Evidence-Based Series 22-1- IN REVIEW

A Quality Initiative of the
Program in Evidence-Based Care (PEBC), Cancer Care Ontario (CCO)

Guideline on Hormone Receptor Testing in Breast Cancer

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Report Date: April 8, 2011

An assessment conducted in April 2014 placed Evidence-based Series (EBS) 22-1 IN REVIEW. This means that it is undergoing a review for currency and relevance.

The Pathology and Laboratory Medicine Group has determined that it is still appropriate for this document to continue to be available while this updating process unfolds.

EBS 22-1 is comprised of 3 sections and is available on the CCO website on the PEBC Pathology & Laboratory Medicine webpage

Section 1: Guideline Recommendations
Section 2: Evidentiary Base
Section 3: EBS Development Methods and External Review Process

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Evidence-Based Series 22-1: Section 1

Guideline on Hormone Receptor Testing in Breast Cancer: Guideline Recommendations

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PURPOSE
The overall purpose of this guideline is to improve the quality and accuracy of hormone receptor (HR) testing and its utility as a prognostic and predictive marker for invasive and in situ breast cancer.

QUESTIONS
1. Clinical Validity of Immunohistochemistry (IHC)
   Can IHC reliably determine the levels of expression of the HR pathway and potentially correlate with the clinical outcome compared to other assays (dextran-coated charcoal [DCC] or ligand-binding assay [LBA], enzyme immunoassay [EIA], enzyme-linked immunosorbent assay [ELISA], and flow cytometry)?

2. Optimizing IHC
   How should HR testing be performed optimally to assess true HR status? This includes evaluating the effect of the following:
   - Preanalytic variables (i.e., variables of testing involving the collection, fixation, and storage of samples)
   - Analytic variables (i.e., variables associated with the method of testing itself)
   - Thresholds to define results
   - Postanalytic variables (i.e., variables associated with handling of the results, such as reporting)

3. Quality Assurance of IHC
   What parameters should be used to assess the proficiency of an individual laboratory performing HR-status testing?
4. Clinical Validity of the Oncotype DX Assay
   Can Oncotype DX reliably determine the levels of expression of the HR pathway?

TARGET POPULATION
   Tissue from adult female patients with primary or metastatic invasive or in situ breast cancer was the focus, since most of the literature includes tissue from female patients. However, the recommendations in this guideline would also apply to tissue from male breast cancer patients.

INTENDED USERS
   Any personnel involved in HR testing and interpretation and any health care provider involved in the management of breast cancer patients.

CONTEXT
   This guideline is not intended to advise clinicians when to perform HR testing for invasive or in situ breast cancer but to improve the quality and accuracy of HR testing. Limited data, based on the retrospective analysis of the National Surgical Adjuvant Breast and Bowel Project (NSABP) B24 study suggest that estrogen receptor (ER) expression is an important predictor of the response to tamoxifen in patients with ER-positive ductal carcinoma in situ (DCIS) (1). The results for ER-negative DCIS were inconclusive (1). Although HR testing for DCIS is not mandatory, some oncologists are seeking the HR status in DCIS when considering the benefit of adjuvant endocrine treatment according to the Cancer Care Ontario treatment guidelines for DCIS (2). If DCIS is tested for HR status, this guideline should be used. In cases of multifocal invasive carcinoma with different histologic types and/or grades, separate HR testing should be conducted.

RECOMMENDATIONS AND KEY EVIDENCE
1. Clinical Validity of IHC
   Can IHC reliably determine the levels of expression of the HR pathway and potentially correlate with the clinical outcome, compared to other assays (DCC/LBA, EIA, ELISA, and flow cytometry)?
   - IHC should be used instead of DCC/LBA, EIA, ELISA, or flow cytometry.
     In 20 out of 22 studies that compared IHC to another test and included patient outcomes, ER and/or progesterone-receptor (PR) using IHC was found to predict patient response to endocrine therapy and/or provide prognostic data such as overall survival, disease-free survival/interval, progression-free survival, metastasis-free interval, and recurrence/relapse-free survival at least as well as or better than the reference assays of DCC or EIA or ELISA (3-24). IHC has certain advantages over older forms of HR testing, which include the requirement of smaller amounts of tissue; the ability to conduct testing on formalin-fixed, paraffin-embedded (FFPE) tissue (allowing for histological examination); and the storage and retrieval of archived, stained slides for retrospective analysis.

2. Optimizing IHC
   How should hormone receptor testing be performed optimally to assess the true HR status?

Preanalytical Variables
Core Biopsies
   - Core biopsies may be used to assess ER and PR status in the primary tumour prior to neoadjuvant therapy or in the case of metastatic disease. However, as they are
derived from only a small sample of a larger tumour where normal ducts and lobules are frequently not present and in view of the heterogeneity in tumour HR expression, it is preferable to test the tumour in the surgical excision specimen. Core biopsies may also be preferentially assessed when the surgical specimen is inadequate due to inadequate fixation or lack of an invasive component.

- If the surgical specimen is adequately fixed and internal and external controls are concordant, it is not mandatory to repeat testing on negative tumours.
  - These recommendations differ from the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) recommendations where core biopsies are preferred (25-27). The potential lack of normal ducts and lobules and tumour heterogeneity are reasons the Working Group preferred excisional specimens. ASCO/CAP preferred core biopsies, because typically cores are immediately placed in formalin, minimizing cold ischemic time, and therefore may be exposed to more uniform and consistent tissue fixation (25-27). In the case of inadequate fixation of surgical specimens, the Working Group preferred core biopsies as well.
  - Eighteen comparative studies found concordance values greater than 83% (median 95%) for ER and greater than 69% (median 88.5%) for PR from core biopsies against standard surgical specimens (28-45).
  - Variability between cores and whole tumours may be dependent on intratumoural heterogeneity or be affected by treatment (33,42).

**Tissue Microarray (TMA)**

- TMA should not be used for diagnostic purposes. The test may be used for quality assurance and as a research tool. At least two cores should be removed from each tumour.
  - Seven comparative studies found concordance values greater than 95% (median 97%) for ER and greater than 85% (median 93.25%) for PR using TMAs against standard whole sections (46-52). Six retrospective studies found prognostic values of HR using TMAs (7,51,53-56). Three studies found good agreement between cores (46,49,55). However, three other studies suggested there may be heterogeneity in the amount of tumour tissue between cores and that cores might be lost (47,50,57). The Working Group recommended against the use of TMAs for diagnostic purposes for several reasons, including the heterogeneity in the cores, the requirement of specialized skill and reading, the possibility for errors with low-positive tumours, and the delay in HR testing caused by waiting for TMAs to be constructed from multiple patients.

**Fine Needle Aspiration (FNA)**

- FNA is not the preferred method to assess HR status; however, FNA may be used to assess ER or PR status on cell blocks using IHC, especially in metastatic disease where a core biopsy may not be a possibility.
- FNAs should be fixed in neutral buffered formalin (NBF), using cell blocks from pellets.
- If not fixed in formalin, the protocol should be validated and have appropriate controls.
- In cases where samples are HR negative, at least 100 nuclei should have been scored from FNA samples, when available.
  - One of the limitations with using FNA is that the distinction between in situ and invasive components of a malignant aspirate is often impossible to make.
Sixteen comparative studies found concordance values greater than 86% (median 94.5%) for ER and greater than 73% (median 80.2%) for PR from FNA against the standard of IHC on paraffin sections (58-73). Two studies showed a high degree of reliability using FNAs (59,74).

The Working Group preferred formalin fixation to maintain consistency with fixative recommendations for surgical sections (please see below).

One study suggested at least 100 nuclei should be used as a criterion to reject technically suboptimal slides for scoring FNAs (71).

**Frozen Sections**

- Paraffin sections should be used instead of frozen sections. However, frozen sections can be used to validate the quality of fixation of paraffin sections.
  - The concordance between frozen and paraffin sections among 21 studies was over 72% (median 91.4%) for ER and over 84% (median 90.8%) for PR (9,21,68,75-92). One study found that ER assessed in paraffin, frozen samples or using DCC were similar in predicting response to endocrine therapy (75), whereas another study found that ER assessed in paraffin sections was a better predictor of endocrine response than ER assessed in frozen samples (21).
  - It is common practice to use paraffin sections, because histological and HR assessment can be determined on the same block. In addition, tumours that are small and/or non-palpable may be difficult to sample for frozen section analysis.

**Fixation Type**

- Ten percent NBF should be used as a fixative for optimal results.
- The formulation of NBF should be confirmed with each lot and if different should be validated.
- Other fixatives may be used if they have been validated against NBF.
- Ethanol should not be used as a fixative.
  - Two studies, using paraffin sections or TMAs, suggest that 10% NBF as a fixative achieves the best results and that ethanol should be avoided as a fixative (93-94). Furthermore, NBF is commonly used in many laboratories. If laboratories use other fixatives, then their results should be validated against tissue fixed in NBF. (See the internal quality assurance [QA] section below.)

**Fixation Time**

- Place tissue into fixative as soon as possible following removal (preferably immediately and not more than one hour afterwards).
- Avoid freezing and cryosectioning before fixing the sample.
- Fix for 24 hours for optimal results.
- With proper antigen retrieval, tissue can be fixed for approximately six to 72 hours, which includes the time the tissue is loaded on the machine to the time that alcohol is introduced.
- Underfixation is more critical than overfixation; however, shorter or longer fixation times do not exclude the specimen for HR testing if proper validation of such protocols is well documented.
  - Two comparative studies found the best results were achieved when tissue was fixed for 24 hours in NBF, immediately after surgery (93-94). Poorer results were found when tissue was frozen and cryosectioned before fixing or when there was a delay in onset of fixation of 12 hours (93). Another study found that paraffin
sections from tissue that had been fixed one to four hours after removal had poorer concordance with frozen tissue than paraffin sections from tissue fixed within a few minutes after removal (76). The Working Group chose to use a conservative estimate of a one-hour threshold for cold ischemic time to fixation.

- Two comparative studies found that tissues fixed for as little as four hours in one study and six to eight hours in another study could achieve similar results to tissue fixed for longer periods of time (76,95-96). Again, the Working Group chose a conservative time of six hours as a minimum requirement for the duration of fixation as well as to be consistent with the HER2/neu-testing guidelines (98). However, this is a controversial issue and any future updates of this guideline should align with any changes made to the ASCO/CAP HR guidelines or HER2/neu-testing guidelines.

- Four comparative studies showed that, with proper antigen retrieval, tissue fixed for several days will continue to immunoreact for HR (80,89,93,97). The longest duration of fixation used was 48 hours for PR, compared to frozen sections, and 96 hours for ER, compared to tissue fixed for 24 hours. The Working Group chose 72 hours for both ER and PR as maximum requirements for the duration of fixation in order to be consistent with HER2/neu testing recommendations (98). In addition, longer fixation does not exclude the specimen for HR testing.

**Tissue Processing**

- Surgical specimens received in the pathology laboratory should be oriented, inked for surgical margin assessment, and carefully sliced at 5-10 mm intervals before being placed into formalin. If the tissue samples are to be further sectioned and placed into tissue cassettes at a later time, gauze pads or paper towels should be placed in between tissue slices to assist with the penetration of the formalin. If a gross tumour is easily identifiable, a section including a portion of the tumour and normal breast tissue from around the tumour can be placed in the same cassette and immediately fixed at the time of the initial gross evaluation. This action will ensure good tissue fixation and that the normal breast tissue, acting as an internal positive control, will be fixed in the same manner as the tumour. For samples obtained remotely from the grossing laboratory, pathologists, in collaboration with the personnel at the remote locations, should ensure the sample is bisected at the level of the tumour and promptly placed in fixative prior to its refrigeration and transport. Although this is less optimal than the rapid gross examination of fresh samples by the pathologist, it is preferable to uncut, fixed or unfixed tissue stored in the refrigerator.

- The block samples from the specimen should be 3-5 mm in thickness. Similarly, the appropriate thickness of the sections from the paraffin block should be 3-5 µm.

- If the laboratory is remote from the site of the surgery then the surgeon should ink and slice the sample and place it in formalin.

  - No published studies were found to inform these recommendations. Therefore, these recommendations were developed through consensus with the Working Group, based on common practices in anatomic pathology laboratories.

**Storage of Slides**

- Use freshly cut sections for optimal results.

  - Two comparative studies using TMAs showed better HR detection was found with freshly cut sections compared with slides stored for longer periods of time (99-100). As well, one study found that longer storage decreased HR associations with
tumour-specific survival (101). Several studies suggested possible methods to preserve antigenicity (paraffin coating and protection from light and heat) (99,102-103); however, the Working Group decided that, for diagnostic purposes, freshly cut sections should be recommended.

**Analytical Variables**

**Controls**
- Controls should include positive and negative breast cancer cases, plus a low positive case if possible.
- The control tissue should be fixed and processed in the same manner as the patient samples.
- Controls should be run with each HR IHC batch run; however, on slide external controls for automated processes are preferred.
- Internal controls of normal breast elements must be evaluated when present on the section. In the majority of breast cancer resection specimens, 5-10% of luminal epithelial cells will express ER in normal breast elements. If normal breast elements are completely ER negative, a false negative result should be considered, and another block should be tested or ER expression may be measured by another method.
- Other types of tissue such as the endometrium or myometrium, or uterine cervix, could be used for external controls. For progesterone receptors, hyperplastic benign prostatic tissue could be used for controls. When selecting controls, attention should be made to the variation in the level of expression during the menstrual cycle in premenopausal women that would give a broad level of expression for HR.
- The controls should use tissue. Cell lines may be used as controls but not in isolation. Cell lines lack stroma and are not subjected to the same processing methods.
  - No articles were found to inform these recommendations. Therefore, these recommendations were developed through consensus with the Working Group based on common practices in their laboratories.

**Antigen Retrieval**
- Antigen retrieval may be used to recover immunoreactivity from tissue; however, the method, reagents and duration must be optimized for each antibody.
- Stringent compliance to validated standard operating procedures developed in assay validation must be adhered to, and quality control (QC) documentation must be in place.
- Any modifications must be validated.
  - Eleven comparative studies were highly variable in their choice of antibody and antigen retrieval method (80,92,104-112). Since no specific recommendations for antigen retrieval could be derived from the evidence, the Working Group recommended compliance with validated standard operating procedures used in the laboratory, either following package inserts or validating against a reference standard (see internal QA section below).

**Reagents**
- Stringent compliance to validated standard operating procedures developed in assay validation must be adhered to, and QC documentation must be in place.
• Any modifications must be validated.
  – Due to the lack of evidence in this area, these recommendations were developed through consensus with the Working Group based on common practices and to be consistent with recommendations developed for HER2/neu-testing (98).

Antibodies
• Currently approved antibodies by Health Canada that have a Class II medical device license may be used.
  – The Working Group chose to recommend antibodies approved by Health Canada, based on the suggestion from external reviewers. Also, the Quality Management Program - Laboratory Services in Ontario recognizes all tests approved and licensed by Health Canada.

Antibody Detection
• Antibody incubation and detection must be optimized for the specific antibodies used and must be validated with every lot of antibody.
  – Although there were comparative studies that identified differences between antibody detection systems for various antibodies (93,112-115), the Working Group did not want to specifically endorse any type of detection system as each protocol needs to be validated or the package inserts need to be followed, regardless of the detection system used (see internal QA section below).

Automated versus Manual Staining
• Correctly operated and validated automated staining protocols and equipment should be used for all steps in the process; however, validated manual staining may be used.
• Maintenance and service records should be regularly updated and filed in the laboratory.
  – There were two comparative studies that found a higher percentage of ER positivity using immunostainers compared to manual staining (116-117). One study found automated staining was more reliable than manual staining but did not find a statistically higher percentage of ER positivity with immunostainers (118). The Working Group decided that automated staining, if available, is recommended, but validated methods should be used.

Image Analysis
• Validated image analysis systems may be used, particularly for low-positive cases, to reduce subjectivity and improve interobserver reliability.
• Pathologists must supervise all image analyses.
  – IHC using image analysis in 29 comparative studies had good reliability and was found to be highly concordant and correlated with manual estimation, but there were no prospective trials with patient outcomes to convince the Working Group that image analysis should replace manual estimation (6,11,13,53-55,76,119-140).

Thresholds to Define Results
• Only nuclear (not cytoplasmic) staining should be scored.
• There are three categories for staining:
  Positive: ≥10% staining for ER or PR
  Low positive: 1% to 9% staining for ER or PR
Negative: < 1% staining for ER and PR

- A statement of intensity can be reported as weak, moderate, or strong; however, this is more important if the tumor is low positive with weak intensity.
  - Different scoring methods were shown to be comparable, but the percent of cells stained positive is the simplest method and is correlated with patient outcomes (5,7,10-11,18,21,53,56,135,141-144). Moreover, six studies found that the proportion of positive staining was at least as good a predictive or prognostic indicator, or better, than the intensity of stained cells or a combination score of the proportion and intensity of stained cells (12-13,16,20,144-145).
  - Eight studies suggest that ER values as low as 10% and possibly as low as 1% can have predictive or prognostic value (9,12,15-16,20,23,143-144). Furthermore, other studies have shown the percentage of PR positivity, to as low as 1%, provides additional predictive or prognostic value (5,19,22,56,143,146), independent of the ER values (20,144). However, all of these studies are retrospective and mainly evaluate the prognostic value of HR in patients treated with endocrine therapy. They do not directly assess the predictive ability of HR at different cutoffs by examining the interaction between patients that receive endocrine therapy versus those patients who do not receive endocrine therapy at various cut points. Since most patients receive endocrine therapy, these studies would require large sample sizes. Therefore, in order to be maximally sensitive and based on the retrospective evidence, the Working Group decided to use 1% positivity for ER or PR as a threshold for defining low-positive samples. However, since there is high interlaboratory variability in assessing low-positive samples resulting in high false-negative rates (94,147), and the nature of the evidence to support a 1% threshold is retrospective, the Working Group felt that clinical judgment should be used when assessing patients with low-positive HR tumors for endocrine therapy, especially when chemotherapy might not be a tolerable option for some patients. Although the Working Group suggested that staining intensity could be included, they felt there was sufficient evidence not to make it a mandatory requirement in the evaluation of HR positive and negative status. Reporting intensity was more valuable in low-positive samples where clinical judgment might be necessary.

Postanalytical Variables

After the standard background patient information (see below), results should be stated clearly and bolded, and the additional information about the quality of the test should follow in synoptic format.

- The following items should be reported.
  To avoid lengthy and exhaustive reports, the Working Group makes a distinction between elements that need to be included in the formal pathology report and items that need to be collected and kept in the laboratory without necessarily being in the final report. Asterisked items should be included in the printed final report, visible to the physician. Other information should be available in the laboratory records.
    - *Standard background patient information
    - *Specimen identification (case and block number)
    - *Specimen site and type
    - Specimen fixative type (*if not 10% NBF)
    - Time to fixation (if available)
    - Duration of fixation (if available)
    - Antibody clone and vendor
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- Method used (test and vendor)
- Image analysis method (if used)
- Adequate controls
- Adequacy of sample for evaluation
- *Results
  o Percentage of positively stained cells for ER and PR
- *Interpretation
  o Low positive 1-9% for ER or PR
  o Positive ≥ 10% for ER or PR
  o Negative < 1% for ER and PR
  o Not interpretable
  o Intensity of staining: weak, moderate or strong

- *Sample exclusion criteria to perform or interpret a HR IHC assay include the following:
  - Tissue fixed using other than 10% NBF unless validated
  - When controls were inappropriate
  - Core needle biopsies with
    o Edge or retraction artifact involving entire core
    o Crush artifact (thin-gauge vacuum-extraction needle samples)
    o Tissues where controls exhibit unexpected results
  - If the assay is negative, then:
    - Look at the histology and grade; some cancer types such as classic lobular and tubular carcinoma or low-grade tumours are only rarely ER negative.
    - For cases where there is incongruence between HR testing and histology and/or grade, repeat the assay or have another laboratory repeat the assay
  - Controlled decalcified specimens with EDTA can be used. When possible, tease out fragments of the tumour so that no decalcification is required.
  - In samples with only DCIS, the diagnosis of DCIS should be mentioned and scored.
  - In tumours that contain both the in situ and invasive components, only the invasive component should be tested and scored.

Reporting elements not supported by evidence described in the answers to question 2, were developed through consensus with the Working Group, based on commonly used practices. These reporting elements are also in keeping with the recommendations for HER2/neu testing (98).

3. Quality Assurance of IHC

What parameters should be used to assess the proficiency of an individual laboratory performing HR-status testing?

**Caseload**

- The evidence to guide exact caseloads per laboratory or pathologist is not available; however, laboratories could use the recommendations for HER2/neu testing, although this is not mandatory. These cases should be carried out by experienced pathologists at laboratories participating in IHC external QA accreditation programs.
- Appropriate training is recommended for pathologists who report on HR status. The appropriate training may be part of a residency or fellowship program, mentorship with an experienced pathologist, or a formal didactic course. Cases should include
at least 20 negative and at least 20 positive specimens, with some being weakly positive. When possible, cases should be reviewed with an experienced pathologist and at least a 90% concordance should be achieved; any discordance should be assessed on a dual-head microscope. In a challenge set of 40, less than three discordant cases are considered acceptable (148).

- Staff should be encouraged to show each other borderline cases; experienced pathologists should be consulted in cases with low-positive staining or weak intensity.
- These activities could be tracked in pathology computer systems.
- The number of tests performed by each pathologist should be considered to ensure competency.
- Test volume should be addressed in conjunction with the laboratory’s adherence to strict QC and QA practices.
- Medical laboratory technologists who perform Class II IHC testing should undergo appropriate training.

**Internal QA**

- Initial test validation should take place together with ongoing QC and equipment maintenance.
- Initial and ongoing education, training, and competency assessment of laboratory personnel should also be implemented and recorded.
- The use of standardized operating procedures, including routine use of control materials, should be enforced, and modified procedures should be revalidated.
- Ongoing competency assessment and education of pathologists should take place.
- When validating a new antibody, at least 40 samples should be tested with known results. Those should include 20 positive, of which at least five are low positive, and 20 negative cases. However, if the laboratory has little experience with performing HR testing, doubling the sample size is advisable (148).
- An assay concordance rate of 90% for ER-positive or PR-positive tumours and a 95% concordance rate for ER-negative or PR-negative tumours should be achieved.
- Adequate validation should be ensured, preferably by using a selection that includes approximately 30% unequivocally positive cases, approximately 30% low positive, and approximately 40% negative.
- Validation documentation must be kept for at least five years.
- Any modification to preanalytical, analytical, or postanalytical procedures requires additional validation to ensure accurate performance.
- At least semiannual trend analysis should be performed by internal audit for each institution and preferably for each pathologist; approximately 70% of samples should be ER positive and approximately 50% of samples should be PR positive; this may vary depending on the referred patient population.

**External QA**

**HR-specific external QA**

- Laboratories are required to participate in at least one external proficiency program assessing analytical and postanalytical components (such as the Canadian IHC Quality Control Group, the Ontario External Quality Assessment program organized by the Quality Management Program - Laboratory Services in Ontario, the United Kingdom National External Quality Assessment Service [UK NEQAS], or CAP) with at least two testing events (mailings) annually (149).
• Unsatisfactory performance results will be addressed according to the regulations of the accreditation program.

IHC external QA
• Each laboratory should be accredited to perform IHC and follow standard operating procedures (according to the Ontario Laboratory Accreditation [http://home.qmpls.org/external/index.html] requirements).
• Onsite inspection for IHC should take place every other year, with an annual requirement for self-inspection.
• A review of the laboratory validation, procedure, QA results and processes, results, and reports for IHC should be put into place.

All recommendations addressing question 3 were developed, through consensus with the Working Group, to be consistent with HER2/neu testing recommendations (98) and the ASCP/CAP recommendations (148). The percentages of ER and PR positivity for periodic trend analysis were derived from the mean frequency observed across 71 laboratories, using a 10% threshold in the UK_NEQAS (150).

4. Clinical Validity of Oncotype DX
Can Oncotype DX reliably determine the levels of expression of the HR pathway?

• Oncotype DX can accompany IHC results but should not replace them.
  – Fourteen studies investigating Oncotype DX have evaluated the predictive validity of the recurrence score in ER-positive breast cancer, and none were found that correlated the expression of the HR-related genes to clinical outcome (151-164). The Working Group decided there was insufficient evidence to suggest that HR assessed with Oncotype DX is a better predictive or prognostic indicator than HR assessed using IHC.

FUTURE RESEARCH
Future research that would provide valuable information for these recommendations would include studies that validated the score derived from the HR-related genes assessed by Oncotype DX, prospective trials that included patient outcomes comparing image analysis with manual estimation, and studies comparing 1% versus 10% thresholds as predictive HR markers.

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