CME/SAM

The Effects of Under 6 Hours of Formalin Fixation on Hormone Receptor and HER2 Expression in Invasive Breast Cancer

A Systematic Review

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Key Words: Fixation time; Estrogen receptor; Progesterone receptor; HER2; Immunohistochemistry; Breast cancer

Am J Clin Pathol July 2014;142:16-22

DOI: 10.1309/AJCP96YDQSTYBXWU

ABSTRACT

Objectives: *A systematic review of the literature was performed to identify whether minimum formalin fixation time may be reduced for reliable immunohistochemical assessment of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2).*

Methods: *PubMed, EMBASE, and the Cochrane Library were systematically searched for studies addressing effects of brief tissue fixation (<6 hours) on the analysis of ER, PR, or HER2 expression in patients with breast cancer.*

Results: *Five publications reported effects of brief fixation on ER, PR, or HER2 expression. Four studies showed similar receptor expression of short fixation compared with recommended fixation time (6-72 hours). One publication found that a minimum fixation time of 6 to 8 hours is necessary for reliable ER results.*

Conclusions: *Available data on the effect of brief fixation on receptor status are limited. However, brief fixation of very highly expressing breast cancers does not seem to alter ER, PR, and HER2 status. Nevertheless, scoring inconsistencies have been observed. Further research is required in larger study populations with more low-expressing cases for future validation.*

Upon completion of this activity you will be able to:

- cite the evidence in the literature regarding effects of under 6 hours of formalin fixation and its impact on reliability of hormone receptor and HER2 expression analysis in invasive breast cancer.
- weigh the findings presented in the literature by number and type of samples used to assess their generalizability to clinical practice.
- name other preanalytical variables that may potentially affect immunohistochemical analysis of receptor expression.
- discuss the implications for clinical practice if currently recommended minimum fixation time for breast cancer samples were revised.

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The authors of this article and the planning committee members and staff have no relevant financial relationships with commercial interests to disclose.

Questions appear on p 133. Exam is located at www.ascp.org/ajcpcme.

Accurate assessment of hormone receptor expression in patients with breast cancer is essential because results determine whether a patient is assigned a specific systemic treatment.[1,2] According to the guideline recommendations of the American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP), breast tissue specimens must be fixed in 10% neutral-buffered formalin (NBF) for a minimum of 6 hours to ensure reliable results for estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression.[1] The ASCO/CAP guideline cites one original paper to back up this recommendation.[3] Recently, the demand for rapid tissue diagnostics in oncology has increased, assuming that shorter waiting times reduce anxiety.[4] Lang et al[5] found highly abnormal patterns

of cortisol secretion in women undergoing large-core breast biopsies when the diagnosis was not reported to the patient on the fifth day after the biopsy. Minimizing the interval between a diagnostic test and reporting of results to the patient may decrease the duration of both psychological and biochemical stress.[6] Same-day diagnosis requires faster tissue processing, in which fixation times are shorter than the recommended minimum of 6 hours. Evidence for the safe reduction of fixation duration with reliable ER, PR, and HER2 assessment may pave the road for same-day tissue diagnosis for patients with suspicious breast lesions.[7]

This study provides an overview of the available evidence on the effect of brief fixation in formalin (<6 hours) on the immunohistochemical analysis of ER, PR, and HER2 expression in invasive breast cancer compared with routine fixation duration (6-72 hours).

Materials and Methods

This study was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement.[8] The main outcomes of this study were ER, PR, and HER2 expression scores of briefly fixed breast cancer specimens (<6 hours) compared with routine fixation (6-72 hours).

Search and Study Selection

A systematic review of the literature was performed using PubMed, EMBASE, and the Cochrane Library up to June 2013. The search terms used were fixation time, ER, PR, and HER2 with their relevant synonyms **Table 1**. Articles were included if they compared immunohistochemistry results of brief fixation (<6 hours) in 10% NBF with recommended fixation duration (6-72 hours) in (tissue samples of) patients with invasive breast cancer. Publications comparing routine fixation with prolonged fixation (>72 hours) were excluded, as were studies assessing cytologic samples. Only publications written in English and Dutch were included. Other exclusion criteria included review articles, abstracts only, letters to the editor, and editorial papers. Two independent reviewers selected studies for inclusion in the review. Reference lists were manually cross checked to ensure that no studies were missed in the initial search.

Data Extraction and Statistical Analysis

Data extraction and evaluation of study quality was independently performed by two reviewers. Data on patient characteristics, tumor morphology, materials, methods, and receptor expression were extracted at different intervals from paired samples. Study quality was evaluated with a modified version of the QUADAS tool.[9] We compared the data extraction and quality scores by the two reviewers, and discrepancies were resolved by consensus **Table 2**. All extracted data were tabulated. Data were not pooled because of the heterogeneity of the reported outcome measures and the small number of studies.

Results

The literature search resulted in 2,010 records and after elimination of 556 duplicates, 1,454 studies remained **Figure 1**. Nine articles focused on the effect of fixation time on the immunohistochemical analysis of ER, PR, or HER2 expression in invasive breast cancer and were eligible for full text screening. Of these, four articles were excluded; one article did not clearly describe fixation times,[10] two articles evaluated prolonged fixation times,[11,12] and one study only described cytology results.[13] Five publications were found suitable

Table 1
Search Strategy Performed on June 3, 2013

Database	Search[a]	Hits
PubMed (MEDLINE)	((((((((((fixation[Title/Abstract]) OR fixative[Title/Abstract]) OR fixatives[Title/Abstract]) OR fix[Title/Abstract]) OR fixed[Title/Abstract]) OR fixated[Title/Abstract])) AND (((((((duration[Title/Abstract]) OR length[Title/Abstract]) OR time[Title/Abstract]) OR quick[Title/Abstract]) OR rapid[Title/Abstract]) OR fast[Title/Abstract]) OR ultrafast[Title/Abstract]))) AND ((((((oestrogen receptor[Title/Abstract]) OR estrogen receptor[Title/Abstract]) OR ER[Title/Abstract])) OR ((((progesteron receptor[Title/Abstract]) OR progesterone receptor[Title/Abstract]) OR PR[Title/Abstract]) OR NR3C3[Title/Abstract])) OR ((((((((((((((((((hormone receptor[Title/Abstract]) OR erbb2[Title/Abstract]) OR erbb-2[Title/Abstract]) OR erb-2b[Title/Abstract]) OR erb2b[Title/Abstract]) OR her2neu[Title/Abstract]) OR her2/neu[Title/Abstract]) OR her2[Title/Abstract]) OR her-2[Title/Abstract]) OR her2-neu[Title/Abstract]) OR her-2/neu[Title/Abstract]) OR neu[Title/Abstract]) OR p-185[Title/Abstract]) OR p185[Title/Abstract]) OR cerbb2[Title/Abstract]) OR c-erbb2[Title/Abstract]) OR c-erb-b2[Title/Abstract]) OR erb-b2[Title/Abstract]))	574
EMBASE	See PubMed	923
The Cochrane Library	See PubMed	513

[a] Search terms were used in title and abstract fields only.

DOI: 10.1309/AJCP96YDQSTYBXWU

for inclusion and critical appraisal.[3,14-17] Cross-referencing revealed no articles missed by the initial search.

Study Characteristics

The studies included were published between 2003 and 2011 **Table 3**. One publication reported results from two distinct original studies,[3] which led to a review of six studies.

Five studies were prospective and were performed on surgical specimens.[3,14-17] One study was retrospective and data were retrieved from core-needle biopsies (CNB), of which receptor expression results were discordant with those of the corresponding surgical specimen.[3] Three publications assessed ER expression,[3,14,15] one PR expression,[14] and two HER2 expression.[16,17] Percentage and intensity of cells stained for ER[14,15] according to the ASCO/CAP guidelines[1] was evaluated in two studies, whereas one study used the Q-score method.[3] This method incorporates intensity and distribution of reactivity within a range of 0 to 7.[18] The study

Table 2
Study Quality Assessment[a]

Authors	Was the Spectrum of Patients Representative of Clinical Practice?	Were Selection Criteria Clearly Described?	Did the Whole Sample Receive Verification by the Same Reference Standard, ie, IHC After Routine Fixation (6-72 h)?	Was the Execution of IHC Analysis of the Briefly Fixed Specimen Described in Sufficient Detail to Permit Replication of the Analysis?	Was the Execution of the Reference Standard Described in Sufficient Detail to Permit its Replication?	Were Results of Brief Fixation Interpreted Without Knowledge of the Results of the Reference Standard?	Were the Reference Standard Results Interpreted Without Knowledge of the Results of Rapid Fixation?	Were Uninterpretable/ Intermediate Test Results Reported?
Apple et al, 2011[14]	N	Y	Y	Y	Y	Y	Y	Y
Moatamed et al, 2011[17]	N	Y	Y	Y	Y	Y	Y	Y
Goldstein et al, 2003[3,b]	N	Y	Y	Y	Y	U	U	N
Goldstein et al, 2003[3,c]	Y	Y	U	Y	Y	U	U	N
Ibarra et al, 2010[15]	N	N	Y	Y	Y	U	U	Y
Ibarra et al, 2010[16]	N	N	Y	Y	Y	U	U	N

IHC, immunohistochemistry; N, no; U, unclear; Y, yes.
[a] Study quality was assessed according to the modified QUADAS tool.[9]
[b] Study part 1.
[c] Study part 2.

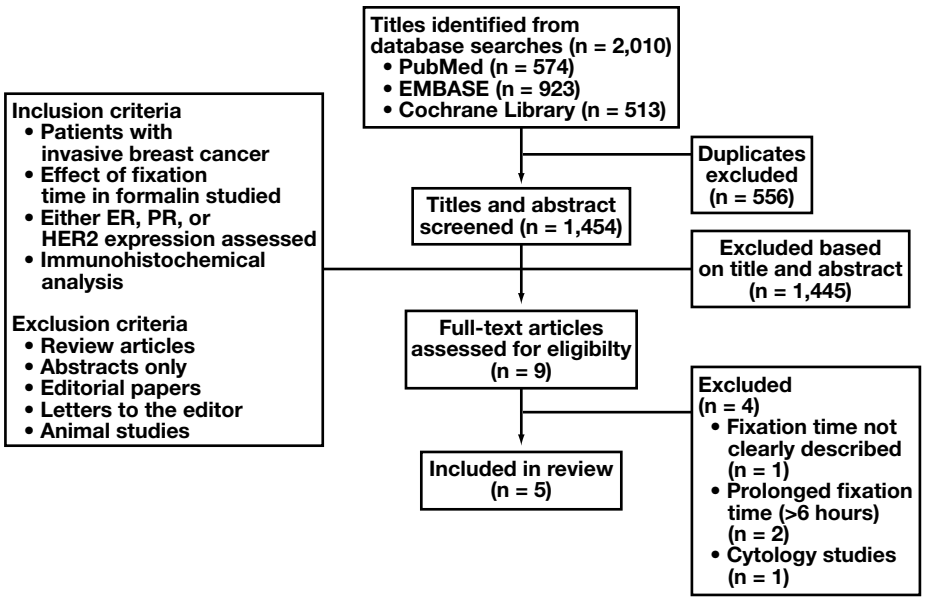

Figure 1 Selection of articles for review. ER, estrogen receptor; PR, progesterone receptor.

DOI: 10.1309/AJCP96YDQSTYBXWU

Table 3
Procedural Data and Outcomes

Authors	Receptor	Design	Specimen Type	Antibody	Fixation Time in 10% NBF	Outcome Measures	Results
Ibarra et al, 2010[15]	ER	Prospective (n = 10)	Resection material ER positive tumors (all >90%) Three 4 × 4 × 2 mm specimens per tumor per time interval studied	Anti-ER monoclonal antibodies: clone SP1 (rabbit), 1D5 and 6F11 (both mouse)	1, 3, 6, and 9-10 h	Percentage and intensity of ER staining[1]	No significant staining difference between varying fixation times
Ibarra et al, 2010[16]	HER2	Prospective (n = 10)	Resection material HER2 positive tumors (all 3+) One 3 mm specimen per tumor per time interval studied	Anti-HER2 monoclonal antibody, clone 4B5 (rabbit)	3, 48, 72, 96, and 120 h	DAKO scoring system[19]	No significant staining difference between varying fixation times
Apple et al, 2011[14]	ER, PR	Prospective (n = 1)	Resection material ER and PR positive tumor (both >90%) One 5-15 mm × 2 mm specimen per time interval studied	Anti-ER monoclonal antibody, clone 6F11 (mouse) and anti-PR monoclonal antibody, clone 636 (mouse)	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 24, 48, 72, and 168 h	Percentage and intensity of ER and PR staining[1]	No significant staining difference between varying fixation times
Moatamed et al, 2011[17]	HER2	Prospective (n = 1)	Resection material HER2 positive tumor (3+) One 5-15 mm × 2 mm specimen per interval studied	HercepTest (DAKO A0485 polyclonal antibody kit)	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 24, 48, 72, and 168 h	DAKO scoring system[19]	The specimen fixed for 1 hour showed weaker and partial membrane staining (1+) compared with the other fixation times (2+/3+)
Goldstein et al, 2003[3,a]	ER	Prospective (n = 24)	Resection material ER-positive tumors (all >90%) One specimen per tumor per interval studied	Anti-ER monoclonal antibody, clone 1D5 (mouse)	3, 6, 8, and 12 h and 1, 2, and 7 d	Q-score method[18]	Mean Q-score for ER status was 2.46 for blocks fixed for 3 h, 5.75 for blocks fixed for 6 h, and 6.70 for blocks fixed for 8 h ($P < .01$)
Goldstein et al, 2003[3,b]	ER	Retrospective (n = 45)	9 ER-negative CNB and ER-positive resection specimen Control group of 36 randomly selected CNB with ER similar results in the resection specimen (33% ER negative, 67% ER positive)	Anti-ER monoclonal antibody, clone 1D5 (mouse)	Cases between July 1999 and December 2001 were retrieved from archives and statistical analyses on approximate fixation times were performed	Mean fixation time of study groups	Mean fixation times for ER-disparate results and ER-similar results were 1.2 and 6.3 h, respectively ($P = .01$).

CNB, core needle biopsy; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; NBF, neutral-buffered formalin; PR, progesterone receptor.
[a] Study part 1.
[b] Study part 2.

DOI: 10.1309/AJCP96YDQSTYBXWU

on PR expression scored percentage and intensity of stained cells.[14] Both HER2 studies[16,17] used the DAKO scoring system as 0, 1+, 2+, and 3+ according to standardized criteria, considering 3+ cases as positive.[19]

Study Quality

Five of six studies included solely (strongly) positive cases (Table 3). Therefore, the spectrum of patients analyzed was not representative for clinical practice. Only two publications clearly described that observers were blinded to the results of routine fixation duration (6-72 hours). In all publications, all tumor samples were immunohistochemically stained after brief fixation and routine fixation duration. Statistical analysis was performed in two studies. One study did not mention the exact size of samples studied.

Study Results

The study by Ibarra et al[15] on ER expression analyzed immunohistochemistry results using different fixation times for surgical specimens of 10 patients with invasive breast cancer. From all 10 (strongly ER+) tumors, 12 small pieces were cut and subsequently placed in 10% NBF for different periods (range, 1-10 hours) before staining. No significant staining difference was found between varying fixation times for different samples from the same tumor (Table 3).

Apple et al[14] compared ER and PR results at different fixation times in samples from the surgical specimen of one patient with invasive breast cancer (ER+, PR+). From this specimen, 16 CNB-sized pieces were fixed in 10% NBF for different periods (range, 1-168 hours) before staining. No differences in both ER and PR expression were observed between samples fixed during different periods.

In part 1 of the publication by Goldstein et al[3] surgical specimens of 24 patients with (strongly ER-positive) invasive breast cancer were stained for ER after varying fixation times (range, 3 hours-7 days). Mean Q-score for ER status was 2.46 for blocks fixed for 3 hours, 5.75 for blocks fixed for 6 hours, and 6.70 for blocks fixed for 8 hours ($P < .01$). In part 2 of the same publication by Goldstein et al[3] a study is described that identifies the minimum time necessary for reliable immunohistochemical assessment of ER expression. In that study, CNB of nine patients with disparate results (ER– CNB and ER+ resection specimens) were identified retrospectively. Fixation times of the CNB with disparate results were compared with those of 36 randomly selected CNB with similar ER results. The means for ER-disparate results and ER-similar results were 1.2 and 6.3 hours, respectively ($P = .01$).

Ibarra et al[16] assessed HER2 expression, and obtained the surgical specimens of 10 patients with (HER2+) invasive breast cancer. From all 10 tumors, five small pieces were cut and subsequently placed in 10% NBF for different periods (range, 3-120 hours) before staining. Results showed no staining difference between samples fixed for 3 hours and those fixed for 6 hours or more.

Moatamed et al[17] fixed 17 samples taken from the same (HER2+) breast tumor for varying periods (range, 0-168 hours) and found a reduction of HER2 expression (ie, from 3+ to 1+) at 1-hour fixation. The other 16 samples showed no significant differences in HER2 expression compared with the clinical specimen (2+ or 3+).

Discussion

This systematic review evaluated the literature to identify whether minimum fixation time in 10% NBF may be reduced to less than 6 hours for reliable immunohistochemical assessment of ER, PR, and HER2 expression in invasive breast cancer. Most of our results showed no evident negative effects of brief fixation on receptor status in high-expressing cases. However, with the evidence currently available it is hard to provide a definite answer on whether short fixation is truly reliable.

Data on brief fixation and receptor status analysis were sparse. Only five publications met the predefined inclusion and exclusion criteria and were included for review. The studies consisted of experimental studies in very small series, often analyzing multiple biopsy findings from one resection specimen. One study reported a significant staining difference for ER between fixation at 3 hours and at 6 or more hours in a relatively high number of cases (n = 24).[3] However, this study did not report the exact size of the tissue samples studied. Larger specimen size may have accounted for the staining differences reported at shorter fixation times.

Regarding the diffusion of formalin into tissue, the literature often makes mention of a general diffusion rate of 1 mm/hour. However, original studies have shown that the diffusion rate of fixatives is a quadratic function rather than a linear one.[20] In this function, the penetrated depth in millimeters is equal to the square root of the fixation time in hours, multiplied by a coefficient of diffusibility (K) for that fixative.[20,21] Early studies evaluating the diffusion rate of formalin reported widely varying K values (0.55-5.5) depending on the methods used.[20-24] The wide range of K values reported makes it difficult to assess the minimum time required for complete formalin penetration of a certain tissue sample. Moreover, sufficient formalin penetration does not imply that the actual chemical reaction of fixation has taken place. The process of cross-linking is thought to affect epitope recognition by antibodies, yet little is known about the time required to complete this chemical reaction. In recommendation 4, the Consensus Recommendations on Estrogen Receptor Testing in Breast Cancer by Immunohistochemistry[25] state that a minimum of 25 hours is required for complete fixation of a 4-mm tissue sample.

DOI: 10.1309/AJCP96YDQSTYBXWU

This recommendation is based on findings by Helander,[26,27] who determined the amount of bound ^{14}C-formaldehyde in animal tissues. Even less is known about potential loss of antigenicity of epitopes because of insufficient cross-linking. As the evidence for theoretical minimum fixation time comes from animal models and is scarce, a more pragmatic and targeted approach may provide answers. All studies included for review examined the empirical evidence for immunoreactivity of specific markers (ER, PR, or HER2) in human breast cancer tissue. Ibarra et al[15] suggested that immunohistochemistry results may be influenced to a greater extent by total time of formalin immersion than by the theoretical time required to complete chemical fixation.

In addition, five of six studies analyzed solely (strongly) positive cases (Table 3). This may have masked the effect of brief fixation on false-negative outcomes of low-expressing cases (eg, 5% cells positive). Reliable conclusions for HER2 expression require the inclusion of intermediate levels of receptor expression.[17] As hormone receptor and HER2 assessment on biopsies determines treatment options, false-negative outcome may have great consequences for a patient's survival. Therefore, accurate testing is essential for the optimal treatment of these patients. We suggest that future studies include both negative and positive cases as well as weakly positive cases.

Related to this, data on true biopsies (as opposed to processed mastectomies) are scarce, and within the framework of same-day diagnostics of breast lesions that is based on CNB, further studies are clearly warranted. Therefore, we recommend an extensive comparative study between briefly fixed CNB and conventionally fixed resection specimens, because this would most accurately reflect clinical practice.

We emphasize that study results cannot simply be extrapolated from one antibody across all antibodies. It is possible that other markers for breast cancer may also be affected by brief formalin fixation. Future studies targeting specific biomarkers will need to address this issue.

In conclusion, data on the effects of brief fixation (<6 hours) on receptor status are scarce. The available evidence suggests that brief fixation of very highly expressing breast cancers does not significantly alter ER, PR, and HER2 status. However, data exist that demonstrate that receptor levels may be altered in some cancers. No data were found on low-expressing breast cancer tissue. Given the high importance of accurate receptor testing, the value of reducing patient anxiety must be balanced against the possibility of obtaining incorrect results that could alter a patient's treatment and survival. For more definite answers regarding the reliability of immunohistochemistry on briefly fixed breast cancer specimens, larger study populations with more low-expressing breast cancers will need to be assessed.

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DOI: 10.1309/AJCP96YDQSTYBXWU