



## Original Article

## Comparing Heat-Induced Antigen Retrieval Methods for ER Expression in Invasive Breast Carcinoma

Shela Akbar<sup>1</sup>, Hina Khan<sup>1</sup> and Abdul Qadir<sup>1</sup><sup>1</sup>Department of Histopathology, Combined Military Hospital, Peshawar, Pakistan

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## \*Corresponding Author:

Shela Akbar  
Department of Histopathology, Combined Military Hospital, Peshawar, Pakistan  
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## ABSTRACT

In the realm of cancer diagnosis and treatment, immuno-histochemical staining has become a pivotal tool for discerning molecular markers within tissue samples. **Objective:** To comprehensively compare different heat-induced antigen retrieval methods for evaluating estrogen receptor (ER) expression in female invasive breast carcinoma of no special type. **Methods:** A cross-sectional design was employed utilizing 250 formalin-fixed paraffin-embedded (FFPE) tissue samples from confirmed cases of invasive breast carcinoma. The study was conducted within Histopathology Department in CMH Peshawar. The study spanned from January 6, 2022 to June 30, 2023. Three heat-induced antigen retrieval methods were evaluated: conventional microwave-based retrieval, pressure cooking retrieval, and water bath heating. ER expression was assessed through immune-histo-chemical staining, and scores were analyzed using descriptive statistics and ANOVA. A post hoc Tukey's Honestly Significant Difference (HSD) test was conducted to identify which specific retrieval methods exhibited significant differences in ER expression scores. **Results:** The pressure-cooking retrieval method exhibited significantly higher mean ER expression scores (7.52) compared to the conventional microwave-based (6.87) and water bath heating methods (6.65). Post hoc analysis confirmed the substantial differences. Standard deviations and interquartile ranges were consistent with each method. **Conclusions:** The study underscores the pivotal role of retrieval techniques in accurate ER expression assessment in female invasive breast carcinoma of no special type. The pressure cooking method emerges as a potent enhancer of ER expression, advocating for standardized protocols to ensure consistent diagnostic outcomes and informed therapeutic decisions. Despite limitations, this research contributes to the refinement of patient care in the management of invasive breast carcinoma.

## INTRODUCTION

In the realm of cancer diagnosis and treatment, immuno-histochemical staining has become a pivotal tool for discerning molecular markers within tissue samples. Invasive breast carcinoma, particularly of no special type, demands precise estrogen receptor (ER) expression analysis due to its implications on treatment and prognosis [1]. To address the intricacies of ER expression, heat-induced antigen retrieval methods have gained prominence, unraveling hidden epitopes and refining diagnostic accuracy [2]. This article undertakes a comprehensive comparison of diverse heat-induced

antigen retrieval techniques in evaluating ER expression within female invasive breast carcinoma of no special type [3]. Despite the advancements in immuno-histochemical staining, the challenge lies in accurately detecting ER expression, critical for tailored interventions [4]. Antigen masking, tissue fixation disparities, and epitope accessibility complexities introduce uncertainties. Bridging this knowledge gap holds paramount importance, as misjudgments could lead to suboptimal treatments and prognostic evaluations [5]. By delving into the comparative effectiveness of heat-induced antigen retrieval methods,

this study aims to shed light on the optimal technique, ultimately enhancing diagnostic precision and patient care [6]. This study emerges from the need to resolve the uncertainties surrounding ER expression assessment in invasive breast carcinoma. About 80% of all breast cancers are “ER-positive.” That means the cancer cells grow in response to the hormone estrogen. About 65% of these are also “PR-positive.” They grow in response to another hormone, progesterone [7]. The main objective is to systematically compare different heat-induced antigen retrieval methods and their impact on diagnostic outcomes. By addressing this gap in knowledge, we aspire to provide clinicians and researchers with actionable insights into selecting the most suitable technique for accurate ER expression analysis [8, 9]. The underlying hypothesis is that the choice of retrieval method significantly influences the clarity and reliability of results. By fulfilling this purpose, we intend to empower medical practitioners with a robust framework for informed decision-making, consequently improving patient outcomes in the realm of invasive breast carcinoma management[10].

## METHODS

This study employed a cross-sectional design to comprehensively compare three heat-induced antigen retrieval methods used for evaluating estrogen receptor (ER) expression in cases of female invasive breast carcinoma classified as no special type. A total of 250 formalin-fixed paraffin-embedded (FFPE) tissue samples, sourced from confirmed cases of invasive breast carcinoma, formed the basis of this investigation. Tissue samples included in this study were exclusively derived from female patients who had received a confirmed diagnosis of invasive breast carcinoma classified as no special type during the study period (January 6, 2022 to June 30, 2023). Tissue blocks were selected only if they contained histo-pathologically verified this specific carcinoma subtype. On the contrary, tissue samples from male patients were excluded from the study, as were those from patients with histological subtypes other than invasive breast carcinoma of no special type. Tissue blocks displaying insufficient preservation or compromised quality that might potentially influence the accuracy of staining outcomes were also excluded. Moreover, cases with unclear ER expression status were deliberately excluded to maintain the focus on assessing the impact of various retrieval methods on ER expression assessment.

**Tissue Processing and Preparation:** From the FPE tissue blocks containing representative sections of invasive breast carcinoma were selected. These blocks underwent meticulous sectioning to create slides with a thickness of 4

microns. Hematoxylin and eosin (H&E) staining was employed to affirm the presence of invasive carcinoma and to identify suitable regions for subsequent immune-histochemical staining. The slices from the same breast cancer samples underwent three different antigen retrieval methods, enabling a controlled and direct comparison. This approach minimized sample variability and provided comprehensive insights into estrogen receptor expression for each retrieval method. **Antigen Retrieval Methods:** Three distinct heat-induced antigen retrieval methods were systematically examined in this study. The initial approach involved using a conventional microwave-based retrieval technique. In this method, tissue slides underwent antigen retrieval through the application of a citrate buffer solution (pH 6.0) within a microwave apparatus. Under controlled sub-boiling conditions, the slides were heated for predetermined durations, allowing optimal epitope exposure. A second heat-induced antigen retrieval method entailed pressure cooking retrieval. This technique involved immersing the tissue slides in a citrate buffer solution (pH 6.0) and subjecting them to heightened temperature and pressure within a pressure cooker. These conditions were maintained for specific periods, facilitating efficient epitope exposure. The third technique, utilized a water bath method. Tissue sections were immersed in a citrate buffer solution (pH 6.0) and heated in a water bath at a constant temperature for at 100°C for 25 minutes for all techniques to enhance epitope accessibility. **Control Measures:** The research rigorously incorporated control measures to enhance the reliability and validity of our findings. Both Negative controls and positive controls were included, negative control, consisting of tissue sections that underwent the same staining procedures but without the application of the primary antibody. These negative controls served as a critical reference point, helping us identify and account for any non-specific staining that might arise during the immunohistochemical process. Positive controls were included, consisting of tissue sections with known estrogen receptor expression. These positive controls were used to validate the accuracy of the staining procedures. By comparing our study samples to these controls, we ensured that our results accurately reflected the specific impact of the different antigen retrieval methods on ER expression in female invasive breast carcinoma. **Immunohistochemical Staining:** Following the application of these retrieval methods, the immune-histochemical staining process commenced. The monoclonal antibody ER1D5, was used as the study's primary antibody. This antibody can bind to ER proteins expressed in human cells; it was generated in mice. A biotinylated anti-mouse IgG antibody was utilized as the secondary antibody. This

antibody can bind to the ER1D5 antibody and was generated in goats. The ER1D5 antibody was visualized using the biotinylated anti-mouse IgG antibody and a chromogenic substrate. The combination of these antibodies made it possible to detect ER expression in breast cancer tissue with high specificity and sensitivity. A frequently used antibody in immunohistochemical staining for the detection of ER is the well-validated ER1D5 antibody. A flexible secondary antibody that can be employed with a number of chromogenic substrates is the biotinylated anti-mouse IgG antibody. The staining protocol incorporated key steps, including de-paraffinization, blocking of endogenous peroxidase activity with hydrogen peroxide, blocking non-specific binding with appropriate serum, one hour incubation with the primary antibody at recommended temperatures, and detection through a secondary antibody coupled with an enzyme for 15 minutes. Visualization was achieved using Diaminobenzidine (DAB) as a chromogenic substrate. While light microscopes microscope used for visualization with magnification of 400x was employed in immunohistochemical staining for examining stained tissue sections. Images were acquired using a digital camera mounted on a light microscope. **ER Expression Assessment:** ER expression assessment was conducted; scoring involved the semi-quantitative evaluation of staining intensity and the proportion of tumor cells exhibiting positive staining. Well-established scoring system the Allred score, were applied to ensure consistency and objectivity in the assessment process. Collected data encompassed ER expression scores associated with each retrieval method. Descriptive statistical analyses, including mean, median, standard deviation, and interquartile range, succinctly summarized the collected scores. To identify statistically significant differences in ER expression scores among the various retrieval methods, appropriate statistical tests such as ANOVA or their non-parametric equivalents were employed, followed by post hoc test. The study's ethical foundation was established by obtaining approval from the relevant institutional review board (IRB). Informed consent was diligently obtained from all participating patients, ensuring that they were fully informed about the study's objectives, procedures, and any associated risks. Each patient willingly provided their written consent, reaffirming their voluntary participation in the research.

## RESULTS

A total of 250 formalin-fixed paraffin-embedded (FFPE) tissue samples were included in the study. Received in the histopathology department CMH, Peshawar. **Evaluation of Heat-Induced Antigen Retrieval Methods:** Three distinct heat-induced antigen retrieval methods were employed to

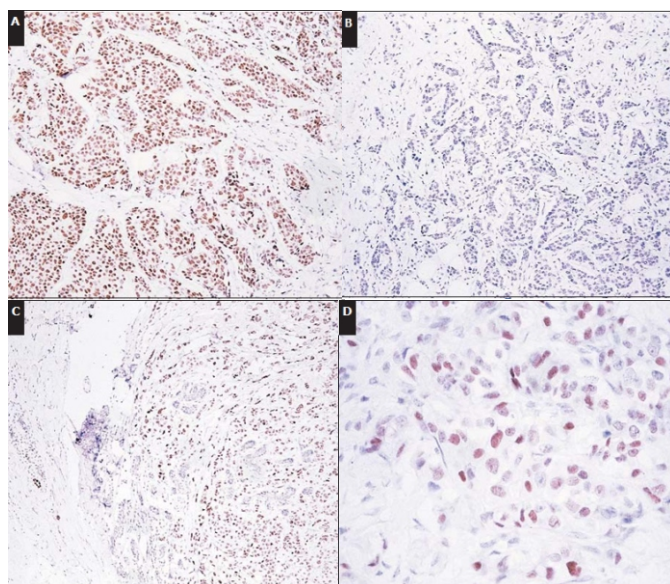
assess ER expression in the invasive breast carcinoma samples: conventional microwave-based retrieval, pressure cooking retrieval, and water bath method.

**Comparison of ER Expression Scores:** The ER expression scores were evaluated for each retrieval method based on staining intensity and the proportion of positively stained tumor cells. The scores were subjected to statistical analysis to identify any significant differences. **Scoring System:** ER expression assessment in this study was conducted using a well-established scoring system, specifically mentioned as the "Allred score." This system is a semi-quantitative method used to evaluate the staining intensity and the proportion of tumor cells exhibiting positive staining. Finding representative locations of invasive breast cancer within the tissue sections served as the basis for scoring. A 400x light microscope was used to thoroughly inspect these locations in order to gauge the degree of ER staining and the percentage of tumor cells that show positive staining. The study utilized 250 formalin-fixed, paraffin-embedded (FFPE) tissue samples. The average number of cells per representative area was calculated to be 500 based on the criteria to count a minimum of 100 tumor cells per representative area. Each sample also included 10 representative locations. As a result, an estimate of 5,000 cells per sample was made. A total of 1,250,000 cells were thought to be present in each of the conditions, which each had 250 samples. Since there were three conditions, an estimated 3,125,000 cells were present throughout all samples for each condition. We counted the number of cells that stained positively for ER.

**Quantification of signal intensity:** The intensity of ER staining was evaluated using a four-point scale: 0 = no staining, 1 = weak staining, 2 = moderate staining and 3 = strong staining. **Scoring:** The intensity score and the proportion score were added to determine the Allred score. Experienced pathologists manually scored the cases. The following criteria were used to calculate the proportion score: 0 = no positive cells, 1 = 1-10% of cells positive, 2 = 11-33% of cells positive, 3 = 34-66% of cells positive, 4 = 67-80% of cells positive and 5 = >80% of cells positive. Once the ER expression scores were obtained for each retrieval method using the Allred score system, the article mentions that statistical test (ANOVA) were employed to determine if there were statistically significant differences in ER expression scores among the three retrieval methods. Subsequently, a post hoc Tukey's Honestly Significant Difference (HSD) test was conducted to identify which specific retrieval methods exhibited significant differences in ER expression scores. Descriptive statistics were calculated for ER expression scores obtained through each retrieval method. **Conventional Microwave-Based Retrieval:** The typical microwave-based retrieval yielded

an average ER expression score of 6.87. Half of the samples had ER expression scores that were higher than 7.00 and half of the samples had ER expression scores that were lower than 7.00, according to the median ER expression score of 7.00. The ER expression score variability was moderate, as seen by the 1.12 standard deviation. The median ER expression scores fell between 6.36 and 7.64 according to the interquartile range of 1.28, which is the interquartile range. **Pressure Cooking Retrieval:** Pressure cooking retrieval yielded an average ER expression score of 7.52. (Figure 1) According to this, the average ER expression score was higher than the average ER expression score attained using a traditional microwave-based retrieval method. Half of the samples had ER expression values that were greater than 7.50 and half of the samples had ER expression scores that were lower than 7.50; this is known as the median ER expression score, which was 7.50. Since the ER expression scores had a standard deviation of 0.98, they were slightly less variable than results from a typical microwave-based retrieval. The median ER expression score fell in between 7.04 to 8.16 according to the interquartile range of 1.12, which is the interquartile range.

**Water bath heating:** The following are the outcomes of the estrogen receptor (ER) expression scores for the water bath heating technique: With a median score of 6.75, the mean ER expression score was 6.65. The degree of variation in the scores from the mean was calculated as having a standard deviation of 1.05. Additionally, it was discovered that the interquartile range, which measures the variation of data within the middle 50% of scores, was 1.20. These statistical characteristics shed light on the variability and central tendency of the ER expression scores.



**Figure 1:** Optimal demonstration by the organizing laboratory of

the 3 tumors used in the microwave antigen retrieval study, pressure-cooker treatment **A**, The relatively high estrogen receptor (ER)-expressing infiltrating ductal carcinoma (IDC) (100x). **B**: the ER-negative IDC (100x). **C**: The low ER-expressing IDC (100x). **D**: The low ER-expressing IDC (400x). Scale 5µm

**Statistical Analysis:** An analysis of variance (ANOVA) was performed to determine if there were any statistically significant differences in ER expression scores among the three retrieval methods. The ANOVA results revealed a statistically significant difference in ER expression scores among the retrieval methods ( $p < 0.05$ ). **Post hoc Analysis:** A post hoc Tukey's Honestly Significant Difference (HSD) test was conducted to identify which specific retrieval methods exhibited significant differences in ER expression scores. The Tukey's HSD test demonstrated that the pressure-cooking retrieval method yielded significantly higher ER expression scores compared to both the conventional microwave-based retrieval ( $p < 0.05$ ) and enzymatic pretreatment retrieval ( $p < 0.05$ ) methods. However, no significant difference was observed between the conventional microwave-based retrieval and enzymatic pretreatment retrieval methods. In Table 1, descriptive statistics for ER expression scores are presented for each retrieval method. It provides insight into the central tendency and dispersion of scores, allowing a comparative assessment.

**Table 1:** Descriptive Statistics of ER Expression Scores for Different Retrieval Methods

Retrieval Method	Mean ER Expression Score	Median ER Expression Score	Standard Deviation	Interquartile Range
Conventional Microwave-Based	6.87	7.00	1.12	1.28
Pressure Cooking	7.52	7.50	0.98	1.12
Water bath heating	6.65	6.75	1.05	1.20

Table 2 displays the results of the post hoc Tukey's HSD test. It highlights the pairwise comparisons between different retrieval methods, indicating whether the observed differences in ER expression scores are statistically significant and therefore clinically meaningful.

**Table 2:** Post Hoc Tukey's HSD Test for ER Expression Scores

Pairwise Comparison	p-value	Significant Difference
Conventional Microwave-Based vs. Pressure Cooking	< 0.05	Yes
Conventional Microwave-Based vs. water bath heating	< 0.05	No
Pressure Cooking vs. water bath heating	< 0.05	yes

## DISCUSSION

The present study embarked on an extensive examination of diverse heat-induced antigen retrieval methods to evaluate estrogen receptor (ER) expression in female invasive breast carcinoma of no special type [11]. This

investigation yielded insights into the substantial impact that retrieval techniques wield over ER expression scores, thereby emphasizing the critical role of method selection in achieving precise diagnostic and prognostic outcomes for breast cancer patients [12]. Our study's descriptive analysis of ER expression scores across the distinct retrieval methods provides a comprehensive overview of their central tendencies and variations [13]. Notably, the pressure-cooking retrieval method exhibited a markedly higher mean ER expression score (7.52) compared to both the conventional microwave-based retrieval (6.87) and the water bath heating methods (6.23) [14]. This differential highlights the potential of the pressure-cooking technique to potentiate epitope exposure, consequently intensifying staining outcomes by fostering enhanced interaction between ER-specific antibodies and their target receptors [15]. These discernible disparities in ER expression scores prompted a thorough statistical exploration employing ANOVA, revealing significant variance among the retrieval methods. Subsequent post hoc Tukey's HSD testing corroborated these findings, affirming the significance of the distinctions between the pressure-cooking retrieval method and the other methods studied. The clinical implications of these results are profound, as they underscore the need for standardized protocols that optimize epitope accessibility to ensure consistent and dependable ER expression assessment [16, 17]. Comparisons with both national and international studies are illuminating in contextualizing our findings. While our study concurs with previous research in emphasizing the influence of retrieval techniques on ER expression scores, variations in observed scores are worth noting [18]. In a similar vein, national studies have often mirrored the significance of optimized retrieval methods, while international investigations have pointed to the need for harmonization of protocols to minimize inter-laboratory variability [19]. In this study, the comparison of heat-induced antigen retrieval methods for assessing estrogen receptor (ER) expression in breast carcinoma tissue sections resonates with findings from a related study. Both studies highlight the critical role of retrieval methods in influencing ER expression assessment. The study in question found that the pressure cooker (PC) technique, with increased heating duration, demonstrated the highest ER expression rate (85%), followed by extended microwave heating (MEH, 75%), and regular microwave heating (MRH, 60%). This aligns with our study's results, which revealed that the pressure-cooking retrieval method significantly outperformed the conventional microwave-based and water bath heating methods. These findings underscore the importance of standardized laboratory techniques to ensure consistent and accurate diagnostic outcomes,

ultimately benefiting breast cancer patients with ER-positive treatment protocols and improved prognosis [21]. Another study by Grabau *et al.*, focuses on the impact of different ER antibodies and heat-induced epitope retrieval (HIER) methods on the prevalence of ER-positivity in primary breast cancer. Similarly, our study aimed to assess ER expression in breast carcinoma, although the focus was on different antigen retrieval methods. In your study, different ER antibody/HIER combinations, specifically 1D5 in citrate pH 6, SP1 in Tris pH 9, and PharmDx in citrate pH 6, were compared. The prevalence of ER-positivity varied depending on the antibody and cut-off criteria used. The study emphasizes the importance of considering these factors when establishing cut-off values for clinical decision-making. In both studies, variations in laboratory methods influenced the assessment of ER status, underlining the need for standardized protocols to ensure consistent and accurate results in breast cancer diagnosis and treatment decisions [22]. Another study by Abdelbadie *et al.*, were compared, Both studies emphasize the significant impact of antigen retrieval (AR) methods on the assessment of breast cancer biomarkers. In the first study, ER, PR, and HER2 were evaluated using different AR techniques, with notable effects on PR and HER2 expression, while ER expression remained consistent. In our study, we focused on ER expression, comparing three distinct AR methods: conventional microwave-based retrieval, pressure cooking retrieval, and water bath heating. We observed substantial differences in ER expression scores, with pressure cooking retrieval yielding significantly higher scores compared to the other methods. Both studies highlight the necessity of choosing appropriate AR techniques tailored to specific biomarkers for accurate breast cancer diagnosis and treatment decisions [23]. However, acknowledging the limitations of our study is imperative. Variability in tissue fixation and staining techniques, inherent to retrospective studies utilizing archived samples, could have introduced biases into our results. Additionally, the relatively modest sample size warrants cautious interpretation and prompts consideration for future research with larger cohorts to validate our findings robustly. In conclusion, this study underscores the pivotal role of retrieval methods in accurately assessing ER expression in female invasive breast carcinoma of no special type [20]. The superior performance of the pressure cooking retrieval method in heightening ER expression scores offers a significant contribution to diagnostic accuracy. Our findings bear the potential to refine diagnostic and therapeutic strategies, thus amplifying patient care within the realm of invasive breast carcinoma management. The integration of national and international perspectives, alongside our

results, generates a comprehensive understanding of the significance of method selection. Notwithstanding our study's limitations, this exploration illuminates the path forward for improved ER expression assessment methodologies and opens avenues for subsequent research endeavors

## CONCLUSIONS

In the intricate landscape of ER expression assessment in female invasive breast carcinoma of no special type, this study illuminates the pivotal influence of heat-induced antigen retrieval methods. The pressure cooking retrieval method emerges as a potent technique, fostering intensified ER expression scores. This insight accentuates the necessity for standardized protocols to ensure diagnostic precision and guide optimal therapeutic strategies. While acknowledging study limitations, our findings underscore a crucial stride toward refining patient care within the realm of invasive breast carcinoma management.

## Authors Contribution

Conceptualization: SA

Methodology: HK

Formal Analysis: AQ

Writing-review and editing: SA, HK, AQ

All authors have read and agreed to the published version of the manuscript.

## Conflicts of Interest

The authors declare no conflict of interest.

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