

Clinical Decision Making in Stage I and II Breast Cancer Patients Based on Gene Profiling

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Abstract

Standard clinical and pathological factors can estimate the risk of recurrence and mortality from early stage breast cancer and also predict the magnitude of benefit of classes of therapy (cytotoxic, hormonal, and HER2-directed biological). Multi-parametric analysis of several proteins or expressed genes can refine these estimates and allow for more precise estimation of risk/benefit calculations that can improve decision-making, particularly for hormone receptor-positive and HER2-negative cases. Recently, the American Society of Clinical Oncology (ASCO) developed guidelines and graded recommendations for commercially available gene profiling assays. This review places these recommendations in the context of the technology used, the clinical series and trials upon which these assays are based, and available data on the utility of these assays. Early results from prospective trials are starting to become available that can provide further support for the relative benefits and limitations of different assays. Ultimately, ongoing developments and refinements in technology as well as the maturation of numerous trials incorporating one or more gene profiling tests will further establish the accuracy and utility of gene expression profiling and other bioassays in decision-making for early stage breast cancer.

Introduction

Breast cancer is a complex disease with heterogeneous presentation and clinical course. Factors that are prognostic for recurrence and mortality risk and predictive of magnitude of risk reduction attributable to specific systemic treatment options are critical to personalize management. The anatomic TNM staging system was used for decades to prognosticate risk and guide treatment. More recently, tumor characteristics like the nuclear grade and the proliferative index as measured by Ki67 immunohistochemical staining also provided risk assessment. However, as specific treatments for breast cancer have evolved, the characterizations of predictive markers that identify the degree of benefit to therapy are more

relevant and useful. Oophorectomy was shown to benefit some patients around 100 years ago but the basis for its activity - the estrogen receptor was discovered many decades later.¹ The estrogen, progesterone and human epidermal growth factor receptors (ER, PR, and HER2) are examples of validated factors that are both prognostic and predictive.^{2,3} For example, HER2 expression predicts a higher risk of recurrence independent of treatment and is also predictive of response to the HER2 antibody trastuzumab. Higher grade and Ki67 score are associated with higher risks independent of treatment and larger relative reduction in recurrence with chemotherapy.⁴

Predictive markers for chemotherapy have been elusive, yet represent a high priority given the significant short and long-term effects of therapy. While tumor grade and hormone receptor negativity are somewhat predictive of chemotherapy benefit, they have not been sufficiently discriminating, as evidenced by consensus recommendations for chemotherapy for most patients with node-negative breast cancer.⁵ With the development of multigene expression profiling, 1 of the first applications sought was to identify patients with lower risk breast cancer who would most benefit from chemotherapy.⁶ The last decade has seen the development, commercialization, and increasing utilization of multigene assays, designed to better assist physicians and patients to make high-quality decisions in early-stage breast cancer.

Recently, the American Society of Clinical Oncology (ASCO) published their first set of guidelines on the use of gene profiling assays and reviewed the literature to provide levels of evidence and recommendations for the use of these assays for both prognostic and predictive (treatment selection) purposes for defined population and clinical scenarios⁷ (**Table 1**). Profiling tests differ in the technological platforms used for studying gene expression; in the number and specific genes that are being tested and in the patient populations used for their development, validation, and assessment of clinical utility. It is important to note that these guidelines were issued prior to publication of initial MINDACT results.

This review focuses on the development, methodology, validation, and most importantly, clinical utility of the assays listed and summarized on (**Table 2**). In the ASCO guidelines, similar levels of evidence are assigned to assays that are performed on samples

TABLE 1. Summary of ASCO Guidelines on Biomarker Assays to Guide Decisions for Early Stage Invasive Breast Cancer*

Key:

- Assists in decisions on the use of adjuvant chemotherapy
- Prognostic, but should not be used for decisions on the use of adjuvant chemotherapy
- Should not be used for decision-making

Patient Characteristics	ER/PR-positive, HER2-negative, Node-negative			ER/PR-positive, HER2-negative, Node-positive			HER2-positive or Triple Negative		
	Type of Recommendation	Evidence Quality	Strength of Recommendation	Type of Recommendation	Evidence Quality	Strength of Recommendation	Type of Recommendation	Evidence Quality	Strength of Recommendation
Oncotype DX	Evidence	High	Strong	Evidence	Intermediate	Moderate	Consensus	Insufficient	Strong
Prosigna (PAM50 ROR)	Evidence	High	Strong	Evidence	Intermediate	Moderate	Consensus	Insufficient	Strong
EndoPredict	Evidence	Intermediate	Moderate	Evidence	Insufficient	Moderate	Consensus	Insufficient	Strong
MammaPrint	Evidence	Intermediate	Moderate	Evidence	Intermediate	Moderate	Consensus	Low/Insufficient	Moderate/Strong
Breast Cancer Index	Evidence	Intermediate	Moderate	Consensus	Insufficient	Strong	Consensus	Insufficient	Strong
IHC4	Evidence	Intermediate	Moderate	Evidence	Intermediate	Moderate	Consensus	Insufficient	Strong
Mammostrat	Evidence	Intermediate	Moderate	Evidence	Intermediate	Moderate	Consensus	Insufficient	Strong

* Adapted from Harris L et al. (Ref 7)

TABLE 2. Commercially[†] Available Gene Profiling Assays for Early Stage Breast Cancer

Assay	Vendor	No. of Genes	Technology	Predictive*/Prognostic	Eligible Patients	Measure/ Categories
Oncotype DX	Genomic Health	16 cancer 5 control	qRT-PCR	+ / +	ER+ and HER2-, T1/2 0-3 nodes	RS: low (<18), intermediate (18-31), high (>31) risk RT-PCR assay for ER, PR and HER2
MammaPrint	Agendia	70	Microarray	- / +	Stage I and Stage II breast cancer	Good risk and poor risk Intrinsic Subtype
PAM50	Prosigna and Nanostring Technologies	50 cancer 22 control/ housekeeping	Digital bar-coded mRNA analysis	- / +	ER+, Stage I/II 0-3 nodes	ROR: Low (<10), intermediate (10-20), high (>20%) risk Intrinsic Subtype
Breast Cancer Index	bioTheranostics	MGI - 5 cell cycle genes H/I - Gene expression ratio	qRT-PCR	+ / +	ER+	Low, intermediate and high risk
IHC4	None	4 (proteins)	IHC, semi-quantitative			Composite formula based on ER, PR, HER2, Ki67 semiquantitative expression
Genomic Grade Index	Ipsogen	70 6	Microarray qRT-PCR	- / +	ER+, intermediate grade	High or low grade
EndoPredict	Myriad/ Sividon Diagnostics	8 cancer 3 control	qRT-PCR	- / +	ER+, HER2-	The test result is composed of the “molecular fingerprint” of a tumor in combination with the established prognostic parameters nodal status and tumor size.
Mammostrat	Clariant	5 (proteins)				

† All except IHC4 – not commercially available

* Based on analysis of tumor tissue from prospective randomized trial

ER indicates estrogen receptor; FFPE, formalin-fixed, paraffin-embedded; HER2, human epithelial receptor 2; IHC, immunohistochemistry; NC, not cleared or approved by the US Food and Drug Administration; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; RS indicates recurrence score.

from randomized trials as are assigned to assays from non-randomized series. Therefore, an additional aim of this review is to provide further perspective on the methodology and utility of gene profiling assays for both prognosis and treatment decisions, especially in the framework of the level of evidence that takes into account study design upon which the assays are based including the more recently validated studies. The purpose of this review is:

- To discuss the available assays in the context of the underlying supportive data and the source of the data, such as large registries or randomized trials.
- To understand what each of these assays brings to clinical decision making.
- To relate and discuss the assays with respect to the early data emerging from prospective randomized clinical trials.

Methodologies

This section provides an overview of the general technologies used to develop an assay and the additional specifics are provided in each individual assay section. Early gene profiling assays required fresh tissue to perform RNA extraction, amplification, and labeling followed by hybridization to oligonucleotide arrays for detection and quantification. Adaptations to use formalin-fixed, paraffin embedded (FFPE) tissue using reverse transcriptase polymerase chain reaction (RT-PCR) have made these assays much more feasible and quantitative. One round of RT-PCR generates complementary DNA from RNA and this is followed by quantitative PCR (qPCR). NanoString's nCounter technology is a modified version of the DNA microarray. It uses molecular "barcodes" and micro-imaging to detect and enumerate hundreds of unique transcripts in one hybridization reaction.⁸ Each color-coded barcode is linked to a single target-specific probe to a gene of interest. Profiling can also be made on the basis of several immunohistochemical assays that are processed and scored and integrated in a consistent and semiquantitative manner, although inter-observer variability remains a limiting factor.

Validation and Utility

Only commercially available assays are included in this review. For all assays, a threshold for the amount of invasive cancer is specified. The tissue should be representative of the tumor. It must be obtained and processed adequately to generate sufficiently high quality RNA for gene expression assays. Regulatory approval requires that a technique and readout be accurate and reproducible, with specific metrics for consistency on repeat testing. In addition, the association of the readout with outcome must be validated using independent datasets of appropriately described patients with specified follow-up, and maintaining constant cut points. In general, a reliably validated test will perform well across the intended population and over a range of variables included in the population such as receptor status, tumor grade, nodal status, and age.

The utility of a test describes its impact on decision-making

and how it affects long-term outcomes. Establishing this utility requires the assay to be performed in a controlled (ideally randomized) trial that tests the treatment for which the assay is intended to help select. Verifying and measuring utility is the most challenging and rarely accomplished milestone for an assay to be prognostic or predictive. The most rigorous proof requires a randomized trial comparing the use of the assay to standard care to make a treatment decision and the demonstration that a clinically relevant outcome (eg, recurrence or quality of life) is improved with a quantification of the benefit and any counterbalancing harm. In the case of breast cancer, most of the data for predictive or prognostic factors is derived from retrospective studies or prospective observational studies, with ongoing large scale prospective trials of different designs ongoing described later in this review.

Therefore, the clinician must ask key questions prior to ordering a test:

- How reliable is the test? Does it accurately and reproducibly measure or estimate the index of interest (eg, risk of recurrence or expected degree of benefit from a given therapy)?
- Does the test provide information that is independent of patient and tumor factors?
- How useful is the test? Will it allow for a decision to maximize benefit and improve cancer-related outcomes, or to avoid a toxic treatment without sacrificing outcome?

This review focuses on the background, performance and utility of the various gene profiling tests available for early stage breast cancer. It provides additional perspective based on more recent supportive literature.

Presently Available Gene Profiling Assays

Oncotype DX

The *Oncotype DX* assay uses RT-PCR to measure gene expression of 16 cancer-related genes (identified in discovery cohorts and also chosen on biological rationale), and 5 housekeeping genes in breast cancer tissue samples.⁹ The test is performed on FFPE tumor samples in a Clinical Laboratory Improvement Amendments (CLIA) and College of American Pathologists (CAP)-regulated central laboratory. A Recurrence Score (RS) result (a continuous variable ranging between 0 and 100) is then calculated by an algorithm for each patient.

Oncotype DX RS provides both prognostic and predictive information. The assay was developed as well as validated in patients with ER-positive early-stage invasive breast cancer who are treated with endocrine therapy and predicts the 10-year risk of distant recurrence in this group of patients.¹⁰⁻¹⁶ The score was found to be prognostic in patients treated with tamoxifen and aromatase inhibitors.^{9,11,13-15} Also, the predictive value of RS with respect to the likelihood of benefit from chemotherapy has been demonstrated in two randomized trials. Paik and colleagues assessed 651 patients with node-negative ER+ breast cancer of which 227 were randomly assigned to tamoxifen and 424 to tamoxifen plus che-

motherapy. The study concluded that patients with high RS had a greater probability of significant benefit from cyclophosphamide/methotrexate/fluorouracil (CMF) chemotherapy as compared to those with a low RS score ($P = .038$).¹⁰ A study by Albain and colleagues assessed patients with node-positive breast cancer who were randomized to receive tamoxifen with or without prior cyclophosphamide, doxorubicin, and fluorouracil (CAF) chemotherapy. The tissue samples consisted of 40% of the 927 patients in the tamoxifen and CAF-T groups that had sufficient RNA for analysis (Total N = 367, tamoxifen, n = 148; CAF-T, n = 219). This study concluded that high RS was associated with substantial benefit from CAF, especially during the first 5 years as compared to those patients with low RS. This difference was statistically significant with a P-value of .029.¹³ One of the shortcomings of this study was the limited number of blocks available for analysis (40% of all patients). This, along with the lower number of events in patients with an intermediate RS in both studies, indicated a possibility that there may be some benefit of chemotherapy in this subgroup of patients.

Several large series have confirmed the ability of RS to provide prognosis independent of conventional risk assessment parameters using multivariable analyses^{9,11,14,15} thereby fulfilling criteria for level I category B evidence for estimating the 10-year risk of distant recurrence and the likelihood of benefit from adjuvant chemotherapy. Consequently, Oncotype DX testing for these indications has been incorporated into international guidelines such as the European Society for Medical Oncology (ESMO), St. Gallen, NCCN (includes 1 to 3 positive nodes) and ASCO (node negative only),⁷ with node-positive cases excluded by ASCO but included by NCCN.¹⁷⁻¹⁹ More recently, larger population-based studies have shown less chemotherapy use and excellent short term outcomes in patients with low recurrence scores.²⁰⁻²² NCCN includes 1-3 positive nodes and ASCO includes node negative only.

The potential benefit of chemotherapy in patients with an intermediate RS result is being addressed in the prospective Trial Assigning Individualized Options for Treatment (TAILORx), in which patients with hormone receptor-positive, HER2-negative, node-negative disease and RS of 11-25 were randomized to chemoendocrine or endocrine therapy alone. Patients with RS 10 or less received hormonal therapy alone and those with RS of 25 or higher were all treated with chemotherapy followed by hormonal therapy. Recently, outcomes from the low-risk cohort treatment only with hormonal therapy were reported, confirming a very low expected rate of distant recurrence of 0.7% at years at a median follow-up of 69 months.²³

To follow-up on the SWOG S8814 study, the recently closed prospective Rx for Positive Node, Endocrine Responsive breast cancer (RxPONDER) trial (or South West Oncology Group [SWOG] S1007 trial) was proposed to study non-inferiority of endocrine treatment in comparison with chemoendocrine treatment in patients with 1 to 3 positive nodes with RS of 25 or less.

These two large prospective trials will validate and explore the utility of several aspects of the 21-gene assay such as:

- 1) Confirm that the low risk group of patients on Oncotype DX is truly at a low risk of recurrence without chemotherapy.
- 2) Assess the impact of chemotherapy in patients with node-negative intermediate RS and 1 to 3 positive nodes with RS <25.
- 3) Allow more robust analyses of the subgroups of patients based on tumor size, tumor stage, grade, age, etc.

MammaPrint

The MammaPrint assay studies the transcription of 70 genes associated with cell cycle, signal transduction, proliferation, invasion, angiogenesis, and metastasis. It uses a DNA microarray platform and is derived from a comparison of expression profile from tumors of patients who developed metastases within 5 years to those who did not within a node-negative cohort who received no systemic therapy.²⁴ The readout is a binary high-risk or low-risk profile corresponding to a 13% or 56% chance of developing distant metastases at 10 years without adjuvant treatment. The US Food and Drug Administration cleared the MammaPrint assay for marketing as a prognostic test but not to select therapy or predict response to therapy. Subsequently, it has been validated by more studies.²⁵⁻²⁷ Hence, it was established that MammaPrint is significantly associated with prognosis in breast cancer patients 1 to 3 positive lymph nodes.²⁶ Additionally, it was also shown by a pooled analysis of patients with tumors lesser than 2 cm that MammaPrint could identify a low-risk group independent of histologic grade, nodal status, treatment, HER2, and ER status.²⁸

The predictive ability of MammaPrint assay was assessed in a retrospective pooled series of 7 studies involving 541 patients who received chemo-endocrine or endocrine therapy alone. Although the analysis did show a significant benefit from chemotherapy in patients with a high risk profile, the hazard ratio for distant metastasis-free survival was similar in both the risk groups at 5 years and the P value was non-significant (0.45) indicating that the assay did not predict chemotherapy benefit.²⁹

The impact of MammaPrint assay on adjuvant treatment decisions was demonstrated by the RASTER observational study that showed that 81% of high-risk patients by MammaPrint received chemotherapy in comparison to only 15% of the low-risk patients by MammaPrint.³⁰ Hence, based on the evidence supporting the prognostic utility of MammaPrint, it was included as a prognostic tool in 2 international guidelines (ESMO and St. Gallen) and in some national guidelines such as those issued by the Arbeitsgemeinschaft Gynäkologische Onkologie (AGO), where it is included as an option with level II category C evidence.

The Microarray In Node-negative and 1 to 3 positive lymph node Disease may Avoid ChemoTherapy (MINDACT) trial has included MammaPrint for risk assessment. The MINDACT trial obtained both clinical prognostic (Adjuvant! Online, a suite of online tools to aid health professionals and patients with early cancer discuss the risks and benefits of getting additional therapy

after surgery), as well as gene profile scores, and assigned those who were low risk by both to no chemotherapy (N=2745) and those who were high risk by both (N=1806) to chemotherapy. Discordant cases (N=2142) were randomized to chemotherapy vs no chemotherapy. Hormonal therapy was given to hormone receptor positive cases. This trial also compared endocrine regimens of 2 years of tamoxifen followed by 5 years of letrozole vs 7 years of letrozole and chemotherapy regimens of FEC (fluorouracil, epirubicin, and cyclophosphamide) followed by docetaxel vs docetaxel-capecitabine treatment. Early results at 5 years of median follow-up confirm higher metastases, relapse, and death rates in high- compared with low-risk groups, and the discordant group exhibited an intermediate metastases-free survival rate of 95% with no difference based on chemotherapy assignment, but with a limited number of events to exclude a benefit, but showing that the assay can potentially reduce the number of patients who would otherwise be prescribed chemotherapy.³¹

The recently published 5 year follow-up shows that the low-risk group (N = 2745) had an excellent outcome with a distant disease-free survival of 97.6% without chemotherapy, whereas this value was 90.6% in the high-risk group (N = 1806) after receiving chemotherapy.³² In the discordant group (N = 2550, or 23.2% of the whole group), the overall distant disease-free survival was 94.7% (95% confidence interval 92.5% to 96.2%) with a non-significant 1.5% lower risk in the group randomized to chemotherapy. These data provide an estimate of potential reduction of patients who would otherwise be prescribed chemotherapy by 46%. More follow up will be needed to determine whether or not chemotherapy had a meaningful impact in the discordant group.³²

Prosigna/PAM50

Prosigna is a gene profiling assay based on the 50-gene intrinsic subtype predictor set, PAM50.³³ The nCounter Dx Analysis system (Nanostring Technologies, Inc., Seattle, WA) is used for the analysis of RNA obtained from FFPE breast tumor tissue in this assay, which measures the expression of 50 genes in the PAM50 panel along with 8 housekeeping genes (for normalization), 6 positive controls, and 8 negative controls by using a hybridization reaction with nucleic acid probes designed specifically for it. A Prosigna score (value between 0 and 100) also called Risk of Recurrence (ROR) Score is then assigned by the Prosigna algorithm. The Prosigna score along with the nodal status is used to determine risk categories (low, intermediate, or high) which represent the 10-year risk of distant recurrence for HR+ post-menopausal women with early-stage breast cancer. The assay is sensitive and uses 250 ng total RNA from FFPE tissue. It can be performed in a local pathology unit or molecular biology laboratory. Prosigna assay was cleared for marketing by the FDA in September 2013 as a prognostic test. However, it is not used to select therapy or to predict/detect response to therapy.

The archived samples from the Austrian Breast & Colorectal Cancer Study Group 8 (ABCSG-8) trial were used to validate

the Prosigna assay. This trial randomly assigned post-menopausal women with HR+ early-stage breast cancer to receive 5 years of tamoxifen versus 2 years of tamoxifen, then 3 years of anastrozole. The cohort used to validate this assay consisted of 1,478 patients (node negative and node positive) and established that the Prosigna score provided significant prognostic information (10-year distant recurrence) and was better than the traditional clinicopathologic characteristics. These findings were applicable to the validation cohort as a whole as well as the node positive and node negative groups separately.³⁴ The assay was further validated for patients with 1 to 3 positive nodes by 2 prospective trials (ABCSG-8 trial and the translational arm of the ATAC [anastrozole or tamoxifen alone or combined] trial [TransATAC]) using data from 2485 patients.^{35,36} Hence, Prosigna met the criteria of level I-II, category B evidence as a prognostic tool and has been included in AGO guidelines as a level II category B evidence.^{37,38} However, its ability to predict response to chemotherapy or impact treatment decisions has not been established by any studies. The use of intrinsic subtype beyond standard evaluation of HR and HER2 receptor status remains under study.

Breast Cancer Index (BCI)

Breast Cancer Index has 2 independent biomarker panels—Molecular Grade Index (MGI) and *HoxB13/IL17BR (H/I)*.³⁹ Both were derived from tumors from patients treated with or without tamoxifen and followed for outcomes, and identifying independently prognostic genes algorithmically. The MGI is prognostic of early and late recurrence and assesses tumor proliferation based on analysis of 5 cell cycle genes. The *H/I* is a gene expression ratio related to estrogen signaling. It is prognostic and predictive of the likelihood patient benefit from extended endocrine therapy. The BCI score (0-10) is a linear combination of MGI and *H/I* which together provide more accurate prognostic power. The BCI score serves as a continuous risk index for prognostication of early and late recurrence. The *H/I* gives a binary result, a “high versus low” BCI Score, which was validated in a retrospective analysis of 2 prospective trials. The population included 1340 patients with early stage estrogen receptor positive and lymph node negative breast cancer across three cohorts (TransATAC, Stockholm, Multi-Institutional).⁴⁰ A large proportion of patients (55% to 65%) in all 3 cohorts were classified as low risk. These patients have continued to exhibit a low risk of recurrence beyond 5 years (<3.5% ROR).⁴⁰ Therefore, the role of the *H/I* biomarker predicting benefit from extended hormone therapy was investigated in the MA.17 trial of extended hormonal therapy that compared letrozole to placebo after the completion of 5 years of adjuvant tamoxifen.⁴⁰ From the 5157 patients in the overall trial, 249 tissue blocks were analyzed using a nested case-control design. High *H/I* was significantly associated with patient benefit from extended endocrine therapy with letrozole ($P = .0061$). Patient characterized as low *H/I* had no significant benefit. There was a significant association between treatment benefit and *H/I* ($P = .03$).

IHC4

The IHC4 assay is based on a multivariate model that uses semi-quantitative scoring from immunohistochemistry for ER, PR, HER2, and Ki67. The assay uses FFPE tumor biopsy specimens and an algorithm calculates a risk score for recurrence.⁴¹ The validation cohort that was followed included 1125 patients from the Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial who were estrogen receptor-positive (ER-positive) who did not receive adjuvant chemotherapy, had the Recurrence Score (RS) calculated, and had sufficient tissue for the IHC measurements of four parameters: estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2), and Ki-67. The primary endpoint being distant recurrence was measured using proportional hazards model with sample splitting to control for overfitting. Additionally, a separate cohort of 786 patients was used to create and assess a prognostic model using the traditional variables and the four IHC markers (IHC4 score). These 4 IHC markers in the presence of classical variables provided independent prognostic information. In sample-splitting analyses, the information in the IHC4 score was found to be similar to that in the Oncotype RS, and little additional prognostic value was seen with the combined use of both scores.

The IHC 4 assay uses the information obtained from ER, PR, and Ki67 differently compared to the classical interpretation of these markers in daily practice. Unlike using these markers as binary categories, for example, ER-positive vs ER-negative, IHC4 uses a mathematical equation that combines the semi-quantitative expression values of these markers into a single risk score. This equation is available to the public however there is some inter-observer variability that is seen with the application of the equation to local pathology. The mathematical equation behind the IHC4 score is public; however, applying the formula to local pathology results could result in considerable inter-observer variability in the absence of standardized quantification of each of the four variables that would match the IHC assay sensitivity and the dynamic ranges used in the original analysis.⁴²

EndoPredict

The EndoPredict test (Sividon Diagnostics GmbH, Koln, Germany), is a RT-PCR-based assay that classifies patients with ER-positive breast cancer being treated with adjuvant endocrine therapy alone into a low risk or a high risk of recurrence. This assay, measuring the expression of 8 cancer genes and 3 housekeeping genes, is available in Europe as a diagnostic kit and is performed by local laboratories. Additionally, a comprehensive risk score called EPclin is calculated by combining the EndoPredict score with nodal status and tumor size. This EPclin score has been validated in 2 randomized phase III trials.⁴⁴ EndoPredict can also be used to determine ER-positive patients at risk for late recurrence.^{42,45}

Of note, OncotypeDx and EndoPredict have developed a clinical scoring system that amends the risk based on clinical factors such as tumor size, grade/proliferative index, and nodal

status.^{44,46,47} In addition, several of the assays provide quantitative levels mRNAs for ER, PR, and HER2, along with threshold for positivity. These readouts have been shown to correlate well with immunohistochemical and other assays, and have also been shown to correlate with response to hormonal and HER2-therapies.⁴⁸⁻⁵⁰ However, these measures have not been shown to over-ride or better predict response compared to conventional measurements for receptors.

Genomic Grade Index

The Genomic Grade Index (GGI) (MapQuant Dx, Ipsogen, France) assay measures the expression of 97 genes and designates a molecular grade by using microarray technique. It was developed by correlating gene expression profiles of histological grade I and grade III tumors.⁵¹ Also, a 6-gene version of this assay is available that uses RT-PCR technology and can be used for FFPE samples.⁵² The GGI can also stratify histologically intermediate-grade ER-positive breast cancers into high or low molecular grade with considerable difference in prognosis.⁴² Additionally, GGI could identify 2 clinically relevant ER+ subtypes with very distinct clinical outcomes in both systemically untreated and tamoxifen only treated BC patients.⁵³

A cohort of 570 patients for which histological grade and relapse-free survival (RFS) was available was used to measure the prognostic information of GGI. The data set pooled from this cohort along with 3 publicly available datasets was used.⁵⁴⁻⁵⁶ A higher rate of relapse was observed in histological grade 3 tumors in comparison with histological grade 1 tumors (HR, 3.18; 95% CI, 2.1-4.8; $P < .001$). The histological grade 2 group (216 patients) was further subdivided into two categories: a grade 1-like gene profile and a grade 3-like gene profile. Here, a higher rate of relapse was observed in the grade3-like gene profile subgroup in comparison to the grade1-like subgroup (HR, 3.61; CI 2.25-5.78; $P < .001$). GGI divided the original cohort of 570 patients into two risk categories (high or low) with significant difference in RFS (HR, 2.83; CI 2.13-3.77; $P < .001$). It is important to note that only tumor size, lymph node status and GGI were statistically significant in multivariate analysis even though GGI histological grade, ER status, lymph node status, and tumor size were all associated with RFS in univariate analysis. In multivariate analysis, histologic grade was not significant (HR, 1.38, 95% CI 0.89-2.14; $P = .11$) whereas GGI showed significant prognostic information (HR, 1.99, 95% CI 1.43-2.78; $P < .001$). Hence, it was established that GGI can improve the precision of grading for prognostic purposes. The prognostic information of GGI was further validated in a large meta-analysis including almost 3000 patients.⁵⁷

Mammostrat

Mammostrat is a 5-protein IHC that assesses 5 functional proteins - SLC7A5, which mediates nutrient transport; p53, a cell cycle checkpoint control; HTF9C, a cell cycle-dependent protein; NDRG1, a stress- and hypoxia-inducible gene product; and CEA-

CAM5, a carcinoembryonic differentiation antigen. This assay was validated in 2 node-negative ER+ trials, NSABP B14 and B20 (same used for Oncotype DX) including 711 cases, and gives low, moderate, and high risk readout, but still a rather high recurrence risk (85% recurrence-free at 10 year) in the low risk category, and did not out-predict the chemotherapy benefit identified by Oncotype DX 21-gene recurrence score.⁵⁸ It was further evaluated in the Tamoxifen versus Exemestane Adjuvant Multicenter (TEAM) trial that included node-positive patients (47%) and those who received adjuvant chemotherapy (36%), with a total of 3837 cases analyzed, and showed an independent impact on 10-year distant recurrence-free survival over and above size, grade, nodal status and ER/PR/HER2 status.⁵⁹ This assay is FDA-cleared although not recommended by either NCCN or ASCO.

Additional Caveats on Clinical Utility of Gene Profiling in Early Stage Breast Cancer

A prospective randomized trial testing the application of an assay compared to standard care, with a clinical relevant outcome as the primary endpoint is the ideal way to formally assess the utility of an assay. However, such trials have rarely been done in the past as the perception of utility from early studies led to rapid adoption, and neither CLIA certification nor FDA approval have required this level of proof. Retrospective analysis of tissues from a prospective trial using a uniform treatment and follow-up protocol can also be very helpful in validating prognostic value as long as there is not a chance of selection bias due to limited specimen availability. However, retrospective series may lead to bias, for example, the blocks available may be from the larger tumors so the smaller tumors are selectively depleted and this is an important component of standards that are being developed for the discovery and validation of prognostic factors and these criteria have been set forward as REMARK criteria.⁶⁰ The optimal validation of predictive value is derived from a controlled randomized trial that is comparing the treatments whose magnitude is predicted by the assay in question – which until recently was best approximated retrospectively.^{10,13} However, we now finally are obtaining data from prospective randomized trials incorporating assays to stratify for treatment, no treatment or randomization to treatment or not.^{23,31} The FDA has signaled that diagnostic assays with therapeutic implications will require prospective trials demonstrating utility.

Another important aspect about most assays is that their prognostic and predictive ability may vary over time.⁶¹ Most statistical analyses assume constant hazard and odds ratios associated with a biomarker, yet studies with longer-term follow suggest that the impact of prognostic markers, including gene profiling scores are strongest in the first 5 years to beyond this time. Hence, the prognostic or predictive value of any assay is likely to be overestimating the effect if extrapolated to 10 years or beyond. In fact, data mining exercises and comparisons using large gene sets and patient samples tend to identify many fewer candidate genes and

signatures that predict recurrences beyond 5 years, as shown with the BCI assay in a trial comparing 5 to 10 years of endocrine therapy.^{62,63} Likewise, the estrogen receptor and associated genes component of the Oncotype assay has been shown to predict late (>5 year) distant recurrence risk.^{48,64} EndoPredict also predicts late recurrence but it is not predictive of benefit from extended hormonal therapy like Breast Cancer Index.⁴⁴ The lack of robust biomarkers for late recurrence may be a general biological phenomenon whereby mutational evolution and other factors introduce more chaos and unpredictability into the clinical trajectory, much like the tracking of weather or a storm becomes less definable at later time-points.

Additionally, intrinsic subtypes (eg, luminal A/B, basal and HER2-enriched) of breast cancer initially described based on unsupervised clustering of expression profiles are reported in some of the multi-gene assays, including PAM50 and Agendia's Blue-Print report.⁶⁵ While there are growing data to suggest that intrinsic subtypes may exhibit specific biological characteristics, there is yet no role in reassigning patients to treatment that is not based on conventional assessment and interpretation of HR and HER2 receptor status.

For quantitative gene expression data, it is ideal to view point estimates with accompanying 95% confidence intervals, the width of which mainly depends on the sample size for the subset of interest. We have much more longitudinal outcome data linked to gene expression signatures since these technologies became available in mid 1990s whereas next generation sequencing (needed to accurately assess mutational status and burden) was not developed until early 2004-2005. Therefore, outcomes data with these technologies are less mature. The next generation of assays may relate to the actual sequence of specific genes or gene sets as opposed to quantitative gene expression.⁶⁶ There are also emerging data that a higher mutational burden may predict a greater impact of chemotherapy. However, while mutational burden may predict short-term response to chemotherapy it may also be associated with worse longer term survival due to higher genomic diversity and emergence of therapeutic resistance.

Conclusion and Summary

Gene profiling studies have been shown to be more reproducible than certain measure such as tumor grade and these can add further prognostic refinement over and above conventional clinical and pathologic features. Prospective validation has been carried out on all the commercially available assays, especially for shorter term recurrences and mortality. Only Oncotype Dx has shown to predict benefit of chemotherapy linked to randomized chemotherapy trials, but biological features that predict low recurrence risk determined by other validated assays may also predict less relative benefit from chemotherapy. Given the long natural history of breast cancer as well as time-dependent nature of both hazards of recurrence and the prognostic/predictive values of most assays, it will be critical to await additional data from prospective con-

trolled trials linked to gene profiles and other bioassays including mutational profiling to further optimize and personalize therapeutic decision-making for early stage breast cancer.

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