenoid cystic carcinoma with high grade transformation
- Secretory carcinoma
- Mucoepidermoid carcinoma
- Polymorphous adenocarcinoma
- Tall cell carcinoma with reverse polarity

Neuroendocrine neoplasms

- Neuroendocrine tumor NOS
- Neuroendocrine tumor, grade 1
- Neuroendocrine tumor, grade 2
- Neuroendocrine carcinoma NOS
- Neuroendocrine carcinoma small cell
- Neuroendocrine carcinoma large cell

Table 1.
Current histologic (morphologic) classification of epithelial breast tumors (WHO, 2019, 5th edition). This classification considers the tumors histologic patterns of tumors. The most common histologic breast cancer subtype is the infiltrating duct carcinoma NOS (or invasive ductal carcinoma non-special type), accounting for 65–80% of all breast cancers. Invasive lobular carcinoma corresponds to around 5% of all breast malignancies.

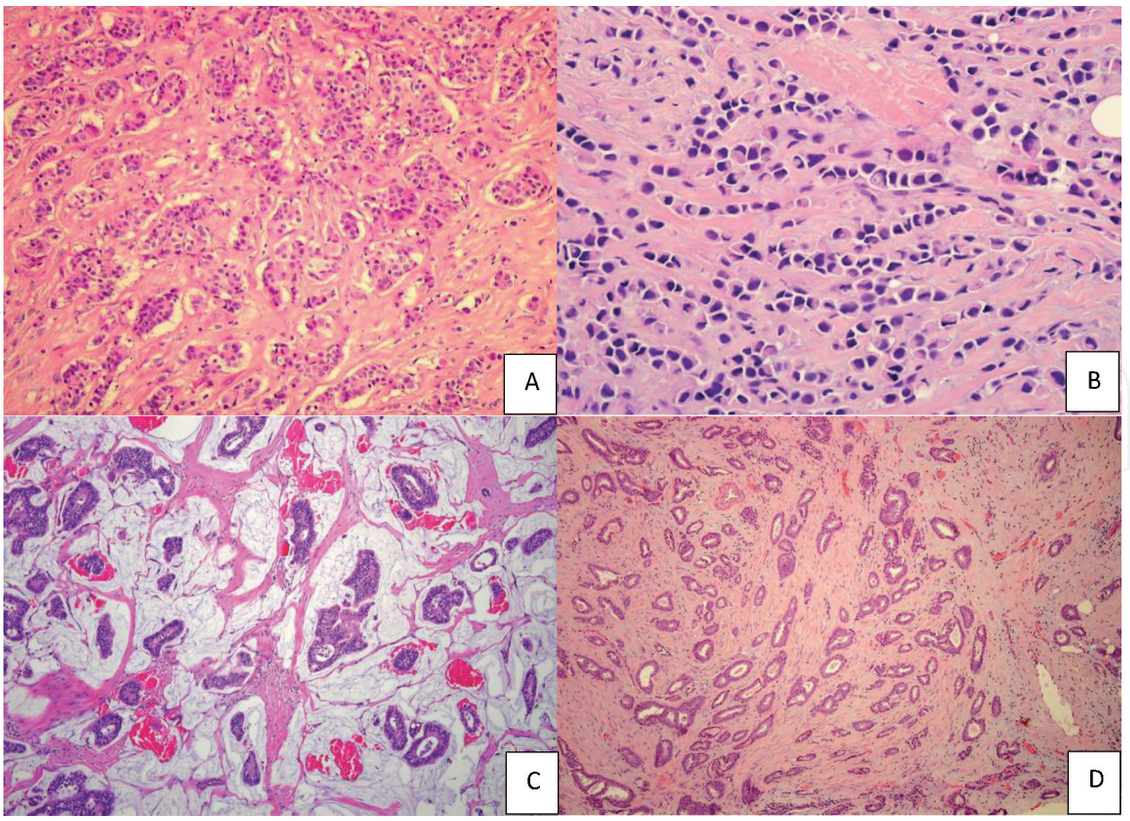


Figure 1.
Photomicrographies of hematoxylin & eosin (H&E) slides illustrating the most frequent histologic subtypes of infiltrating (invasive) breast cancer. (A) Infiltrating duct carcinoma (Invasive ductal carcinoma NOS) is the most frequent histologic subtype of breast cancer (nearly 75–80% of all invasive breast cancer), constituted of cohesive cancer cells forming infiltrative ductal and ribbons structures (4×); (B) Lobular invasive carcinoma is the second most frequent invasive breast cancer (5–15% of all invasive breast cancer), composed of infiltrating cancer cells with diffuse single-file pattern (10×). In this subtype, the cancer cells lose the cohesion (e-cadherin, an immunomarker important for cell adhesion evaluation, is negative on immunohistochemistry); (C) Mucinous carcinoma represents approximately 2% of breast invasive cancer, composed of groups of cancer cells outling ductal structures, immersed in mucin pools, with delicate fibrous strands containing capilaries (10×); (D) Tubular carcinoma represents around 2% of invasive breast cancer, composed of haphazard arrangement of small well-differentiated duct structures, forming tubules (4×). The other listed invasive breast cancers are uncommon, with each one histologic subtype representing 1% or less (figures extracted from [11]).

luminal, *HER-2* overexpressed and "triple negative" (**Table 2**). This new classification has demonstrated better correlation with the breast cancer behavior. Thus, it was adopted on diagnostic routine of breast cancer. Since this study was published, besides of evaluate the histologic patterns and report the pathologic tumor stage, the pathologist has been required to determine the molecular cancer profile, which has became indispensable to therapy planning [12, 13].

The luminal subtype cancer is the hormonal positive tumors. This kind of cancer is frequently well or moderately differentiated on histology, formed by lower grades of cells, with lower proliferative index, which is evaluated by antibody *Ki-67/MIB-1* on immunohistochemistry. The majority of breast cancers are classified as this subtype (**Figure 2**). Eventually, luminal cancer can overexpress or amplify at the same time the protein called human epithelial growth factor receptor 2 (*HER-2*), codified by the oncogen *ERBB2* [14, 15].

ERBB2 is a oncogen localized in chromosome 17, which codifies the *HER-2* protein, a type I transmembrane protein with an extracellular and an intracellular domains, activating signaling pathways from extracellular signals. In last instance, the overexpression/amplification of *HER-2* overactivates the intracellular protein kinases, dysregulating the cell cycle, disrupting the cell adhesion and cell polarity and promoting the invasive phenotype [16].

Molecular subtype	Biomarkers profile	Incidence
Luminal	Hormone receptors positive (ER+ and/or PR+)	50–70%
• Luminal A	• with Ki-67 ≤ 14% of cancer cells	35–50%
• Luminal B	• with Ki-67 > 14% of cancer cells	5–15%
○ Luminal B1	○ HER-2 negative	
○ Luminal B2	○ HER-2 positive	
HER-2 overexpressed	Hormone receptors negative (ER and PR negative) and HER-2 positive	10–20%
"Triple negative"	Hormone receptors negative (ER and PR negative) and HER-2 negative	15–30%

Table 2.
Molecular subtypes of breast carcinoma. The reported absolute incidences of each molecular subtype of breast carcinoma are variable among several studies.

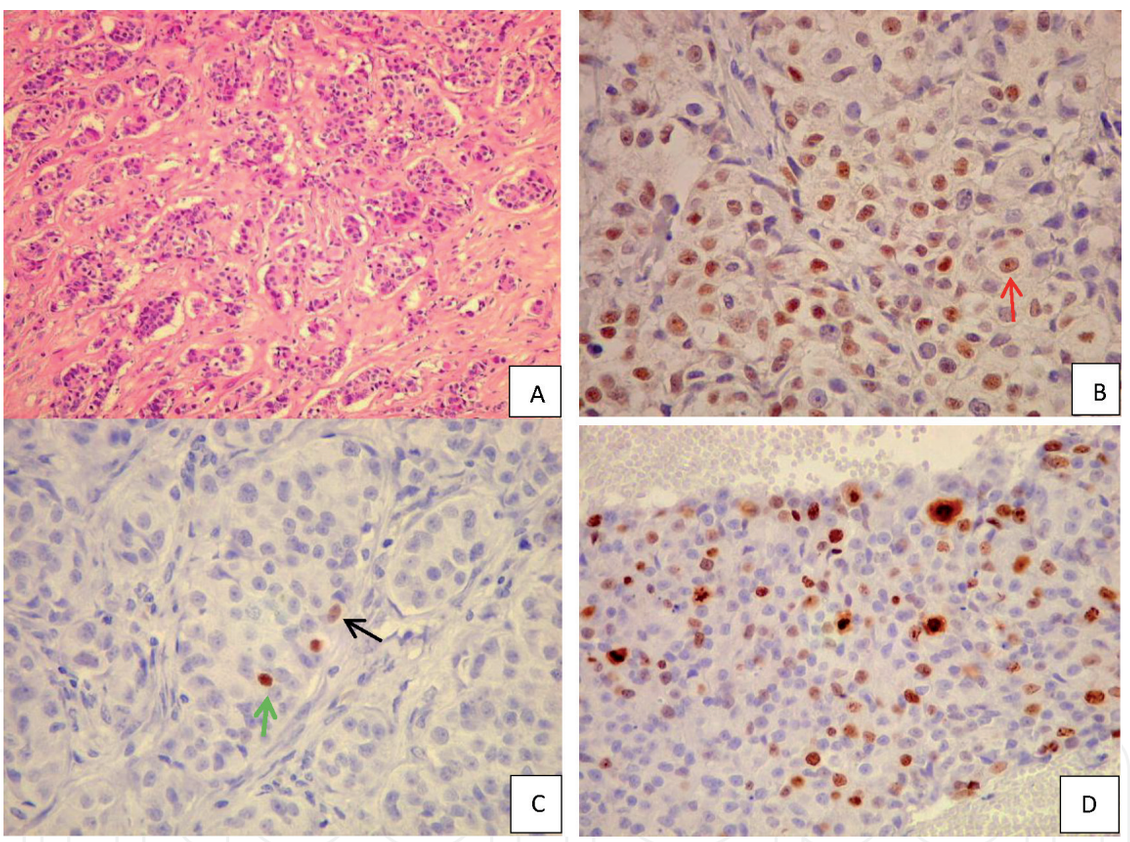
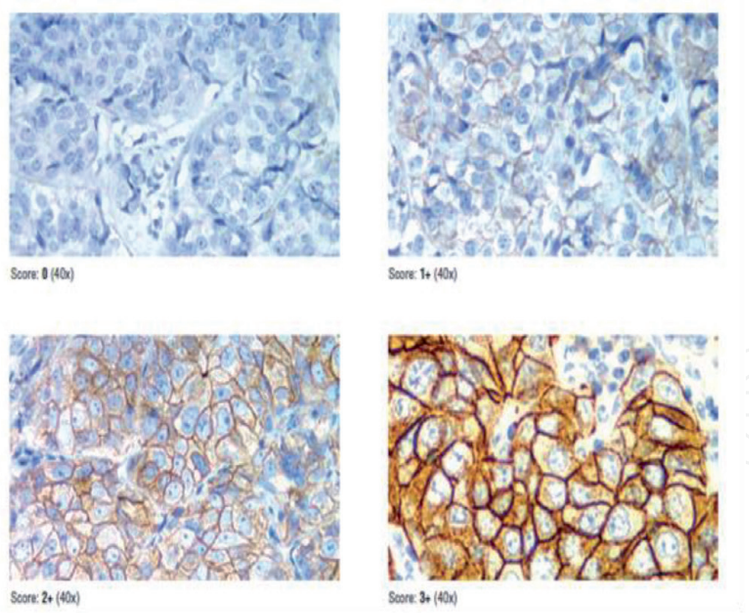
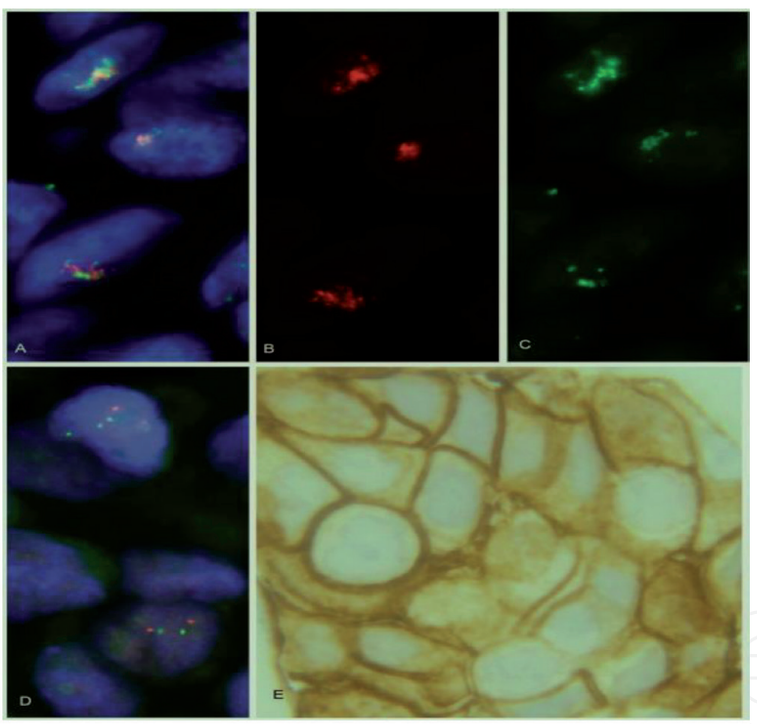


Figure 2.
Photomicrographies of immunohistochemical assessment of invasive breast cancer hormonal expression, in an example of infiltrating duct carcinoma (Invasive ductal carcinoma NOS, WHO 2019), which is the most frequent histologic subtype of breast cancer, constituted by ductal and ribbons structures of cancer cells infiltrating the breast stroma (A). Any kind of nuclear positivity of estrogen receptor (B) and progesterone receptor (C) allows to consider the tumor as positive to hormonal receptor on immunohistochemistry, even when rare cells are positive (C). The hormonal receptors positivities on immunohistochemistry are evaluated for intensity (mild, moderate or strong) and percentages of positive cells (0–100%). Examples of mild positivity (black arrow, C), moderate positivity (red arrow, B) and strong positivity (green arrow, C). Ki-67/ MIB-1 assesses the tumor proliferative index (D), its positivity is nuclear and is expressed in percentages of positive cells (0–100%).

The breast cancer classified as *HER-2* subtype is necessarily negative for hormonal receptors and is featured by overexpression or amplification of *HER-2*. This subtype is frequently less differentiated than the luminal ones on histology, constituted by high grades of cancer cells, with high proliferative index. The presence of elevated concentration of intratumoral lymphocytes (TIL) is not an uncommon finding in these tumors [17].



(a)



(b)

Figure 3.
(a) Photomicrographies of immunohistochemical assessment of HER-2 expression status by tumoral cells in histologic paraffinized specimen of breast cancer. Score 0 (negative): none tumoral cell is labeled. Score 1+ (negative): incomplete positivity with low intensity in part of tumoral cells. Score 2+ (equivocal): complete positivity with low intensity in majority of tumoral cells. Score 3+ (positive): complete positivity with strong intensity in majority of tumoral cells. (b) Photomicrographies of amplification of HER-2 gene performed through fluorescence in situ hibridization (FISH) in a HER-2-overexpressed breast carcinoma on immunohistochemistry (Score 3+, E). HER-2 gene copies are the orange signals (B) and chromosome 17 centromeres (CEP17) are the green signals (C). The signals of HER-2 gene and CEP17 are present in tumoral cell nuclei (blue, A and D). CEP17 is an internal control on the same chromosome to compare with HER-2 signals in tumoral cell nucleus. According to American Society of Clinical Oncology/College of American Pathologists (ASCO-CAP) guidelines, a HER-2/CEP17 ratio ≥ 2.0 defines a positive result for amplification of HER-2 gene. If HER-2/CEP17 ratio is < 2.0, an average HER-2 copy number ≥ 6.0 signals/cell defines a positive result for amplification of HER-2 gene, an average HER-2 copy number < 4.0 signals/cell defines a negative result for amplification of HER-2 gene and an average HER-2 copy number ≥ 4.0 and < 6.0 signals/cell defines an equivocal result for amplification of HER-2 gene (extracted from [20]).

This new receptor was one of the pioneers for target therapy in molecular era of breast cancer approaching, as it was developed a new class of drug, called trastuzumab, with selective action against the cancer cells overexpressing/amplifying *HER-2*. Besides the *HER-2* subtype tumors, this drug is also recommended for the luminal ones with positive status for *HER-2* [18, 19].

The status of *HER-2* expression is analyzed through immunohistochemistry of paraffin-embedded specimens of the breast cancer (**Figure 3a**). The tumor is considered negative for *HER-2* if it is not labeled (score zero) or the cell membrane is partially labelled for the *HER-2* antibody (score 1+). The tumor is positive for *HER-2* if all the cell membranes outlines are strongly labeled for this antibody (score 3+). Finally, in part of the cases, the *HER-2* antibody can label totally the cancer cell membrane, but with low intensity or can label partially the cell membrane with high intensity. In these situations, the *HER-2* status is considered equivocal (score 2+). The confirmation of overexpression/amplification must be evaluated through fluorescence "in situ" hybridization (FISH) (**Figure 3b**) [21, 22].

The "triple negative" breast cancer is negative for hormonal receptors and *HER-2*. It is the less differentiated tumor subtype on histology, formed by highest grades cancer cells, with highest proliferative index, presenting the worst prognosis among the 3 molecular subtypes. This tumor still does not present an specific therapy, which is chosen depending on the clinicopathological stage. In metastatic disease, the treatment focuses on quality of life and palliation. In "triple negative" tumors, the evaluation of *BRCA* status is mandatory [8, 21].

3. Germline mutations of *BRCA-1/BRCA-2* genes: increased risk of breast cancer development during the life

Identified in 1994, *BRCA-1*/*BRCA-2* are tumoral suppressor genes, respectively located in chromosome 17 and 13. Mutations of these genes are related to hereditary breast cancer, estimated in 5–10% of all breast malignancies. *BRCA-1/BRCA-2* play a central role in DNA repair [23, 24]. Mutations of these genes increase the susceptibility for DNA damages. "Triple negative" subtypes carry more frequently mutations of *BRCA-1* and mutations of *BRCA-2* increase the risk for luminal subtypes of breast cancer. *HER-2* overexpression is inversely correlated to *BRCA* mutations [24, 25].

It was observed in some studies that "triple negative" breast cancers with *BRCA* mutations present more chemosensitivity than the ones without *BRCA* mutations. Chemotherapy with DNA-damaging drugs, like the alkylating agents and anthracycline, can prolong the free of disease survival for tumors of triple negative phenotypes. This found is expected, since *BRCA* mutation prejudices the DNA repair and, consequently, increase the sensibility to DNA damages of cancer cells by these drugs. Neither therapeutic response nor free of disease survival of luminal subtypes of breast cancer seems to be influenced by *BRCA* mutations [8, 24, 26].

Regarding on prognosis, multiples studies present conflicting results. The prognosis depends on tumor features, especially the molecular subtypes and the clinicopathologic stage. The predictive value depends on the administrated therapy. Thus, *BRCA-1* mutated breast cancer probably present worse prognosis than the *BRCA-2* mutated ones, since *BRCA-1* mutated tumors are mainly of "triple negative" phenotype, therefore intrinsically more aggressive than the luminal subtypes harboring *BRCA-2* mutations [24, 27].

The tumoral suppressor proteins codified by *BRCA-1/BRCA-2* act on homologous recombination repair of double stranded DNA breaks. Homologous recombination mechanism protect the integrity of genome in proliferating cells. *BRCA-1* recognize DNA damage and recruit DNA repair proteins. *BRCA-2* mediates the

recruitment of another protein, called *RAD51*, to double stranded DNA breaks, allowing for homologous recombination repair [24, 28].

In *BRCA*-mutant breast tumors, the base excision repair pathway is important for cancer cell survival, in response to single stranded DNA breaks. Polyadenosine diphosphate-ribose (*PARP*) is a family of DNA repair enzymes, playing a key role in base excision repair mechanism. These enzymes are recruited to the site of DNA damage and add ADP-ribose to target nuclear proteins, causing post-translational modifications and restarting stalled DNA replication. *BRCA*-mutant breast cancer presents deficiency of homologous recombination repair, with overactivated *PARP*, leading the cancer cell to avoid apoptosis [24, 26, 28].

The inhibition of *PARP* cause persistance of single stranded break, resulting in stalled replication and double strand breaks. This mechanism leads to accumulation of DNA damage, causing cell cycle arrest and apoptosis. The *PARP* inhibitors form an emerging class of drugs, which have been recommended to chemotherapy for *BRCA*-mutant breast cancer and empirically for metastatic breast cancer, with promising results [24, 25, 28].

4. Cancer stem-cell hypothesis: impact in breast cancer prognosis

In the last two decades, experimental evidences in several studies of neoplastic tissues have revealed a population of cancer cell with properties of self-renewal, differentiation to multiple lineages ability and low proliferative index. These properties have been considered cancer stem-cell like features and attributed to a possible cancer stem-cell lineage present in the tumor bulk [29, 30].

Cancer stem-cell has awaked interest in the context of breast cancer because of its characteristic heterogeneity of biological behavior and therapeutic response. It has been hypothesized that cancer stem-cell might be one of the causes of the high variability of biological and prognostic spectrum of breast cancer. Cancer-stem cells might play an important role on therapeutic resistance and progression of disease, affecting the overall and free of disease survival [31, 32].

Thus, an important feature which allows possible cancer stem-cell resistance to chemotherapy is its low expression of surface proteins. Because of its self-renewal properties, cancer stem-cell does not depends on signaling from other cells to proceed its functions in tumoral tissues. Furthermore, for its low antigenicity and low proliferation index, there are few alternatives for drug interactions. DNA damage agents are poor effective against these cells possibly for a lack of proliferation, as well new classes of drugs, like *PARP* inhibitors, which better act on cells in proliferative phase [31, 33].

One of possible pathways for breast cancer therapeutic resistance acquired along the time might be explained by populations of cancer stem-cells not eliminated, selected by multiple chemotherapy cycles. Tumoral cells in active proliferation phase are more hitten, increasing the proportion of indolent cells with stem-like features in cancer cell population. Through the capacity of multilineage differentiation, cancer stem cells might generate new daughter cells with more aggressiveness and chemoresistance [32, 34].

The identification of cancer stem-cells is challenging. First, because of its irregular distribution in selected tumor amounts. Second, for definition, these cells are frequently scarces in tumor bulk. In this way, these cells are better identified through "in vitro" methods, like cellular cultures. However, the mainly disadvantage of this technique is the fact of stem cells behave in a different fashion in artificial environment, since the cell phenotype expression depends on their interactions [32, 35].

Thus, several studies with cancer stem-cells in different neoplastic tissues have been accomplished with conflicting results. An interesting method to identify these cells in their original environment is the immunohistochemistry performed on amounts of paraffin-embedded neoplastic tissues, with the advantages to allow the evaluation of phenotype expression next to the reality and to be easily performed and cost-effectiveness in diagnostic routine [35].

In the last years, some putative stem-cell markers detected by immunohistochemistry have been tested in paraffinized tissues of breast cancer. Multiple studies have demonstrated that expression of putative stem-cell markers by tumoral cells seems to worse the prognosis and survival in breast cancer. The most frequent studied stem-cell markers are *CD24*, *CD44*, *CD133* and *EPCAM*, with two identified putative stem-cell phenotypes: *CD24* low/*CD44* enriched and co-expression of *CD133* and *EPCAM* (**Figure 4**). Besides of the scarcity of stem-cells in neoplastic tissues, the conflictous results of these studies might be explained by a necessity to qualitative analysis of these markers expression, exactly for the rarity of stem-cells [32, 36].

In some studies, identification of a stem-cell like phenotype *CD24* low/*CD44* enriched have prejudiced the free of disease survival, especially in cases of early stages of breast cancer, with more occurrence of distant metastasis and cancer recurrence after surgical and adjuvant treatments. The presence of cancer cells with positivity for cancer stem-cell phenotype *CD133/EPCAM* is has been related to poor overall survival in breast cancer, with more adjuvant therapeutic fail [32].

For the moment, these putative stem-cell phenotypes seems to be independent prognostic factors in breast cancer. "Triple negative" breast cancer and *BRCA-1* mutant breast cancer have been associated to stem-cell like phenotype *CD24* low/*CD44* enriched. These putative stem-cell markers may become possible future targets for new drugs in the future [30, 32].

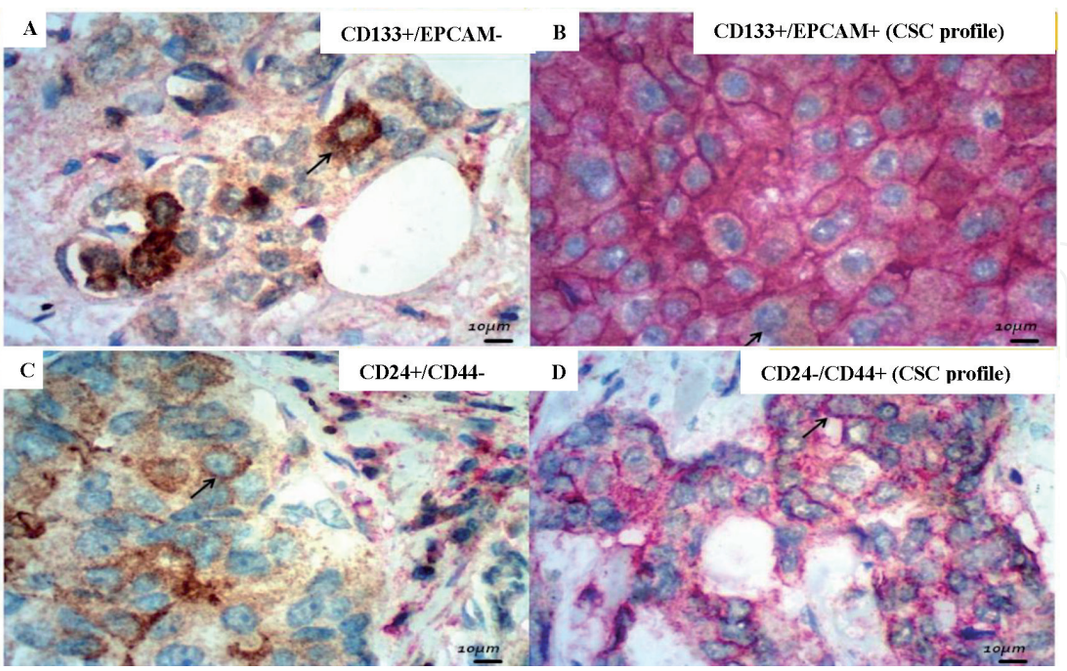


Figure 4.
Photomicrographies of double-labeled simple stained putative CSC antibodies (400×, original magnification, immunoperoxidase and DAB). (A) CD133: cytoplasm positivity (immunoperoxidase); (B) EPCAM: membrane positivity (DAB); (B) CD133+/EPCAM+: CSC profile (black arrow: membrane positivity to DAB and cytoplasm positivity to immunoperoxidase at the same cell); (C) CD24: cytoplasm positivity (immunoperoxidase); (D) CD24−/CD44+: CSC profile (black arrow: membrane positivity only to DAB)

5. Immunologic aspects related to breast cancer

In the context of cancer, the immune system can suppress the tumor growth by the destruction of cancer cells or inhibition of their outgrowth. On the other hand, immune system can play a role on tumor progression by the selection of tumor cells which are adapted to survive in an immunocompetent host or modifying the tumor environment to facilite the tumor outgrowth [37] .

Elevated levels of *CD4+* regulatory T lymphocytes (*Tregs*) found in many cancers are associated to poor prognosis. *Tregs* create a favorable immunosuppressive microenvironment to the outgrowth and progression of the tumor. On this way, *FOXP3* is expressed by the *Tregs* and can be detected by immunohistochemistry. *FOXP3* is responsible for induction and maintenance of tolerance to self antigens in normal cells, as well this immunotolerance can be performed by the *Tregs* with cancer cell antigens [37, 38].

Another example of cancer cell escape mechanism from the immune system is *caspase-8* mutations present in "triple negative" breast cancers and other solid malignant tumors. These mutations abolish the death induced by cytotoxic lymphocytes *CD8+* in tumoral cells [37, 39].

The activation of T lymphocytes by foreign antigens occurs by concomitant major histocompatibility complex (*MHC*) antigen presentation and co-expression of T-cell receptor (*TCR*). At the same time, a family of T-cell transmembrane proteins *CD28/B7*, called "immune checkpoints", produces co-inhibitory or co-stimulatory signals. The immune checkpoints regulates the T-cell immunotolerance to protect the tissues from undesirable damages. Cancer cells may produce signals to inhibit T-cell action, through cytotoxic T-lymphocyte associated antigen-4 (*CTLA-4*), programmed cell death-1 (*PD-1*) and its ligands (*PDL-1*) [37, 40].

PD-1 is an inhibitory "immune checkpoint" expressed on the surface of T-cells, B-cells and NK-cells. When T-cells have been activated by their *TCR*, the cells express at the same time *PD-1*, which is a possibility to the attacked cell to escape from the immune reaction (**Figure 5**). Cancer cells express the ligand *PDL-1* on their surfaces, activating *PD-1* of T-cells, escaping from the attack [37, 40].

PD-L1 expression has been associated with large tumor size, high grade, high proliferation, estrogen receptor (*ER*)-negative status, and human epidermal growth factor receptor-2 (*HER2*)-positive status in breast cancer. Survival in breast cancer is inversely related to *PD-1/PDL-1* levels. *PDL-1* expression increases tumor

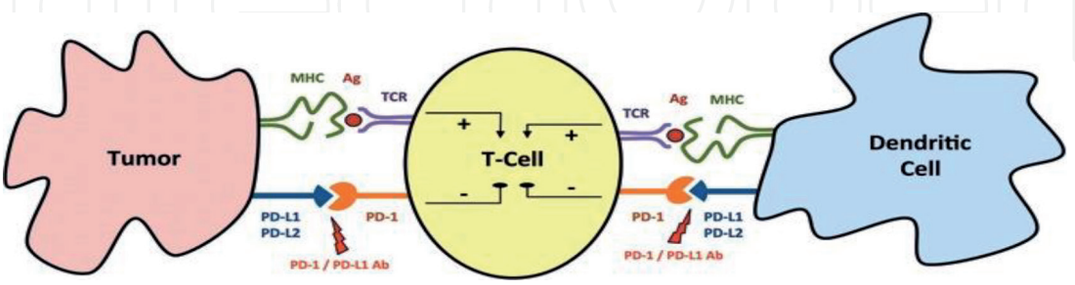


Figure 5.
Simplified schematic illustration of PD-1/PDL-1 interactions in immune responses against cancer cell. Tumoral antigens (Ag) are presented via T-cell by major histocompatibility complex (MHC) of dendritic cells. T-cell recognize tumoral Ag via TCR (T-cell receptor). Interaction Ag-TCR induces an positive immune response against tumoral Ag. Though, there is a scape mechanism of cancer cell from the T-cell attack: interaction of programmed death cell ligands (PDL-1/2) expressed by cancer cell with PD-1 expressed by T-cell inhibit the T-cell action. This scape mechanism of cancer cell mimics the regulation action to avoid immune responses of T-cell against self antigens. The principle of immune therapy is the inhibition of PD-1/PDL-1 (extracted from [40]).

aggressiveness, stimulating tumorigenesis, invasiveness and ability to escape from cytotoxic T *CD8+* lymphocytes attacks [39, 41]. The immunohistochemical evaluation of *PDL-1* is shown in **Figure 6**.

Immune therapies with anti-*CTLA-4* and anti-*PD1*/anti-*PDL-1* agents have been promising for treating several cancers. In breast cancer, some researches reported positive results around 20% of breast tumors on treatment with these agents, mainly the "triple negative" and *HER-2* subtypes, for their higher antigenicity. In general, breast cancer present lower immunogenicity than other cancers and breast cancer cells frequently create an immunosuppressor tumor microenvironment by signaling [37, 43].

The presence of tumor infiltrating lymphocytes (TIL) in some breast cancers has been related to a favorable prognosis, especially in "triple negative" and *HER-2* subtypes. TIL are formed mainly by T-cells *CD3+/CD56* negative, which are either *CD4+* or *CD8+*. A minority component of B-cells *CD20+* and *NK-cells* may be present. The attraction of TIL by cancer cells have been related to their expression of some chemokines, like *CXCL9* and *CXCL13* [37, 44].

In "triple negative" and *HER-2* subtypes of breast cancer, the presence of TIL is related to a better response to neoadjuvant therapy, as well neoadjuvant treatment may modify the tumor microenvironment to attract TIL to tumor site. Furthermore, when the TIL are not attracted instead of neoadjuvance, it is indicative for bad prognosis [44].

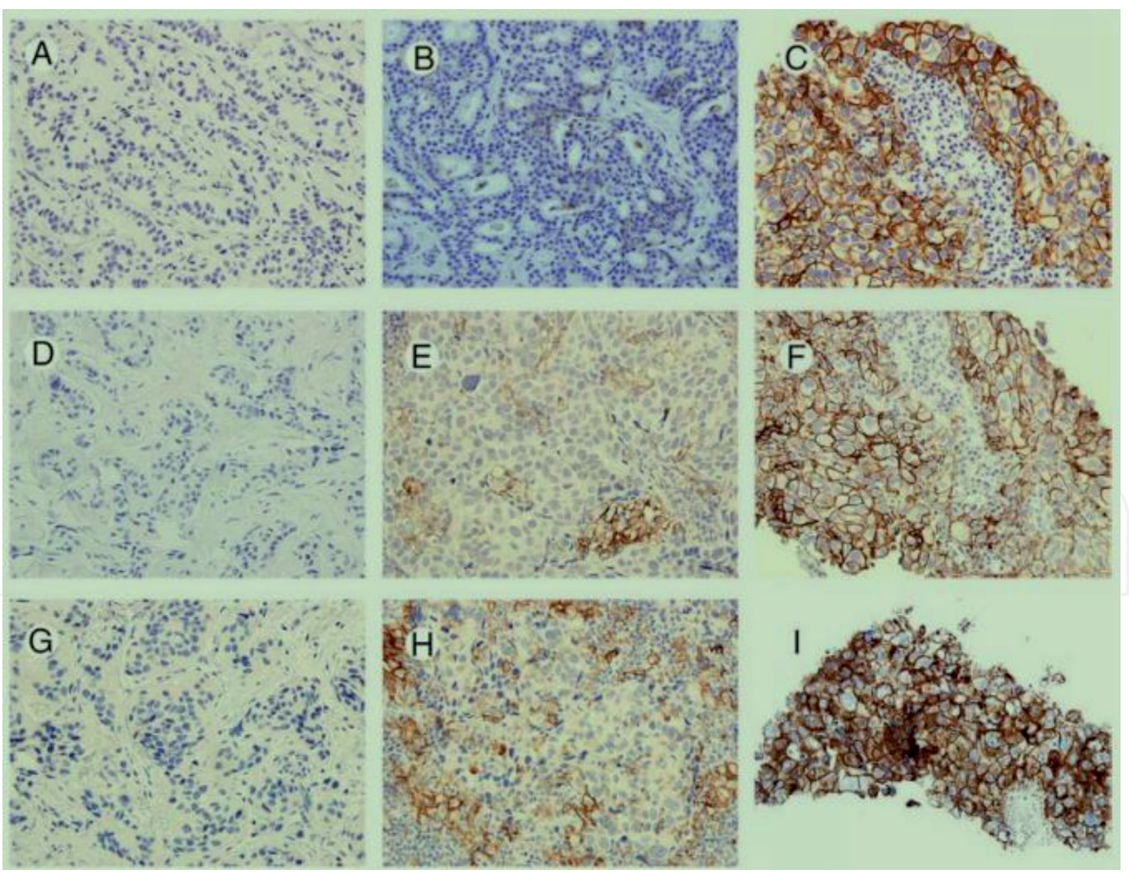


Figure 6.
Examples of PDL-1 expression in breast cancer using 3 different antibodies: Dako 22C3 (D,E and F), Ventana SP263 (G,H and I) and BioCare RbM CAL10 (A, B and C). PDL-1 scoring is divided into 3 groups: zero staining is negative, 1–49% of positive cells are considered "low PDL-1 expression" and 50% of more positive cells are considered "high PDL-1 expression". Examples of negative, low and high PDL-1 expression are represented on A, B and C for BioCare antibody (extracted from [42]).

6. Advanced stage breast cancer: considerations under current approach and futures perspectives

Metastatic breast cancer is considered incurable nowadays with currently therapies. Therapy of metastatic disease aims to guarantee quality of life, palliation of symptoms and prolongation of the patient survival. Advanced stage disease is becoming increasingly chronic, controlled by sequencial therapies, with more personalized approach than the early stage breast cancer [8].

Systemic therapy is frequently the first choice of metastatic disease. Before the new therapeutic decision, it is necessary to consider the previous treatments. If possible, it is recommended to re-evaluate the histologic features and molecular subtype status of the metastatic lesion through a new biopsy, with new immunohistochemical study for hormonal receptor and *HER-2* status. Some studies reported until 40% of discrepances of metastatic lesion histologic features and molecular subtype status *versus* primary tumor histologic and immunohistochemical aspects [45].

The metastatic disease therapeutic choices search for positive targets to hit more effectively the neoplastic cells. Thereby, expression of hormonal receptors by the metastatic lesion is elective for endocrine therapy. Endocrine drugs include tamoxifen, aromatase inhibitors, fulvestrant and progestins. The use of these drugs in metastasis with hormone receptor positive status have demonstrated increase of free of disease survival in several studies [8, 45].

Furthermore, new generation of drugs which inhibit the cyclin dependant kinase (*CDK*) have been successful in prolongation of free of disease survival in luminal subtype *HER-2* negative metastatic disease. *CDK4/6* is a holoenzyme responsible for several extracellular signaling pathways to cell cycle transitions. *CDK4/6* fosforilates and inactivates retinoblastoma tumor supressor protein (*Rb*). Extracellular signals regulate the expression of cyclins and *CDK* inhibitors, like $p16^{INK4a}$ [46].

In human cancer, this circuit is dysregulated by either overexpression of cyclin D1, loss of $p16^{Ink4a}$, the mutation of *CDK4* to an *Ink4*-refractory state, or the loss of *Rb* itself. The primary target of *CDK4* is the *Rb* protein, though this holoenzyme either can phosphorylate factors involved in cell differentiation affecting their transcriptional activity, apoptotic factors affecting their activity and other factors that can directly affect mitochondrial function [8, 46, 47].

Therefore, *CDK* inhibitors act in tumor microenvironment, blocking *Rb* phosphorilation and leading to cell cycle exit. Moreover, *CDK* have kinase activity towards *SPOP*, an ubiquitin protein that interacts with *PDL-1*. *CDK* inhibitors lead to inhibition of *SPOP* phosphorilation with blockade of *PDL-1* and stimulus to *PD-1* expression by T-cells, attracting T-cell infiltration to the tumor. In this way, the combined use of *CDK* inhibitors and *PDL-1/PD-1* inhibitors may be promising, requiring more future studies [46–48].

For the moment, hormonal receptors and *HER-2* status are the few validated molecular targets of clinical importance on metastatic breast cancer approaching through chemotherapy and endocrine therapy. For *HER-2* positive metastatic disease, anti-HER-2 treatment with trastuzumab is well established and is recommended as soon as possible. Immune therapy is not standardized for metastatic breast cancer, since metastatic breast disease is highly heterogeneous. Though, it is a promising therapy for the future, as well the target molecular therapies, which become more effective with discovery of novel pathways and mutations by new studies to be developed [8].

A resume of main biomarkers of clinicopathologic importance for breast cancer management is shown in **Table 3** and a proposal of a algorithm for clinicopathologic evaluation of breast cancer is presented in **Table 4**.

Biomarker	Detection technique	Nature	Clinicopathologic importance
Hormonal receptors/ HER2	IHC[1]/FISH	Biomarkers of molecular subtypes of breast cancer	Targets for endocrine and anti-HER2 therapies; prognostic predictors
BRCA1/BRCA2	PCR sequencing	Biomarker of hereditary breast cancer	Target for PARP inhibitors; indication for other malignancies screening
CD24, CD44, CD133, EPCAM	IHC	Putative stem-cell biomarkers	Prediction of poor prognosis, risk of tumor progression and reduction of survival
PD-1/PDL-1	IHC	Biomarker of possible inhibited immune response of T-cell against cancer cell	Target for immune therapy with PD-1/PDL-1 antagonists
TILs[2]	Histologic assessment and IHC	Marker of better cellular mediated immune response against cancer cell	Prediction of better therapeutic responses, mainly of neoadjuvant therapies

[1]*IHC = Immunohistochemistry.*
[2]*TILs = Tumoral infiltrating lymphocytes.*

Table 3.
Resume of main biomarkers of clinicopathologic importance for breast cancer management.

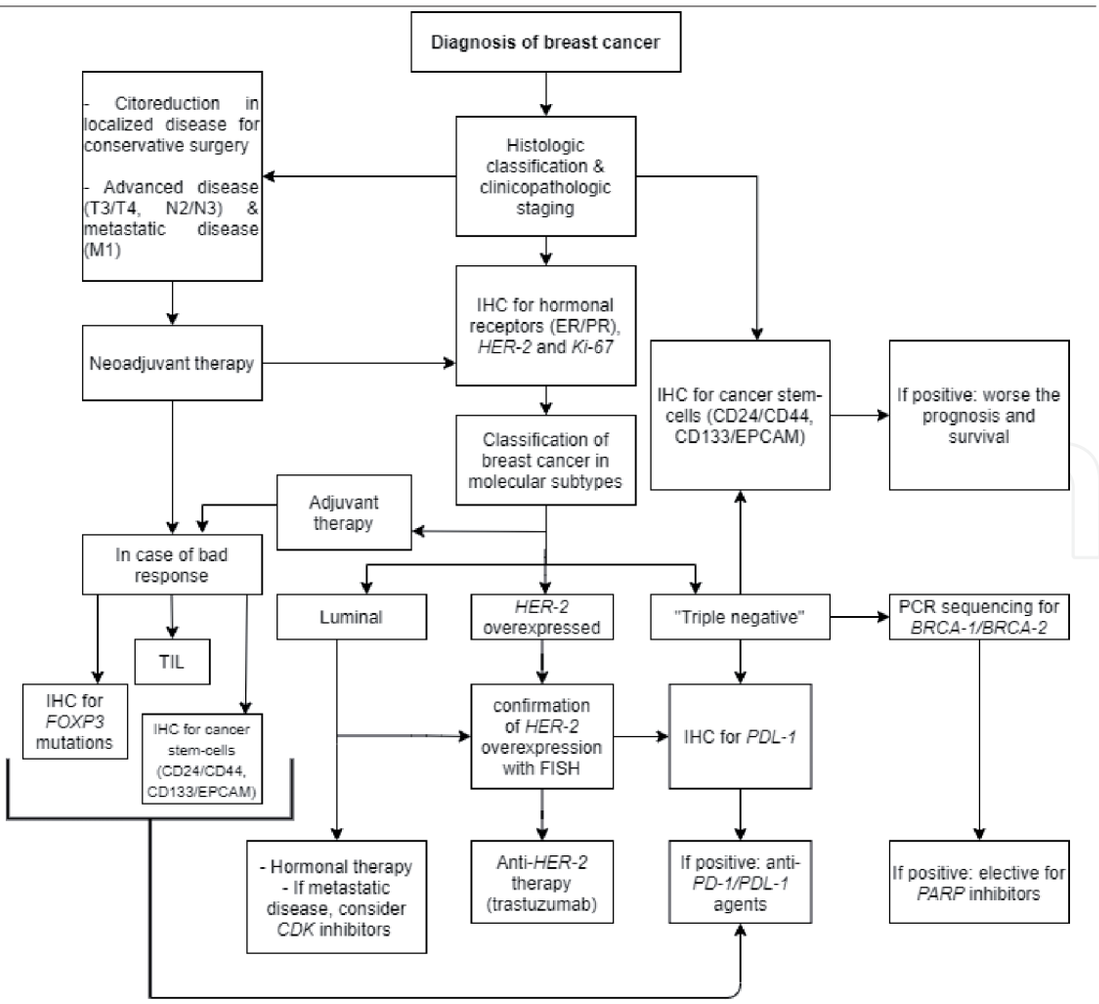


Table 4.
Proposal of an algorithm for clinicopathologic evaluation of breast cancer.

7. Conclusion and final considerations

In the 21st century, breast cancer classification and diagnosis advanced considerably from a purely morphologic/histologic approaching to a immune and molecular basis, with remarkable improvement of the correlation between classification and prediction of biological behavior and prognosis.

The adoption of a clinicopathologic classification based on molecular subtypes of breast cancer in the last decade has modified decisively the management of the disease in the way of molecular era, opening new ways to discovering of multiple targets for novel therapies.

Innovative concepts related to immune reactions related to human cancers, which have been unveiled in the recent years, particularly the immune checkpoints, have offered new treatment tools for several human cancers with promising results, although not still established for breast cancer.

In the molecular era of cancer, the integration of novel knowledges in a direction of more accurated diagnosis and prediction of prognosis to allow personalized therapies is the key to future human cancer management, including the breast cancer.

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