ASCO/CAP
Guideline Recommendations for IHC Testing of ER and PgR in Breast Cancer

Arch Pathol Lab Med, 134:907-22, 2010
Journal of Clinical Oncology, 28:2784-2795, 2010

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SCHOOL OF MEDICINE
ASCO/CAP Guidelines for IHC Testing of ER and PgR in Breast Cancer

Invasive Breast Cancers (Mandatory)
Ductal Carcinoma In Situ (Optional)

IHC Assays for ER and PgR
Must be Comprehensively Validated

Quantitative Scoring of Results
Percent or Proportion of Positive Cells
Intensity of Positive Cells

Interpretation of Results
Calibrated to Response to Endocrine Therapy

<1% Expressing Cells
"Negative"
Expect 20-30%
Not Responsive to Endocrine Therapy

Retest and Confirm If:
External Control Negative
Internal Control Negative
Low Histological Grade
Lobular Subtype
Tubular Subtype
Mucinous Subtype
Other…

≥ 1% Expressing Cells
"Positive"
Expect 70-80%
Responsive to Endocrine Therapy

Report Results
Scores
Interpretation

Comprehensive Ongoing:
Quality Assurance
Proficiency Testing

*Probably single most helpful recommendation for improving accuracy
**Not required if internal control positive
What is Comprehensive Validation?

**Technical:** The assay should be specific, sensitive, reproducible, calibrated to clinical outcome, interpreted, and reported in a relatively uniform manner. There should be comprehensive ongoing quality assurance.

**Clinical:** The factor should identify groups of patients with significantly different risks of relapse, survival, or treatment response – demonstrated in multiple large studies (ideally randomized clinical trials).

**Useful:** Actually used by physicians to make important treatment decisions.

### Comprehensively Validated IHC Assays for Measuring ER\(\alpha\) and PgR in Breast Cancer

*(identified in ASCO/CAP Guidelines)*

<table>
<thead>
<tr>
<th><strong>Estrogen Receptor</strong></th>
<th><strong>Reference</strong></th>
<th><strong>Antibody</strong></th>
<th><strong>Cutpoint for &quot;Positive&quot;</strong></th>
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<tbody>
<tr>
<td></td>
<td>Harvey. J Clin Oncol 17:1474, 1999</td>
<td>6F11</td>
<td>Allred Score ≥3 (1-10% weakly positive cells)</td>
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<td>Cheang. J Clin Oncol 24:5637, 2006</td>
<td>SP1</td>
<td>≥1%</td>
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<td></td>
<td>Phillips. Appl IHC Molec Morphol15:325, 2007</td>
<td>ER.2.123 + 1D5</td>
<td>Allred Score ≥3 (1-10% weakly positive cells)</td>
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<tr>
<td></td>
<td>Dowsett. J Clin Oncol 26:1059, 2008</td>
<td>6F11</td>
<td>H-score &gt;1 (≥1%)</td>
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</table>

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<th><strong>Antibody</strong></th>
<th><strong>Cutpoint for &quot;Positive&quot;</strong></th>
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<td></td>
<td>Mohsin. Modern Pathol 17:1545, 2004</td>
<td>1294</td>
<td>Allred Score ≥3 (1-10% weakly positive cells)</td>
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<tr>
<td></td>
<td>Dowsett. J Clin Oncol 26:1059, 2008</td>
<td>312</td>
<td>≥10%</td>
</tr>
</tbody>
</table>
A Few Words about Validation in Your Laboratory

It is NOT mandatory to use the same validated antibodies and assays identified in the Guidelines.

It IS mandatory to get the same results.

It IS mandatory to provide ongoing proof of equivalent results.

Using the same validated antibodies and assays is a very reasonable thing to do.

FDA approval does not necessarily mean that there has been comprehensive validation of the assay.
A Few Words About Scoring Results

Examples of Satisfactory Methods:

H-Score (McCarty, Arch Pathol Lab Med 109:716, 1985)
Allred-Score (Allred, Mod Pathol 11:155, 1998)
Quick-Score (Rhodes, J Clin Pathol 53:125, 2000)
Percent and intensity positive – by computer (many methods)
Percent and intensity positive – by human (absolute, point-counting)

Clinical Relevance:

Tam = Tam+Chemo in postmenopausal patients with HIGH ER
  e.g. Albain. Lancet Oncol. 11:55, 2010
Neoadjuvant endocrine therapy = restricted to patients with HIGH ER
  e.g. Ellis. JNCI 100:1380, 2008

⇒ Must avoid using IHC assays that are too sensitive (saturated)
A Few Words about Controls

Multiple purposes

- confirm satisfactory performance of assay
- confirm satisfactory condition of sample

Perfect control does not currently exist

Certain normal tissues are satisfactory practical external controls
(e.g. endometrium; normal breast = external and internal control)

- readily available
- relatively stable reactivity
- broad range of expression
- ~90% normal breast tissue with positive epithelial cells...but not 100%
- do NOT use breast cancers (abnormal variable expression)

Routinely score and record results to illuminate problems

- utilizing same control daily can be very helpful

Every batch of cases must have positive and negative controls

Every pathologist must review the controls before scoring case
Essential Elements of Quality Assurance and Proficiency Testing

Confirm accuracy of results
- reasonable distribution (70-85% ER-positive; 60-75% PgR+)
- stable with time (e.g. day of week; quarter to quarter)
- >90% concordance vs. independent expert

Demonstrate reproducibility of results
- >90% vs. in-house or outside expert

Demonstrate concordance of results
- >90% between pathologists
- >90% by same pathologist

Comprehensive training of pathologists
- educational conferences (e.g. review important new publications)
- training conferences (e.g. calibrate scoring between pathologists)

Comprehensive training of other laboratory personnel
- educational and training conferences
- monitor reagents (e.g. lot numbers; expiration dates)
- monitor and regulate fixation time

Designate an in-house expert Medical Director
- with true expertise (requires substantial training and commitment)
- monitor quality of slides daily before disseminating to other pathologists
- go-to person for general oversight; problem solving; feedback, etc.

Advertise quality of performance
- reassurance to others (e.g. annual report at tumor board)

Comprehensive record keeping
- all above and more
Example of Comprehensive Report

LEFT BREAST MASS, CORE BIOPSY:
Invasive ductal carcinoma

<table>
<thead>
<tr>
<th>Factor</th>
<th>Proportion Score (PS)</th>
<th>Intensity Score (IS)</th>
<th>Total Score (TS)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen Receptor</td>
<td>5</td>
<td>2</td>
<td>7</td>
<td>Positive</td>
</tr>
<tr>
<td>Progesterone Receptor</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Comments:
Estrogen receptor (antibody 6F11) and progesterone receptor (antibody 1294) were evaluated by immunohistochemistry (IHC) on routine formalin-fixed paraffin-embedded tissue utilizing comprehensively validated assays (J Clin Oncol 17;1474, 1999; Mod Pathol 17:1545, 2004) in compliance with ASCO/CAP guidelines (Arch Pathol Lab Med 134:907, 2010; J Clin Oncol 28:2784, 2010).

The results were scored and interpreted using the Allred Score (J Clin Oncol 17;1474, 1999). PS (proportion of positive tumor cells): 0=none; 1<1/100th; 2=1/100th-1/10th; 3>1/10th-1/3rd; 4>1/3rd-2/3rds; 5>2/3rds. IS (average intensity of positive tumor cells): 0=none; 1=weak; 2=intermediate; 3=strong. TS = PS+IS (range 0-8); **positive >2**.

Formalin fixation time: 20 hours.
Essential Reading


Current issues in ER and HER2 testing by IHC in breast cancer. Mod Pathol. 2008 May;21 Suppl 2:S8-S15


Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol. 1999 May;17(5):1474-81

Re-evaluating adjuvant breast cancer trials: assessing hormone receptor status by immunohistochemical versus extraction assays. JNCI. 2006 Nov 1;98(21):1571-81.


Resistance to Hormone Therapies in Breast Cancer: Mechanisms and Clinical Implications

C. Kent Osborne
Dan L. Duncan Cancer Center
Lester and Sue Smith Breast Center
Baylor College of Medicine, Houston, TX
Estrogen Action in Normal and Cancer

- Requires binding of E to the estrogen receptor (ER).
- ER is predominantly a nuclear protein that acts as a transcription factor.
- ER also has a non-nuclear function to activate growth factor signaling.
- ER controls hundreds of genes important for proliferation, cell survival, angiogenesis, other.
- Blocking ER is very effective treatment in appropriate tumors.
- Proper measurement of ER is crucial.
Types of Endocrine Therapy (Block ER)

1. Estrogen deprivation
   - ovarian ablation/suppression in premen
   - aromatase inhibitors in postmen

2. ER blockade (SERMS)
   - tamoxifen

3. ER downregulators
   - fulvestrant
Benefits of Endocrine Therapy

• Metastatic breast cancer (macrometastasis)
  - Clinical benefit rate of 50-60% in ER+
  - Median survival of 5-6 years (rare 10+ yrs)

• Adjuvant therapy after primary surgery (micrometastasis)
  - Reduction of recurrence of 50% if ER+
  - Probably cures in many patients

But, de novo and acquired resistance common
General Types of Resistance

1. De Novo.
2. Acquired, with ER still present but not functioning.
3. Acquired drug resistance but ER still functioning.
4. Acquired with ER lost.

Drug resistance vs loss of E dependence and acquisition of an alternate survival pathway.
Clinical Clues

- Loss of ER expression.
- Multiple responses to sequential endocrine therapies over time; “drug” resistance but not loss of E dependence.
- ER level decreases over time; gradual loss of E dependence.
- PR is lost 50% of time with resistance to tam; tumor progresses faster when lost.
- Tumors with high HER2 or EGFR have lower ER and PR and less response to endo therapy.
Mechanism of Resistance

• The tumor evolves and activates other survival pathways (escape pathways) to bypass the E block.
Driver and Escape Pathways in Cancer

Survival / Proliferation

3

a b c

1

2
Biological Systems in Normal and Cancer Cells

1. Complex
2. Multiple control mechanisms
3. Fine tuning
4. Redundant
5. Evolvable

Makes cells difficult to kill!
Escape Pathways

- Little data
- Few resistant/metastatic tumors to study
- Reliance on preclinical models
Molecular Action of E2/ER

Estrogen

Membrane

RTKs: EGFR, HER2, IGFR

GF

INGs

Stress

Microenvironment

ER

TFS

TFs

ER

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Endocrine Therapy

GF

RTKs:
EGFR, HER2, IGFR

Akt
Src
PI3K
FAK
Src
PELP1

Membrane

Stress Responses:
Treatments
Cytokines
Hypoxia
ROS
PAK

Cellular/signaling Kinases:
MEK/MAPK
PTEN/PI3K/Akt
P38/JNK
Src
FAK

Microenvironment

INGs

Possible Escape Pathways to Endocrine Resistance

Stress

ERα
variants,
ERβ: low
AR
CoA
HAT

CoA/R
High P160 (SRC1/3)
Low NCoR/SMART

TFs

Pathways:
Myc
Cyclin D1, E1, E2
BCL-2, MYC
Cyclins D1, E1, E2

Resistance: Growth, Proliferation, Survival, Angiogenesis

NR:
α: variants,
β: low
CoA/R

AP1
NFκB

HAT

Adapted from:
Musgrove & Sutherland, Nature Reviews Cancer, 2009
Acquired Resistance to Tamoxifen is Associated with Increased Levels of EGFR and HER2

Massarweh et al., SABCS 2004

(Benz et al., Breast Cancer Res Treat, 1992)
Overcoming Tam Resistance with Gefitinib in HER2-Positive Tumors

Hypothesis: GefitinibR is due to incomplete blockade of the HER signaling pathway (all HER dimer pairs).

Shou J, JNCI 2004
Massarweh S, ASCO 2002
Reversal of Tam Resistance with Gefitinib in HER2-Negative Tumors

Massarweh et al., SABCS 2002
## Conversion from HER2\(^-\) to HER2\(^+\)

<table>
<thead>
<tr>
<th>Study</th>
<th>% Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gutierrez (tumor)</td>
<td>12</td>
</tr>
<tr>
<td>Uhr (CTCs)</td>
<td>37</td>
</tr>
<tr>
<td>Lipton (serum)</td>
<td>26</td>
</tr>
</tbody>
</table>

Most converters had intervening endo Rx
Patients

- Postmenopausal women
- Age ≥ 18 years
- Stratum 1: Newly diagnosed ER- and/or PgR-positive MBC or disease recurring after adjuvant tamoxifen, completed ≥1 year before study entry
- Stratum 2: Disease recurring during or after AI therapy or who have failed first-line AI therapy for MBC
- PS 0-2
- No prior chemotherapy for metastatic disease

N=290 (206 in Stratum 1 & 84 in Stratum 2)

Response variables

Primary
- PFS in Stratum 1
- CBR (CR + PR + SD for ≥24 weeks using RECIST) in Stratum 2

Secondary
- CBR in Stratum 1
- PFS in Stratum 2
- ORR
- PFS in patients with HER2-expressing tumours
- Safety and tolerability
- PK

Until disease progression or other event requiring discontinuation

Osborne et al, manuscript submitted
0225 trial: PFS in Stratum 1 patients

PFS similar over first 200 days and KM curves then diverge compatible with a delay in the development of resistance similar to that in preclinical models.

A more substantial difference in PFS observed for the HER2 positive subset of patients (n=37).

Osborne et al, manuscript submitted
0713 trial: study design

Patients
- Postmenopausal women
- Age ≥ 18 years
- Newly diagnosed ER- and / or PgR-positive metastatic breast cancer
- No prior hormonal therapy, or development of metastatic disease during / after adjuvant tamoxifen
- Measurable or non-measurable disease (via RECIST)

N=94

1:1 randomisation

anastrozole 1 mg / day + gefitinib 250 mg / day

anastrozole 1 mg / day + placebo

Response variables
Primary
- PFS
Secondary
- ORR
- CBR
- OS
- Safety and tolerability
- Expression of biomarkers in tumour tissue sections

Until disease progression or other event requiring discontinuation

Cristofanilli et al, Abstract 1012, oral presentation, ASCO 2008
Progression-free survival

<table>
<thead>
<tr>
<th>Events</th>
<th>Gefitinib + anastrozole (n = 43)</th>
<th>Placebo + anastrozole (n = 50)</th>
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<tbody>
<tr>
<td>Median PFS (months)</td>
<td>14.5</td>
<td>8.2</td>
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</table>

HR (95% CI) = 0.55 (0.32, 0.94)

At risk:

<table>
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<th>Placebo</th>
<th>Gefitinib</th>
</tr>
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<tr>
<td>50</td>
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</table>
Reversal of Tam Resistance with Gefitinib in HER2- Tumors

Massarweh et al., SABCS 2002
Other Escape Pathways Identified

1. Oxidative stress/increased AP1 activity
2. Integrin signaling/increased pSrc and pFac
3. PI3K/AKT pathway activation
Oxidative stress and cancer

- Oxidative stress has been defined as “a disturbance in the pro-oxidant-antioxidant balance in favor of the former, leading to potential damage” (Sies, 1991)

- There is increasing evidence that malignant cells are in a pro-oxidant state due to:
  
  - increased formation of reactive oxygen species
  - decreased antioxidant defenses (Halliwell B, Biochem J. 2007)
Endocrine Resistance: ER/Signaling Networks Crosstalk

Integrins → GFRs (HER/IGFR) → Cytokines (PI3K/Akt) → Stress

ER/Signaling Networks:
- ERK1/2
- p38
- JNK

CoA

TFs

Proliferation Invasiveness

ER

ERE

CoA

Proliferation Invasiveness

Endocrine resistance?

Proliferation Invasiveness
Oxidative stress and endocrine resistance

• Tamoxifen can alter the redox status of the cell both as an oxidant and as an antioxidant

• Resistance to endocrine treatment is associated with oxidative stress (Schiff R et al., JNCI, 2000)
Stress-related pathways and endocrine resistance

- The stress-related kinase pathways Jun N-terminal kinase (JNK)/AP-1 and p38 MAPK are key mediators of cell response to oxidative stress

- JNK overexpression is a poor prognostic factor (Yeh et al. Int J Cancer 2006)

- cJun overexpression in ER+ breast cancer cells results in endocrine resistance (Smith et al. Oncogene 1999)

- JNK activity and AP-1 levels are higher in Tam resistant human tumors (Johnston et al. Clinical Caner Res, 1999)
Genetic approach (DN cJun)

**MCF7 Tet-Off**  
DN cJun (Tam 67)

Inhibits AP-1 activity in different cell lines

(Ludes-Meyers JH et al., Oncogene, 2001)
DN cJun enhances response to Tam in vivo

**DNcJun Clone 67**
- Proportion Not Responders: 40%
- Control: p = .04

**DNcJun Clone 62**
- Proportion Not Responders: 30%
- Control: p = .014

**Time to Response**
- Survival Probability: 85% non-complete responders
- Control: p = .0034
- DN cJun: p = .001

**Time to Complete Response**
- Survival Probability: 60% non-complete responders
- Control: p = .001
DN cJun delays TamRes onset in vivo

![Graph showing survival probability over time to tumor size doubling for DNcJun Clone 67. The graph compares Control and DNcJun groups, with a 50% tumor progression marker. The survival probability is shown on the y-axis, and time to tumor size doubling is on the x-axis. The legend indicates a p-value of 0.0028.](image-url)
DN cJun does not affect E2 stimulated growth *in vivo*

**DNcJun Clone 67**

- Control
- DN cJun

\( p = 0.31 \)

**DNcJun Clone 62**

- Control
- DN cJun

\( p = 0.6 \)
Summary and Conclusions

1. Resistance to endo therapy:
   - reactivation of the ER pathway or
   - activation of another escape pathway
2. Growth factor receptors, integrins, and stress pathways are prime candidates for escape from ER blockade.
3. Identification or anticipation of which pathways will take over when ER is blocked will be crucial for personalized treatment.
4. Treatment will require combination therapy.
5. Biopsies of resistant tumors in patients are crucial for study.
Molecular Classification of Oestrogen Receptor (ER) Positive/ Luminal Breast Cancers
Jorge S Reis-Filho 1, MD PhD FRCPath & Britta Weigelt 2, PhD
Email: Jorge.Reis-Filho@icr.ac.uk

INTRODUCTION
Breast cancer comprises a complex and heterogeneous group of diseases. Although the heterogeneity of breast cancer was known for a long time, it was only after seminal studies using high throughput transcriptomic methods that this concept was brought to the forefront of breast cancer research, and, most importantly, clinical practice [1-4]. It is currently accepted that the heterogeneity of breast cancer is such that it encompasses different diseases that have distinct risk factors, clinical presentation, histopathological features, molecular characteristics, response to therapies and clinical behaviour, which happen to affect the same anatomical site and to originate from cells in the same microanatomical structure (i.e. terminal duct-lobular unit) [1-4].

Despite this heterogeneity, the management of breast cancers is currently based on a constellation of clinicopathological features that are derived from careful histopathological analysis of primary cancers, including tumour size, histological subtype and grade, lymph node metastases, and lymphovascular invasion [1-5]. In addition, three predictive markers have been incorporated in breast cancer patient care, namely oestrogen (ER) and progesterone (PR) receptors, which are the predictive markers of response to endocrine therapy, and HER2, which is the predictive marker and molecular target of Trastuzumab and Lapatinib [2-5]. This information is then used according to guidelines or using multiple parameter algorithms for clinical decision making (e.g. Adjuvant! Online) [1-5]. These approaches, albeit simplistic, have been proven to work, given that the mortality of breast cancer has declined over the last two decades despite the increase in the incidence of breast cancer. Furthermore, the predictions made by multiparameter algorithms largely meet the actual outcome of breast cancer patients [6]. It has become blatantly clear, however, that these approaches only provide information about the best therapy for the average breast cancer patient with a given constellation of clinicopathological features, and is not sufficient for the implementation of individualised therapies.
In addition to the development of the concept of personalised medicine, the advent of high throughput technologies that could be applied to the study of solid malignancies have led to a rediscovery of the heterogeneity of cancers [7-12]. In particular, these methods have led to the development of a molecular taxonomy for breast cancers, which was initially believed to have histogenetic implications [7, 9, 13]. Microarray-based gene expression profiling studies have also provided direct evidence to demonstrate that ER-positive and negative breast cancers have remarkably distinct transcriptomes [1-3]. This has led to the realisation that ER-positive and negative diseases are fundamentally different. Furthermore, multi-gene predictors, which have been suggested as alternatives for the current clinicopathological algorithms for treatment decision making, have been developed.

Oestrogen receptor positive disease comprises the vast majority of breast cancers diagnosed, therefore it is not surprising that numerous potential prognostic and predictive markers that can be used for the management of patients with ER-positive disease have emerged [1, 2, 5]. In this manuscript, I will review the impact of the molecular classification of breast cancers, its impact on our understanding of ER-positive disease, and the current multi-gene predictors that can be used for the management of patients with ER-positive breast cancer.

**BREAST CANCER MOLECULAR CLASSIFICATION: EMPEROR’S NEW CLOTHES?**

Back in the late 90s, the promise of microarrays was of apocalyptic dimensions, with one of the proponents of this technology suggesting that treatments or cures for all human illnesses would be found by applying this technology to the study of human cancers. In fact, a paradigm shift in terms of our understanding of breast cancer took place with the publication of the seminal class discovery studies published by the Stanford group [7], where the heterogeneity and complexity of breast cancers were re-discovered at the molecular level.

Perou et al. [7] analysed 38 invasive breast cancers (36 invasive ductal and 2 lobular carcinomas), 1 ductal carcinoma *in situ*, 1 fibroadenoma and 3 normal breast samples, and a number of biological replicates of tumours from the same patients with cDNA microarrays, and defined an ‘intrinsic gene’ list (i.e., the genes that vary more among tumours from different patients than in samples from the same tumour) [7]. The approach employed at that time may now sound quaint to the average microarrayer, but in early 00s they had a major impact on how breast cancer was perceived. Hierarchical cluster analysis using this ‘intrinsic’ gene list revealed that ER-positive and ER-negative breast cancers are fundamentally distinct at the transcriptomic level [7-11], and also demonstrated the existence of molecular
subtypes of breast cancer: luminal, normal breast-like, HER2 and basal-like [2, 8-10, 14-16]. In the original publication, ER-positive cancers were classified as luminal tumours, whereas ER-negative cancers comprised the so-called basal-like, HER2 and normal breast-like cancers. When this approach was applied to additional breast cancer microarray datasets linked to outcome information, it was demonstrated that i) similar molecular subtypes of breast cancer could be identified in multiple cohorts of breast cancers [8-10, 15], ii) that luminal cancers could be subclassified into two (luminal A and B) [8] or three groups (luminal A, B and C) [10], and iii) that different molecular subtypes were associated with distinct clinical outcomes [8, 15, 17]. According to this classification, luminal cancers are characterised by the expression of ER and ER-related genes, and can be subclassified into two groups (i.e. luminal A and luminal B) according to the expression levels of proliferation related genes. Luminal A tumours express the highest levels of ER and ER-related genes, and the lowest levels of proliferation-related genes; on the other hand, luminal B cancers express the opposite gene expression pattern [1, 2, 10, 18]. Basal-like breast cancers are characterised by the expression of genes usually found in ‘basal’/myoepithelial cells of the normal breast, and express high levels of proliferation related genes. HER2 or HER2-enriched breast cancers are characterised by the expression of HER2 and genes mapping to the HER2 amplicon. Normal breast-like cancers are still poorly understood, however their defining feature is that they consistently cluster together with samples of normal breast and fibroadenomas. In terms of outcome, luminal A cancers were shown to have the best prognosis, whereas basal-like tumours to have the worst outcome [8-10].

This molecular taxonomy for breast cancers has been enthusiastically embraced by surgeons, oncologists and scientists alike. Several groups have demonstrated that some of these subtypes (e.g. basal-like) have distinct risk factors, clinical presentation, histological features, response to therapy and outcome [2, 19-25]. The data accumulated have led to some experts in the field to suggest that traditional clinicopathological features and immunohistochemical markers should be replaced by this molecular taxonomy [26].

To quote Amos Elon, “if hindsight really is twenty-twenty it is important to try as often as we can to analyse, embrace and employ it as we make our way through life”. In 2011, if we look back at the ‘intrinsic’ molecular subtype classification, it would be fair to temper the enthusiasm with this taxonomy, as it has numerous limitations [4, 27]. The initial approach employed for the identification of the molecular subtypes was based on hierarchical clustering analysis. It should be noted, however, that this approach requires large datasets, is to some extent subjective, and cannot be employed for the classification of individual
samples prospectively [28, 29]. Therefore, the proponents of the molecular taxonomy developed 'single sample predictors' (SSPs). These SSPs are based on the correlation between the expression profile of a given sample with the centroids for each molecular subtype (i.e. average expression profile of each molecular subtype) [10]. Our group [18] and others [30] have recently demonstrated that the identification of molecular subtypes of breast cancer by SSPs depends on the methodology employed, and only basal-like cancers can be reliably identified. In fact, the subclassification of luminal tumours into A and B subclasses was strongly dependent on the SSP used and different patients were classified as A or B depending on the methodology employed [18, 30]. In fact, even when the authors of the molecular taxonomy themselves classified the same cohort of breast cancer patients (that is, NKI-295 [17]) using two different methods [9, 10], one by Sorlie et al.[9, 31] and the other by Hu et al.[10, 32], the agreement was only moderate (Kappa scores = 0.527 (95% confidence interval 0.456 to 0.597)). Second, there are several lines of evidence to suggest that normal breast-like cancers may constitute an artefact of gene expression profiling (that is, samples with a disproportionately high content of normal breast epithelial cells and stromal cells) [2, 11, 12, 18]. Third, and perhaps most importantly for the main topic of this session, given that the subdivision of luminal tumours into A and B is driven by the levels of expression of proliferation-related genes and that several studies have demonstrated that the expression levels of proliferation-related genes in ER-positive cancers are a continuum and do not display a bi-modal distribution, the subclassification of luminal cancers is likely to be arbitrary [2, 18, 30, 33, 34]. Fourth, the HER2 molecular subtype neither comprises all cases classified as HER2-positive by FDA approved methods (i.e. immunohistochemical analysis and chromogenic/fluorescence in situ hybridisation) and not all HER2-positive cancers by FDA approved methods are classified as HER2 subtype by microarrays [11, 18, 35]. Finally, contrary to the initial belief that luminal tumours would originate from luminal cells and basal-like cancers from 'basal' cells of the normal breast, recent studies [36, 37] have demonstrated that the likeliest cell of origin of basal-like breast cancer resides in the luminal progenitor compartment.

Therefore, I argue that the 'intrinsic' molecular subtype classification of breast cancer is not yet ready for clinical use in prognostic models or otherwise. Standardisation of the definitions and the methodologies for the identification of the molecular subtypes and prospective clinical trials to validate the contribution of these five molecular subtypes in addition to the current clinicopathological parameters for the management of breast cancer patients are still required [18, 38]. Although the qRT-PCR based test for the identification of the subtypes (i.e.
PAM50 [11, 26]) is an interesting approach, independent validation of its robustness and its prognostic and predictive values have yet to be published.

**PROGNOSTIC GENE SIGNATURES: PROLIFERATION BY ANOTHER NAME**

Microarray-based prognostic gene signatures were heralded as a major breakthrough for the management of breast cancer patients, as initial studies claimed that these signatures would provide a more objective assessment of the risk of relapse of breast cancer patients and would be more reproducible than the methods currently used. The first prognostic gene signatures (70-gene signature also known as MammaPrint® [14, 17] and the 76-gene signature [39, 40]) were developed to be applied to all breast cancer patients. Numerous studies provided evidence to demonstrate that the prognostic information provided by these signatures is indeed independent of the information provided by tumour size, presence of lymph node metastasis and histological grade [14, 17, 39, 40]. Subsequent to these initial stories of success, several groups developed their own prognostic signatures either employing bottom-up or top-down approaches (for reviews, see [1, 2]). Furthermore, microarray signatures to capture the information provided by histological grade were developed and shown to be independent predictors of outcome [41, 42].

Following the initial over-hyping of microarray-based prognostic gene signatures, the enthusiasm with this approach waned. Furthermore, re-analyses of the initial studies on cancer prognosis with microarrays demonstrated that the overlap between gene signatures was negligible [1, 2]; that the gene composition of first generation signatures was not stable [43, 44]; and that these gene signatures were strongly time dependent [39]. The wave of often unjustified scepticism that followed, was possibly an over-reaction. One expert in the field of biomarker discovery and validation stated that “on close scrutiny, in five of the seven largest studies on cancer prognosis, this technology performs no better than flipping a coin. The other two studies barely beat horoscopes” [45]. Again, hindsight is a beautiful thing, in particular when it comes to the contribution of new technologies to science. With the availability of microarray datasets in public repositories, meta-analyses performed by independent groups revealed that:

i) different gene signatures do identify similar (but not necessarily identical) groups of patients as of poor outcome [32, 34, 46];

ii) the assignment of cases as of poor outcome is based on the expression of proliferation-related genes [18, 33, 34];
iii) the discriminatory power of first generation signatures is restricted to ER-positive breast cancers; in fact, <5% of ER-negative breast cancers are classified as of good prognosis using these approaches [33, 34];

iv) first generation prognostic gene signatures do not identify prognostically significant groups of ER-negative disease [33, 34];

v) that rare types of ER-negative breast cancers that have an indolent outcome (e.g. adenoid cystic carcinomas) are consistently classified as of poor prognosis [47];

vi) proliferation is perhaps the strongest determinant of outcome in ER-positive disease. In fact, when first generation prognostic gene signatures are divided into sub-signatures composed of ‘proliferation-related genes’ and ‘non-proliferation-related genes’, and only the former are used, the overall performance was not degraded and, improved for some signatures. On the other hand, sub-signatures composed of ‘non-proliferation-related genes’ display suboptimal performance [34];

vi) within ER-positive breast cancers, there is a correlation between the poor prognosis groups ascribed by Mammaprint® and OncotypeDX™ and luminal B cancers [1, 2, 32]; however, the level of agreement between these methods for the identification of poor prognosis ER-positive cancers is yet to be determined.

In parallel with the development of first generation microarray-based prognostic gene signatures, a 21-gene signature based on quantitative real time RT-PCR was developed through a re-analysis of microarray datasets and a review of the literature [48, 49]. OncotypeDX™(Genomic Health, Redwood, CA, USA) was developed and validated through a retrospective analysis of formalin-fixed, paraffin-embedded material from the prospective clinical trials B-20 and B-14 (for reviews, see [50, 51]). The signature includes the expression assessment of 5 reference genes for the standardisation of the relative quantification and 16 prognostic genes that are related to proliferation (KI-67, STK15, Survivin, CCNB1 and MYBL2), invasion (Stromelysin 3 and Cathepsin L2), HER2 group (HER2 and GRB7), oestrogen receptor signalling (ER, PR, Bcl2 and SCUBE2), and GSTM1, BAG1 and CD68 [48]. The expression of these genes is presented as a Recurrence Score (RS) ranging from 0 to 100 providing an estimate of 10-year distant recurrence-risk. For clinical use, patients are separated in 3 categories: low RS (RS<18), intermediate RS (RS≥ 18 and <31) and high RS (RS≥31) [48]. OncotypeDX™ was developed and validated in patients with ER-positive, node-negative breast cancers using retrospectively the material of 2 randomised trials (i.e. NSABP-B-20 and NSABP-B-14), and this signature was shown to outperform standard clinico-pathological parameters for the prediction of 10-year distant recurrence-risk [48]. The 21-gene has been subsequently evaluated in other cohorts of breast cancer patients [52] and
shown to be an independent prognostic parameter in patients with ER-positive tumours with up to 3 positive-nodes receiving adjuvant chemotherapy [53], and in postmenopausal patients with ER-positive tumours treated with anastrozole [54].

OncotypeDx™ RS has also been shown to be correlated with the benefit patients derive from adjuvant chemotherapy in samples from clinical trials [49, 55, 56]. In fact, patients with tumours displaying high RSs, despite their poor prognosis, derive significantly more benefit from chemotherapy than those with low RS tumours. In addition, patients with low RS cancers appear to derive negligible benefit from the addition of chemotherapy to tamoxifen. Therefore, OncotypeDx™ has also been used as a predictive marker of benefit from chemotherapy.

Level II evidence in support of the prognostic role of OncotypeDx™ has already been accrued. Therefore, it has received the approval from the American Society of Clinical Oncology (ASCO) [57] and was included in the National Comprehensive Cancer Network guidelines (NCCN guidelines Breast Cancer version 1.2011 - http://www.nccn.org/) as an option to evaluate prognosis and to predict response to chemotherapy for ER-positive, node-negative breast cancer patients, as a complement to clinico-pathological features. None of the other prognostic signatures has been endorsed by these professional bodies.

One important point that should not be overlooked is the fact that there is evidence to suggest that the prognostic power of OncotypeDx™, in a way akin to the other first generation signatures, largely if not exclusively stems from the quantitative analysis of the levels of expression of proliferation-related genes.

Hence, one could claim that first generation prognostic signatures only have prognostic power in ER-positive disease [27], and that this prognostic information is derived from the analysis of the expression levels of proliferation-related genes. In fact, recent comparisons of the prognostic information provided by OncotypeDx™ or four immunohistochemical markers (i.e., ER, PR, HER2 and Ki67 - a proliferation marker) semi-quantitatively analysed in the material from the ATAC (Arimidex, Tamoxifen, Alone or in Combination) prospective trial demonstrated that these four markers would provide prognostic information that is at least be equivalent to OncotypeDx™ [58].

Given the importance of proliferation, and the fact that multiple studies have provided level III evidence in support of Ki67 as an independent predictor of outcome, why has Ki67, a
proliferation marker routinely used in surgical pathology laboratories, not been incorporated in immunohistochemistry-based prognostic panels? The answer to this question lies in the definition of biomarkers (i.e. “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”) [59]. The assessment of Ki67 in different pathology laboratories has yet to be standardised. Multiple antibodies are available and different laboratories perform this test using different antigen retrieval methods and antibody dilutions; furthermore, the quantification of Ki67 labelling indices has some degree of subjectivity, as it is based on the assessment of ‘hot-spot’ areas. However, international consortia of experts in the field of biomarker discovery and validation have now turned their attention to pre- and analytical parameters required for a standardised Ki67 immunohistochemical test. Guidelines should be available in 2011.

In any case, acknowledgement of the existence of a continuum of proliferation levels in the group of ER-positive breast cancer [1, 2, 18, 33, 34] and that the extremes of this continuum have dramatically different outcomes and probably responses to chemotherapy is of utmost importance and clinical trials testing the therapeutic efficacy of new agents should take this information into account.

PREDICTIVE SIGNATURES: CAN THEY BE INCORPORATED IN CLINICAL PRACTICE?

First generation prognostic signatures can also be used as predictors of response to multi-drug chemotherapy regimens in ER-positive breast cancers. Studies have demonstrated that OncotypeDx™ can be used to define which patients should receive chemotherapy in addition to endocrine therapy [49]. There is also evidence to suggest that Mammaprint® [14, 60] and Genomic Grade Index [42, 61] can also be used in this context. The clinical utility of these signatures in the context of prediction of response to multi-drug chemotherapy regimens stems from the fact that they are surrogates of proliferation and that proliferation is associated with response to multi-drug chemotherapy.

In terms of predictive markers of endocrine therapy, some considerations need to be made. ER status has a strong negative predictive value for response to endocrine therapy (i.e. patients with ER-negative disease do not respond to endocrine therapy). ER expression, however, is not sufficient to predict which ER-positive tumours will respond to hormone therapies [5, 62]. Using microarrays technologies, gene expression signatures have been developed in several studies to predict outcome in tamoxifen treated. A promising approach is the sensitivity to endocrine therapy (SET) index, which was developed through the
transcriptomic analysis of a large series of ER-positive breast cancers [63]. The SET index is based on the principle that expression of genes correlated with ER may predict better response to endocrine treatment than ER expression alone. Microarray analysis was used to identify 165 genes co-expressed either positively (n=109) or negatively (n=59) with ER in a discovery cohort of 437 breast cancers. Cut-off points were determined in a validation cohort of 245 patients to define 3 categories of sensitivity (low, intermediate and high). Association between SET and outcome was then analysed in 3 types of ER-positive cohorts receiving either adjuvant tamoxifen for 5 years or neo-adjuvant chemotherapy followed by endocrine therapy (i.e. tamoxifen or aromatase inhibitors) or no adjuvant systemic treatment. The SET index was significantly associated with the outcome of patients receiving any type of endocrine treatment (tamoxifen or chemo-endocrine treatment) but had no prognostic value in untreated patients. Unlike other multi-gene signatures evaluating proliferation in ER-positive tumours, the SET index seems to be predictive of benefit from endocrine therapy independently of the inherent prognosis of the tumour. A potential clinical application of the SET index is in the identification of a subset of ER-positive tumours associated with an excellent prognosis and no relapse in the tamoxifen-treated group (high SET index tumours) and in the chemo-endocrine group (high and intermediate SET index) [63]. This type of predictive signatures may constitute one of the ways forward for the molecular stratification of ER-positive cancers.

**MOLECULAR GENETICS OF OESTROGEN RECEPTOR POSITIVE BREAST CANCERS**

Studies investigating the patterns of gene copy number aberrations and mutations in breast cancer have demonstrated that the pattern and type of gene copy number aberrations segregates with the ER-status breast cancers [64-68]. In fact, while the approximately 80% of grade I ER-positive breast cancers and 50% of grade III ER-positive tumours harbour concurrent deletions of 16q and gains of 1q, these changes are found in a small minority of ER-negative tumours [69] (for a review see [70]). Even when present in ER-negative cancers, the mechanisms leading to 16q losses in ER-positive and –negative diseases differ: in ER-positive disease, 16q losses and 1q gains often stem from an unbalanced chromosomal translocation [i.e. der(16)t(1;16)/der(1;16)], whereas in ER-negative cancers, deletion of 16q often results from loss of chromosome 16 (i.e. 16p and 16q losses) [69-72]. These lines of evidence have been interpreted as evidence that ER-positive and ER-negative breast cancers are distinct at the genetic level and that progression from ER-positive to ER-negative disease is an uncommon biological phenomenon.
Contrary to initial observations that progression from low- to high-grade breast cancer would be incredibly rare [73], the available genomic data suggest that progression from grade I to grade III ER-positive tumours may happen. In fact, approximately 50% of grade III ER-positive cancers harbour the typical pattern of gene copy number aberrations found in grade I tumours (i.e. deletion of 16 and gain of 1q) [69, 70]. Grade III ER-positive cancers, however, harbour additional genetic changes and more often harbour gene amplifications rarely seen in grade I ER-positive disease (e.g. 8p11.2, 11q13-q14, 17q21, 17q23.2, and 20q13) [64-69].

Given that histological grade correlates with proliferation in ER-positive breast cancers, that high proliferation ER-positive breast cancers have a poor prognosis, and that the so-called luminal B cancers overlaps significantly with the group of ER-positive grade III patients, it is not surprising that luminal B cancers have been shown to have more complex molecular karyotypes than luminal A cancers [67, 68, 74].

Importantly, although grade I ER-positive cancers seem to have less genomic aberrations than high grade ER-positive disease, it should be noted that these tumours do display varying levels of genetic instability [70]. Furthermore, recent massively parallel sequencing studies have demonstrated that there is intra-tumour genetic heterogeneity within ER-positive cancers and that some ER-positive tumours harbour fusion genes [75]; however, no fusion gene in ER-positive disease has been shown to be recurrent as yet.

ER-positive disease has been shown to harbour numerous gene mutations and the repertoire is quite vast; it should be emphasised, however, that the majority of mutations identified so far are present in a minority of lesions. One of the most prevalently mutated genes in ER-positive breast cancers is PIK3CA [76], which is an integral component of the PI3K-AKT-mTOR pathway. Interestingly, in vitro and clinical studies have suggested that tumours with PIK3CA mutations may be sensitive to inhibitors of mTOR (e.g. rapalogs) and small molecule inhibitors that inhibit PIK3CA, TORC1 and TORC2 [77].

**CONCLUSION**

ER positive disease comprises a spectrum of tumours, with varying degrees of proliferation and levels of genetic aberrations. Proliferation as defined by microarray-based gene signatures and OncotypeDx™ has been shown to be one of the main independent prognostic markers for patients with ER-positive disease, and also to be a predictive marker of benefit for addition of multi-drug chemotherapy to endocrine therapy. As a group, these tumours respond to endocrine therapies, however a substantial proportion of cases are either de novo resistant or develop resistance over time. Active research using high throughput methods is
currently being performed to identify the potential mechanisms of resistance to hormone therapies and ways to circumvent them. Despite the enthusiasm with the use of the molecular taxonomy for breast cancers and the terminology luminal A and luminal B [26], recent studies have called into question the reproducibility of these subtypes [18, 30, 38]. In fact, their clinical utility remains to be determined.

With the advent of massively parallel sequencing and the ability to characterise the entire genomes of cancers, it is likely that the drivers of ER-positive disease will soon be identified. It is anticipated that the repertoire of mutations in breast cancer will be vast with few recurrent genetic lesions. Nevertheless, this deluge of information in conjunction with functional genomic approaches may expedite the development of predictive classification systems for ER-positive disease.

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