

# **Supplemental Digital Content**

## **Estrogen and Progesterone Receptor Testing in Breast Cancer: ASCO/CAP Clinical Practice Guideline Update**

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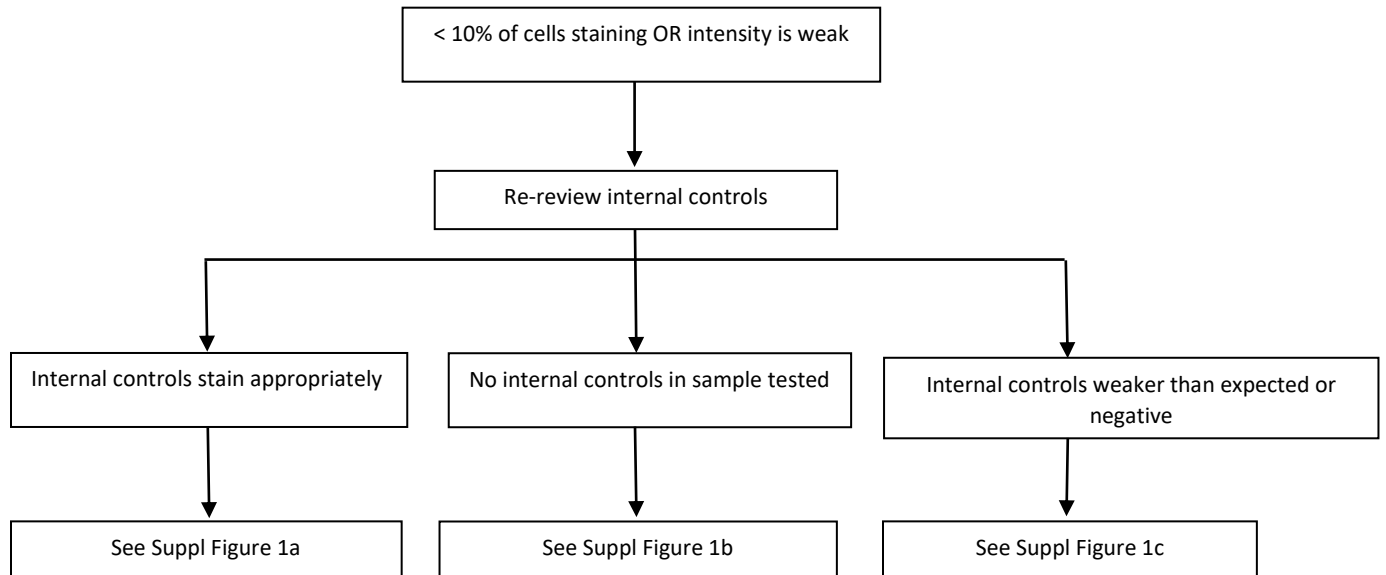
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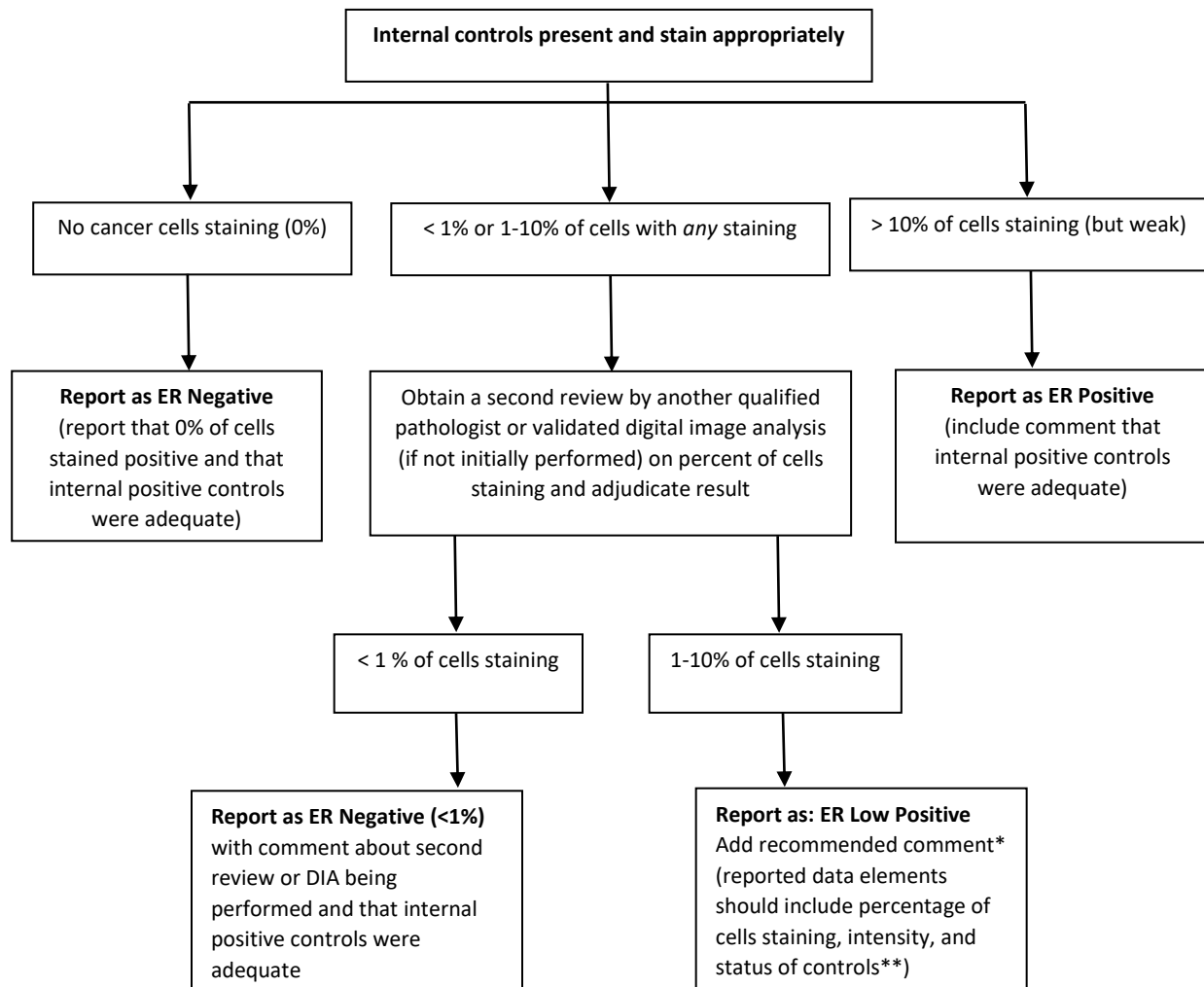
**Data Supplement 1: Quantitative Image Analysis Principles:**

1. Use only QIA systems that have been validated for diagnostic purposes
2. Validate QIA results before offering this test using an alternative validated method such as manual IHC interpretation using approved reagents
3. Monitor and document the reproducibility and precision of the results using:
  - a. Same case, different batches
  - b. Same case, different operators or pathologists
4. Create standard procedures and monitor their use for training:
  - a. New operators and pathologists in finding region of interest (ROI)
  - b. New operators and pathologists in using the annotated data to produce a result
  - c. Pathologists in reviewing ROI, annotated data and result
5. Revalidate the QIA system if changes are made
6. Document QIA results in the report
7. Maintain images and metadata for future review according to local regulations

**Data Supplement 2: Figure 1. Example of a Lab-Specific Standard Operating Procedure for cases with initial ER IHC result with < 10% of cells staining or stain intensity is weak**

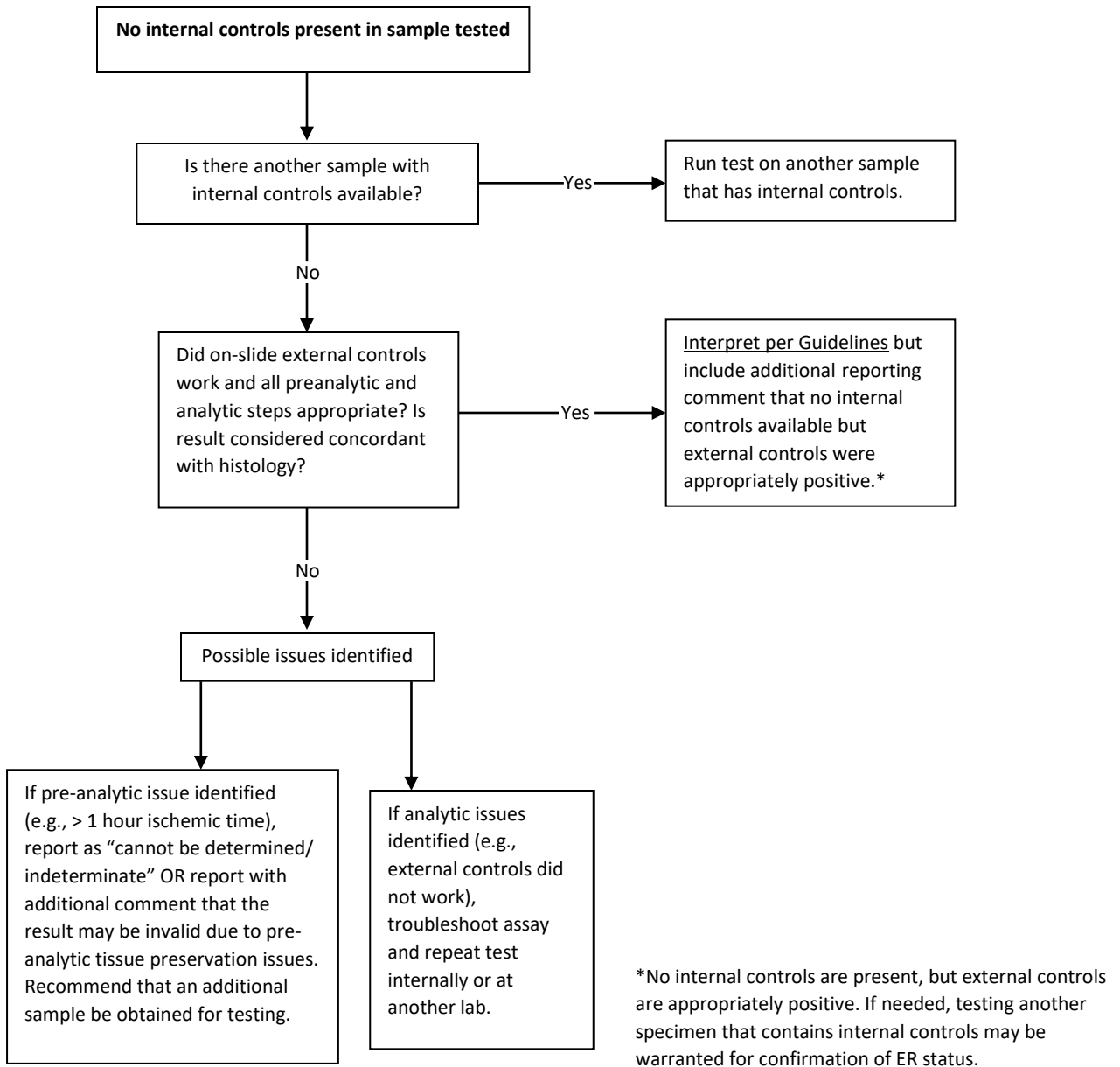


**Figure 1a. Internal controls present and stain appropriately**

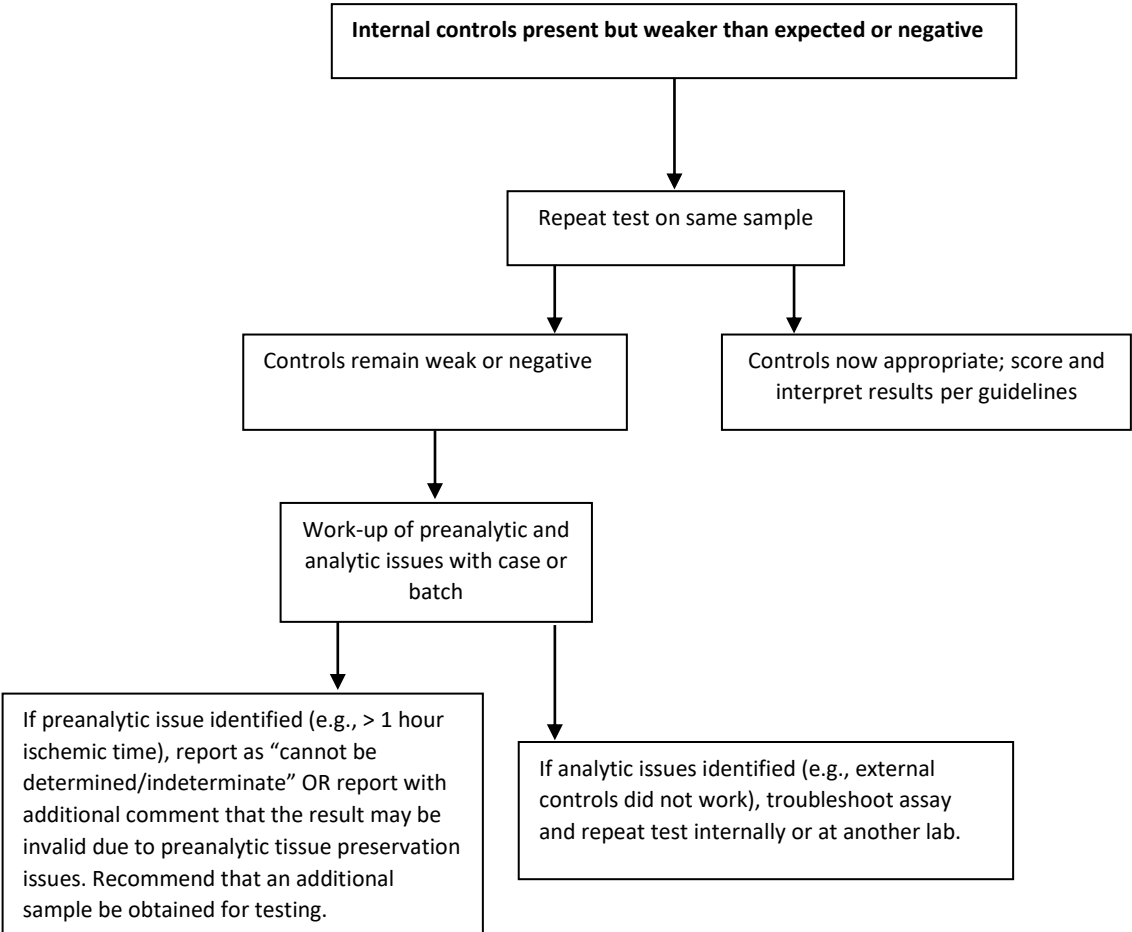


Report comments: \*Recommended comment for low positive results: The cancer in this sample has a low level (1-10%) of ER expression by IHC. There are limited data on the overall benefit of endocrine therapies for patients with these results, but they currently suggest possible benefit, so patients are considered eligible for endocrine treatment. There are data that suggest invasive cancers with these results are heterogeneous in both behavior and biology and often have gene expression profiles more similar to ER negative cancers. \*\*If the test results are either ER negative or low positive and no internal controls are present, the following comment should be included in the report: No internal controls are present, but external controls are appropriately positive. If needed, testing another specimen that contains internal controls may be warranted for confirmation of ER status.

**Figure 1b. No internal controls present in sample tested**



**Figure 1c. Internal controls present but weaker than expected or negative**



### Data Supplement 3: Search Strategy String and Dates

#### Question 1: What is the optimal testing algorithm for the assessment of ER/PR status?

1/1/2008 – 02/10/2016: 1051 results

02/10/2016 -04/30/2019: 719 results

("humans"[MeSH Terms] OR "humans"[All Fields] OR "human"[All Fields]) AND ("breast neoplasms"[MeSH Terms] OR ("breast"[All Fields] AND "neoplasms"[All Fields]) OR "breast neoplasms"[All Fields]) AND (("receptors, estrogen"[MeSH Terms] OR ("receptors"[All Fields] AND "estrogen"[All Fields]) OR "estrogen receptors"[All Fields] OR ("receptors"[All Fields] AND "estrogen"[All Fields]) OR "receptors, estrogen"[All Fields]) OR ("receptors, progesterone"[MeSH Terms] OR ("receptors"[All Fields] AND "progesterone"[All Fields]) OR "progesterone receptors"[All Fields] OR ("receptors"[All Fields] AND "progesterone"[All Fields]) OR "receptors, progesterone"[All Fields]) OR ("tumour markers"[All Fields] OR "biomarkers, tumor"[MeSH Terms] OR ("biomarkers"[All Fields] AND "tumor"[All Fields]) OR "tumor biomarkers"[All Fields] OR ("tumor"[All Fields] AND "markers"[All Fields]) OR "tumor markers"[All Fields]) OR ("biology"[MeSH Terms] OR "biology"[All Fields] OR "biological"[All Fields])) AND (("algorithms"[MeSH Terms] OR "algorithms"[All Fields]) OR ("decision support techniques"[MeSH Terms] OR ("decision"[All Fields] AND "support"[All Fields] AND "techniques"[All Fields]) OR "decision support techniques"[All Fields]) OR ("computational biology"[MeSH Terms] OR ("computational"[All Fields] AND "biology"[All Fields]) OR "computational biology"[All Fields]) OR ("immunohistochemistry"[MeSH Terms] OR "immunohistochemistry"[All Fields]) OR ("staining and labelling"[All Fields] OR "staining and labeling"[MeSH Terms] OR ("staining"[All Fields] AND "labeling"[All Fields]) OR "staining and labeling"[All Fields]) OR ("reference standards"[MeSH Terms] OR ("reference"[All Fields] AND "standards"[All Fields]) OR "reference standards"[All Fields]) OR ("laboratories"[MeSH Terms] OR "laboratories"[All Fields] OR "laboratory"[All Fields]) AND ("methods"[Subheading] OR "methods"[All Fields] OR "techniques"[All Fields] OR "methods"[MeSH Terms] OR "techniques"[All Fields]) AND ("methods"[Subheading] OR "methods"[All Fields] OR "procedures"[All Fields] OR "methods"[MeSH Terms] OR "procedures"[All Fields])) AND ("disease-free survival"[MeSH Terms] OR ("disease-free"[All Fields] AND "survival"[All Fields]) OR "disease-free survival"[All Fields] OR ("disease"[All Fields] AND "free"[All Fields] AND "survival"[All Fields]) OR "disease free survival"[All Fields]) OR ("survival rate"[MeSH Terms] OR ("survival"[All Fields] AND "rate"[All Fields]) OR "survival rate"[All Fields]) OR ("neoplasm recurrence, local"[MeSH Terms] OR ("neoplasm"[All Fields] AND "recurrence"[All Fields] AND "local"[All Fields]) OR "local neoplasm recurrence"[All Fields] OR ("neoplasm"[All Fields] AND "recurrence"[All Fields] AND "local"[All Fields]) OR "neoplasm recurrence, local"[All Fields]) OR ("prognosis"[MeSH Terms] OR "prognosis"[All Fields]) OR ("treatment outcome"[MeSH Terms] OR ("treatment"[All Fields] AND "outcome"[All Fields]) OR "treatment outcome"[All Fields]) OR ("outcome and process assessment (health care)"[MeSH Terms] OR ("outcome"[All Fields] AND "process"[All Fields] AND "assessment"[All Fields] AND "(health"[All Fields] AND "care)"[All Fields]) OR "outcome and process assessment (health care)"[All Fields] OR ("outcome"[All Fields] AND "process"[All Fields] AND "assessment"[All Fields]) OR "outcome and process assessment"[All Fields]) AND ("delivery of health care"[MeSH Terms] OR ("delivery"[All Fields] AND

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**Question 2: What strategies can help ensure optimal performance, interpretation, and reporting of established assays?**

1/1/2008 – 02/10/2016: 1597 results

02/10/2016 -04/30/2019: 983 results

("humans"[MeSH Terms] OR "humans"[All Fields] OR "human"[All Fields]) AND ("breast neoplasms"[MeSH Terms] OR ("breast"[All Fields] AND "neoplasms"[All Fields]) OR "breast neoplasms"[All Fields]) AND (("receptors, estrogen"[MeSH Terms] OR ("receptors"[All Fields] AND "estrogen"[All Fields]) OR "estrogen receptors"[All Fields] OR ("receptors"[All Fields] AND "estrogen"[All Fields]) OR "receptors, estrogen"[All Fields]) OR ("receptors, progesterone"[MeSH Terms] OR ("receptors"[All Fields] AND "progesterone"[All Fields]) OR "progesterone receptors"[All Fields] OR ("receptors"[All Fields] AND "progesterone"[All Fields]) OR "receptors, progesterone"[All Fields]) OR ("tumour markers"[All Fields] OR "biomarkers, tumor"[MeSH Terms] OR ("biomarkers"[All Fields] AND "tumor"[All Fields]) OR "tumor biomarkers"[All Fields] OR ("tumor"[All Fields] AND "markers"[All Fields]) OR "tumor markers"[All Fields]) OR ("biology"[MeSH Terms] OR "biology"[All Fields] OR "biological"[All Fields])) AND (("laboratories"[MeSH Terms] OR "laboratories"[All Fields]) OR ("laboratories, hospital"[MeSH Terms] OR ("laboratories"[All Fields] AND "hospital"[All Fields]) OR "hospital

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**Question 2b: What are the optimal external quality assurance methods to ensure ongoing accuracy in ER/PR testing?**

1/1/2008 – 02/10/2016: 1012 results

02/10/2016 -04/30/2019: 731 results

("humans"[MeSH Terms] OR "humans"[All Fields] OR "human"[All Fields]) AND ("breast neoplasms"[MeSH Terms] OR ("breast"[All Fields] AND "neoplasms"[All Fields]) OR "breast neoplasms"[All Fields]) AND (("receptors, estrogen"[MeSH Terms] OR ("receptors"[All Fields] AND "estrogen"[All Fields]) OR "estrogen receptors"[All Fields] OR ("receptors"[All Fields] AND "estrogen"[All Fields]) OR "receptors, estrogen"[All Fields]) OR ("receptors, progesterone"[MeSH Terms] OR ("receptors"[All Fields] AND "progesterone"[All Fields]) OR "progesterone receptors"[All Fields] OR ("receptors"[All Fields] AND "progesterone"[All Fields]) OR "receptors, progesterone"[All Fields]) OR ("tumour markers"[All Fields] OR "biomarkers, tumor"[MeSH Terms] OR ("biomarkers"[All Fields] AND



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**Question 2c: How can these efforts be implemented and the effects measured?**

1/1/2008 – 02/10/2016: 202 results

02/10/2016 -04/30/2019: 198 results

("humans"[MeSH Terms] OR "humans"[All Fields] OR "human"[All Fields]) AND ("breast neoplasms"[MeSH Terms] OR ("breast"[All Fields] AND "neoplasms"[All Fields]) OR "breast neoplasms"[All Fields]) AND (("receptors, estrogen"[MeSH Terms] OR ("receptors"[All Fields] AND "estrogen"[All Fields]) OR "estrogen receptors"[All Fields] OR ("receptors"[All Fields] AND "estrogen"[All Fields]) OR "receptors, estrogen"[All Fields]) OR ("receptors, progesterone"[MeSH Terms] OR ("receptors"[All Fields] AND "progesterone"[All Fields]) OR "progesterone receptors"[All Fields] OR ("receptors"[All Fields] AND "progesterone"[All Fields]) OR "receptors, progesterone"[All Fields]) OR ("tumour markers"[All Fields] OR "biomarkers, tumor"[MeSH Terms] OR ("biomarkers"[All Fields] AND "tumor"[All Fields]) OR "tumor biomarkers"[All Fields] OR ("tumor"[All Fields] AND "markers"[All Fields]) OR "tumor markers"[All Fields]) OR ("biology"[MeSH Terms] OR "biology"[All Fields] OR "biological"[All Fields]) AND (("guideline"[Publication Type] OR "guidelines as topic"[MeSH Terms] OR "guideline"[All Fields]) OR ("practice guideline"[Publication Type] OR "practice guidelines as topic"[MeSH Terms] OR "practice guideline"[All Fields]) OR ("evaluation studies as topic"[MeSH Terms] OR ("evaluation"[All Fields] AND "studies"[All Fields] AND "topic"[All Fields]) OR "evaluation studies as topic"[All Fields]) OR ("programme evaluation"[All Fields] OR "program evaluation"[MeSH Terms] OR ("program"[All Fields] AND "evaluation"[All Fields]) OR "program evaluation"[All Fields]) OR ("outcome and process assessment (health care)"[MeSH Terms] OR ("outcome"[All Fields] AND "process"[All Fields] AND

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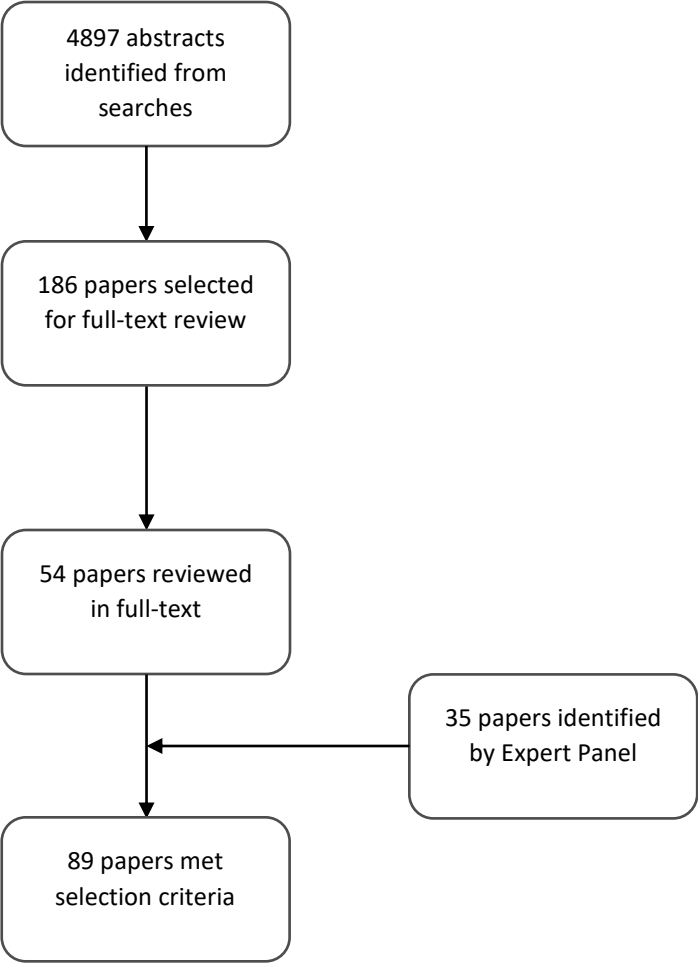
### **New Testing Methods**

("breast neoplasms"[MeSH Terms] OR ("breast"[All Fields] AND "neoplasms"[All Fields]) OR "breast neoplasms"[All Fields]) AND (("receptors, estrogen"[MeSH Terms] OR ("receptors"[All Fields] AND "estrogen"[All Fields]) OR "estrogen receptors"[All Fields] OR ("receptors"[All Fields] AND "estrogen"[All Fields]) OR "receptors, estrogen"[All Fields]) OR ("receptors, progesterone"[MeSH Terms] OR ("receptors"[All Fields] AND "progesterone"[All Fields]) OR "progesterone receptors"[All Fields] OR ("receptors"[All Fields] AND "progesterone"[All Fields]) OR "receptors, progesterone"[All Fields]) OR ("tumour markers"[All Fields] OR "biomarkers, tumor"[MeSH Terms] OR ("biomarkers"[All Fields] AND "tumor"[All Fields]) OR "tumor biomarkers"[All Fields] OR ("tumor"[All Fields] AND "markers"[All Fields]) OR "tumor markers"[All Fields]) OR ("biology"[MeSH Terms] OR "biology"[All Fields] OR "biological"[All Fields])) AND (ARRAY[All Fields] OR RT-qPCR[All Fields] OR nCounter[All Fields] OR ("rna, messenger"[MeSH Terms] OR ("rna"[All Fields] AND "messenger"[All Fields]) OR "messenger rna"[All Fields] OR "mrna"[All Fields])) AND ("2008/11/11"[PDAT] : "2018/11/08"[PDAT]) AND ((Clinical Trial[ptyp] OR Review[ptyp]) AND "humans"[MeSH Terms])

2008/11/11-2018/11/08: 387 results

2018/11/08-2019/04/30 : 4 results

**Data Supplement 4: QUOROM Diagram**



**Data Supplement 5: Evidence Tables**

**Endocrine Therapy Response**

First Author, Journal, Year	Title	Study Design	Conclusions
Regan, JCO, 2016 <sup>2</sup>	Absolute Benefit of Adjuvant Endocrine Therapies for Premenopausal Women With Hormone Receptor–Positive, Human Epidermal Growth Factor Receptor 2–Negative Early Breast Cancer: TEXT and SOFT Trials	The TEXT and SOFT hormone receptor–positive, HER2-negative analysis population included 4,891 women. The end point was breast cancer–free interval (BCFI), defined as time from random assignment to first occurrence of invasive locoregional, distant, or contralateral breast cancer. A continuous, composite measure of recurrence risk for each patient was determined from a Cox model incorporating age, nodal status, tumor size and grade, and estrogen receptor, progesterone receptor, and Ki-67 expression levels. Subpopulation treatment effect pattern plot methodology revealed differential treatment effects on 5-year BCFI according to composite risk.	SOFT patients who remained premenopausal after chemotherapy experienced absolute improvement of 5% or more in 5-year BCFI with exemestane plus OFS versus tamoxifen plus OFS or tamoxifen alone, reaching 10% to 15% at intermediate to high composite risk; the benefit of tamoxifen plus OFS versus tamoxifen alone was apparent at the highest composite risk. The SOFT no-chemotherapy cohort—for whom composite risk was lowest on average—did well with all endocrine therapies. For TEXT patients, the benefit of exemestane plus OFS versus tamoxifen plus OFS in 5-year BCFI ranged from 5% to 15%; patients not receiving chemotherapy and with lowest composite risk did well with both treatments.
Spring, JAMA Oncol, 2016 <sup>3</sup>	Neoadjuvant Endocrine Therapy for Estrogen Receptor-Positive Breast Cancer: A Systematic Review and Meta-analysis	To evaluate the effect of neoadjuvant endocrine therapy (NET) on the response rate and the rate of breast conservation surgery (BCS) for ER+ breast cancer. Based on PRISMA guidelines, a librarian-led search of PubMed and Ovid MEDLINE was performed to identify eligible trials published from inception to May 15, 2015. The search was performed in May 2015. Study Selection: Inclusion criteria were prospective, randomized, neoadjuvant clinical trials that reported response rates with at least 1 arm incorporating NET (n = 20). Two authors independently analyzed the	The analysis included 20 studies with 3490 unique patients. Compared with combination chemotherapy, NET as monotherapy with aromatase inhibitors had a similar clinical response rate (OR, 1.08; 95% CI, 0.50-2.35; P = .85; n = 378), radiological response rate (OR, 1.38; 95% CI, 0.92-2.07; P = .12; n = 378), and BCS rate (OR, 0.65; 95% CI, 0.41-1.03; P = .07; n = 334) but with lower toxicity. Aromatase inhibitors were associated with a significantly higher clinical response rate (OR, 1.69; 95% CI, 1.36-2.10; P < .001; n = 1352), radiological response rate (OR, 1.49; 95% CI, 1.18-

		<p>studies for inclusion. Data Extraction and Synthesis: Pooled odds ratios (ORs), 95% CIs, and P values were estimated for end points using the fixed- and random-effects statistical model.</p>	<p>1.89; P &lt; .001; n = 1418), and BCS rate (OR, 1.62; 95% CI, 1.24-2.12; P &lt; .001; n = 918) compared with tamoxifen. Dual combination therapy with growth factor pathway inhibitors was associated with a higher radiological response rate (OR, 1.59; 95% CI, 1.04-2.43; P = .03; n = 355), but not clinical response rate (OR, 0.76; 95% CI, 0.54-1.07; P = .11; n = 537), compared with endocrine monotherapy. The incidence of pathologic complete response was low (&lt;10%).</p>
<p>Early Breast Cancer Trialists' Collaborative Group, Lancet, 2011<sup>5</sup></p>	<p>Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomized trials</p>	<p>We undertook a collaborative meta-analysis of individual patient data from 20 trials (n=21 457) in early breast cancer of about 5 years of tamoxifen versus no adjuvant tamoxifen, with about 80% compliance. Recurrence and death rate ratios (RRs) were from log-rank analyses by allocated treatment.</p>	<p>In estrogen receptor (ER)-positive disease (n=10 645), allocation to about 5 years of tamoxifen substantially reduced recurrence rates throughout the first 10 years (RR 0.53 [SE 0.03] during years 0–4 and RR 0.68 [0.06] during years 5–9 [both 2p&lt;0.00001]; but RR 0.97 [0.10] during years 10–14, suggesting no further gain or loss after year 10). Even in marginally ER-positive disease (10–19 fmol/mg cytosol protein) the recurrence reduction was substantial (RR 0.67 [0.08]). In ER-positive disease, the RR was approximately independent of progesterone receptor status (or level), age, nodal status, or use of chemotherapy. Breast cancer mortality was reduced by about a third throughout the first 15 years (RR 0.71 [0.05] during years 0–4, 0.66 [0.05] during years 5–9, and 0.68 [0.08] during years 10–14; p&lt;0.0001 for extra mortality reduction during each separate time period). Overall non-breast-cancer mortality was little affected, despite small absolute increases in thromboembolic and uterine cancer mortality (both only in</p>

			women older than 55 years), so all-cause mortality was substantially reduced. In ER-negative disease, tamoxifen had little or no effect on breast cancer recurrence or mortality.
Khoshnoud, Breast Cancer Research and Treatment, 2011 <sup>6</sup>	Immunohistochemistry compared to cytosol assays for determination of estrogen receptor and prediction of the long-term effect of adjuvant tamoxifen	The Stockholm Breast Cancer Study Group conducted a randomized trial during 1976 through 1990 comparing adjuvant tamoxifen versus control. The patients were stratified according to tumor size and lymph node status in high-risk and low-risk groups. In this study we evaluated 683 patients with “low risk” breast cancer (size B30 mm, lymph node negative) for whom ER status had been determined by both the cytosol assays and IHC at one pathology laboratory.	The median follow-up was 17 years. Six hundred eighty-three patients had tumors with ER determined by both methods, 536 (78.5%) were ER-positive by cytosol assays using the cutoff level at C0.05 fmol/lg DNA and 539 patients were ER-positive (79%) by IHC using the cutoff level at C10% cell stained. Thirty-nine tumors (5.7%) were ER-positive by cytosol but not by IHC, whereas the opposite pattern was found for 42 cases (6.1%). Only seven tumors had stained cells between 0 and 9% by IHC. The concordance between IHC and cytosol assays was high (88%). The kappa statistic was 0.65, 95% CI 0.58–0.72. Among patients classified as ER-negative no therapeutic benefit from tamoxifen was observed. Among patients with ER-expressing tumors, tamoxifen resulted in significantly better recurrence-free survival irrespective of the method (IHC: HR, 0.53, P<0.001; cytosol: HR, 0.53, P<0.001). The effect on overall survival was not statistically significant probably due to the limited sample size.

<p>Kim, JCO, 2011<sup>7</sup></p>	<p>Estrogen Receptor (ESR1) mRNA Expression and Benefit From Tamoxifen in the Treatment and Prevention of Estrogen Receptor-Positive Breast Cancer</p>	<p>We performed gene expression profiling of paraffin-embedded tumors from National Surgical Adjuvant Breast and Bowel Project (NSABP) trials that tested the worth of tamoxifen as an adjuvant systemic therapy (B-14) and as a preventive agent (P-1). This was a retrospective subset analysis based on available materials.</p>	<p>In B-14, ESR1 was the strongest linear predictor of tamoxifen benefit among 16 genes examined, including PGR and ERBB2. On the basis of these data, we hypothesized that, in the P-1 trial, a lower level of ESR1 mRNA in the tamoxifen arm was the main difference between the two study arms. Only ESR1 was downregulated by more than two-fold in ER-positive cancer events in the tamoxifen arm (P &lt; .001). Tamoxifen did not prevent ER-positive tumors with low levels of ESR1 expression.</p>
<p>Eljertsen, Ann Oncol, 2011<sup>8</sup></p>	<p>Prognostic and predictive role of ESR1 status for postmenopausal patients with endocrine-responsive early breast cancer in the Danish cohort of the BIG 1-98 trial</p>	<p>ESR1 was assessed in 1129 (81%) of 1396 postmenopausal Danish women with early breast cancer randomly assigned to receive 5 years of letrozole, tamoxifen or a sequence of these agents in the Breast International Group 1-98 trial and who had ER <math>\geq</math> 1% after central review.</p>	<p>By FISH, 13.6% of patients had an ESR1-to-Centromere-6 (CEN-6) ratio <math>\geq</math> 2 (amplified), and 4.2% had ESR1-to-CEN-6 ratio &lt;0.8 (deleted). Deletion of ESR1 was associated with significantly lower levels of ER (P &lt; 0.0001) and PgR (P = 0.02) and more frequent HER2 amplification. ESR1 deletion or amplification was associated with higher-Ki-67 than ESR1-normal tumors. Overall, there was no evidence of heterogeneity of disease-free survival (DFS) or in treatment effect according to ESR1 status. However, significant differences in DFS were observed for subsets based on a combination of ESR1 and HER2 status (P = 0.02).</p>

Dowsett, JCO, 2008 <sup>9</sup>	Relationship between quantitative estrogen and progesterone receptor expression and human epidermal growth factor receptor 2 (HER-2) status with recurrence in the Arimidex, Tamoxifen, Alone or in Combination trial	Formalin-fixed, paraffin-embedded tumor blocks were retrospectively collected from patients in the monotherapy arms of the Arimidex, Tamoxifen Alone or in Combination (ATAC) trial and centrally tested for ER, PgR and HER-2. ER and PgR were scored using continuous scales and HER-2 was scored as 0 to 3+ with 2+ cases being analyzed by fluorescence in situ hybridization.	Blocks were collected from 2,006 of 5,880 eligible patients. Tissue was assessable and ER and/or PgR positivity confirmed centrally in 1,782 cases. In these, TTR was longer for anastrozole than for tamoxifen by a similar extent to that in the overall trial. None of the three biomarkers identified a set of patients with differential benefit from anastrozole over tamoxifen. Patients with low ER, low PgR, and high HER-2 expression had a poorer prognosis with either drug. Only 2.6% of patients in the highest quartile of PgR experienced recurrence after 5 years, compared with 13.2% in the lowest quartile.
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#### Low ER Positivity

First Author, Journal, Year	Title	Study Design	Conclusions
Landmann, Am J Clin Pathol, 2018 <sup>23</sup>	Low Estrogen Receptor (ER)-Positive Breast Cancer and Neoadjuvant Systemic Chemotherapy: Is Response Similar to Typical ER-Positive or ER-Negative Disease?	Human epidermal growth factor receptor 2-positive cases, cases without semiquantitative ER score, and patients treated with neoadjuvant endocrine therapy alone were excluded.	The pCR rate of low ER+ tumors was similar to the pCR rate of ER- tumors (37% and 26% for low ER and ER- respectively, P = .1722) but significantly different from the pCR rate of moderately ER+ (11%, P = .049) and high ER+ tumors (4%, P < .001). Patients with pCR had an excellent prognosis regardless of the ER status. In patients with residual disease (no pCR), the recurrence and death rate were higher in ER- and low ER+ cases compared with moderate and high ER+ cases.



Chen, Clinical Breast Cancer, 2017 <sup>24</sup>	Borderline ER-Positive Primary Breast Cancer Gains No Significant Survival Benefit From Endocrine Therapy: A Systematic Review and Meta-Analysis	We aimed at investigating differences in endocrine responsiveness, prognosis, and clinicopathological characteristics between the ER+ (1%-9%) cohort and the ER- cohort or ER+ (≥10%) cohort. Eligible literature published from inception to November 20, 2016 was retrieved from the PubMed database on the basis of Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. Data on survival outcomes were extracted and pooled odds ratios (ORs), 95% confidence intervals (CIs), and 2-tailed P values are reported. P values of the c2 test for comparison of clinicopathological characteristics among included patients in the ER+ (1%-9%) cohort and the other 2 cohorts were calculated respectively.	The analysis included 6 studies with 16,606 patients. Significant differences were detected between the ER+ (1%-9%) cohort and the other 2 cohorts on the basis of clinicopathological characteristics respectively. When taking all of the patients into analysis without consideration of treatment modality, the ER+ (1%-9%) cohort presented better prognosis than the ER- group in terms of 5-year disease-free survival (OR, 1.47; P = .046) and 5-year overall survival (OR, 1.23; P = .046). However, patients with ER+ (1%-9%) breast cancer who received endocrine therapy seemed to have a prognosis similar to those without any endocrine therapy (P = .684) and those with ER-carcinoma who received endocrine therapy (P = .145). Patients with ER+ (≥10%) tumors had better endocrine responsiveness compared with their ER+ (1%-9%) counterparts (OR, 0.52; P = .034, ER+ [1%-9%] vs. ER+ [≥10%]).
Zhang, Histopathology, 2014 <sup>25</sup>	Pathological features and clinical outcomes of breast cancer according to levels of oestrogen receptor expression	Analyzed clinicopathological features in five subgroups based on ER expression levels in 1700 consecutive invasive breast cancer patients diagnosed and treated at our institution between 2000 and 2011.	Of the cases, 24% had ER expression <1%, 2% were ER 1–10%, 5% were 11–50%, 5% were 51–70% and 64% were 71–100%. We observed four subgroups of patient cohorts (ER <1%, 1–10%, 11–70% and 71–100%) that were unique in Nottingham grade, nuclear grade, progesterone receptor expression and disease-free survival. Of the 341 patients with follow-up data, we found no significant differences in pathological features between patients in the ER 11–50% and ER 51–70% subgroups.

<p>Gloyeske, AJCP, 2014<sup>26</sup></p>	<p>Low ER+ Breast Cancer Is This a Distinct Group?</p>	<p>Forty-nine ER+/HER2- invasive tumors with low ER expression (H-scores of 1-100, representing approximately 5% of all tumors) were studied for various morphologic parameters, progesterone receptor (PR), and Ki-67 IHC.</p>	<p>Eighteen of 49 patients received neoadjuvant chemotherapy. The morphologic analysis showed that these tumors are often grade 3 and frequently demonstrate a sheet-like growth pattern, an intratumoral lymphocytic inflammatory infiltrate, and necrosis. Eighty percent of tumors showed a Ki-67 proliferation index of more than 50%, and 94% were PR-. Of the 18 patients who received neoadjuvant chemotherapy, six (33%) achieved pathologic complete response.</p>
<p>Balduzzi, Clinical Breast Cancer, 2014<sup>27</sup></p>	<p>Survival Outcomes in Breast Cancer Patients With Low Estrogen/Progesterone Receptor Expression</p>	<p>We retrospectively analyzed 1424 consecutive patients with HER2/neu-negative and low endocrine receptors expression early breast cancer, submitted to surgery at the European Institute of Oncology between January 1995 and December 2009. Patients were classified according to the percentage of ER/PgR expression using immunohistochemistry. Group 1 with ER/PgR &lt; 1%, and group 2 with ER/PgR 1% to 10%.</p>	<p>Group 1 (ER/PgR &lt; 1%) included 1300 patients, and group 2 (ER/PgR 1%-10%) 124 patients. Median follow-up time was 74 months (range, 3-192 months). The 5-year disease-free survival (DFS) rate was 74% (95% confidence interval [CI], 72%-77%) for group 1, and 79% (95% CI, 70%-86%) for group 2 (P = .16). The 5-year overall survival (OS) rate was 86% (95% CI, 84%-88%) in group 1 and 90% (95% CI, 83%-95%) in group 2 (P = .13). In patients without lymph node involvement, the 5-year OS rate was 92% (95% CI, 89.5%-93.6%) for group 1 and 98% (95% CI, 90.2%-99.8%) for group 2 (P = .061). One hundred ten patients received endocrine therapy with no significant effect on DFS (P = .36) and OS (P = .30).</p>

<p>Yi, Annals of Oncology, 2014<sup>28</sup></p>	<p>Which threshold for ER positivity? a retrospective study based on 9639 patients</p>	<p>The study population consisted of patients with primary breast carcinoma treated at our center from January 1990 to December 2011 and whose records included complete data on ER status. Patients were separated into three groups: ≥10% positive staining for ER (ER-positive ≥10%), 1%–9% positive staining for ER (ER-positive 1%–9%) and &lt;1% positive staining (ER-negative).</p>	<p>Of 9639 patients included, 80.5% had tumors that were ER-positive ≥10%, 2.6% had tumors that were ER-positive 1%–9% and 16.9% had tumors that were ER-negative. Patients with ER-positive 1%–9% tumors were younger with more advanced disease compared with patients with ER-positive ≥10% tumors. At a median follow-up of 5.1 years, patients with ER-positive 1%–9% tumors had worse survival rates than did patients with ER-positive ≥10% tumors, with and without adjustment for clinical stage and grade. Survival rates did not differ significantly between patients with ER-positive 1%–9% and ER-negative tumors.</p>
<p>Deyarmin, Annals of Surgical Oncology, 2013<sup>29</sup></p>	<p>Effect of ASCO/CAP Guidelines for Determining ER Status on Molecular Subtype</p>	<p>Clinicopathological characteristics were compared between ER-negative, ER-positive, and low-ER staining (1–10 %) tumors using chi-square analysis with P&lt;0.05 defining statistical significance. Gene expression data were generated for 26 low-ER-staining tumors, and their intrinsic subtype determined. Immunohistochemistry (IHC)-defined surrogate subtypes, using the threshold of positivity defined by ASCO/CAP guidelines, were compared with molecular subtypes.</p>	<p>Low-ER-staining tumors were clinicopathologically more similar to ER-negative than to ER-positive tumors; 88 % of low-staining tumors were basal like or HER2 enriched. Only those tumors expressing 10 % ER-positive cells were classified as luminal A subtype.</p>
<p>Reisenbichler, AJCP, 2013<sup>30</sup></p>	<p>Interobserver Concordance in Implementing the 2010 ASCO/CAP Recommendations for Reporting ER in Breast Carcinomas</p>	<p>We report interobserver concordance manually measuring ER in 264 breast cancers using ER-SP1 and 1D5 and 2 scoring methods (H-score and Allred score).</p>	<p>With both antibodies, 3% to 4% of cases have a low level of ER expression (1%-10%), more than previously reported (&lt;1%). We find a high level of paired observer concordance with both antibodies and scoring methods (k = 0.892-0.943) with no significant difference with method of scoring. Despite excellent concordance, positive/negative</p>

			discordance was almost 5% among 3 observers using either antibody, an underappreciated clinically significant rate.
Iwamoto, JCO, 2012 <sup>31</sup>	Estrogen Receptor (ER) mRNA and ER-Related Gene Expression in Breast Cancers That Are 1% to 10% ER-Positive by Immunohistochemistry	ER status was determined by IHC in 465 primary breast cancers and with the Affymetrix U133A gene chip. We compared expressions of ESR1 mRNA and a 106-probe set ER-associated gene signature score between ER-negative (n = 183), 1% to 9% (n = 25), 10% (n = 6), and more than 10% (n = 251) ER-positive cancers. We also assessed the molecular class by using the PAM50 classifier and plotted survival by ER status.	Among the 1% to 9%, 10%, and more than 10% ER IHC-positive patients, 24%, 67%, and 92% were also positive by ESR1 mRNA expression. The average ESR1 expression was significantly higher in the ≥ 10% ER-positive cohorts compared with the 1% to 9% or ER-negative cohort. The average ER gene signature scores were similar for the ER-negative and 1% to 9% IHC-positive patients and were significantly lower than in ≥ 10% ER-positive patients. Among the 1% to 9% ER-positive patients, 8% were luminal B and 48% were basal-like; among the 10% ER-positive patients, 50% were luminal. The overall survival rate of 1% to 9% ER-positive patients with cancer was between those of patients in the ≥ 10% ER-positive and ER-negative groups.
Raghav, Cancer, 2012 <sup>32</sup>	Impact of Low Estrogen/Progesterone Receptor Expression on Survival Outcomes in Breast Cancers Previously Classified as Triple Negative Breast Cancers	In a retrospective review, 1257 patients were categorized according their ER/PR percentages into 3 groups, ER/PR <1% (group A), ER/PR 1% to 5% (group B), and ER/PR 6% to 10% (group C). Kaplan-Meier product limit method was used to estimate survival outcomes. Cox proportional hazards models were used to adjust for patient and tumor characteristics.	Groups A, B, and C had 897 (71.4%), 241 (19.2%), and 119 (9.4%) patients, respectively. After a median follow-up of 40 months there was no significant difference in 3-year recurrence-free survival (RFS): 64%, 67%, and 77% (P = .34) or overall survival (OS): 79%, 81%, and 88% (P = .33) for groups A, B, and C, respectively. ER/PR expression was not an independent predictor for RFS (hazard ratio [HR], 1.10; 95% confidence interval [CI], 0.86-1.39; P = .46 for group B, and HR, 0.96; 95% CI, 0.66-1.38; P = .81 for group C, compared with group A), or OS (HR, 1.11; 95% CI, 0.84-1.46; P =

			.46 for group B, and HR, 0.94; 95% CI, 0.63-1.42; P = .78 for group C, compared with group A). Endocrine therapy had no impact on survival outcomes (RFS: P = .10; OS: P = .45) among groups.
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ER-/PgR+ or ER+/PgR-

First Author, Journal, Year	Title	Study Design	Conclusions
Kuroda, Breast Cancer, 2019 <sup>33</sup>	Oestrogen receptor-negative/progesterone receptor-positive phenotype of invasive breast carcinoma in Japan: re-evaluated using immunohistochemical staining	We selected patients who underwent surgery for primary breast carcinoma from our databases at Dokkyo Medical University Hospital and Kameda General Hospital. Among the 9844 patients, the largest series in Japan, 27 (0.3%) were initially diagnosed as ER-/PgR+ breast carcinomas and we re-evaluated by IHC.	The re-evaluated IHC showed that of the 27 patients with the initial results of ER-/PgR+, 12 were ER+/PgR+, 8 were ER-/PgR-, and 7 were ER-/PgR+. ER was negative in 12 of 27 patients (44.4%), and PgR was positive in 8 of 27 patients (29.6%). In our seven re-evaluated and confirmed as ER-/PgR+ cases, the staining proportions of tumor cells were 0% in ER and 1-69% (average 15.8%) in PgR. The average staining proportion of PgR in the re-evaluated ER-/PgR+ phenotype was lower than the initial diagnosis. Histological grading was as follows: grade I, one case; grade II, two cases; grade III, four cases. There were two lymph-node-positive cases.
Foley, Pathol Oncol Res, 2018 <sup>34</sup>	Re-Appraisal of Estrogen Receptor Negative/Progesterone Receptor Positive (ER-/PR+) Breast Cancer Phenotype: True Subtype or Technical Artefact?	The aim of this study was to investigate the true incidence and clinico-pathological features of ER-/PR+ breast cancers in a tertiary referral symptomatic breast unit. Clinico-pathological data were collected on invasive breast cancers diagnosed between 1995 and 2005. IHC for ER and PR receptors was repeated on all cases which were ER-/PR+, with the same paraffin block used for the initial diagnostic testing. Concordance between the diagnostic	Complete data, including ER and PR status were available for 697 patients diagnosed during the study period. On diagnostic IHC, the immunophenotype of the breast tumors was: ER+/PR+ in 396 (57%), ER-/PR- in 157 (23%), ER+/PR- in 88 (12%) and ER-/PR+ in 56 (8.6%) patients. On repeat IHC of 48/56 ER-/PR+ tumors 45.8% were ER+/PR+, 6% were ER+/PR- and 43.7% were ER-/PR-. None of the cases were confirmed to be ER-/PR+. The ER-/PR+ phenotypic breast cancer is likely to be the result of technical artefact.

		and repeat IHC was determined using validated testing.	
Ahmed, J Clin Pathol, 2017 <sup>35</sup>	Clinicopathological characteristics of oestrogen receptor negative, progesterone receptor positive breast cancers: re-evaluating subsets within this group	We investigated 267 archival documented ER(-)/PR(+) BCs diagnosed between January 1994 and July 2009. Histological slides were retrieved and reviewed. Tissue microarrays were constructed by selecting two 1 mm cores of tumour per case. Repeat immunohistochemistry was performed for confirmation of the ER(-)/PR(+) status. Clinicopathological parameters including age, ethnicity, tumour size, histological grade, histological subtype, associated ductal carcinoma in situ, lymphovascular invasion and lymph node status were evaluated.	On repeat immunohistochemistry, 92 tumors were confirmed as ER(-)/PR(+) BCs. This phenotype accounted for 1.1% of all BC phenotypes and exhibited different clinicopathological features and survival outcome when compared with other phenotypes. ER(-)/PR(+) tumors showed a trend for an early recurrence and poorer overall survival as compared with the patients with ER(+)/PR(+) tumors and similar to ER(-)/PR(-) tumors.
Bae, BMC Cancer, 2015 <sup>36</sup>	Poor prognosis of single hormone receptor- positive breast cancer: similar outcome as triple-negative breast cancer	We examined the clinical and biological features of 6,980 women with invasive ductal carcinoma, and these patients were stratified according to ER and PR expression as double HR+ (ER + PR+), single HR+ (ER + PR- and ER-PR+) and double HR-negative (HR-, ER-PR-) tumors.	In this study, 571 (8.2%) cases were single HR+ tumors, of which 90 (1.3%) were ER-PR+ tumors and 481 (6.9%) were ER + PR- tumors. Our multivariate analysis showed that in patients without HER2 overexpression ER + PR- tumors were associated with an increased risk of recurrence and death compared with ER + PR+ tumors, with a hazard ratio of 2.12 for disease-free survival (DFS) and 4.79 for overall survival (OS). In patients without HER2 overexpression ER-PR+ tumors had increased risk of recurrence and death compared with ER + PR+ tumor, with a hazard ratio of 4.19 for DFS and 7.22 for OS. In contrast, in patients with HER2 overexpression, the difference in survival between single HR+ tumors and double HR+ HR- tumors was not statistically significant. In patients

			without HER2 overexpression the DFS and OS of ER + PR- and ER-PR+ tumors were not significantly different from those of ER-PR- tumors.
Knoop, Eur J Cancer, 2014 <sup>37</sup>	Estrogen receptor, Progesterone receptor, HER2 status and Ki67 index and responsiveness to adjuvant tamoxifen in postmenopausal high-risk breast cancer patients enrolled in the DBCG 77C trial	Between 1977 and 1982, 1716 postmenopausal patients with tumours larger than 5cm or positive axillary nodes were randomly assigned to no systemic therapy or tamoxifen 30mg daily for one year. Archival tumour tissue from 1515 patients was analysed and the hormone receptor positive (estrogen receptor (ER) and/or progesterone receptor (PR)) cancers were defined as luminal A if Ki67 low and HER2-negative; as luminal B if Ki67 high or HER2-positive; and otherwise as non-luminal-HER2 positive or triple negative.	In the intent-to-treat (ITT) population one year of tamoxifen improved the disease-free-survival (DFS) (hazard ratio (HR)=0.87; 95% confidence interval (CI) 0.77-0.98), the Breast Cancer Recurrence Rate (BCRR) (HR=0.79; 0.69-0.90) and reduced the breast-cancer-specific-mortality (BCM) (HR=0.83; 0.73-0.93). BCRR were improved significantly by tamoxifen in luminal A (HR=0.66; 0.53-0.84) and luminal B/HER2- (HR=0.54; 0.39-0.74) but not in the other subsets, and with similar results for BCM with 30 years follow-up.
Cserni, Pathol Oncol Res, 2011 <sup>38</sup>	Estrogen receptor negative and progesterone receptor positive breast carcinomas-how frequent are they?	The authors were asked to collect 500 to 1,000 breast carcinoma cases with ER and PR status from institutional databases of 8 Hungarian pathology or related oncology departments. These were classified according to their receptor statuses and the ER-PR+ cases were looked at again.	A total of 205/6587 (3.1%; range of the rate per department: 0.3–7.1%.) cases reported to have the ER-negative and PR-positive status by immunohistochemistry were collected from 9 Hungarian departments. After careful reevaluation of the tumor slides and control tissues with a 1% cut-off for positivity and restaining of the questionable cases, all but 1 of the reevaluable 182 cases changed their original phenotype. Most cases converted to dual positives (n=124) or dual negatives (n=31) or unassessable / questionable.
Albert, Cancer, 2011 <sup>39</sup>	Patients With Only 1 Positive Hormone Receptor Have Increased Locoregional Recurrence Compared With Patients With Estrogen Receptor-Positive Progesterone Receptor-	The authors retrospectively reviewed records of 635 patients with T1a,bN0 disease who received definitive treatment at their institution between 1997 and 2002 and had archival tissue blocks for prospective assessment of	LR recurrence rates were higher in patients with 1 receptor positive compared with ER $\beta$ /PR $\beta$ (7-year rate: 8.8% vs 2.5%, P = .024). There was no difference between the 2 groups in the rates of distant metastasis (DM) (P

	Positive Disease in Very Early Stage Breast Cancer	ER/PR expression. They compared clinical outcomes of the 479 patients with ER+/PR+ disease to the 156 patients with ER+/PR- or ER-/PR+ disease.	= .531) or overall survival (P = .491). One positive receptor predicted for LR recurrence in patients who did not receive hormonal therapy (P = .046), but not in patients who received hormonal therapy (P = .296). On multivariate analysis, 1 positive receptor predicted for LR recurrence in the overall group (hazard ratio, 2.81; 95% confidence interval, 1.06-7.48; P = .038).
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PgR Testing

First Author, Journal, Year	Title	Study Design	Conclusions
Ahn, Endocrine-Related Cancer, 2019 <sup>40</sup>	Low PR in ER(+)/HER2(-) breast cancer: high rates of TP53 mutation and high SUV	This study included 272 patients surgically treated for ER-positive, HER2-negative breast cancer and who had undergone TP53 gene sequencing. Of these, 229 patients also underwent 18F-FDG PET or PET/CT. Mutational analysis of exons 5–9 of the TP53 gene was conducted using PCR amplification and direct sequencing. The SUVs were measured using 18F-FDG-PET scan images.	Twenty-eight (10.3%) tumors had a somatic TP53 mutation. The TP53 mutation rate was significantly higher in low-PR tumors than in high-PR tumors (17.1% vs 7.9%, P = 0.039). Low-PR tumors had significantly higher median SUVs than high-PR tumors (P = 0.046). The multivariable analysis revealed that SUV and age remained independent variables associated with low PR expression. An adverse impact of low PR expression on recurrence-free survival was observed in the multivariable Cox regression hazard model.



Prat, JCO, 2012	Prognostic Significance of Progesterone Receptor–Positive Tumor Cells Within Immunohistochemically Defined Luminal A Breast Cancer	Gene expression and pathologic features were collected from primary tumors across five independent cohorts: British Columbia Cancer Agency (BCCA) tamoxifen-treated only, Grupo Espanol de Investigacion en Cancer de Mama 9906 trial, BCCA no systemic treatment cohort, PAM50 microarray training data set, and a combined publicly available microarray data set. Optimal cutoffs of percentage of progesterone receptor (PR) –positive tumor cells to predict survival were derived and independently tested. Multivariable Cox models were used to test the prognostic significance.	Clinicopathologic comparisons among luminal A and B subtypes consistently identified higher rates of PR positivity, human epidermal growth factor receptor 2 (HER2) negativity, and histologic grade 1 in luminal A tumors. Quantitative PR gene and protein expression were also found to be significantly higher in luminal A tumors. An empiric cutoff of more than 20% of PR-positive tumor cells was statistically chosen and proved significant for predicting survival differences within IHC-defined luminal A tumors independently of endocrine therapy administration. Finally, no additional prognostic value within hormonal receptor (HR) –positive/HER2-negative disease was observed with the use of the IHC4 score when intrinsic IHC-based subtypes were used that included the more than 20% PR-positive tumor cells and vice versa.
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#### Tissue Microarray

First Author, Journal, Year	Title	Study Design	Conclusions
Viale, Breast Cancer Res Treat, 2017 <sup>41</sup>	Immunohistochemical versus molecular (BluePrint and MammaPrint) subtyping of breast carcinoma. Outcome results from the EORTC 10041/BIG 3-04 MINDACT trial	MS classified patients in the following subtypes: Luminal A, Luminal B, HER-2-, and Basal-type. IHC/FISH for pathological subtyping (ER, PgR, HER-2, and Ki67) was centrally assessed in the European Institute of Oncology (n = 5806). Hazard ratios for distant-metastasis-free survival (DMFS) by subtype were adjusted for chemotherapy and endocrine therapy administration and thus independent of adjuvant treatment allocation.	PS Luminal cancers classified as HER-2+ or Basal-type by MS did not have a significantly lower DMFS than the Luminal-type cancers by MS (95.9%): HR = 1.40, 95% CI 0.75-2.60 (p = 0.294). More patients were identified with Luminal A disease by MS (63%) as compared with PS (47%) with comparable 5-year DMFS (>/=96.0%). Among the 500 patients with PS TN cancers, MS identified 24 (5%) patients as Luminal-type with 5-year DMFS estimated at 100% versus 71.4%

			for MS HER-2+ or 90.1% for MS Basal-type.
Zarella, Laboratory Investigation, 2016 <sup>42</sup>	Automated measurement of estrogen receptor in breast cancer: a comparison of fluorescent and chromogenic methods of measurement	Here, we compare three methods of ER detection and assessment on two retrospective tissue microarray (TMA) cohorts of breast cancer patients: estimates of percent nuclei positive by pathologists and by Aperio's nuclear algorithm (standard chromogenic immunostaining), and immunofluorescence as quantified with the automated quantitative analysis (AQUA) method of quantitative immunofluorescence (QIF).	Reproducibility was excellent (R240.95) between users for both automated analysis methods, and the Aperio and QIF scoring results were also highly correlated, despite the different detection systems. The subjective readings show lower levels of reproducibility and a discontinuous, bimodal distribution of scores not seen by either mechanized method. Kaplan–Meier analysis of 10-year disease-free survival was significant for each method (Pathologist, P= 0.0019; Aperio, P= 0.0053, AQUA, P= 0.0026); however, there were discrepancies in patient classification in 19 out of 233 cases analyzed. Out of these, 11 were visually positive by both chromogenic and fluorescent detection. In 10 cases, the Aperio nuclear algorithm labeled the nuclei as negative; in 1 case, the AQUA score was just under the cutoff for positivity (determined by an Index TMA). In contrast, 8 out of 19 discrepant cases had clear nuclear positivity by fluorescence that was unable to be visualized by chromogenic detection, perhaps because of low positivity masked by the hematoxylin counterstain.
Viale, Breast Cancer Research and Treatment, 2016 <sup>43</sup>	Discordant assessment of tumor biomarkers by histopathological and molecular assays in the EORTC randomized controlled 10041/BIG 03-04 MINDACT trial breast cancer : Intratumoral heterogeneity and DCIS or normal tissue components are	The purpose of this preplanned translational research is to investigate the correlation of central IHC/FISH assessments with microarray mRNA readouts of ER, PgR, and HER-2 status in the MINDACT trial and to determine if any discordance could be attributed to intratumoral heterogeneity or the	Gene-expression data were obtained by TargetPrint; IHC and/or FISH were assessed centrally (n = 5788; 86 %). Macroscopic and microscopic evaluation of centrally submitted FFPE blocks identified 1427 cases for which the very same sample was submitted for gene-expression analysis.

	unlikely to be the cause of discordance	DCIS and normal tissue components in the specimens. MINDACT is an international, prospective, randomized, phase III trial investigating the clinical utility of MammaPrint in selecting patients with early breast cancer for adjuvant chemotherapy (n = 6694 patients).	TargetPrint ER had a positive agreement of 98 %, and a negative agreement of 95 % with central pathology. Corresponding figures for PgR were 85 and 94 % and for HER-2 72 and 99 %. Agreement of mRNA versus central protein was not different when the same or a different portion of the tumor tissue was analyzed or when DCIS and/or normal tissue was included in the sample subjected to mRNA assays.
Wesseling, Virchows Arch, 2016 <sup>44</sup>	An international study comparing conventional versus mRNA level testing (TargetPrint) for ER, PR, and HER2 status of breast cancer	To compare results from messenger RNA (mRNA)-based TargetPrint testing with those from immunohistochemistry (IHC) and in situ hybridization (ISH) conducted according to local standard procedures at hospitals worldwide. Tumor samples were prospectively obtained from 806 patients at 22 hospitals. The mRNA level of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) was assessed by TargetPrint quantitative gene expression readouts. IHC/ISH assessments were performed according to local standards at the participating hospitals.	TargetPrint readout showed a high concordance with IHC/ISH of 95 % (kappa 0.81) for ER, 81 % (kappa 0.56) for PR, and 94 % (kappa 0.76) for HER2. The positive/negative agreement between TargetPrint and IHC for ER, PR, and HER2 was 96 %/87 %, 84 %/74 %, and 74 %/98 %, respectively. The concordance rate in IHC/ISH results between hospitals varied: 88-100 % for ER (kappa 0.50-1.00); 50-100 % for PR (kappa 0.20-1.00); and 90-100 % for HER2 (kappa 0.59-1.00). mRNA readout of ER, PR, and HER2 status by TargetPrint was largely comparable to local IHC/ISH analysis. However, there was substantial discordance in IHC/ISH results between different hospitals.
Dekker, Breast Cancer Research and Treatment, 2015 <sup>45</sup>	Quality assessment of estrogen receptor and progesterone receptor testing in breast cancer using a tissue microarray-based approach	Formalin-fixed paraffin-embedded (FFPE) tumor blocks were collected for TMA construction from nine laboratories in the Netherlands. The tissue blocks contained invasive breast carcinomas that were previously tested for ER, PR, and/or HER2 expression by immunohistochemistry as part of routine pathological diagnostics.	When a discordant result was found between the local and TMA result, the original testing slide was revised and staining was repeated on a whole-tissue block. Sensitivity and specificity of individual laboratories for testing estrogen receptor expression were high, with an overall sensitivity of 99.7 and 95.4 %, respectively. Overall sensitivity and specificity of

			<p>progesterone receptor testing were 94.8 and 92.6 %, respectively. Out of 96 discordant cases, 36 cases would have been concordant if the recommended cut-off value of 1 % instead of 10 % was followed. Overall sensitivity and specificity of estrogen and progesterone receptor testing were high among participating laboratories.</p>
<p>Viale, Annals of Oncology, 2014<sup>46</sup></p>	<p>High concordance of protein (by IHC), gene (by FISH; HER2 only), and microarray readout (by TargetPrint) of ER, PgR, and HER2: results from the EORTC 10041/BIG 03-04 MINDACT trial</p>	<p>Data from local (N = 800) and central (N = 626) assessments of receptor status were collected and compared with TargetPrint results.</p>	<p>For ER, the positive agreement (the percentage of central pathology positive assessments that were also TargetPrint/local laboratory positive) for TargetPrint in comparison to centralized assessment was 98% with a negative agreement (the percentage of central pathology negative assessments that were also TargetPrint/local laboratory negative) of 96%. For PgR, the positive agreement was 83% with a negative agreement of 92%. For HER2, the positive agreement was 75% with a negative agreement of 99%. Even though the local assessment showed higher positive agreement for PgR (89%) and higher positive agreement for HER2 (85%), the range of discordant local versus central assessments were as high as 6.7% for ER, 12.9% for PgR, and 4.3% for HER2.</p>
<p>Karn, Breast Cancer Research and Treatment, 2010<sup>47</sup></p>	<p>Data driven derivation of cutoffs from a pool of 3,030 Affymetrix arrays to stratify distinct clinical types of breast cancer</p>	<p>We have analyzed influences of these strategies using a pool of 3,030 Affymetrix U133A microarrays from breast cancer samples. We present data on the resulting concordance with biochemical assays of well known parameters and highlight critical pitfalls. We further propose a method for the inference of cutoff values</p>	<p>The cutoffs derived by this method displayed high specificity and sensitivity. Markers with a bimodal distribution like ER, PgR, and HER2 discriminate different biological subtypes of disease with distinct clinical courses. In contrast, markers displaying a continuous distribution like proliferation markers as Ki67</p>

		directly from the data without prior knowledge of the true result.	rather describe the composition of the mixture of cells in the tumor.
Bordeaux, PLoS One, 2012 <sup>48</sup>	Quantitative In Situ Measurement of Estrogen Receptor mRNA Predicts Response to Tamoxifen	Messenger RNA for ER (ESR1) and Ubiquitin C (Ubc) were visualized using RNAscope probes and levels were quantified by quantitative in situ hybridization (qISH) on two Yale breast cancer cohorts on tissue microarrays. ESR1 levels were compared to ER protein levels measured by QIF using the SP1 antibody.	ESR1 mRNA is reproducibly and specifically measurable by qISH on tissue collected from 1993 or later. ESR1 levels were correlated to ER protein levels in a non-linear manner on two Yale cohorts. High levels of ESR1 were found to be predictive of response to tamoxifen.
Welsh, JCO, 2011 <sup>49</sup>	Standardization of Estrogen Receptor Measurement in Breast Cancer Suggests False-Negative Results Are a Function of Threshold Intensity Rather Than Percentage of Positive Cells	An assay was developed to quantify ER by using a control tissue microarray (TMA) and a series of cell lines in which ER immunoreactivity was analyzed by quantitative immunoblotting in parallel with the automated quantitative analysis (AQUA) method of quantitative immunofluorescence (QIF). The assay was used to assess the ER protein expression threshold in two independent retrospective cohorts from Yale and was compared with traditional methods.	Two methods of analysis showed that change in percentage of positive cells from 10% to 1% did not significantly affect the overall number of ER-positive patients. The standardized assay for ER on two Yale TMA cohorts showed that 67.9% and 82.5% of the patients were above the 2-pg/ $\mu$ g immunoreactivity threshold. We found 9.1% and 19.7% of the patients to be QIF-positive/IHC-negative, and 4.0% and 0.4% to be QIF-negative/IHC-positive for a total of 13.1% and 20.1% discrepant cases when compared with pathologists' judgment of threshold. Assessment of survival for both cohorts showed that patients who were QIF-positive/pathologist-negative had outcomes similar to those of patients who had positive results for both assays.

#### mRNA

First Author, Journal, Year	Title	Study Design	Conclusions
Wilson, Breast Cancer Res Treat, 2014 <sup>50</sup>	Development of a robust RNA-based classifier to accurately determine ER, PR, and HER2 status in breast cancer clinical samples	We developed a Random Forests-based algorithm using a training set of 158 samples with centrally confirmed IHC status, and subsequently validated	We observed a strong correlation between target mRNA expression and IHC assays for HER2 and ER, achieving an overall accuracy of 97 and 96 %,

		<p>this algorithm on multiple test sets with known, locally determined IHC status.</p>	<p>respectively. For determining PR status, which had the highest discordance between central and local IHC, incorporation of expression of co-regulated genes in a multivariate approach added predictive value, outperforming the single, target gene approach by a 10 % margin in overall accuracy.</p>
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RT-PCR

First Author, Journal, Year	Title	Study Design	Conclusions
<p>Cai, Breast Cancer Research and Treatment, 2018<sup>51</sup></p>	<p>A qualitative transcriptional signature to reclassify estrogen receptor status of breast cancer patients</p>	<p>From the gene pairs with significantly stable REOs in ER+ samples and reversely stable REOs in ER- samples, concordantly identified from four datasets, we extracted a signature to determine a sample's ER status through evaluating whether the REOs within the sample significantly match with the ER+ REOs or the ER- REOs.</p>	<p>A signature with 112 gene pairs was extracted. It was validated through evaluating whether the reclassified ER+ or ER- patients could benefit from tamoxifen therapy or neoadjuvant chemotherapy. In three datasets for IHC-determined ER+ patients treated with post-operative tamoxifen therapy, 11.6–12.4% patients were reclassified as ER- by the signature and, as expected, they had significantly worse recurrence-free survival than the ER+ patients confirmed by the signature. On another hand, in two datasets for IHC-determined ER- patients treated with neoadjuvant chemotherapy, 18.8 and 7.8% patients were reclassified as ER+ and, as expected, their pathological complete response rate was significantly lower than that of the other ER- patients confirmed by the signature .</p>
<p>Wu, Breast Cancer Research and Treatment, 2018<sup>52</sup></p>	<p>Comparison of central laboratory assessments of ER, PR, HER2, and Ki67 by IHC/FISH and the corresponding mRNAs (ESR1, PGR, ERBB2, and MKi67) by RT-qPCR on an automated, broadly deployed diagnostic platform</p>	<p>FFPE tissue sections from 523 patients were sent to a College of American Pathologists-certified central reference laboratory to evaluate concordance between IHC/FISH and STRAT4 using the laboratory's</p>	<p>Concordance between STRAT4 and IHC was 97.8% for ESR1, 90.4% for PGR, 93.3% for ERBB2 (IHC/FISH for HER2), and 78.6% for MKi67. Receiver operating characteristic curve (ROC) area under the curve (AUC) values of</p>

		standard of care methods. A subset of 155 FFPE specimens was tested for concordance with STRAT4 using different IHC antibodies and scoring methods.	0.99, 0.95, 0.99, and 0.85 were generated for ESR1, PGR, ERBB2, and MKI67, respectively. Minor variabilities were observed depending on the IHC antibody comparator used.
Hyeon, Journal of Breast Cancer, 2017 <sup>53</sup>	NanoString nCounter® Approach in Breast Cancer: A Comparative Analysis with Quantitative Real-Time Polymerase Chain Reaction, In Situ Hybridization, and Immunohistochemistry	Data on IHC/FISH results for ER, PR, and HER2 in 240 patients from a single tertiary hospital in Korea were collected and compared with NanoString nCounter® and qRT-PCR results at a single institution.	Expression levels for each gene using NanoString nCounter® showed good correlation with the corresponding data for protein expression by IHC ( $p < 0.001$ ) and gene amplification status for HER2 ( $p < 0.001$ ). Comparisons between gene expression and IHC data showed good overall agreement with a high area under the curve (AUC) for ESR1/ER (AUC=0.939), PgR/PR (AUC= 0.796), and HER2/HER2 (AUC=0.989) ( $p < 0.001$ ).
Varga, Breast Cancer Research, 2017 <sup>54</sup>	An international reproducibility study validating quantitative determination of ERBB2, ESR1, PGR, and MKI67 mRNA in breast cancer using MammaTyper®	Ten international pathology institutions participated in this study and determined messenger RNA expression levels of ERBB2, ESR1, PGR, and MKI67 in both centrally and locally extracted RNA from formalin-fixed, paraffin-embedded breast cancer specimens with the MammaTyper® test. Samples were measured repeatedly on different days within the local laboratories, and reproducibility was assessed by means of variance component analysis, Fleiss' kappa statistics, and interclass correlation coefficients (ICCs).	Total variations in measurements of centrally and locally prepared RNA extracts were comparable; therefore, statistical analyses were performed on the complete dataset. Intersite reproducibility showed total SDs between 0.21 and 0.44 for the quantitative single-marker assessments, resulting in ICC values of 0.980–0.998, demonstrating excellent agreement of quantitative measurements. Also, the reproducibility of binary single-marker results (positive/negative), as well as the molecular subtype agreement, was almost perfect with kappa values ranging from 0.90 to 1.00.
Wirtz, Breast Cancer Res Treat, 2016 <sup>55</sup>	Biological subtyping of early breast cancer: a study comparing RT-qPCR with immunohistochemistry	We compared RT-qPCR with IHC in the assessment of Ki-67 and other standard factors used in breast cancer subtyping. RNA was extracted from archival breast tumour tissue of 769 women randomly assigned to the	The results were correlated with distant disease-free survival (DDFS) and overall survival (OS). qPCR-based and IHC-based assessments of ER and PgR showed good concordance. Both low tumour MKI67 mRNA (RT-qPCR)

		<p>FinHer trial. Cancer ESR1, PGR, ERBB2 and MKI67 mRNA content was quantitated with an RT-qPCR assay. Local pathologists assessed ER, PgR and Ki-67 expression using IHC.</p>	<p>and Ki-67 protein (IHC) levels were prognostic for favourable DDFS (hazard ratio [HR]) 0.42, 95 % CI 0.25–0.71, P = 0.001; and HR 0.56, 0.37–0.84, P = 0.005, respectively) and OS. In multivariable analyses, cancer MKI67 mRNA content had independent influence on DDFS (adjusted HR 0.51, 95 % CI 0.29–0.89, P = 0.019) while Ki67 protein expression had not any influence (P = 0.266) whereas both assessments influenced independently OS. Luminal B patients treated with docetaxel-FEC had more favourable DDFS and OS than those treated with vinorelbine-FEC when the subtype was defined by RT-qPCR (for DDFS, HR 0.52, 95 % CI 0.29–0.94, P = 0.031), but not when defined using IHC. Breast cancer subtypes approximated with RT-qPCR and IHC show good concordance, but cancer MKI67 mRNA content correlated slightly better with DDFS than Ki-67 expression.</p>
<p>Sheffield, Breast Cancer Research and Treatment, 2016<sup>56</sup></p>	<p>Molecular subtype profiling of invasive breast cancers weakly positive for estrogen receptor</p>	<p>Consecutive cases of breast cancer treated by primary surgical resection were retrospectively identified from 4 centers that engage in routine external proficiency testing for breast biomarkers. ER-negative (Allred 0 and 2) and ER weakly positive (Allred 3–5) cases were included. Gene expression profiling was performed using qRT-PCR. Intrinsic subtype prediction was made based upon the PAM50 gene expression signature.</p>	<p>148 cases were included in the series: 60 cases originally diagnosed as ER weakly positive and 88 ER negative. Of the cases originally assessed as ER weakly positive, only 6 (10 %) were confirmed to be of luminal subtype by gene expression profiling; the remaining 90 % of cases were classified as basal-like or HER2-enriched subtypes. This was not significantly different than the fraction of luminal cases identified in the IHC ER-negative cohort (5 [5%] luminal, 83 [95%] nonluminal). Recurrence-free, and overall, survival rates were similar in both groups (p = 0.4 and 0.5, respectively) despite adjuvant</p>



			hormonal therapy prescribed in the majority (59 %) of weakly positive ER cases.
Laible, BMC Cancer, 2016 <sup>57</sup>	Technical validation of an RT-qPCR in vitro diagnostic test system for the determination of breast cancer molecular subtypes by quantification of ERBB2, ESR1, PGR and MKI67 mRNA levels from formalin fixed paraffin-embedded breast tumor specimens	Tumor RNA was extracted with the novel RNXtract RNA extraction kit. Synthetic RNA was used to assess the sensitivity of the RNXtract kit. DNA and RNA specific qPCR assays were used so as to determine analyte specificity of RNXtract. For the assessment of limit of blank, limit of detection, analytical measurement range and PCR efficiency of the MammaTyper kit serial dilutions of samples were used. Analytical precision studies of MammaTyper were built around two different real time PCR platforms and involved breast tumor samples belonging to different subtypes analyzed across multiple sites and under various stipulated conditions. The MammaTyper assay robustness was tested against RNA input variations, alternative extraction methods and tumor cell content.	Individual assays were linear up to at least 32.33 and 33.56 Cqs (quantification cycles) for the two qPCR platforms tested. PCR efficiency ranged from 99 to 109 %. In qPCR platform 1, estimates for assay specific inter-site standard deviations (SD) were between 0.14 and 0.20 Cqs accompanied by >94 % concordant single marker assignments for all four markers. In platform 2, the inter-site SD estimates were between 0.40 and 0.66 Cqs while the concordance for single marker assignments was >94 % for all four markers. The agreement reached between the two qPCR systems located in one site was 100 % for ERBB2, 96.9 % for ESR1, 97.2 % for PGR and 98.6 % for MKI67. RT-qPCR for individual markers was stable up to a 64-fold dilution for a typical clinical sample. There was no change in assay performance detected at the level of individual markers or subtypes after using different RNA isolation methods. The presence of up to 80 % of surrounding non-tumor tissue including in situ carcinoma did not affect the assay output. Sixteen out of 20 RNXtract eluates yielded more than 50 ng/μl of RNA (average RNA output: 233 ng/μl), whereas DNA contamination per sample was restricted to less than 15 ng/μl. Median recovery rate of RNA extraction was 91.0 %.

<p>Cheang, The Oncologist, 2015<sup>58</sup></p>	<p>Defining Breast Cancer Intrinsic Subtypes by Quantitative Receptor Expression</p>	<p>We merged 1,557 cases from three randomized phase III trials into a single data set. These breast tumors were centrally reviewed in each trial for quantitative ER, PR, and HER2 expression by immunohistochemistry (IHC) stain and by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), with intrinsic subtyping by research-based PAM50 RT-qPCR assay.</p>	<p>Among 283 HER2-negative tumors with &lt;1% HR expression by IHC, 207 (73%) were basal-like; other subtypes, particularly HER2-enriched (48, 17%), were present. Among the 1,298 HER2-negative tumors, borderline HR (1%–9% staining) was uncommon (n = 39), and these tumors were heterogeneous: 17 (44%) luminal A/B, 12 (31%) HER2-enriched, and only 7 (18%) basal-like. Including them in the definition of triple negative breast cancer significantly diminished enrichment for basal-like cancer (P &lt; .05). Among 106 HER2-positive tumors with &lt;1% HER expression by IHC, the HER2-enriched subtype was the most frequent (87, 82%), whereas among 127 HER2-positive tumors with strong HR (&gt;10%) expression, only 69 (54%) were HER2-enriched and 55 (43%) were luminal (39 luminal B, 16 luminal A). Quantitative HR expression by RT-qPCR gave similar results. Regardless of methodology, basal-like cases seldom expressed ER/ESR1 or PR/PGR and were associated with the lowest expression level of HER2/ERBB2 relative to other subtypes.</p>
<p>Tramm, Virchows Arch, 2013<sup>59</sup></p>	<p>Reliable PCR quantitation of estrogen, progesterone and ERBB2 receptor mRNA from formalin-fixed, paraffin-embedded tissue is independent of prior macro-dissection</p>	<p>The aim was to test if mRNA from tissue surrounding breast cancer affected quantification of estrogen receptor <math>\alpha</math> (ESR1), progesterone receptor (PGR) and human epidermal growth factor receptor 2 (ERBB2), by comparing gene expression from whole slide and tumor-enriched sections, and correlating gene expression from whole slide sections with corresponding immunohistochemistry.</p>	<p>Gene expression, based on mRNA extracted from a training set (36 paraffin blocks) and two validation sets (133+1,083 blocks), were determined by quantitative reverse transcription polymerase chain reaction for all samples, as well as by microarray for 133 validation samples. In the training set, agreement between high vs. low mRNA expression from whole slide and tumor-enriched sections was absolute for ESR1 and ERBB2, and 83 % for</p>

			PGR. Overall agreements, when comparing mRNA expression to immunohistochemistry, were 100 % (ERBB2), 89% (ESR1) and 83% (PGR), which was confirmed in the validation sets. Percentage of tumor in the sections did not influence the results.
Bastien, BMC Medical Genomics, 2012 <sup>60</sup>	PAM50 Breast Cancer Subtyping by RT-qPCR and Concordance with Standard Clinical Molecular Markers	We used the PAM50 RT-qPCR assay to expression profile 814 tumors from the GEICAM/9906 phase III clinical trial that enrolled women with locally advanced primary invasive breast cancer. All samples were scored at a single site by IHC for estrogen receptor (ER), progesterone receptor (PR), and Her2/neu (HER2) protein expression. Equivocal HER2 cases were confirmed by chromogenic in situ hybridization (CISH). Single gene scores by IHC/CISH were compared with RT-qPCR continuous gene expression values and “intrinsic” subtype assignment by the PAM50. High, medium, and low expression for ESR1, PGR, ERBB2, and proliferation were selected using quartile cut-points from the continuous RT-qPCR data across the PAM50 subtype assignments.	ESR1, PGR, and ERBB2 gene expression had high agreement with established binary IHC cut-points (area under the curve [AUC] ≥ 0.9). Estrogen receptor positivity by IHC was strongly associated with Luminal (A and B) subtypes (92%), but only 75% of ER negative tumors were classified into the HER2-E and Basal-like subtypes. Luminal A tumors more frequently expressed PR than Luminal B (94% vs 74%) and Luminal A tumors were less likely to have high proliferation (11% vs 77%). Seventy-seven percent (30/39) of ER-/HER2+ tumors by IHC were classified as the HER2-E subtype. Triple negative tumors were mainly comprised of Basal-like (57%) and HER2-E (30%) subtypes. Single gene scoring for ESR1, PGR, and ERBB2 was more prognostic than the corresponding IHC markers as shown in a multivariate analysis.
Kraus, Modern Pathology, 2012 <sup>61</sup>	Semi-quantitative immunohistochemical assay versus oncotype DXs qRT-PCR assay for estrogen and progesterone receptors: an independent quality assurance study	As part of an ongoing quality assurance program at our institution, we reviewed 464 breast cancer cases evaluated by both immunohistochemistry and oncotype DX assay for estrogen and PR.	We found good correlation for ER status between both assays (98.9% concordance), with immunohistochemistry being slightly more sensitive. Concordance for PR was 94.2% between immunohistochemistry and qRT-PCR with immunohistochemistry again more sensitive than RT-PCR. The results also showed linear correlation between immunohistochemistry H-

			scores and qRT-PCR expression values for ER (correlation coefficient of 0.579), and PR (correlation coefficient of 0.685).
Muller, Diagn Mol Pathol, 2011 <sup>62</sup>	Quantitative determination of estrogen receptor, progesterone receptor, and HER2 mRNA in formalin-fixed paraffin-embedded tissue--a new option for predictive biomarker assessment in breast cancer	We investigated a novel, fully automated, and xylene-free method for RNA isolation and biomarker determination using formalin-fixed paraffin-embedded (FFPE) tissue. The aim was to show that this approach is feasible and gives results that are comparable to the current gold standards. Expression of the breast cancer biomarkers ESR1, PGR, and HER2 was measured in a total of 501 FFPE tissue samples from 167 breast carcinomas, which had been stored for up to 21 years.	Total RNA was extracted from tissue sections and biomarker expression was measured by kinetic RT-PCR (RT-kPCR). The results of the new method were compared with immunohistochemistry as the current gold standard. RNA was successfully isolated from all samples, with a mean yield of 1.4 mug/sample and fragment lengths of at least 150 bp in 99% of samples. RT-kPCR analysis of ESR1, PGR, and HER2 was possible in all samples. Comparing RT-kPCR results with standard IHC, we found a good concordance for ESR1 (agreement: 98.4%), PGR (84.4%), and HER2 (89.8%). We observed a low section-to-section variability of kPCR results for all 3 biomarkers (root of mean squared errors: 0.2 to 0.5 Ct values).
Nielson, Clinical Cancer Research, 2010 <sup>63</sup>	A Comparison of PAM50 Intrinsic Subtyping with Immunohistochemistry and Clinical Prognostic Factors in Tamoxifen-Treated Estrogen Receptor-Positive Breast Cancer	Quantitative real-time reverse transcription-PCR (qRT-PCR) assays for 50 genes identifying intrinsic breast cancer subtypes were completed on 786 specimens linked to clinical (median follow-up, 11.7 years) and IHC (ER, progesterone receptor [PR], HER2, and Ki67) data. Performance of predefined intrinsic subtype and risk-of-relapse scores was assessed using multivariable Cox models and Kaplan-Meier analysis. Harrell's C-index was used to compare fixed models trained in independent data sets, including proliferation signatures.	Despite clinical ER positivity, 10% of cases were assigned to nonluminal subtypes. qRT-PCR signatures for proliferation genes gave more prognostic information than clinical assays for hormone receptors or Ki67. In Cox models incorporating standard prognostic variables, hazard ratios for breast cancer disease-specific survival over the first 5 years of follow-up, relative to the most common luminal A subtype, are 1.99 (95% confidence interval [CI], 1.09-3.64) for luminal B, 3.65 (95% CI, 1.64-8.16) for HER2-enriched subtype, and 17.71 (95% CI, 1.71-183.33) for the basal-like

			subtype. For node-negative disease, PAM50 qRT-PCR-based risk assignment weighted for tumor size and proliferation identifies a group with >95% 10-year survival without chemotherapy. In node-positive disease, PAM50-based prognostic models were also superior.
Badve, JCO, 2008 <sup>64</sup>	Estrogen- and Progesterone-Receptor Status in ECOG 2197: Comparison of Immunohistochemistry by Local and Central Laboratories and Quantitative Reverse Transcription Polymerase Chain Reaction by Central Laboratory	A case-control sample of 776 breast cancer patients from Eastern Cooperative Oncology Group (ECOG) study E2197 was evaluated. Central IHC Allred score for ER and PR was obtained using tissue microarrays and 1D5 ER antibody and 636 PR antibody. Quantitative RT-PCR for ER and PR in whole sections was performed using the 21-gene assay.	For ER, the concordance between local and central IHC was 90% (95% CI, 88% to 92%), between local IHC and central RT-PCR was 91% (95% CI, 89% to 93%), and between central IHC and central RT-PCR was 93% (95% CI, 91% to 95%). For PR, the concordance between local IHC and central IHC was 84% (95% CI, 82% to 87%), between local IHC and central RT-PCR was 88% (95% CI, 85% to 90%), and between central IHC and central RT-PCR was 90% (95% CI, 88% to 92%). Although concordance was high, IHC ER-negative cases that were RT-PCR positive were more common than IHC ER-positive cases that were RT-PCR negative. In ER-positive patients, ER expression by central IHC Allred score was marginally associated with recurrence (P= .091), and ER expression by central RT-PCR was significantly associated with recurrence (P = .014). However, recurrence score, which incorporates additional genes/pathways, was a highly significant predictor of recurrence (P< .0001).

#### Image Analysis

First Author, Journal, Year	Title	Study Design	Conclusions
Bui, Arch Pathol Lab Med, 2019 <sup>1</sup>	Quantitative Image Analysis of Human Epidermal Growth Factor Receptor 2	To develop evidence-based recommendations to improve	Eleven recommendations were drafted: 7 based on CAP laboratory

	<p>Immunohistochemistry for Breast Cancer: Guideline From the College of American Pathologists</p>	<p>accuracy, precision, and reproducibility in the interpretation of human epidermal growth factor receptor 2 (HER2) immunohistochemistry (IHC) for breast cancer where QIA is used. The College of American Pathologists (CAP) convened a panel of pathologists, histotechnologists, and computer scientists with expertise in image analysis, immunohistochemistry, quality management, and breast pathology to develop recommendations for QIA of HER2 IHC in breast cancer. A systematic review of the literature was conducted to address 5 key questions. Final recommendations were derived from strength of evidence, open comment feedback, expert panel consensus, and advisory panel review.</p>	<p>accreditation requirements and 4 based on expert consensus opinions. A 3-week open comment period received 180 comments from more than 150 participants. To improve accurate, precise, and reproducible interpretation of HER2 IHC results for breast cancer, QIA and procedures must be validated before implementation, followed by regular maintenance and ongoing evaluation of quality control and quality assurance. HER2 QIA performance, interpretation, and reporting should be supervised by pathologists with expertise in QIA.</p>
<p>Rimm, Mod Pathol, 2019<sup>65</sup></p>	<p>An international multicenter study to evaluate reproducibility of automated scoring for assessment of Ki67 in breast cancer</p>	<p>The International Ki67 in Breast Cancer Working Group investigated whether Ki67 immunohistochemistry can be analytically validated and standardized across laboratories using automated machine-based scoring. Sets of pre-stained core-cut biopsy sections of 30 breast tumors were circulated to 14 laboratories for scanning and automated assessment of the average and maximum percentage of tumor cells positive for Ki67. Seven unique scanners and 10 software platforms were involved in this study. Pre-specified analyses included evaluation of reproducibility between all laboratories (primary) as well as among those using scanners from a single vendor (secondary). The primary reproducibility metric was</p>	<p>Intraclass correlation coefficient for automated average scores across 16 operators was 0.83 (95% credible interval: 0.73-0.91) and intraclass correlation coefficient for maximum scores across 10 operators was 0.63 (95% credible interval: 0.44-0.80). For the laboratories using scanners from a single vendor (8 score sets), intraclass correlation coefficient for average automated scores was 0.89 (95% credible interval: 0.81-0.96), which was similar to the intraclass correlation coefficient of 0.87 (95% credible interval: 0.81-0.93) achieved using these same slides in a prior visual-reading reproducibility study. Automated machine assessment of average Ki67 has the potential to achieve between-laboratory</p>

		intraclass correlation coefficient between laboratories, with success considered to be intraclass correlation coefficient >0.80.	reproducibility similar to that for a rigorously standardized pathologist-based visual assessment of Ki67.
Peck, J Clin Pathol, 2018 <sup>66</sup>	Review of diagnostic error in anatomical pathology and the role and value of second opinions in error prevention	A literature review of diagnostic accuracy in selected specimen categories was undertaken and was compared with data on metropolitan and regional pathologist diagnostic proficiency performance in an external quality assurance programme from surveys provided 2015-2017. For each specimen category, cases having attracted a diagnostic inaccuracy (ie, major discordance) of >=20% and cases attracting a combined error rate (ie, major and minor discordance) of >=30% are reviewed and discussed.	The rate of inaccurate diagnoses (assessed as a major discordance) ranged from 3% to 9% among the different specimen groups, with highest mean percentage of inaccurate diagnoses in gynecology, dermatopathology and gastrointestinal specimens
Tosteson, Breast Cancer Res Treat, 2018 <sup>67</sup>	Second opinion strategies in breast pathology: a decision analysis addressing over-treatment, under-treatment, and care costs.	Decision analysis examining 12-month outcomes of breast biopsy for nine breast pathology interpretation strategies in the U.S. health system. Diagnoses of 115 practicing pathologists in the Breast Pathology Study were compared to reference-standard-consensus diagnoses with and without second opinions. Interpretation strategies were defined by whether a second opinion was sought universally or selectively (e.g., 2nd opinion if invasive). Main outcomes were the expected proportion of concordant breast biopsy diagnoses, the proportion involving over- or under-interpretation, and cost of care in U.S. dollars within one-year of biopsy.	Without a second opinion, 92.2% of biopsies received a concordant diagnosis. Concordance rates increased under all second opinion strategies, and the rate was highest (95.1%) and under-treatment lowest (2.6%) when all biopsies had second opinions. However, over-treatment was lowest when second opinions were sought selectively for initial diagnoses of invasive cancer, DCIS, or atypia (1.8 vs. 4.7% with no 2nd opinions). This strategy also had the lowest projected 12-month care costs (\$5.907 billion vs. \$6.049 billion with no 2nd opinions).
Ahern, J Clin Pathol, 2017 <sup>68</sup>	Continuous measurement of breast tumour hormone receptor expression: a comparison of two computational pathology platforms	Breast tumour microarrays from the Nurses' Health Study were stained for ER (n=592) and PR (n=187). One expert pathologist scored cases as	Both platforms showed considerable overlap in continuous measurements of ER and PR between positive and negative groups classified by expert

		<p>positive if <math>\geq 1\%</math> of tumour nuclei exhibited stain. ER and PR were then measured with the Definiens Tissue Studio (automated) and Aperio Digital Pathology (user-supervised) platforms. Platform-specific measurements were compared using boxplots, scatter plots and correlation statistics. Classification of ER and PR positivity by platform-specific measurements was evaluated with areas under receiver operating characteristic curves (AUC) from univariable logistic regression models, using expert pathologist classification as the standard.</p>	<p>pathologist. Platform-specific measurements were strongly and positively correlated with one another (<math>r \geq 0.77</math>). The user-supervised Aperio workflow performed slightly better than the automated Definiens workflow at classifying ER positivity (AUC<sub>Aperio</sub>=0.97; AUC<sub>Definiens</sub>=0.90; difference=0.07, 95% CI 0.05 to 0.09) and PR positivity (AUC<sub>Aperio</sub>=0.94; AUC<sub>Definiens</sub>=0.87; difference=0.07, 95% CI 0.03 to 0.12).</p>
<p>Maeda, J Clin Pathol, 2017<sup>69</sup></p>	<p>Effectiveness of computer-aided diagnosis (CADx) of breast pathology using immunohistochemistry results of core needle biopsy samples for synaptophysin, oestrogen receptor and CK14/p63 for classification of epithelial proliferative lesions of the breast</p>	<p>Two sets of 100 consecutive core needle biopsy (CNB) specimens were collected for test and validation studies. All 200 CNB specimens were stained with antibodies targeting oestrogen receptor (ER), synaptophysin and CK14/p63. All stained slides were scanned in a whole-slide imaging system and photographed. The photographs were analyzed using software to identify the proportions of tumour cells that were positive and negative for each marker. In the test study, the cut-off values for synaptophysin (negative and positive) and CK14/p63 (negative and positive) were decided using receiver operating characteristic (ROC) analysis. For ER analysis, samples were divided into groups with <math>&lt;10\%</math> positive or <math>&gt;10\%</math> positive cells and decided using receiver operating characteristic (ROC) analysis. Finally, these two groups categorized as ER-low, ER-intermediate (non-low and non-high) and ER-high groups. In the validation</p>	<p>The cut-off values for synaptophysin, <math>&lt;10\%</math> ER positive, <math>&gt;10\%</math> ER positive and CK14/p63 were 0.14%, 2.17%, 77.93% and 18.66%, respectively. The positive predictive value for malignancy (PPV) was 100% for synaptophysin-positive/ER-high/(CK14/p63)-any or synaptophysin-positive/ER-low/(CK14/p63)-any. The PPV was 25% for synaptophysin-positive/ER-intermediate/(CK14/p63)-positive. For synaptophysin-negative/(CK14/p63)-negative, the PPVs for ER-low, ER-intermediate and ER-high were 100%, 80.0% and 95.8%, respectively. The PPV was 4.5% for synaptophysin-negative/ER-intermediate/(CK14/p63)-positive.</p>



		study, the second set of immunohistochemical slides were analyzed using these cut-off values.	
Barnes, Laboratory Investigation, 2017 <sup>70</sup>	Whole tumor section quantitative image analysis maximizes between-pathologists' reproducibility for clinical immunohistochemistry-based biomarkers	In this study, we implemented a novel solely morphology-based whole tumor section annotation strategy to maximize image analysis quantitation results between readers. We first compare the field-of-view image analysis annotation approach to digital and manual-based modalities across multiple clinical studies (~120 cases per study) and biomarkers (ER, PR, HER2, Ki-67, and p53 IHC) and then compare a subset of the same cases (~40 cases each from the ER, PR, HER2, and Ki-67 studies) using whole tumor section annotation approach to understand incremental value of all modalities.	Between-reader results for each biomarker in relation to conventional scoring modalities showed similar concordance as manual read: ER field-of-view image analysis: 95.3% (95% CI 92.0–98.2%) vs digital read: 92.0% (87.8–95.8%) vs manual read: 94.9% (91.4–97.8%); PR field-of-view image analysis: 94.1% (90.3–97.2%) vs digital read: 94.0% (90.2–97.1%) vs manual read: 94.4% (90.9–97.2%); Ki-67 field-of-view image analysis: 86.8% (82.1–91.4%) vs digital read: 76.6% (70.9–82.2%) vs manual read: 85.6% (80.4–90.4%); p53 field-of-view image analysis: 81.7% (76.4–86.8%) vs digital read: 80.6% (75.0–86.0%) vs manual read: 78.8% (72.2–83.3%); and HER2 field-of-view image analysis: 93.8% (90.0–97.2%) vs digital read: 91.0 (86.6–94.9%) vs manual read: 87.2% (82.1–91.9%). Subset implementation and analysis on the same cases using whole tumor section image analysis approach showed significant improvement between pathologists over field-of-view image analysis and manual read (HER2 100% [97–100%]), P= 0.013 field-of-view image analysis and 0.013 manual read; Ki-67 100% (96.9–100%), P= 0.040 and 0.012; ER 98.3% (94.1–99.5%), p = 0.232 and 0.181; and PR 96.6% (91.5–98.7%), p = 0.012 and 0.257).
Liu, Tumor Biol., 2016 <sup>71</sup>	Application of multispectral imaging in quantitative immunohistochemistry study of breast cancer: a comparative study	We analyzed and compared the utility of multispectral (MS) versus conventional red–green–blue (RGB) images for immunohistochemistry	The MS images acquired of IHC-stained membranous marker human epidermal growth factor receptor 2 (HER2), cytoplasmic marker

		<p>(IHC) staining to explore the advantages of MSI in clinical-pathological diagnosis.</p>	<p>cytokeratin5/6 (CK5/6), and nuclear marker estrogen receptor (ER) have higher resolution, stronger contrast, and more accurate segmentation than the RGB images. The total signal optical density (OD) values for each biomarker were higher in MS images than in RGB images (all <math>P &lt; 0.05</math>). Moreover, receiver operator characteristic (ROC) analysis revealed that a greater area under the curve (AUC), higher sensitivity, and specificity in evaluation of HER2 gene were achieved by MS images (AUC=0.91, 89.1 %, 83.2 %) than RGB images (AUC=0.87, 84.5, and 81.8 %). There was no significant difference between quantitative results of RGB images and clinico-pathological characteristics (<math>P &gt; 0.05</math>). However, quantifying MS images, the total signal OD values of HER2 positive expression were correlated with lymph node status and histological grades (<math>P = 0.02</math> and <math>0.04</math>). Additionally, the consistency test results indicated the inter-observer agreement was more robust in MS images for HER2 (inter-class correlation coefficient [ICC]=0.95, <math>r_s = 0.94</math>), CK5/6 (ICC=0.90, <math>r_s = 0.88</math>), and ER (ICC= 0.94, <math>r_s = 0.94</math>) (all <math>P &lt; 0.001</math>) than that in RGB images for HER2 (ICC=0.91, <math>r_s = 0.89</math>), CK5/6 (ICC=0.85, <math>r_s = 0.84</math>), and ER (ICC=0.90, <math>r_s = 0.89</math>) (all <math>P &lt; 0.001</math>).</p>
<p>Stålhammar, Modern Pathology, 2016<sup>72</sup></p>	<p>Digital image analysis outperforms manual biomarker assessment in breast cancer</p>	<p>In this study, 3 cohorts of primary breast cancer specimens (total <math>n = 436</math>) with up to 28 years of survival data were scored for Ki67, ER, PR, and HER2 status manually and by digital image analysis (DIA). The results were then compared for sensitivity and</p>	<p>The DIA system used was the Visiopharm Integrator System. DIA outperformed manual scoring in terms of sensitivity and specificity for the Luminal B subtype, widely considered the most challenging distinction in surrogate subclassification, and</p>

		<p>specificity for the Luminal B subtype, concordance to PAM50 assays in subtype classification and prognostic power.</p>	<p>produced slightly better concordance and Cohen's <math>\kappa</math> agreement with PAM50 gene expression assays. Manual biomarker scores and DIA essentially matched each other for Cox regression hazard ratios for all-cause mortality. When the Nottingham combined histologic grade (Elston–Ellis) was used as a prognostic surrogate, stronger Spearman's rank-order correlations were produced by DIA. Prognostic value of Ki67 scores in terms of likelihood ratio <math>\chi^2</math> (LR <math>\chi^2</math>) was higher for DIA that also added significantly more prognostic information to the manual scores (LR-<math>\Delta\chi^2</math>).</p>
<p>Elmore, BMJ, 2016<sup>73</sup></p>	<p>Evaluation of 12 strategies for obtaining second opinions to improve interpretation of breast histopathology: simulation study</p>	<p>Misclassification rates for individual pathologists and for 12 simulated strategies for second opinions. Simulations compared accuracy of diagnoses from single pathologists with that of diagnoses based on pairing interpretations from first and second independent pathologists, where resolution of disagreements was by an independent third pathologist. 12 strategies were evaluated in which acquisition of second opinions depended on initial diagnoses, assessment of case difficulty or borderline characteristics, pathologists' clinical volumes, or whether a second opinion was required by policy or desired by the pathologists. The 240 cases included benign without atypia (10% non-proliferative, 20% proliferative without atypia), atypia (30%), ductal carcinoma in situ (DCIS, 30%), and invasive cancer (10%). Overall misclassification rates and agreement</p>	<p>Misclassification rates significantly decreased (<math>P &lt; 0.001</math>) with all second opinion strategies except for the strategy limiting second opinions only to cases of invasive cancer. The overall misclassification rate decreased from 24.7% to 18.1% when all cases received second opinions (<math>P &lt; 0.001</math>). Obtaining both first and second opinions from pathologists with a high volume (<math>\geq 10</math> breast biopsy specimens weekly) resulted in the lowest misclassification rate in this test set (14.3%, 95% confidence interval 10.9% to 18.0%). Obtaining second opinions only for cases with initial interpretations of atypia, DCIS, or invasive cancer decreased the over-interpretation of benign cases without atypia from 12.9% to 6.0%. Atypia cases had the highest misclassification rate after single interpretation (52.2%), remaining at more than 34% in all second opinion scenarios.</p>

		<p>statistics depended on the composition of the test set, which included a higher prevalence of difficult cases than in typical practice.</p>	
<p>Khazai, J Surg Oncol, 2015<sup>74</sup></p>	<p>Breast pathology second review identifies clinically significant discrepancies in over 10% of patients</p>	<p>We retrospectively studied all 1,970 breast pathology referral cases reviewed during one calendar year. The variables studied were histologic classification; tumor grade, necrosis, size, margin status, lymphatic/vascular invasion, dermal involvement, and biomarker profile (ER, PR, and Her-2). Each variable was rated as "agree," "disagree," "missing information," or "not applicable."</p>	<p>A significant discrepancy, defined as a disagreement that affected patient care, was found in 226 cases (11.47%). Additionally, in 418 resection cases (31.6%), some CAP-checklist specific required information was missing. The most common areas of significant discrepancy were histologic category (66 cases; 33%) and biomarker reporting (50 cases; 25%). The most problematic diagnostic categories were intraductal lesions, lobular carcinoma, metaplastic carcinomas, and phyllodes tumors. Most disagreements in the biomarker-profile category were interpretive, but in 20% of discrepant cases, findings were supported by repeat immunohistochemical analysis.</p>
<p>Engelberg, Hum Pathol, 2015<sup>75</sup></p>	<p>"Score the Core" Web-based pathologist training tool improves the accuracy of breast cancer IHC4 scoring</p>	<p>We developed a Web-based training tool, called "Score the Core" (STC) using tissue microarrays to train pathologists to visually score estrogen receptor (using the 300-point H score), progesterone receptor (percent positive), and Ki-67 (percent positive). STC used a reference score calculated from a reproducible manual counting method.</p>	<p>Pathologists in the Athena Breast Health Network and pathology residents at associated institutions completed the exercise. By using STC, pathologists improved their estrogen receptor H score and progesterone receptor and Ki-67 proportion assessment and demonstrated a good correlation between pathologist and reference scores. In addition, we collected information about pathologist performance that allowed us to compare individual pathologists and measures of agreement. Pathologists' assessment of the proportion of positive cells was closer to the reference than their</p>

			assessment of the relative intensity of positive cells.
Gertych, Diagnostic Pathology, 2014 <sup>76</sup>	Effects of tissue decalcification on the quantification of breast cancer biomarkers by digital image analysis	Tissues were prospectively decalcified for up to 24 hours and stained by immunohistochemistry (IHC) for ER, PR, Ki-67 and p53. HER2 positive breast cancer sections were retrieved from the pathology archives, and annotated with the categorical HER2 expression scores from the pathology reports. Digital images were captured with Leica and Aperio slide scanners. The conversion of the digital to categorical scores was accomplished with a Gaussian mixture model and tested for accuracy by comparison to clinical scores.	We observe significant effects of the decalcification treatment on common breast cancer biomarkers that are used in the clinic. ER, PR and p53 staining intensities decreased 15 – 20%, whereas Ki-67 decreased > 90% during the first 6 hrs of treatment and stabilized thereafter. In comparison with the Aperio images, pixel intensities generated by the Leica system are lower. A novel statistical model for conversion of digital to categorical scores provides a systematic approach for conversion of nuclear and membrane stains and demonstrated a high concordance with clinical scores.
Ali, British Journal of Cancer, 2013 <sup>77</sup>	Astronomical algorithms for automated analysis of tissue protein expression in breast cancer	We report image analysis algorithms adapted from astronomy for the precise automated analysis of IHC in all subcellular compartments. The power of this technique is demonstrated using over 2000 breast tumours and comparing quantitative automated scores against manual assessment by pathologists.	All continuous automated scores showed good correlation with their corresponding ordinal manual scores. For oestrogen receptor (ER), the correlation was 0.82, P<0.0001, for BCL2 0.72, P<0.0001 and for HER2 0.62, P<0.0001. Automated scores showed excellent concordance with manual scores for the unsupervised assignment of cases to 'positive' or 'negative' categories with agreement rates of up to 96%
Jorns, Arch Pathol Lab Med, 2013 <sup>78</sup>	Review of estrogen receptor, progesterone receptor, and HER-2/neu immunohistochemistry impacts on treatment for a small subset of breast cancer patients transferring care to another institution	To determine the frequency of interinstitutional discordance for the interpretation of ER/PR and HER-2/neu immunohistochemical slides and assess the resulting clinical significance. DESIGN: One thousand one hundred thirty-nine ER, 1111 PR, and 663 HER-2/neu immunohistochemistry stains from 1139 cases were reviewed at	Interinstitutional concordance for individual stains was excellent (ER: kappa = 0.93; PR: kappa = 0.90; HER-2/neu: kappa = 0.93). One hundred four (9.1%) had interinstitutional discordance in 1 or more stains; however, the majority of the discordance was clinically insignificant. Seven patients (0.6%) had a clinically significant change in treatment

		contributing and referral centers and compared for concordance and clinical impact of discordance.	recommendation based on review and 2 (0.2%) had interpretation changes that would likely have resulted in treatment change had they not already completed therapy. Two patients (0.2%) had change in treatment despite concordant interpretations.
Nassar, Anatomic Pathology, 2011 <sup>79</sup>	A Multisite Performance Study Comparing the Reading of Immunohistochemical Slides on a Computer Monitor With Conventional Manual Microscopy for Estrogen and Progesterone Receptor Analysis	A total of 520 formalin-fixed breast tissue specimens were assayed at 3 clinical sites for ER and PR (260 each). Percentage and average staining intensity of positive nuclei were assessed. At each site, 3 pathologists performed a blinded reading of the glass slides using their microscopes initially and later using digital images on a computer monitor.	Comparable percentages of agreements were obtained for manual microscopy (MM) and manual digital slide reading (MDR) (ER, percentage of positive nuclei with cutoffs: MM, 91.3%-99.0%/MDR, 91.3%-100.0%; PR, percentage of positive nuclei with cutoffs: MM, 83.8%-99.0%/MDR, 76.3%-100.0%).
Slodkowska, Folia Histochem Cytobiol., 2010 <sup>80</sup>	Study on breast carcinoma Her2/neu and hormonal receptors status assessed by automated images analysis systems: ACIS III (Dako) and ScanScope (Aperio)	The aims of our study were: to evaluate the scoring reproducibility of Her-2 /neu ihc expression tested by two automated systems: ACIS (Dako) and ScanScope (Aperio); to estimate the ER/PR expression in ihc staining methods with different anti-ER/anti-PR antibodies (the monoclonal and the ER/PR pharmDx TM Kit ) by the ACIS system. Her-2/neu ihc expression was measured in 114 primary invasive breast carcinomas by the manual and the automated scoring (ACIS and Aperio system). 106 slides stained ihc with two types of anti-ER/anti-PR antibodies entered the quantisation.	The results of our investigations showed very high reproducibility of Her-2/neu scores in intra- and interobserver analysis by ACIS evaluation. The major concordance was present in strong 3+ ihc cases; very small discordance was shown by cases with low expression of Her-2/neu. The accuracy of scoring by the Aperio was little lower in comparison to ACIS but it might result from the smaller and variable series of samples analysed by Aperio. The concordance in scoring of two automated systems was 86.5% (p<0.0001; $\gamma$ =0.887); the discordance was referred only to the lower expression of Her-2/neu. The concordance in manual scoring performed by the single observer and the panel was 84.2% (p<0.0001, $\gamma$ = 0.99); the discordance comprised a few cases with strong expression (2+ vs 3+). Very high intra- and

			interobserver reproducibility of the ER/PR ihc measurements was present in the readers results (referred to the percentage of immunoreactive carcinomatous cell population in the breast carcinomas acc. to the ACIS algorithm). No differences were disclosed in the percentage of ER-immunoreactive and PR-immunoreactive carcinomatous cell populations when used 2 different type of antibodies, in the ACIS automated method.
Tuominen, Breast Cancer Research, 2010 <sup>81</sup>	ImmunoRatio: a publicly available web application for quantitative image analysis of estrogen receptor (ER), progesterone receptor (PR), and Ki-67	The application, named ImmunoRatio, calculates the percentage of positively stained nuclear area (labeling index) by using a color deconvolution algorithm for separating the staining components (diaminobenzidine and hematoxylin) and adaptive thresholding for nuclear area segmentation. ImmunoRatio was calibrated using cell counts defined visually as the gold standard (training set, n = 50). Validation was done using a separate set of 50 ER, PR, and Ki-67 stained slides (test set, n = 50). In addition, Ki-67 labeling indexes determined by ImmunoRatio were studied for their prognostic value in a retrospective cohort of 123 breast cancer patients.	The labeling indexes by calibrated ImmunoRatio analyses correlated well with those defined visually in the test set (correlation coefficient r = 0.98). Using the median Ki-67 labeling index (20%) as a cutoff, a hazard ratio of 2.2 was obtained in the survival analysis (n = 123, P = 0.01). ImmunoRatio was shown to adapt to various staining protocols, microscope setups, digital camera models, and image acquisition settings. The application can be used directly with web browsers running on modern operating systems (e.g., Microsoft Windows, Linux distributions, and Mac OS). No software downloads or installations are required. ImmunoRatio is open source software, and the web application is publicly accessible on our website.
Lloyd, J Pathol Inform, 2010 <sup>82</sup>	Using image analysis as a tool for assessment of prognostic and predictive biomarkers for breast cancer: How reliable is it?	Whole slide images of 33 invasive ductal carcinoma (IDC) (10 ER and 23 HER2) were scored by pathologist under the light microscope and confirmed by another pathologist. The HER2 results were additionally confirmed by fluorescence in situ	For HER2 positive group, each algorithm scored 23/23 cases within the range established by the pathologist. For ER, both algorithms scored 10/10 cases within range. The performance of each algorithm varies somewhat from the percentage of

		<p>hybridization (FISH). The scoring criteria were adherent to the guidelines recommended by the American Society of Clinical Oncology/College of American Pathologists. Whole slide stains were then scored by commercially available image analysis algorithms from Definiens (Munich, Germany) and Aperio Technologies (Vista, CA, USA). Each algorithm was modified specifically for each marker and tissue. The results were compared with the semi-quantitative manual scoring, which was considered the gold standard in this study.</p>	<p>staining as compared to the pathologist's reading.</p>
<p>Aitken, Annals of Oncology, 2009<sup>83</sup></p>	<p>Quantitative analysis of changes in ER, PR and HER2 expression in primary breast cancer and paired nodal metastases</p>	<p>A total of 385 patients with invasive primary breast carcinomas and paired lymph nodes (n = 211) were assessed for ER, PR and HER2 expression using quantitative immunofluorescence. Cut-points were defined by comparison with tumours scored by immunohistochemistry (IHC) and FISH. Differences in expression for each of the markers and molecular phenotype were analysed.</p>	<p>Quantitative receptor expression shows a wide dynamic range compared with IHC. Overall, 46.9% cases had disparate breast/node receptor status of at least one receptor. Many of the differences in expression between primary tumour and node are large magnitude (greater than fivefold) changes. Triple-negative phenotype changes in 23.1% of cases.</p>

### DCIS

First Author, Journal, Year	Title	Study Design	Conclusions
<p>Chaudhary, WMJ, 2018<sup>84</sup></p>	<p>Does Progesterone Receptor Matter in the Risk of Recurrence for Patients With Ductal Carcinoma in Situ?</p>	<p>Six hundred ninety-three patients diagnosed and treated for DCIS at Froedtert and Medical College of Wisconsin Cancer Center (February 2002 to March 2015) were studied to determine if the recurrence rates were significantly different between ER+/PR- and ER+/PR+ tumors. Recurrence was defined as either noninvasive or invasive ipsilateral, contralateral, or distant disease.</p>	<p>Median follow-up was 5.2 years. The 5-year recurrence-free survival (RFS) was 91% (95% CI, 88.2-93.3) while estimated 7-year RFS was 86% (95% CI, 81.9-89.2). Seventy-five patients had a recurrence during their follow-up. Patients with ER-/PR- tumors (n = 118) had a significantly higher risk of recurrence (Hazard Ratio 3.7, 95% CI, 1.9-7.2, P = 0.0001) whereas those with ER+/PR- subtype (n = 77) did not</p>



		Probabilities of recurrences were calculated using Kaplan-Meier estimator. Cox proportional hazards model was used to evaluate the effect of prognostic factors on DCIS recurrence.	have a significant difference in recurrence risk (HR 1.75, 95% CI, 0.92-3.32, P = 0.085) when compared to ER+/PR+ tumors (n = 482). No endocrine therapy for ER+ DCIS and lumpectomy alone were also significant predictors of recurrence (P = 0.0073 and P = 0.005, respectively).
Hwang, Breast Cancer Research and Treatment, 2018 <sup>85</sup>	Tamoxifen therapy improves overall survival in luminal A subtype of ductal carcinoma in situ: a study based on nationwide Korean Breast Cancer Registry database	Data of 14,944 patients with DCIS were analyzed. Molecular subtypes were classified into four categories based on expression of estrogen receptor (ER)/progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Kaplan-Meier estimator was used for overall survival analysis while Cox proportional hazards model was used for univariate and multivariate analyses.	Luminal A subtype (ER/PR+, HER2-) showed higher (P = .009) survival rate than triple-negative (TN) subtype. Tamoxifen therapy group showed superior (P < .001) survival than no-tamoxifen therapy group. It had survival benefit only for luminal A subtype (P = .001). Tamoxifen therapy resulted in higher survival rate in subgroups with positive ER (P = .006), positive PR (P = .009), and negative HER2 (P < .001). In luminal A subtype, tamoxifen therapy showed lower hazard ratio (HR) compared to no-tamoxifen therapy (HR, 0.420; 95% CI 0.250-0.705; P = .001). Tamoxifen therapy was a significant independent factor by multivariate analysis (HR, 0.538; 95% CI 0.306-0.946; P = .031) as well as univariate analysis.
Ravaioli, International Journal of Experimental Pathology, 2017 <sup>86</sup>	Androgen and oestrogen receptors as potential prognostic markers for patients with ductal carcinoma in situ treated with surgery and radiotherapy	A series of 42 DCIS patients treated with quadrantectomy and radiotherapy were followed for a period of up to 95 months. Of these, 11 had recurrent DCIS or progressed to invasive cancer. All tumors were analyzed for clinical pathological features. Conventional biomarkers and androgen receptor expression were determined by immunohistochemistry.	Results showed that AR was higher in tumors of relapsed patients than non-relapsed patients (P value: 0.0005). Conversely, estrogen receptor (ER) was higher, albeit not significantly, in non-relapsed patients than in relapsed patients. AR/ER ratio was considerably different in the two subgroups (P value: 0.0033). Area under the curve (AUC) values were 0.85 for AR and 0.80 for the AR/ER ratio.
Allred, JCO, 2012 <sup>87</sup>	Adjuvant Tamoxifen Reduces Subsequent Breast Cancer in Women	Estrogen (ER) and progesterone receptors (PgR) were evaluated in 732	ER was positive in 76% of patients. Patients with ER-positive DCIS treated

	<p>With Estrogen Receptor–Positive Ductal Carcinoma in Situ: A Study Based on NSABP Protocol B-24</p>	<p>patients with DCIS (41% of original study population). An experienced central laboratory determined receptor status in all patient cases with available paraffin blocks (n = 449) by immunohistochemistry (IHC) using comprehensively validated assays. Results for additional patients (n = 283) determined by various methods (primarily IHC) were available from enrolling institutions. Combined results were evaluated for benefit of tamoxifen by receptor status at 10 years and overall follow-up (median, 14.5 years).</p>	<p>with tamoxifen (v placebo) showed significant decreases in subsequent breast cancer at 10 years (hazard ratio [HR], 0.49; P &lt; .001) and overall follow-up (HR, 0.60; P = .003), which remained significant in multivariable analysis (overall HR, 0.64; P= .003). Results were similar, but less significant, when subsequent ipsilateral and contralateral, invasive and noninvasive, breast cancers were considered separately. No significant benefit was observed in ER-negative DCIS. PgR and either receptor were positive in 66% and 79% of patients, respectively, and in general, neither was more predictive than ER alone.</p>
<p>Cuzick, Lancet Oncol, 2011<sup>88</sup></p>	<p>Effect of tamoxifen and radiotherapy in women with locally excised ductal carcinoma in situ: long-term results from the UK/ANZ DCIS trial</p>	<p>Women with completely locally excised DCIS were recruited into a randomized 2x2 factorial trial of radiotherapy, tamoxifen, or both. Randomization was independently done for each of the two treatments (radiotherapy and tamoxifen), stratified by screening assessment center, and blocked in groups of four. The recommended dose for radiation was 50 Gy in 25 fractions over 5 weeks (2 Gy per day on weekdays), and tamoxifen was prescribed at a dose of 20 mg daily for 5 years. Elective decision to withhold or provide one of the treatments was permitted. The endpoints of primary interest were invasive ipsilateral new breast events for the radiotherapy comparison and any new breast event, including contralateral disease and DCIS, for tamoxifen. Analysis of each of the two treatment comparisons was restricted to patients who were randomly assigned to that treatment. Analyses</p>	<p>Between May, 1990, and August, 1998, 1701 women were randomly assigned to radiotherapy and tamoxifen, radiotherapy alone, tamoxifen alone, or to no adjuvant treatment. Seven patients had protocol violations and thus 1694 patients were available for analysis. After a median follow-up of 12.7 years (IQR 10.9-14.7), 376 (163 invasive [122 ipsilateral vs 39 contralateral], 197 DCIS [174 ipsilateral vs 17 contralateral], and 16 of unknown invasiveness or laterality) breast cancers were diagnosed. Radiotherapy reduced the incidence of all new breast events (hazard ratio [HR] 0.41, 95% CI 0.30-0.56; p&lt;0.0001), reducing the incidence of ipsilateral invasive disease (0.32, 0.19-0.56; p&lt;0.0001) as well as ipsilateral DCIS (0.38, 0.22-0.63; p&lt;0.0001), but having no effect on contralateral breast cancer (0.84, 0.45-1.58; p=0.6). Tamoxifen reduced the incidence of all new breast events</p>

		<p>were by intention to treat. All trial drugs have been completed and this study is in long-term follow-up. This study is registered, number ISRCTN99513870.</p>	<p>(HR 0.71, 95% CI 0.58-0.88; p=0.002), reducing recurrent ipsilateral DCIS (0.70, 0.51-0.86; p=0.03) and contralateral tumors (0.44, 0.25-0.77; p=0.005), but having no effect on ipsilateral invasive disease (0.95, 0.66-1.38; p=0.8). No data on adverse events except cause of death were collected for this trial.</p>
<p>Lin, Biotech Histochem, 2010<sup>89</sup></p>	<p>Tissue microarray-based immunohistochemical study can significantly underestimate the expression of HER2 and progesterone receptor in ductal carcinoma in situ of the breast</p>	<p>Our study was designed to investigate the concordance of expression in TMA and whole sections of estrogen receptor (ER), progesterone receptor (PR) and HER2 using IHC analysis for ductal carcinoma in situ (DCIS) of the breast. Seventy-five consecutive cases of DCIS were retrieved, reviewed and used to construct the TMA. IHC analysis of the expression of ER, PR, and HER2 were performed on TMA and whole sections of the corresponding cases, and the results were compared.</p>	<p>The specificity and sensitivity for TMA-based assays were 87.0, 75.9, 90.6 and 90.4%, and 76.1, 27.3 for ER, PR and HER2, respectively. The concordance and discordance were 89.3, 76.0 and 72.0%, and 6.7, 13.3 and 16.0% for ER, PR, HER2, respectively. The kappa values were 0.83, 0.89 and 0.42 for ER, PR and HER2, respectively. The non-concordance rates were inversely related to core number, with 46.67, 22.67 and 11.56% for one core, two cores, and three cores, respectively, per marker per case ( p 0.001), but not associated with tumor size.</p>

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