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Pre-analytical conditions affect histomorphology and biomarker assessment in invasive breast carcinoma

Marcelo Araújo Buzelin[1], Cristiana Buzelin Nunes[1], Débora Balabram[2], Rodrigo Fonseca Abreu[3] and Helenice Gobbi[3*]

Abstract

Background Standardization of the pre-analytical phase, including prompt specimen sectioning and appropriate fixation, is essential for preserving tissue morphology and protein integrity. However, these practices are not consistently implemented in many pathology laboratories, particularly in resource-limited settings. This study aimed to compare histopathological features and predictive biomarkers in invasive breast carcinomas processed under optimal conditions versus routine conditions.

Methods A total of 49 surgical fresh specimens of breast cancer were grossly examined and bisected. A representative tumor fragment from each specimen (1.0 × 1.0 × 0.2 cm) was immediately fixed in 10% neutral buffered formalin for 24 h (Group 1: early sectioning with buffered formalin fixation). The remaining specimen was reassembled, placed in non-buffered formalin, and transported to the laboratory according to routine workflow, with delayed sectioning (24–48 h; Group 2: delayed sectioning with non-buffered formalin fixation). A second sample of tumor was obtained from each delayed sectioned specimen (total: 49 samples) on the opposite side of previous sectioning. Histopathological evaluation included autolysis-related morphological changes, histological tumor type, and tumor grade. Immunohistochemical analysis of the estrogen receptor (ER), progesterone receptor (PR), and HER2 were performed.

Results Autolysis-related morphological changes were significantly more frequent in Group 2 than in Group 1 (33.3% vs. 12.5%, $p = 0.02$). Special histological types were more frequently identified in Group 1 ($p = 0.03$). No significant differences were observed in histological grade or ER and PR expression. HER2-negative status was more frequent in Group 2, whereas equivocal (2+) cases were more common in Group 1, although without statistical significance.

Conclusions Delayed sectioning with non-buffered formalin fixation is associated with increased autolysis-related morphological alterations and may affect histological tumor classification of invasive breast cancer. ER and PR expressions appear relatively stable, while HER2 is more susceptible to pre-analytical variability, underscoring the importance of optimized specimen handling.

Keywords Breast neoplasms, Immunohistochemistry, Preanalytical phase, Tissue fixation, HER2, Estrogen receptor, Progesterone receptor

*Correspondence:
Helenice Gobbi
helenice.gobbi@uftm.edu.br
[1]Department of Anatomic Pathology and Forensic Medicine, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil
[2]Department of Surgery, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil
[3]Discipline of Special Pathology, Institute of Health Sciences, Federal University of Triangulo Mineiro, Uberaba, MG, Brazil

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Introduction

Proper fixation of surgical specimens is a critical step in preserving tissue morphology and ensuring the reliability of histopathological evaluation. Formalin has long been the most widely used fixative in pathology practice due to its ability to maintain structural integrity and provide consistent staining results (Goldstein et al. 2007; Grizzle 2009; Khoury 2012; Fitzgibbons 2019).

With the increasing integration of molecular techniques into diagnostic pathology, the analysis of proteins, RNA, and DNA in formalin-fixed paraffin-embedded (FFPE) tissues has become essential for both diagnosis and therapeutic decision-making. This has heightened the need for optimal preservation of tumor samples throughout the pre-analytical phase (Groenen et al. 2011; Khoury 2018; Allison & Krishnamurti, 2025; Chen and Qiu 2025). Following the incorporation of immunohistochemistry (IHC) into routine practice, it became evident that pre-analytical variables significantly influence assay performance and interpretation (Agrawal et al. 2018; Allison & Krishnamurti, 2025; Chen and Qiu 2025).

Key pre-analytical factors include cold ischemia time, type of fixative, and duration of fixation. Prolonged cold ischemia time—particularly beyond one hour—has been shown to adversely affect tissue integrity and reduce antigen detectability by IHC (Apple et al. 2011; Siddiqui et al., 2010; Neumeister et al. 2012; Yildiz-Aktas et al. 2012; Khoury 2018). In addition, both underfixation and overfixation may impair antigen preservation, with the latter potentially leading to epitope masking. Neutral buffered formalin is considered the standard fixative for most IHC applications, particularly when combined with heat-induced epitope retrieval, which helps reverse formalin-induced cross-linking and restore antigenicity (Grizzle 2009; Koonmee et al. 2022).

In invasive breast carcinoma (IBC), accurate assessment of prognostic and predictive factors is essential for therapeutic decision-making. Expression of estrogen (ER) and progesterone (PR) receptors serves as both prognostic indicators and predictors of response to endocrine therapy, while HER2 status is a key determinant for targeted therapies, including trastuzumab and antibody–drug conjugates (Modi et al. 2022; Venetis et al. 2022). Immunohistochemistry remains the standard method for evaluating ER, PR, and HER2; however, its accuracy is highly dependent on pre-analytical, analytical, and post-analytical variables (Li et al. 2013; Nunes et al. 2013; Cree et al. 2014; Fitzgibbon et al., 2023; Wolff et al. 2023; Ellis et al. 2024).

Current guidelines from the American Society of Clinical Oncology/College of American Pathologists (Wolff et al. 2023; Allison & Krishnamurti, 2025), international (Rakha et al. 2023) and Brazilian groups (Buzaid et al. 2020; Leite et al. 2021; Gobbi et al. 2022, 2024) recommend prompt tissue sectioning followed by fixation in neutral buffered formalin for a duration between 6 and 72 h to ensure optimal IHC performance in breast cancer specimens.

Despite established recommendations, optimal pre-analytical practices—such as prompt specimen sectioning and fixation in buffered formalin for adequate durations—are not consistently implemented in many pathology laboratories in Brazil (Buzaid et al. 2020; Leite et al. 2021; Gobbi et al. 2022, 2024).

Therefore, this study aims to compare histopathological features, as well as prognostic and predictive biomarkers, in invasive breast carcinoma specimens processed under optimal conditions (early sectioning with adequate fixation) versus routine conditions involving delayed sectioning of mastectomy and lumpectomy specimens.

Materials and methods

Study design and sample collection

A total of 98 tumor samples were prospectively collected from 49 consecutive surgical specimens of invasive breast carcinoma from patients who had not received neoadjuvant chemotherapy. All patients underwent surgery at the Hospital das Clínicas, Federal University of Minas Gerais (Belo Horizonte, Brazil). The samples were prospectively collected over 3 consecutive months. The specimens included mastectomies ($n = 20$) and lumpectomies ($n = 29$).

After surgical excision, all specimens were handled in the operating room by the same pathologist, inked, and grossly examined in the fresh state. Each specimen was bisected, and a representative tumor fragment measuring $1.0 \times 1.0 \times 0.2$ cm was carefully obtained to avoid compromising routine diagnostic evaluation. This fragment was immediately immersed in 10% neutral buffered formalin, using a fixative volume at least 10 times greater than the tissue volume, and fixed for 24 h. These samples were assigned to **Group 1 (early sectioning with buffered formalin fixation)**. The remaining specimen was reassembled without additional sectioning or interposition of absorbent material. It was then placed in a plastic container containing an uncontrolled volume of 10% non-buffered formalin and transported to the pathology laboratory according to the institution's routine workflow, with a delay of 24–48 h after surgery. Upon arrival, the specimen underwent gross examination and sectioning. A second tumor sample was taken from the opposite side of the previous section and assigned to **Group 2 (delayed sectioning with non-buffered formalin fixation)**.

For Group 2, the interval between surgical excision and specimen sectioning in the pathology laboratory was defined as the "cold ischemia time". Tumor samples from both groups were processed using the same routine

histological protocol. Serial 4-µm sections were obtained from paraffin-embedded blocks and stained with hematoxylin and eosin ("H&E") for morphological and immunohistochemical analyses.

Histopathological analysis

Histomorphological evaluation included assessment of nuclear, cytoplasmic, and cell membrane features. The following findings were considered indicative of autolysis-related changes: retraction of tumor cells from the surrounding stroma, loss of cellular cohesion, nuclear chromatin margination, and cell membrane disruption. Morphological changes were analyzed across the entire area of tumor samples from Groups 1 and 2.

Tumors were classified into two categories according to the 2019 World Health Organization (WHO) Classification of Breast Tumors: invasive breast carcinoma of no special type (NST) and special types (including mixed and specific histological subtypes) (WHO Editorial Board, 2019).

Histological grading was performed using the Nottingham grading system, based on tubule formation, nuclear pleomorphism, and mitotic count (WHO Editorial Board, 2019).

Immunohistochemistry

Immunohistochemical (IHC) analysis for estrogen receptor (ER), progesterone receptor (PR), and HER2 was performed on histological sections from one tumor block from Group 1 and from one block from Group 2 for each case.

Tissue Sections (4 µm thick) were mounted on charged slides, deparaffinized, and rehydrated through graded alcohols. Immunostaining was performed using the HercepTest™ and Dako PharmaDx™ kits (Dako, Carpinteria, CA, USA), according to the manufacturers' instructions. Antigen retrieval methods, detection systems, and antibody specifications are summarized in Table 1.

Slides were counterstained with hematoxylin and mounted. Appropriate positive and negative controls were included in all staining runs.

Immunohistochemical evaluation

All immunostained slides were evaluated independently and blindly by the same pathologist (CBN). ER and PR expression were assessed according to Allred scoring criteria. Tumors with ≥ 1% of positively stained cells were considered positive. Both the percentage of positive cells and staining intensity were recorded, in accordance with WHO recommendations (WHO Editorial Board, 2019).

HER2 expression was evaluated following ASCO/CAP guidelines (Wolff et al. 2023): **0**: no staining or incomplete membrane staining in < 10% of tumor cells; **1+**: faint/incomplete membrane staining in ≥ 10% of tumor cells; **2+**: weak to moderate complete membrane staining in ≥ 10% of tumor cells, or strong complete staining in < 30%; **3+**: strong complete membrane staining in ≥ 30% of tumor cells. Scores of 0 and 1 + were considered negative, 3 + positive, and 2 + equivocal.

Statistical analysis

Statistical analyses were performed using SPSS software (version 19.0). Paired categorical variables (e.g., histological grade and IHC status within the same tumor) were compared using the McNemar–Bowker test. Continuous variables were analyzed using the paired Student's *t*-test. The association between fixation conditions and the presence of autolysis was assessed using Fisher's exact test. A p-value < 0.05 was considered statistically significant. A formal sample size and power calculation was performed using the Power and Sample Size software (Dupont & Plummer, 1990; https://cqsclinical.app.vumc.org/ps/), considering an alpha level of 0.05. The calculated sample size provided adequate statistical power (> 99%) to detect a difference of two units.

Results

Specimen characteristics

Of the 49 cases included, one was excluded due to the absence of invasive breast carcinoma (IBC) in the sample of Group 2, resulting in 48 evaluable cases. Tumor size ranged from 0.5 to 10.0 cm (median: 3.0 cm).

In Group 1 (early sectioning), the time from surgical excision to fixation ranged from 30 to 60 min (median: 44 min). In Group 2 (delayed sectioning), the interval between surgical excision and specimen sectioning in the pathology laboratory (defined as cold ischemia time) ranged from 24 to 48 h (median: 36 h).

Histomorphological findings

Histomorphological analysis (Table 2) demonstrated variable degrees of tissue alteration in both groups, including stromal retraction, loss of cellular cohesion, nuclear chromatin margination, and cell membrane disruption,

Table 1 Primary antibodies and immunohistochemical conditions

Marker	Clone/Kit	Manufacturer (City, Country)	Detection system	Antigen retrieval method	Dilution
HER2	HercepTest™	Dako, Carpinteria, USA	Polymer-based	Citrate buffer, pH 6.0	Prediluted
ER	1D5 (PharmaDx™)	Dako, Carpinteria, USA	Polymer-based	High pH buffer	Prediluted
PR	PgR 1294 (PharmaDx™)	Dako, Carpinteria, USA	Polymer-based	High pH buffer	Prediluted

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2

Table 2 Histomorphological findings in invasive breast carcinomas according to pre-analytical conditions

Variable	Category	Group 1 ($n=48$) n (%)	Group 2 ($n=48$) n (%)	p value
Autolysis-related morphological changes	Absent	42 (87.5)	32 (66.7)	**0.02**
	Present	6 (12.5)	16 (33.3)	
Histological tumor type	NST	32 (66.7)	38 (79.2)	**0.03**
	Special types	16 (33.3)	10 (20.8)	
Histological grade (Nottingham)	Grade I	9 (18.8)	15 (31.2)	0.08
	Grade II	26 (54.2)	18 (37.6)	
	Grade III	13 (27.0)	15 (31.2)	
Tubule formation	Score 1	2 (4.2)	3 (6.2)	0.10
	Score 2	16 (33.3)	21 (43.8)	
	Score 3	30 (62.5)	24 (50.0)	
Nuclear pleomorphism	Score 1	4 (8.3)	5 (10.4)	0.25
	Score 2	20 (41.7)	18 (37.5)	
	Score 3	24 (50.0)	25 (52.1)	
Mitotic count	Score 1	25 (52.1)	27 (56.2)	0.51
	Score 2	16 (33.3)	14 (29.2)	
	Score 3	7 (14.6)	7 (14.6)	

Abbreviations: NST, no special type. Notes: Group 1, early sectioning with buffered formalin fixation; Group 2, delayed sectioning with non-buffered formalin fixation. Paired comparisons were performed using the McNemar–Bowker test. Statistically significant p values are shown in bold

consistent with autolysis-related changes. These alterations were significantly more frequent in Group 2 compared to Group 1 (16/48 vs. 6/48 cases, respectively; $p=0.02$) (Fig. 1).

Autolysis-related changes according to type of surgery

The frequency of autolysis did not differ significantly according to the type of surgical procedure. In Group 1, autolysis was observed in 5/29 lumpectomy specimens and 1/20 mastectomy specimens ($p=0.379$). In Group 2, autolysis was present in 11/28 lumpectomy specimens and 5/20 mastectomy specimens ($p=0.363$).

Histological tumor type and grade

Special histological types of breast carcinoma were more frequently identified in Group 1 (16/48 cases; $p=0.03$), including mucinous (colloid), tubular, lobular, and carcinomas with medullary or apocrine features. In contrast, Group 2 showed a higher proportion of invasive breast carcinoma of no special type (NST) or mixed patterns (38/48 cases) (Fig. 2).

No statistically significant differences were observed between groups regarding histological grade, including tubule formation, nuclear pleomorphism, and mitotic count (Table 2).

Immunohistochemical findings

The results of ER, PR, and HER2 immunohistochemical analyses are summarized in Table 3. No significant differences were observed between Group 1 and Group 2 in ER and PR status. However, in one case, ER and PR expression were positive in the early-sectioned sample and negative in the corresponding delayed-sectioned sample (Fig. 3, case 22). Comparative analysis of ER and PR staining intensity and percentage of positive cells also showed no statistically significant differences between groups. The mean ER Allred score was 6.35 in Group 1 (standard deviation, SD, ± 2.72) and 6.15 in Group 2 (SD ± 2.81), with a mean difference of 0.20 (SD, ± 1.00, $p=0.192$). The mean PR Allred Score was of 5.62 in group 1 (SD ± 3.16) and 5.77 in group 2 (SD ± 3.05), with a mean difference of -0.149 (SD ± 1.41, $p=0.474$) for PR. Two cases were excluded due to the absence of residual invasive tumor in the respective tissue blocks.

A higher number of HER2-negative cases were observed in Group 2 compared to Group 1. In group 1, 28/47, HER2-negative, and 8/47 were HER2-1+. In group 2, 31/47 cases were HER2-negative, and 8 cases were HER2-1+. Equivocal (2+) HER2 expression was more frequent in Group 1 (5/47 cases) than in Group 2 (2/47 cases), although this difference did not reach statistical significance ($p=0.22$). The mean difference between HER2-categories was 0.128 ($p=0.083$).

All equivocal cases showed no gene amplification by dual color in situ hybridization (DDISH). Notably, one case demonstrated discordant HER2 results, with overexpression (3+) in Group 2 and equivocal expression (2+) in Group 1; this case showed no gene amplification by DDISH and exhibited heterogeneous staining (Fig. 4).

Discussion

In the present study, we systematically evaluated the impact of pre-analytical control on both histomorphological features and IHC biomarkers in invasive breast carcinoma. Although initial specimen handling was similar for both groups, the effective cold ischemia time differed substantially due to delayed sectioning in Group 2. Importantly, immersion in fixative does not equate to immediate fixation, as formalin penetration occurs at an approximate rate of 1 mm per hour, and fixation only begins once the fixative reaches the target tissue (Khoury, 2014). Consequently, specimens processed under delayed sectioning conditions were exposed to prolonged ischemia, contributing to the observed increase in autolysis-related changes.

These findings are consistent with prior studies demonstrating that delayed fixation compromises tissue integrity (Khoury 2012, 2018; Cree et al. 2014). Morphological alterations—such as stromal retraction, loss of cellular cohesion, and nuclear chromatin

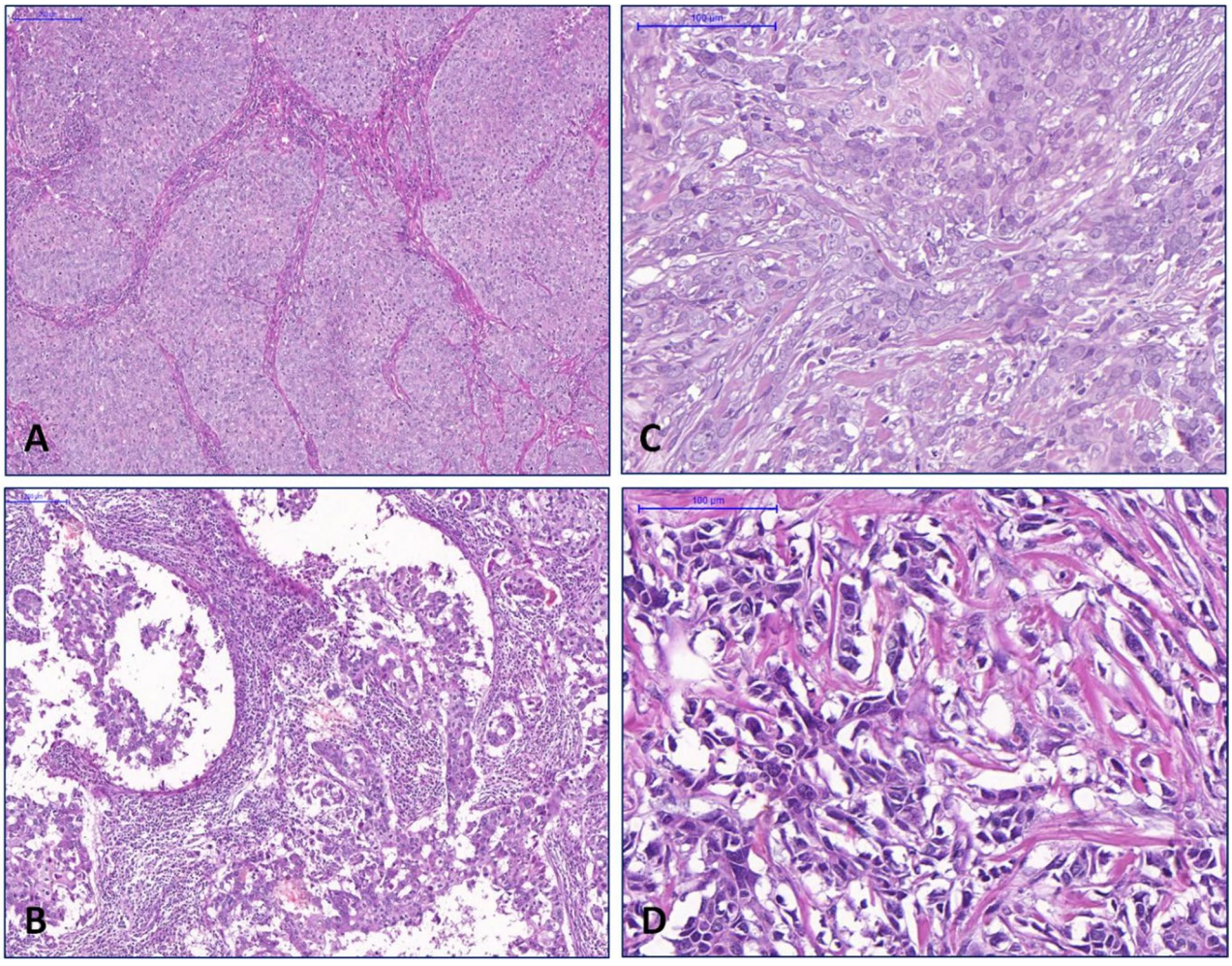

Fig. 1 Histomorphological changes in tissue samples from the same cases. Case 17: Group 1 (**A**) and Group 2 (**B**); Case 44: Group 1 (**C**) and Group 2 (**D**). Hematoxylin and eosin staining. Group 1, early sectioning with buffered formalin fixation; Group 2, delayed sectioning with non-buffered formalin fixation

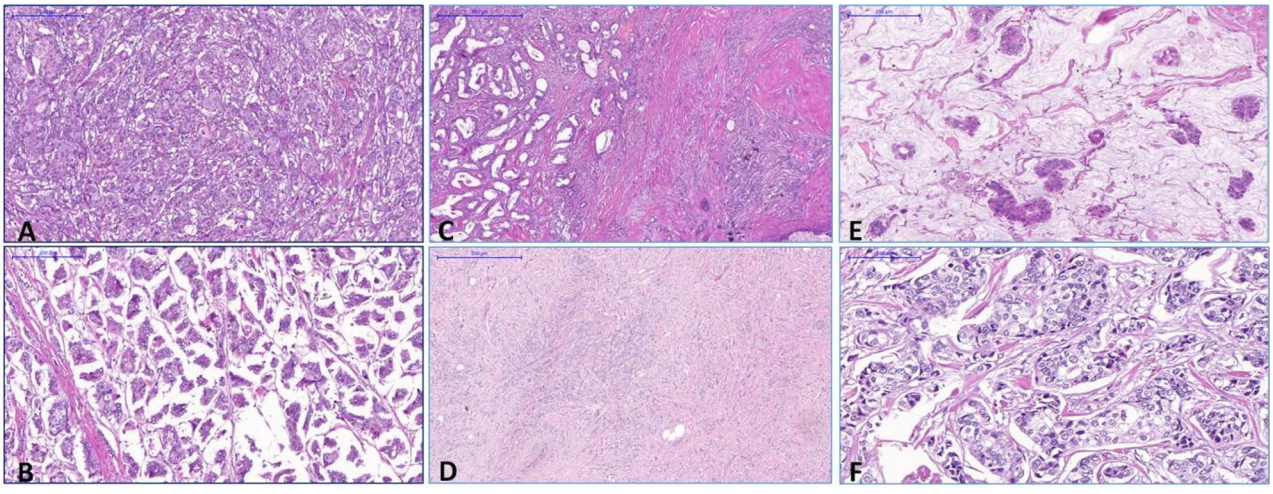

Fig. 2 Different classification in histological types in Group1 and Group 2 of case 21 (**A** and **B**), case 37 (**C** and **D**), case 48 (**E** and **F**), respectively. "H&E" staining (Hematoxylin and eosin staining. Group 1, early sectioning with buffered formalin fixation; Group 2, delayed sectioning with non-buffered formalin fixation

Table 3 Immunohistochemical biomarkers in invasive breast carcinomas according to pre-analytical conditions

Marker	Category	Group 1 *n/N* (%)	Group 2 *n/N* (%)	*p* value
HER2	0	28/47 (59.6)	31/47 (66)	0.22
	1+	8/47 (17.0)	8/47 (17.0)	
	2+ (equivocal)	5/47 (10.6)	2/47 (4.3)	
	3+	6/47 (12.8)	6/47 (12.8)	
Estrogen receptor (ER)	Negative	6/46 (13.0)	7/46 (15.2)	1.00
	Positive	40/46 (87.0)	39/46 (84.8)	
Progesterone receptor (PR)	Negative	10/47 (21.3)	9/47 (19.1)	1.00
	Positive	37/47 (78.7)	38/47 (80.9)	

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2

Notes: Group 1, early sectioning with buffered formalin fixation; Group 2, delayed sectioning with non-buffered formalin fixation. Paired comparisons were performed using the McNemar–Bowker test. Statistically significant *p* values are shown in bold

margination—were significantly more frequent in the delayed group and may hinder accurate histopathological interpretation. From a diagnostic perspective, distinguishing true stromal retraction (retraction clefts) from artifactual changes induced by autolysis is critical, as true retraction clefts have been associated with adverse prognostic features, including increased lymphovascular invasion and tumor aggressiveness (Acs et al. 2015).

We also observed a statistically significant difference in histological tumor type classification between groups, with a higher detection of special histological types in specimens early sectioned and fixed in buffered formalin. This finding likely reflects the impact of tissue preservation on the recognition of subtle morphological features. However, intratumoral heterogeneity and sampling variability may also have contributed to this difference, as previously described in comparative studies of breast core biopsies and surgical specimens (Andrade and Gobbi 2004; Arnedos et al. 2009; Robertson et al. 2019; Slostad et al. 2022). This represents an inherent limitation of the study design, as only two samples per tumor were analyzed.

Histological grading did not differ significantly between groups, suggesting that the components of the Nottingham grading system may be relatively robust to moderate pre-analytical variation. Nevertheless, caution is warranted in borderline cases, where subtle morphological degradation may influence grading reproducibility (Khoury 2012; Fitzgibbons 2019; Gobbi et al. 2022).

Regarding IHC biomarkers, ER and PR expression remained largely stable across pre-analytical conditions, in agreement with prior reports indicating relative resistance of hormone receptors to moderate fixation

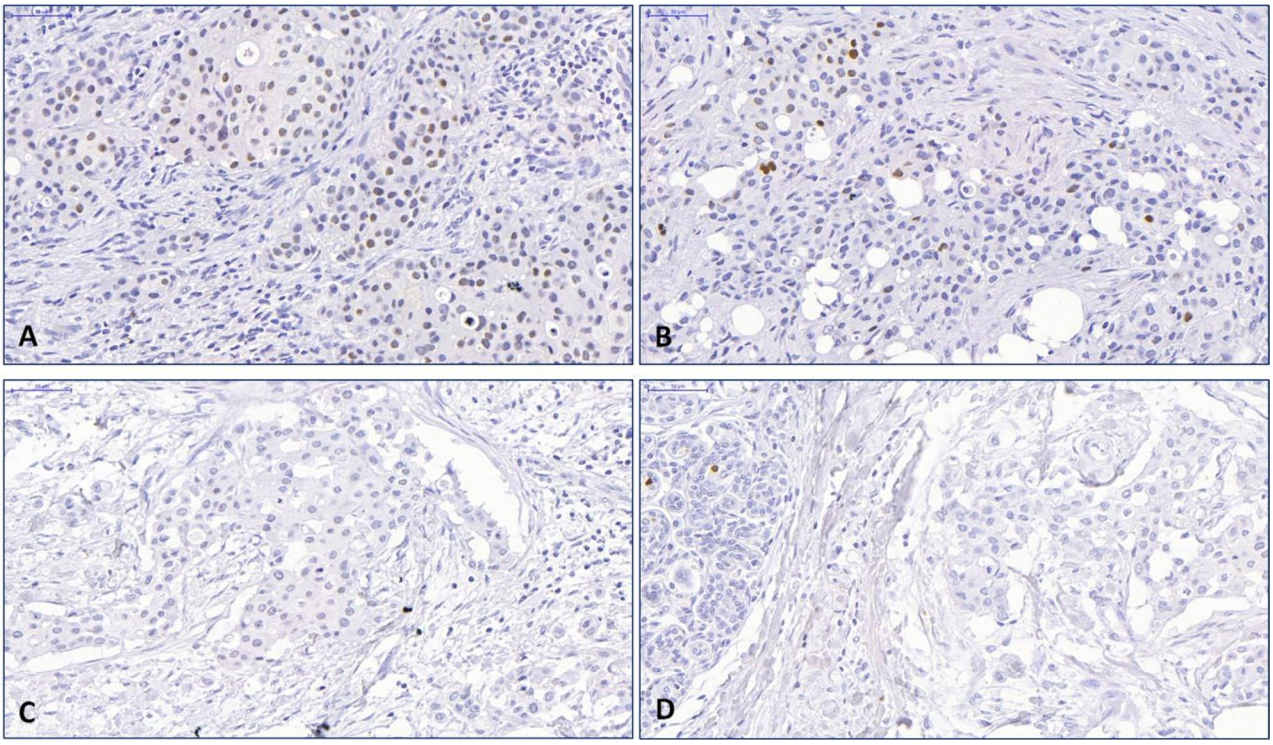

Fig. 3 ER and PR nuclear immunohistochemical staining in case 22: positive in Group 1 (**A** and **B**) and negative in Group 2 (**C** and **D**). Group 1, early sectioning with buffered formalin fixation; Group 2, delayed sectioning with non-buffered formalin fixation

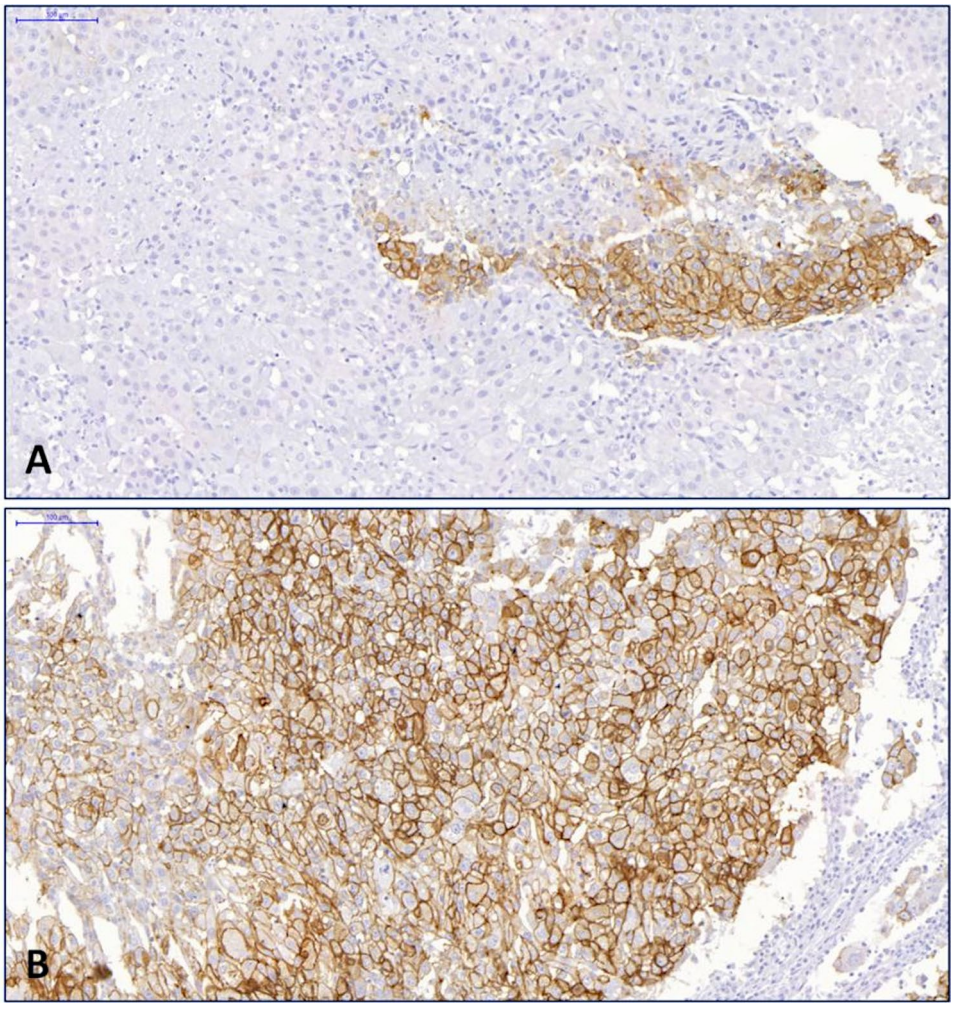

Fig. 4 HER2 membranous staining in case 16: equivocal (score 2+) in Group 1 (**A**) and positive (score 3+) in Group 2 (**B**), showing heterogeneous pattern, contrasting positive and negative areas of the same tumor sample. Group 1, early sectioning with buffered formalin fixation; Group 2, delayed sectioning with non-buffered formalin fixation

variability. It is also possible that the prior bisection of the specimen allowed some amount of formalin to penetrate the tumor and preserve the tissue proteins. However, the occurrence of a discordant ER and PR results in one case underscores that clinically relevant discrepancies may arise, even under controlled conditions. Given the typically homogeneous expression pattern of hormone receptors, it is possible that such discrepancies are unlikely to be solely attributable to sampling and may reflect pre-analytical effects on antigen preservation (Siddiqui et al. 2010; Moatamed et al, 2011; Uy et al, 2010; Tong et al. 2011; Li et al. 2013; Khoury 2018).

HER2 evaluation demonstrated greater variability, with a trend toward increased negativity and reduced equivocal results in the delayed sectioning group, although without statistical significance. A notable discordant case, showing HER2 overexpression in the delayed sample and equivocal expression in the early sample without gene amplification, highlights the combined influence of pre-analytical factors and intratumoral heterogeneity, that might affect the concordance in HER2 expression between samples from different areas of the same tumor (Yildiz-Aktas et al., 2012; Portier et al., 2013; Acs et al., 2021). HER2 is known to be particularly sensitive to fixation conditions, and variability in protein expression may occur independently of gene amplification status, reinforcing the importance of confirmatory in situ hybridization in equivocal cases (Rakha et al., 2023; Wolff et al., 2023; Allison &, Krishnamurti, 2025).

HER2 accurate stratification in low and ultra-low protein expression has become increasingly relevant with the advent of antibody–drug conjugates (Modi et al. 2022; Venetis et al. 2022). The mean difference between groups regarding HER2 IHC was 0.128 ($p = 0.083$). Although our sample had enough size to detect a change of categories between groups, the difference was smaller than a unit, unable to achieve significance. Differences between groups, if they occur, might not be sufficient to change

HER2 classification, and thus would not affect treatment decisions. Optimization of pre-analytical conditions, careful reagent selection, and pathologist training are essential to enhance test performance and reduce diagnostic variability in cases with low or ultra-low expression of HER2 (Wolff et al. 2023; Ellis et al. 2024; Gobbi et al. 2024; Joaquim et al. 2025). In the current era of antibody–drug conjugate (ADC)-based therapies, optimization of pre-analytical conditions is essential to improve the accurate assessment of different levels of HER2 expression for therapeutic decision-making.

From a mechanistic standpoint, formaldehyde fixation induces protein cross-linking through the formation of methylene bridges involving amino acid residues. While this process preserves tissue architecture, it may also mask antigenic epitopes, particularly under conditions of prolonged fixation or suboptimal pH. Buffered formalin provides a more stable environment for antigen preservation, whereas non-buffered solutions may lead to pH fluctuations that affect protein structure and antigenicity (Goldstein et al. 2007; Khoury 2012, 2018). Routine practice, particularly in low- and middle-income settings, pre-analytical conditions are often heterogeneous and difficult to control. Factors such as specimen size, tissue composition, fixative volume, and delays in transport and gross examination all contribute to variability in fixation quality (Buzaid et al. 2020; Gobbi et al. 2022).

The presence of multiple factors (including time to fixation, fixation duration, use of buffered versus unbuffered formalin, and formalin volume) makes it difficult to determine which specific variable accounts for the observed differences, representing a limitation of our study. However, these conditions reflect the reality under which many surgical specimens are routinely received in pathology laboratories in Brazil. Our findings highlight that improving pre-analytical standardization requires a multidisciplinary approach involving surgeons, operating room staff, and pathologists, as emphasized by international and national guidelines (Rakha et al. 2023; Gobbi et al. 2024; Allison & Krishnamurti, 2025). Further studies evaluating individual pre-analytical variables in a controlled manner are needed to clarify the specific contribution of each factor to the optimization of histomorphological assessment and immunohistochemistry-based molecular classification of tumors.

Conclusions

This study demonstrates that lack of pre-analytical control, is associated with a significant increase in autolysis-related morphological alterations and may affect histological tumor classification invasive of breast carcinomas. Although ER and PR immunohistochemical expression appears to be relatively preserved, HER2 assessment is more susceptible to pre-analytical variability. These findings reinforce the importance of minimizing cold ischemia time and ensuring adequate fixation in buffered formalin to optimize both morphological evaluation and biomarker reliability.

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Not applicable.

Author contributions
HG conceived the study design and supervised all tissue analysis, paper writing and reviewing. MAB was responsible for collecting the samples and perfoming the immunohistochemistry reactions. CBN held the morphological and immunohistochemistry analysis. RFA participated in the data collection and analysis. DB was responsible for the statistics. All authors contributed to the writing and reviewing of the manuscript.

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Data availability
All the data generated or analyzed during this study are included in this published article.

Declarations

Ethical approval
Federal University of Minas Gerais, (ETIC: 0297.1.203.000–10) and Federal University of Triangulo Mineiro (CAAE: 64420016.5.0000.5154).

Competing interests
The authors declare no competing interests.

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