# Predictive factors of breast cancer evaluated by immunohistochemistry

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## *Fatores preditivos do câncer de mama avaliados pela imuno-histoquímica*

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**key words**

**Estrogen receptor**

**Progesterone receptor**

**Her2/*neu***

## abstract

Hormone receptor and Her2 protein overexpression evaluated by immunohistochemistry (IHC) is widely validated as a predictive factor in breast cancer. The quality of the IHC reaction is influenced by tissue fixation and processing. Over- and underfixation deeply affect IHC results. Antigen retrieval may improve IHC but it does not recover tissue from autolysis or overfixation. The choice of primary antibody for IHC as to its sensitivity and specificity in relation to therapeutic response represents an important stage. Apart from mouse monoclonal antibodies, new rabbit monoclonal antibodies are commercially available, such as clones anti-ER SP1 and B644, anti-PR SP2 and B645 and anti-Her2 SP3 and 4B5. They represent an alternative to hormone receptor and Her2 evaluation by IHC. New polymeric non-biotinylated detection systems are also available and allow accurate and strong marking with no stromal and no non-specific cytoplasmic staining due to endogenous biotin. The most recommended cut off for estrogen and progesterone receptors (ER and PR) is more than 1% of positive cells with moderate or strong staining intensity (Allred's scoring system). New guidelines for Her2 evaluation by IHC show a cut off of more than 30% of positive cells with strong intensity (3+) that correlates better with gene amplification. The 2+ cases are now considered indeterminate and should be confirmed by fluorescence *in situ* hybridisation (FISH) or chromogenic *in situ* hybridisation CISH. A quality control of pre-analytical, analytical and post-analytical phases of IHC is recommended in order to optimize results.

**unitermos**

***Receptor de estrógeno***

***Receptor de progesterona***

***Her2/neu***

## resumo

*A superexpressão de receptores hormonais e Her2 avaliada pela imuno-histoquímica (IHQ) é amplamente validada como fator preditivo em câncer de mama. A qualidade da reação imuno-histoquímica é influenciada pela fixação do tecido e seu processamento. A fixação insuficiente ou demasiada afeta profundamente os resultados da IHQ. A reativação antigênica pode melhorar os resultados da IHQ, porém não recupera tecidos com autólise ou com excessiva fixação. A escolha do anticorpo primário para a IHQ, considerando sua sensibilidade e sua especificidade de acordo com a resposta terapêutica, representa uma importante etapa. Além de anticorpos monoclonais de camundongo, novos anticorpos monoclonais de coelho são comercialmente disponíveis, tais como clones SP1 e B644 anti-RE, SP2 e B645 anti-RP, e SP3 e 4B5 anti-Her2. Eles representam uma alternativa para avaliação de receptores hormonais e Her2 através da IHQ. Novos sistemas de detecção poliméricos não-biotinilados também são disponíveis e permitem marcação exata e forte sem marcação estromal ou citoplasmática inespecífica devido à biotina endógena. O* cut off *mais recomendado para receptor de estrogênio (RE) e receptor de progesterona (RP) é acima de 1% de células positivas com marcação moderada ou forte (sistema de escore de Allred). Novas recomendações para avaliação de Her2 através da IHQ apontam um* cut off *de mais de 30% de células positivas com marcação forte (3+), que melhor se relaciona com amplificação gênica. Os casos 2+ são agora considerados indeterminados e devem ser confirmados por hibridação* in situ *por fluorescência (FISH) ou hibridização* in situ *colorimétrica (CISH). Um controle de qualidade de fases pré-analítica, analítica e pós-analítica da IHQ é recomendado para a otimização dos resultados.*

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## Introduction

Prognostic factors are clinic, pathologic, and biologic features of cancer patients and their tumors that forecast clinical outcome, *i.e.* the likelihood of disease recurrence or patient survival, in an untreated setting. Predictive factors, in contrast are clinic, pathologic, and biologic features that are used to estimate the likelihood for a response to a particular type of adjuvant therapy[1, 15]. Pathologists measure and interpret certain prognostic and predictive factors of breast cancers. Oncologists use the results supplied by pathologists in combination with other important information to make decisions about the treatment of their patients[1, 15].

To be useful a predictive factor should be technically and clinically validated and should influence clinical decision-making. Technical validation means that the assay used to measure the factor is sensitive, specific, reproducible, and interpreted in a uniform manner from laboratory to laboratory. Clinical validation means that the test identifies subsets of patients with significantly different risks for recurrence, survival or treatment response that are independent from other factors[1, 15].

Although predictive factors of therapy response have more clinical value, the only broadly validated predictive factors for the routine clinical use are estrogen (ER) and progesterone (PR) receptors and Her2[3].

The immunohistochemical technique for hormone receptors and Her2 evaluation in breast cancer will be discussed along this review including tissue processing, antigen retrieval, choice of primary antibody and detection system, and evaluation of immunostaining.

## Estrogen and progesterone receptors

Hormone receptors exert their physiological effects of regulating the proliferation and differentiation of normal breast epithelium by binding to estrogen receptors, which are members of the nuclear receptor superfamily of ligand-inducible transcription factors[1]. The estrogen receptor (ER)α regulates the differentiation and maintenance of neutral, skeletal, cardiovascular, and reproductive tissues. Compounds that modulate ERα transcriptional activity are currently being used to treat osteoporosis, cardiovascular disease, and breast cancer[21]. All ERα ligands bind exclusively with the C-terminal ligand-binding domain. Synthetic ligands such as tamoxifen and raloxifene belong to a growing class of molecules known as selective estrogen receptor modulators (SERM), which function as antagonists in specific tissue and promoter contexts[37].

The discovery of a second ER, termed ERβ[37], indicates that the mechanism of action of estrogens is more complex than anticipated. Human ERβ has a structure highly homologous to the previously known ERα. ERβ is expressed by normal ductal epithelium and a majority of breast cancers[26]. ERβ positive breast cancers are predominantly ERα and PR-positive, node negative, well differentiated and slowly proliferating. The coexpression of ERβ with ERα and PR as well as its association with indicators of low biological aggressiveness suggest that ERβ-positive tumors are likely to respond to hormonal therapy. The independent predictive value of ERβ remains to be established[26].

PRs are ligand-activated transcription factor members of the steroid hormone family of nuclear receptors. They exist naturally as two isoforms, PR-B and PR-A, transcribed from two promoters on a single gene[14]. Molecular basis for functional differences between both PR isoforms demonstrate that in breast cancer cells, although some genes are regulated by progesterone through both PR isoforms, most genes are uniquely regulated through one or the other isoform and predominantly through PR-B[14]. In breast cancers, total PR levels are routinely measured as a guide to hormone therapy and as markers of disease prognosis together with ER[43].

ER may be the best example of a tumor biomarker with an assay that drives therapeutic decision-making. Since ER and PR represent a well established predictor of response to endocrine therapy in breast cancer, their measurements improve the predictive value further by defining the ER-positive/PR-negative tumor type, which is less likely to respond to therapy than tumors that are positive for both receptors[1]. ER and PR assays have been routinely used in the selection of appropriate therapy for breast cancer patients for more than 30 years[1]. However, it is well known that up to 30% to 40% of breast tumors with positive hormone receptor status do not respond to endocrine therapy[15]. Reasons for the lack of response have remained poorly understood, although steroid-independent growth factor signaling (e.g. via HER-2/*neu*), functionally deficient splicing variants of the ER gene[15], and heterogeneity of ER expression[28] may partly explain poor therapy outcome of ER-positive tumors.

## ER and PR antibodies

Up to the last decade, quantification of ER and PR was made by biochemical methods that consist of a dextrane-coated charcoal assay (DCC) and a minimum of 0.5 $mm^3$

of the extirpated tumor was necessary. The cut-off point for positivity/negativity was generally established at 10 fmol/mg cytosol protein[15]. With the development of monoclonal antibodies against nuclear estrogen and progesterone nuclear epitopes, immunohistochemical measurements have been increasingly validated by several studies[1, 15, 23]. Since 1990, ER and PR have been evaluated almost exclusively by immunohistochemistry, using formalin-fixed paraffin-embedded tissue samples.

The most used antibodies for ER and PR evaluation by immunohistochemistry in formalin-fixed paraffin-embedded tissue have been the mouse monoclonal antibodies, including clones 1D5 and 6F11 anti-ER and the PgR 636 and PgR 312 anti-PR. Recently, a new generation of rabbit monoclonal antibodies has been developed and is commercially available, such as clones SP1 and B644 anti-ER and SP2 and B645 anti-PR[24, 25, 46]. The technology to prepare these antibodies from a single hybridoma allows the production of antibodies with high sensitivity and specificity, high working dilutions and better cost versus benefits. Results from comparative studies have shown that the rabbit clone SP1 may be more sensitive than clone 1D5 and has the same specificity as clone 1D5 in immunohistochemistry[24].

The new rabbit antibody SP1 and the mouse antibody 1D5 were recently evaluated and compared to the biochemical ER assay results and clinical data on survival and adjuvant systemic therapy. The authors detected 69.5% positivity when using the rabbit SP1 and 63.1%, using the mouse antibody 1D5. Rabbit antibody SP1 was also a better independent prognostic factor than 1D5 in multivariate analysis, including age, tumor size, grade, lymphovascular invasion, and nodal status. SP1 was considered by the authors an improved standard for ER immunohistochemistry assessment in breast cancer[8].

The rabbit monoclonal antibodies against ER and PR were also evaluated on alcohol-fixed smears of breast cancers obtained by fine needle aspiration biopsy. The results showed advantages, such as high sensitivity and specificity of the reaction, stronger immunostaining and shorter procedure times[6]. The rabbit antibodies appear to offer increased sensitivity with no apparent loss of specificity and allow a higher working dilution[24].

We carried out a study evaluating new rabbit monoclonal antibodies anti-ER (SP1 and B644) and anti-PR (SP2 and B645), comparing them to mouse antibodies anti-ER (1D5 and 6F11) and anti-PR (PgR 636 and PgR 312). Our results showed that the rabbit antibodies could be used in higher working dilutions when using antigen retrieval (**Figure 1**). They represent an alternative for estrogen and progesterone receptor evaluation in clinical tests[44, 45].

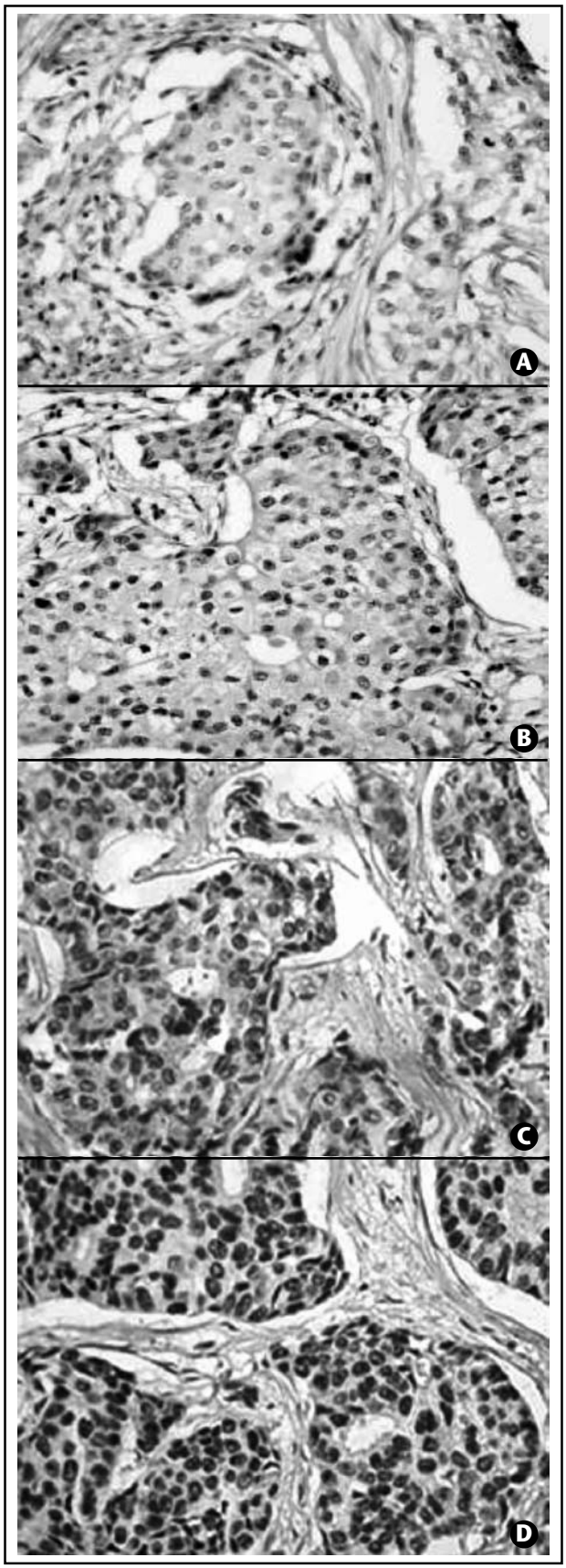


**Figure 1** – *Immunohistochemical staining carried out in our laboratory in the same tumor: ER and PR staining using mouse monoclonal antibodies anti-ER, clone 1D5 (A); and anti-PR, clone PgR 636 (C); and rabbit monoclonals anti-ER, clone SP1 (B); and anti-PR, clone SP2 (D)*

## Interpretation of ER and PR immunostainings

ER and PR staining interpretation and cut off rely basically on subjective visual estimates, yielding only qualitative or at best semi-quantitative results. New studies have applied semi-quantitative scores to assess nuclear staining intensity as a marker of the number of receptors per cell[1, 15]. Although in some cases a cytoplasm staining is demonstrated, its biological or clinical meaning is not well established, and only nuclear staining has been considered[5]. Diverse computerized image analysis systems have been employed and were found to correlate well with semi-quantitative visual scoring methods and with biochemical data. However, the high cost and the complexity of these image analysis systems, requiring major hardware and software investments, severely limit their practicability in routine diagnostic laboratory[11, 31].

The initial studies that validated the assessment of ER by immunohistochemistry established a cut off of 10% positivity that correlates to 10 fmol/mg of protein detected by biochemical method. The 10% level for positivity, independent on the staining intensity, has been accepted and has been the most used cut off for ER and PR[15, 17, 48]. However, further studies showed that patients whose tumors expressing ER in more than 1% of neoplastic cells with moderate or strong intensity presented response to anti-estrogen therapy[1, 20]. The 2005 Saint Gallen Consensus Conference recommended the cut off proposed by Allred (**Table**) in which a total score of more than 3 is considered positive[18].

## Her2/*neu*

*HER2* gene (*c-erbB-2* or *HER2-neu)* is a human analogue of the gene neu identified in rat neuroblastomas. The proto-oncogene *HER2* is located on chromosome 17 and encodes a 185-kd transmembrane glycoprotein with tyrosine-kinase activity, which is a member of the epithelial HER family that also includes the epidermal growth factor receptor (EGFR) or HER1, HER3 and HER4. Normal cells and the majority of breast cancers carry two copies of the *HER2* gene on chromosome 17 and express low levels of p185 (Her2 protein)[7, 9, 13, 15]. *HER2* gene amplification or protein overexpression is seen in approximately 20% to 30% of invasive breast cancers in humans, most commonly in high-grade invasive ductal carcinomas[2, 9, 15, 41].

Studies have reported an association with Her2 overexpression and other adverse prognostic factors, such as positive lymph nodes, larger tumor size, high histological grade, high proliferation rate, and lack of expression of estrogen and progesterone receptors[15, 40]. But the importance of this gene is higher as a predictive than a prognostic factor. The overexpression of Her2 is implied in the resistance to tamoxifen and the chemotherapy with cyclophosphamide, metothrexate and 5-fluorouracil. Therefore, breast cancers with Her2 overexpression are more sensitive to antracyclines, specifically to trastuzumab. There is resistance to tamoxifen, but replacing of tamoxifen with aromatase inhibitors in patients with breast cancer ER and Her2 positive have demonstrated promising clinical results[7, 9]. The American Society of Clinical Oncology (ASCO) Tumor Marker Guidelines Panel has recommended routine testing of HER2 on newly diagnosed and metastatic breast cancer since 2001[7, 33].

Different techniques have been used to assess the Her2 status in biopsies and surgical specimens, but currently the most frequently used methods are immunohistochemistry (IHC) to assess Her2 protein overexpression and *in situ* hybridisation to assess *HER2* gene amplification[29, 34, 41].

IHC is relatively easy to perform, has a short turnaround time and relatively low cost. However, immunohistochemical analysis is highly influenced by technical procedures, such as fixation affecting the quality of antigen epitopes in the formalin-fixed paraffin-embedded (FFPE) tissue samples

**Table** Allred's scoring system for ER evaluation in breast carcinomas obtained by the combination of intensity and proportion of stained tumor nuclei*

| Intensity score | Proportion score (%) | | | | |
|---|---|---|---|---|---|
| | 0 | > 0 to 10 | > 10 to 33 | > 33 to 66 | > 66 to 100 |
| Weak | 0 | 2 | 4 | 5 | 6 |
| Intermediate | 0 | 3 | 5 | 6 | 7 |
| Strong | 0 | 3 | 6 | 7 | 8 |

**A total score (TS) is obtained combining scored proportion plus intensity score. A TS of 0 to 2 is considered negative and a TS of 3 to 8, positive[1, 23].*

and choice of IHC reagents and protocols. Selection of specific antibodies and scoring methods are very important parameters for the accurate evaluation of protein expression[21, 41, 43, 52]. The HercepTest™ kit has overcome some of these problems by using standardized methodology and reagents and by the inclusion of cell line controls. However, the reported sensitivity and specificity vary between different centres. The HercepTest™ kit is more expensive and shows more false positive cases than other monoclonal antibodies[21, 35]. Despite the commercially variety of IHC antibodies available, there is no consensus about the best antibody for Her2 testing. The most common used antibodies for HER2 testing have been the HercepTest™ and A0485 (rabbit polyclonal antibody), and the CB11 and TAB250 (mouse monoclonal antibodies). Recently, a new generation of rabbit monoclonal antibodies was released including the anti-Her2 rabbit clone SP3 (LabVision™) and 4B5 (Ventana™)[24].

Several *in situ* hybridisation techniques are available to evaluate the *HER2* gene amplification. Fluorescence *in situ* hybridisation (FISH) is thought to be an accurate technique for quantitative evaluation of *HER2* gene status in breast cancer cells. FISH methodology requires a fluorescence microscopy equipped with high quality immersion objectives and fluorescence filters. As the fluorescence signals can fade within several weeks, the hybridisation results must be recorded with digital cameras or modern scanner systems. Therefore, analysis and recording of FISH data is expensive and time consuming. Most important, tissue section morphology is not optimal in FISH on FFPE, a particular problem in distinguishing invasive breast cancer and intraductal carcinoma[50, 53]. To overcome these practical limitations, chromogenic *in situ* hybridisation (CISH) has been introduced, in which the DNA probe is detected using a simple immunohistochemical-like peroxidase reaction[56]. CISH is faster to analyse than FISH, does not require any equipment other than those used in routine histopathology laboratories, and allows for a simultaneous analysis of gene copy number and histological features of the lesions[4, 49, 54]. CISH has been validated and compared to FISH, with a high concordance rate. CISH is reported to have the same accuracy of FISH when a high level amplification is detected, and 93%-99% agreement when low level of amplification is detected[4, 12]. In case of low-level amplification, some authors suggest that CISH chromosome 17 probe should be used, or dual probe FISH is recommended for confirmation[4].

The most common scoring system for Her2 overexpression has been that recommended in the HercepTest™ manufacturer's protocol[10]. This immunohistochemical Her2 scoring is based on the proportion and the intensity of cell membrane staining. But inter and intraobserver variations have been demonstrated on determination of Her2 overexpression, especially on intermediate categories[38]. When IHC results are compared with those obtained by FISH analysis, it appears that the IHC technique is associated with a significant number of false positives, particularly those represented by grade 2+ scored using the HercepTest system[21].

Given the rather poor predictive value of weakly positive results by IHC, two new consensus (UK Guidelines and the ASCO/College of American Pathologists [CAP] recommendations for Her2 testing in breast cancer) considered that immunohistochemistry for Her2 protein overexpression represents a valuable screening test. The ASCO/CAP guidelines proposed that cases interpreted as grade 2+ should be considered as indeterminate or borderline, and not weakly positive. Strongly positive (3+) and completely negative (0) results appear to correlate well with gene amplification status[2, 19, 21], but *in situ* hybridization should confirm all 2+ results[13, 54, 55]. Only the invasive component of a tumor (not *in situ* disease) should be scored. For IHC, membranous reactivity only should be considered positive in a good quality assay. Her2 staining should not be observed in adjacent stroma or inflammatory cells, nor should benign epithelium show membranous reactivity. If staining is observed in benign components, the assay may be considered indeterminate; reporting should include an estimate of the percentage of immunopositive invasive cancer cells[13, 30, 51].

A threshold of more than 30% of tumor (rather than the originally specified 10% of the HercepTest) should show strong complete or circumferential membrane staining for a positive result. When less than 30% of the cells show circumferential staining, the result is considered indeterminate or equivocal and the tumor subjected to confirmatory FISH testing[27]. The visualization of a "train track" pattern that results from the staining apposing cell membranes has also been emphasized[32]. A cut off of more than 30% reflects the cumulative experience that usually a high percentage of the cells will be positive if it is a true IHC 3+, compared to the cut-off values higher than 10%. The goal of the 30% cut off is to decrease the incidence of false positive 3+[54].

The ASCO/CAP Conference recommends for Her2 testing in breast cancer that HER2 status should be determined for all invasive breast carcinomas. A positive Her2 result is IHC

staining of 3+ (uniform, intense membrane staining of > 30% of invasive tumor cells), a FISH result of more than six *HER2* gene copies per nucleus, or FISH ratio (*HER2* gene signals to chromosome 17 signals) of more than 2.2. A negative result is an IHC staining of 0 or 1+, a FISH result of less than 4.0 *HER2* gene copies per nucleus, or FISH ratio of less than 1.8. Equivocal results require additional action for final determination[22, 54].

In our laboratory, we compared the "sensitivity" and "specificity" of SP3 with those of mouse monoclonal and rabbit polyclonal antibodies to detect Her2. We also compared the immunohistochemical protein over-expression of protein with the gene amplification using CISH[20, 38, 39]. The antibody SP3 displayed an optimal "sensitivity", similar to that obtained with anti-Her2 polyclonal antibodies (i.e. HercepTest and A0485) when comparing IHC with CISH results. The SP3, HercepTest and A0485 identified all cases with *HER2* amplification using the current guidelines for Her2 assessment. However, the SP3 and the polyclonal antibodies, including the HercepTest, detected more false-positive cases than mouse monoclonal antibodies CB11 and 4D5[20, 38, 39].

In our experience, SP3 shows higher sensitivity than mouse monoclonal antibodies (**Figure 2**). However the HercepTest, CB11 and 4D5 show higher "specificity" than SP3 for the identification of *HER2* gene amplification[20, 38]. Ricardo *et al.*[43] also compared SP3 with CB11, having CISH as the gold standard in breast cancer tissue microarrays, however they showed a high specificity and a moderate sensitivity of SP3[43]. We also evaluated interobserver variation in Her2 interpretation in IHC slides. The quality of immunostainings and the experience of the observers influence Her2 evaluation in tissue sections[38].

## Limitations of predictive factors evaluation in breast cancer by immunohistochemistry in formalin-fixed paraffin-embedded tumors

The success of immunohistochemistry is largely a result of a development of reliable markers for ER, PR and Her2 and of highly sensitive detection procedures. However, pre-analytical factors, such as unsatisfactory tissue fixation (under- or overfixation), a common problem in Brazil, represent an important drawback in immunohistologic evaluation of the neoplasia. Formalin has several advantages over alcohol, particularly the superior preservation of morphological detail. A major cause of variation in the

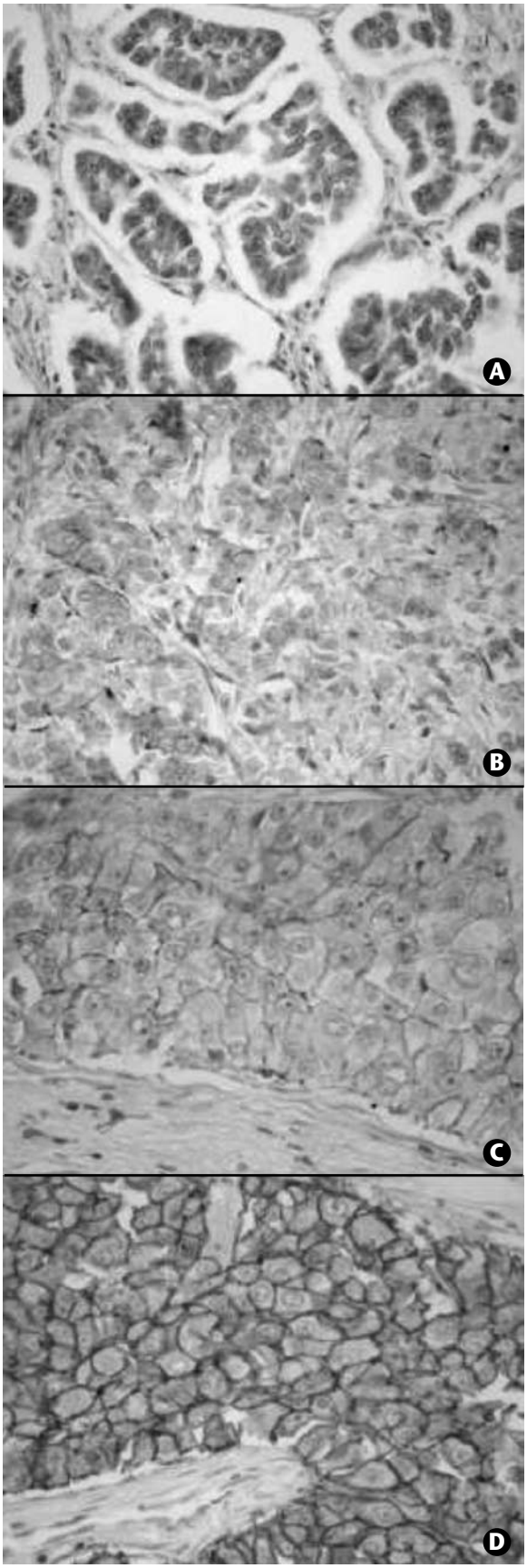


**Figure 2 –** *Immunohistochemical staining carried out in our laboratory: Her2 staining using rabbit monoclonal SP3: 0 (A); 1+ (B); 2+ (C); 3+ (D)*

reproducibility of immunohistochemical staining is induced by tissue fixation and, to a lesser degree, tissue processing. Most laboratories in USA use neutral-buffered formalin (10%) for tissue fixation that introduces cross-links, whereas coagulative fixatives are less popular[42]. However, in Brazil the use of buffered formalin is not a routine in the majority of the laboratories. Problems with formalin fixation comprise delay of fixation and variations in time of fixation. Overfixation leading to antigen masking probably through aldehydic linkage between proteins and formalin, and long-term storage of sections on slides before staining have been blamed for false-negative results[51].

One of the challenges of immunohistochemistry is to develop methods that reverse changes produced during fixation such as high-temperature heating using the most suitable buffered solution. High-temperature heating is the most important factor for retrieval of antigens masked by formalin fixation. Higher temperature in general yields better results of antigen retrieval. Different heating methods have been used for antigen retrieval, such as autoclaving, pressure cooking, water bath, microwaving plus plastic pressure cooking, and steam heating. The temperature achieved by these methods appears to be the critical variable[47].

Although the antigen retrieval step is more time consuming, the total cost of each test drops greatly when this technique is used. For example, the rabbit monoclonal antibody SP1 can be used 10 times less concentrated than the 6F11 when using antigen retrieval[6]. However, antigen retrieval may cause false-positive stains or non-specific background due to endogenous biotin. Although problems with endogenous biotin have previously been negligible in formalin-fixed tissue, antigen demasking also makes endogenous biotin more accessible[5, 47]. High temperature, long heating time and high pH of the retrieval solution also increase the reactivity of endogenous biotin although they give the most efficient epitope retrieval[5, 47]. In our experience, the antigen retrieval for ER and PR using high pH (EDTA, pH = 9) increases the reactivity, but also produces more non-specific and background staining. Different alternatives to avoid or reduce the effects of biotin/peroxidase are offered by different methods from different suppliers. Antigen retrieval methods have been improved[5] and more sensitive non-biotin polymeric detection systems for the antigen-antibody reaction have also been developed[39].

A generation of polymer-based, biotin-free detection reagent is based on polymeric technology, which uses compact enzyme-antibody conjugates resulting in markedly increased penetrative ability of the reagent with improved detection sensitivity and efficiency[39]. Besides allowing cheap immunohistochemical assays – as the cost per test can be the same or even better than the non-polymeric detection systems – the high dilutions of the primary antibody achieved by the polymeric conjugate detection systems also enable more reliable results to be obtained[16]. High dilutions prevent background staining, formation of electrostatic or other non-immunological non-specific bonds, or unexpected cross reactivities (especially with polyclonal antibodies)[36, 39]. In our laboratory, we compared the new polymer detection systems with the streptavidin-biotin system in the assessment of ER in breast carcinomas. Our results showed that staining intensity of non-biotinylated polymeric detection systems is superior compared to conventional streptavidin-biotin detection system. Background and non-specific cytoplasm staining was also lower or completely absent when using the non-biotinylated polymeric systems[45].

## Take-home messages

1. Fixation: cut sections of breast carcinoma should be fixed in buffered formalin for no longer than 24 hours. Under- and overfixation deeply affect immunohistochemical results.

2. Antigen retrieval: may improve IHC, but is not a magic tool to recover autolysis or overtime fixation. The most common methods are heat-induced epitope retrieval in citrate buffer (pH = 6) or EDTA (pH = 9). High pH improves antigen retrieval, but also increases background and non-specific cytoplasmic staining.

3. Choice of primary antibody: besides mouse monoclonal antibodies, novel rabbit monoclonals are available, allow high working dilutions and are cost effective.

4. Immunodetection systems: novel non-biotinylated polymer detection systems represent an alternative to the streptavidin-biotin systems. They show high staining intensity, low background and non-specific cytoplasmic staining. They allow higher working dilution of primary antibody than streptavidin-biotin systems.

5. Interpretation of immunohistochemistry – the recommended cut offs are:

• ER and PR Allred's scoring system is recommended. Tumors with more than 1% of positive cells with moderate or strong staining (total score ≥ 3) are considered positive;

• Her2: tumors with > 30% of neoplastic cells showing strong complete circumferential staining are considered

positive. When < 30% of cells show strong circumferential staining, the result is considered indeterminate and should be confirmed by FISH or CISH;

• quality control: laboratories should carry out quality control of pre-analytical, analytical and post-analytical phases of IHC in order to optimize quality and reproducibility of results.

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