

DR ANDREW R GREEN (Orcid ID : 0000-0002-0488-5913)

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## **Current issues with luminal subtype classification in terms of prediction of benefit from endocrine therapy in early breast cancer**

**Running title:** Luminal breast cancer and benefit of endocrine therapy

Lutfi Alfarsi,<sup>1</sup> Simon Johnston,<sup>1</sup> Dong-Xu Liu,<sup>3</sup> Emad Rakha<sup>1,2</sup> and Andrew Green<sup>1</sup>

<sup>1</sup>*Academic Pathology, Division of Cancer and Stem Cells, School of Medicine, University of Nottingham, Nottingham City Hospital, Hucknall Road, Nottingham NG5 1PB;* <sup>2</sup>*Cellular Pathology, Nottingham University Hospitals NHS Trust, Hucknall Road, Nottingham NG5 1PB;* <sup>3</sup>*The Centre for Biomedical and Chemical Sciences, School of Science, Faculty of Health and Environmental Sciences, Auckland University of Technology, Auckland 1010, New Zealand.*

### **Correspondence:**

Dr Andrew R. Green. Academic Pathology, Division of Cancer and Stem Cells, School of Medicine, University of Nottingham, Nottingham City Hospital, Hucknall Road, Nottingham NG5 1PB

Tel: (44) 115 8231407

Email: andrew.green@nottingham.ac.uk

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### **Abstract**

Endocrine therapy for oestrogen receptor-positive (ER+) breast cancer (BC) is arguably the most successful targeted cancer therapy to date. Nevertheless, resistance to endocrine therapy still occurs in a significant proportion of patients, limiting its clinical utility. ER+ or luminal

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BC, which represents around three quarters of all breast malignancies, are biologically heterogeneous with no distinct, clinically defined sub-classes able to predict the benefit of endocrine therapy in early settings. To improve patient outcomes, there is a clear need for improved understanding of the biology of the luminal BC, with subsequent translation into more effective methods of diagnosis to identify potential predictive biomarkers for endocrine therapy. This review summarises current knowledge of factors predictive of benefit of endocrine therapy, and discusses why molecular classification systems of BC have yet to be translated into the clinic.

Keywords: breast cancer, luminal, endocrine therapy response, resistance, oestrogen receptor, predictive biomarker

## **Introduction**

Oestrogen receptor (ER) is the driving transcription factor in up to three-quarters of all BC and its protein expression by immunohistochemistry classifies patients as either having ER+ or ER-negative (ER-) disease. Endocrine therapy is one of the most effective and well-established targeted anti-cancer treatments for ER+ BC. However, despite its undisputed efficacy, up to one third of patients will relapse after treatment for early stage disease, whilst in the advanced setting, all will eventually progress and become resistant [1]. It is therefore desirable to be able to predict, at an early stage of treatment, which ER+ patients will benefit from endocrine therapy, and which are likely to develop resistance, so that alternative or additional therapies can be offered to prevent relapse and reduce BC specific mortality. The identification of biomarkers to predict endocrine therapy benefit in addition to ER status is therefore of crucial importance in stratifying ER+ patients for targeted therapy. During the past two decades, the molecular era has promised much with respect to prediction of benefit

from endocrine therapy in early BC, but clinicians remain unable to predict which of their patients will benefit from endocrine therapy.

ER and progesterone receptor (PR) measurements are currently used for both clinical diagnosis to classify BC patients and as a guide to endocrine therapy [2]. Although the absence of ER expression is well known predictor of lack of hormonal therapy effect, the response of ER+ tumours to hormone therapy is heterogeneous. The role of PR measurement to predict benefit to endocrine therapy remains controversial. Some studies suggests its measurement lacks clinical value in BC management [3], while other reports considered that PR expression is a positive prognostic factor in ER+ BC [4, 5]. There is therefore a clear unmet clinical need for further biomarkers, beyond ER and PR, to help in predicting the benefit from endocrine therapy.

Global gene expression profiling (GEP) studies of BC have identified a distinct molecular subtype called 'luminal-like'. The current accepted molecular classification of luminal subtype divides them into two subgroups: luminal A and luminal B [6]. Luminal B disease demonstrates increased expression of proliferation-related genes which make it more aggressive in its clinical behaviour [7]. This subtype has a higher recurrence rate and lower survival rates after relapse [8], and is considered as a more aggressive subtype of ER+ disease, while luminal-A disease tends to result in better clinical outcomes [7]. However, such molecular classification is currently not routinely used in the clinic to guide endocrine therapy.

Thus, there remains an urgent need to identify biomarkers, singularly or in combination, and clinically relevant subclasses within ER+ BC to refine classification of luminal tumours, particularly with a view to predicting the benefit from the endocrine therapy in early BC. In this article, we provide a comprehensive review of the current modalities with potential

applicability in predicting benefit from endocrine therapy. The article focusses on why molecular classification systems have yet to be translated successfully into the clinic for this purpose, and how the field can move forward in order to improve patient outcomes.

### **Endocrine therapy**

Endocrine therapy is a well-established standard treatment for ER+ BC patients. Classes of endocrine therapy include selective ER modulators (SERMs) (e.g. tamoxifen), aromatase inhibitors (AI) (e.g. anastrozole, exemestane and letrozole) and selective ER down-regulators (SERDs) (e.g. fulvestrant). The key clinical questions for those with ER+ BC are 1) which endocrine therapy should be offered and 2) what is the role of biomarkers in selecting patients for the most effective therapy option?

### ***Mechanisms of action***

Current endocrine therapy works either by blocking or lowering the action of ER, or inhibiting peripheral synthesis of oestrogen from its precursors in the circulation. Tamoxifen, a SERM, is non-steroidal synthetic agent that exhibits tissue-specific ER agonist or antagonist activity. The mechanism of action of tamoxifen involves a competitive inhibition of oestrogen binding to the ER via its ligand-binding domain. This induces a conformational change in the nuclear receptor. In ER+ breast tumours, binding of tamoxifen to the ER results in inhibition of oestrogen dependent gene transcription, cell proliferation and tumour growth [1]. Tamoxifen causes apoptosis and cell cycle arrest in G0/G1 phase *via* modulating growth factors. However, tamoxifen also exerts an agonist effect on the endometrium, thus increasing the risk of developing endometrial cancer as a side effect of BC treatment [1].

AIs reduce oestrogen levels by blocking the peripheral conversion of androgen to oestrogens catalysed by the aromatase enzyme. They are classified by their mechanisms of action into two types: steroidal agents such as exemestane, which irreversibly inactivates the enzyme, and non-steroidal such as anastrozole and letrozole, which inhibit aromatase through reversible binding [9]. AIs are recommended above tamoxifen for postmenopausal women with ER+ BC [10].

Fulvestrant, a SERD, is known as a “pure” anti-oestrogen as it does not have any agonist activity in other tissue contexts. It binds competitively to ER, inhibits its dimerisation and ultimately results in downregulation of ER expression [11]. Although fulvestrant is not currently approved by the National Institute for Health and Excellent Care (NICE) in the UK for the treatment of ER+ BC, it is widely used in the advanced setting and is approved by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) [12, 13].

### *Success*

Tamoxifen is considered the gold standard endocrine therapy for premenopausal women with early and advanced ER+ BC, and post-menopausal women who have contra-indications to AIs [10, 14]. The 2011 meta-analyses from the Early Breast Cancer Trialists’ Collaborative Group (EBCTCG) showed that, for ER+ patients, 5 years treatment with tamoxifen reduced the risk of BC recurrence and mortality by 39% and 30%, respectively [15]. A further reduction of the recurrence risk in ER+ BC can be achieved by extending the duration of tamoxifen treatment to 10 years [16]. However, whilst there has been a significant fall in mortality rates with the use of tamoxifen, a third of patients experience resistance to tamoxifen. Resistance is classified as either primary (relapse within the first 2 years of

adjuvant endocrine therapy) or secondary (relapse after 2 years on endocrine therapy, or within 12 months of completing adjuvant endocrine therapy) [17].

In postmenopausal women with ER+ BC, treatment with AIs is marginally superior to tamoxifen in efficacy both in terms of the risk of recurrence and overall survival [10, 18].

Recently, it has been reported that in premenopausal women with ER+ BC, clinical outcomes for patients with high recurrence risk may be improved by treatment with AIs and ovarian function suppression compared to tamoxifen alone [19].

### **Cyclin-dependent kinases 4 and 6 inhibitors**

Cyclin-dependent kinases 4 and 6 (CDK4/6) are important drivers of the cell cycle and are required for the initiation and progression of various tumours [20, 21]. Inhibitors of CDK4/6 lead to cell cycle arrest in the G1 phase by preventing phosphorylation of retinoblastoma protein, and thereby prevent tumour progression [22]. Currently, there are three selective inhibitors of CDK4/6 (palbociclib, abemaciclib and ribociclib) have been tested in clinical BC trials. Palbociclib has been approved by the FDA and EMA, in combination with an AI as endocrine based therapy in postmenopausal women with ER+/HER2-negative advanced or metastatic BC [23, 24]. Ribociclib has also been approved by the FDA, as initial treatment for postmenopausal women with advanced ER+/HER2-negative BC [24].

In the near future, the role of CDK4/6 inhibitors is likely to expand into the setting of early BC. For example, abemaciclib is currently being explored in combination with standard endocrine therapy for patients at higher risk of relapse [25].

## ER and PR measurement

The determination of ER protein expression has been used in the clinic for over 30 years to identify patients likely to benefit from endocrine therapy. Despite this, ER positivity is insufficient to predict whether an individual patient with early breast cancer is likely to experience disease relapse.

In addition to ER, there is some additional predictive benefit in measuring progesterone receptor (PR) expression, as recommended by the Royal College of Pathologists [2]. Immunohistochemistry (IHC) remains the most commonly used diagnostic technique for determination of ER and PR status as it is relatively quick, cheap and can be performed on formalin-fixed paraffin embedded (FFPE) tissue. Although the St. Gallen Guidelines recommended IHC for use in clinical practice in 2013 [14], there is no gold standard assay available for determination of ER and PR status by IHC with regard to the prediction of benefit from endocrine therapy. Until the recent decade, ER positivity was defined in routine clinical practice using a cut-off value for ER nuclear staining of 10%. By this criterion, patients with  $\geq 10\%$  nuclear staining of ER were deemed eligible for endocrine therapy [26].

The American Society of Clinical Oncology (ASCO) Reported Guidelines for ER and PR IHC assays was an attempt to make the benefit of endocrine therapy available to the widest range of patients. ASCO recommends 1% as a universal cut-off point to distinguish between ER positivity and negativity to help determine likelihood of patients responding to endocrine therapy. Thus, they considered “1% or more of cells staining for ER” as the cut-off for a specimen to be considered positive. Tumours with less than 1% of cells staining for ER are considered ER negative based on data that patients with such tumours do not receive meaningful benefit from endocrine therapy [27]. However, lowering the threshold for ER

positivity increases the proportion of patients in whom endocrine therapy is indicated. This has raised uncomfortable questions about the low ER+ (1-9%) tumours.

Subsequent studies have confirmed the suspicion that a significant proportion of these low-ER+ are in fact non-luminal tumours. It is therefore suggested that the most expeditious therapeutic approach may be to use both endocrine therapy and chemotherapy in this subset of patients [28, 29]. It is reported that patients with low ER tumours (1–9%), in contrast to those with ER+  $\geq 10\%$ , have clinicopathological characteristics more similar to those of the ER- negative tumours and did not appear to benefit from endocrine therapy [26]. A retrospective study of early breast cancer including patients with HER2-negative and low hormone (ER and PR) receptor expression tumours reported no clear effect on survival outcomes with the addition of endocrine therapy for patients with low ER/PR (1-9%) [30]. A further study supported this finding in early breast cancer, reporting that this subgroup behaves clinically like triple-negative BC in terms of pathological complete response and survival outcomes [31].

All of these results suggest that BC with low ER expression 1-9% and those with  $\leq 1\%$  of ER may have similar molecular features and clinical prognoses, but differ in terms of efficacy of endocrine therapy compared to patients who have tumours with  $\geq 10\%$  of cells staining positive for ER.



Whilst the measurement of ER is useful as a predictive biomarker to determine the efficacy of endocrine therapy, the role of PR in predicting response to endocrine therapy for patients with ER+ tumours is controversial. Thus, while it has been reported that benefit from tamoxifen is similar between PR+ and PR- patients [32] and also that PR status does not affect the relative efficacy of AI over tamoxifen [33], other studies report that PR status is an independent predictive indicator of endocrine therapy response and is associated with better overall survival and less relapse [34, 35]. A recent mechanistic study demonstrated that, in the presence of progesterone, PR guides ER to genomic binding sites associated with more favourable clinical outcomes [36]. Therefore PR positivity can be considered a biomarker of good response to endocrine therapy. Positive PR expression could, in future, be utilised to augment endocrine therapy response by the addition of progesterone to an ER-antagonist. In the meantime, there is still a need to find biomarkers beyond ER and PR status to predict endocrine therapy benefit, due to the complex biology and heterogeneity of luminal BC.

### **ER-negative BC and benefit from endocrine therapy**

Breast tumours that are ER negative but PR+ by current criteria are difficult to interpret and present a clinical challenge. Typically, patients with ER-/PR+ tumours will be given endocrine therapy. Although some studies do suggest the existence of a PR-positive class within ER-negative tumours, in up to 2–8% of cases, and that patients with these tumours may benefit from endocrine therapy [34, 37-39], there is controversy over whether the ER-/PR+ phenotype is a real phenomenon. Several recent studies including those from our group have indicated that ER-/PR+ phenotype represents a technical failure, resulting from either false-negative ER or false-positive PR result, at least in the majority of cases [27, 40-42].

In routine practice we have seen rare cases showing ER-/PR+ phenotype despite repeat staining of both markers. However, the frequency is less than 1%, with 4 cases seen in 9000 breast cancers (unpublished data). As expected these tumours exhibit more aggressive behaviour than the double positive tumours (ER+/PR+) [37, 39, 43]; however, the benefit of endocrine therapy in patients with these rare tumours featuring a genuine ER-/PR+ phenotype remains to be confirmed [15, 27, 44, 45]. The very low incidence of the tumours is likely to limit the ability to evaluate the benefit from endocrine therapy in clinical trials.

### **Gene expression profiling**

Gene expression profiling (GEP) studies over the past two decades have led to the molecular classification of BC into at least four subtypes. Computational methods have been used to subclassify BC based on gene expression pattern across clinical samples. The first study identified four subtypes based on expression of ESR1 (the gene encoding ER), ER-related genes and HER2. In this classification system, ER+ tumours comprise the largest class characterised by expression of not only ESR1 and ER-related genes, but also of low molecular weight cytokeratins and other genes characteristic of luminal epithelial cells. This intrinsic subtype was termed luminal class with no further sub-classification [46].

A subsequent study interrogated GEP data obtained from 78 BC cases and seven non-malignant breast samples, and further defined the intrinsic molecular subtypes of BC. This study classified luminal tumours into 3 subclasses including luminal-A, B and C. Luminal-B and C were associated with poor prognosis and outcomes compared to luminal-A, which had the highest expression of ER and ER-regulated genes. In contrast to luminal-A and B, luminal-C shared a high expression of genes with HER2 and basal-like subtypes, but with an unknown function [47].

Subsequent study by the same investigators using hierarchical clustering based on patterns of expression of 534 “intrinsic” genes failed to robustly identify luminal-C, and it subdivided luminal-like BC into luminal-A and luminal-B subtypes. Thus, some of the genes that previously clustered in the luminal-C subtype were clustered in the luminal-B and basal-like [48]. Classification of luminal BC differs between studies, most likely because of the identification and use of distinct intrinsic gene sets for cluster analysis. Most studies using GEP support the sub-classification of luminal tumours into luminal-A and B [6].

Although some luminal-B tumours have a similar signature to the HER2+ subtype, they are discriminated mainly by proliferation-related genes such as MKI67, CCNB1A and MYBL2. Luminal-A disease is a low proliferation phenotype associated with good prognosis and outcome, whereas the proliferative phenotype luminal-B is associated with worse clinical outcome [49]. Luminal-B tumours, though ER+, have a distinct molecular phenotype from the good-prognosis luminal-A subtype. Indeed, luminal-B tumours share many molecular features with ER-negative subtypes. Furthermore, the luminal-B subtype is defined by the expression of genes associated with endocrine therapy resistance such as CCNB1, MKI67, MYBL2, FGFR1 and ZNF703 [49]. However, classification of BC into luminal-A or B does not differentiate tumours according to endocrine therapy response and has not been incorporated into routine clinical practice.

### **Multigene expression signatures**

Multigene signatures can be valuable as prognostic tools with regards to recurrence and the stratification of risk, but so far studies have not validated their value in predicting benefit from endocrine therapy. Additionally, most of these gene signatures are focused on association with outcome following adjuvant treatment with endocrine therapy with or

without chemotherapy. Clinically, they have found useful application as biomarkers to pick out patients at high risk of relapse who would benefit most from the addition of chemotherapy prior to their endocrine treatment. However, they have not yet found a role in predicting benefit from endocrine therapy itself.

It is important to investigate the value of multigene signatures in terms of predicting benefit from endocrine treatment, as this may also guide clinical decision making. Table 1 summarises these multigene assays and recommendations for their use in ER+ BC.

Improving upon the current panel of two biomarkers (ER and PR expression) to predict endocrine therapy benefit may in future identify patients who could be spared endocrine therapy and its associated side effects. Alternatively, better biomarkers could be used to identify patients who would benefit from alternative approaches such as CDK4/6 or MTOR inhibition, either alone or in combination with current endocrine therapy.

Specific multigene signatures will now be discussed, with particular reference to their potential utility as predictors of benefit from endocrine therapy.

#### *PAM50 Prosigna*

The PAM50 Prosigna assay was developed to classify breast tumours into one of four intrinsic subtypes (Luminal-A, Luminal-B, HER2-enriched, and Basal-like), based on the expression measurement of 50 genes [50], which were first described in early GEP microarray studies [46-48]. This assay is performed on RNA isolated from FFPE breast tumour tissue and generates a risk of recurrence (ROR) score by weighting the molecular subtype correlations, pathologic tumour size and a subset of proliferation genes [50, 51]. PAM50 ROR score and intrinsic subtype can identify clinically relevant prognostic

subgroups of patient with a limited risk of metastasis after endocrine treatment only, for whom chemotherapy can be spared [52]. Additionally, another study reported that PAM50 ROR provided significant additional prognostic information with respect to late distant recurrence (DR)-free survival compared with clinicopathological risk factors [53]. The ASCO guidelines recommend that this assay may be used for patient with ER/PR+, HER2-negative (node-negative) early stage BC, in conjunction with other clinicopathologic variables, for predicting outcome and aiding decisions on adjuvant systemic chemotherapy [54].

PAM50 can potentially be utilised as a tool to determine whether extended endocrine therapy is required (beyond 5 years), although the assay has not yet been validated for this purpose. However, PAM50 lacks the ability to predict benefit from endocrine therapy as a first line treatment in early ER+ BC. Moreover, despite the fact that studies have confirmed that the PAM50 score can be used to determine which patients carry a high risk of late DR, this ability is more prognostic than predictive.

#### *Oncotype DX*

The Oncotype DX recurrence score (RS) is based on measuring the mRNA expression levels of 16 cancer related-genes and 5 housekeeping genes from FFPE tissue using the real-time qRT-PCR. This assay was developed for patients with ER+ lymph-node negative BC treated with tamoxifen to predict the risk of DR. The RS classifies patients into three groups: low, intermediate or high risk [55, 56]. Several reports have shown that the RS assay not only quantifies the likelihood of BC recurrence in ER+ patients, but also predicts the magnitude of chemotherapy benefit; thus low-risk patients derive minimal, if any, benefit from chemotherapy, while high-risk patients require more aggressive regimens [55, 57, 58].

Despite the clinical validation of Oncotype DX, there remain questions as to whether this assay provides more independent prognostic information than can be gained from immunohistochemical assay of known markers such as ER, PR, HER2 and Ki67 (see later section on IHC4 score) [59]. Thus there may be a simpler and cheaper way of obtaining the same prognostic information. However, in favour of Oncotype DX, it is argued that immunohistochemical determination of Ki67 lacks standardisation among laboratories and there is a lack of quality assurance programmes. PAM50 ROR, in comparison to the Oncotype DX RS, was found to be a more useful prognostic assay for predicting the risk of DR after endocrine treatment in ER+ patients, with better differentiation of intermediate- and higher-risk groups [60]. The Oncotype DX assay can be useful in predicting the benefit from chemotherapy, but is not currently helpful in the clinical setting for predicting the benefit from endocrine therapy for early stage ER+ BC. The St. Gallen Group and ASCO guidelines recommend Oncotype DX RS for newly diagnosed BC patients with ER+/HER2-negative to determine prognosis and aiding decision of chemotherapy [14, 54].

#### ***MammaPrint***

The MammaPrint assay measures the expression of 70 genes involved in metastasis, proliferation, tumour stroma and invasion in BC, irrespective of ER status. This assay is a gene microarray-based prognostic score that stratifies patients into low-risk or high-risk groups and utilises fresh tissue that has had its RNA stabilised [61, 62]. Again, like the other gene tests, it is able to demonstrate that only the high-risk ER+ patients could potentially benefit from chemotherapy, whereas low-risk group could be spared this type of treatment [63, 64]. In ER+/PR+ (HER2-negative) patients, this assay was shown to be an independent prognostic marker and it may be integrated into a selection of strategies for patients who are candidates for more aggressive therapeutic approaches [65].

The clinical utility of MammaPrint was recently confirmed in an international, prospective, randomised trial (MINDACT). The study found that women with early-stage BC, who were at a high clinicopathological risk and a low genomic risk according to MammaPrint for recurrence, could be spared chemotherapy [66]. The ASCO guidelines recommends the use of MammaPrint for patient with ER+/PR+, HER2-negative (node-negative) early stage BC, to guide decisions on adjuvant systemic chemotherapy [67], while St. Gallen group and EGTM do not differentiate between lymph node-negative and lymph node-positive disease [14, 68].

The requirement for fresh tissue has limited the clinical uptake of MammaPrint, as this is not always routinely available. The performance, validation and optimisation of MammaPrint using FFPE tissue remains to be validated [69].

#### ***Breast cancer index (BCI)***

The BCI is an algorithmic combination of a molecular grade index (MGI), consisting of the average expression of five cell cycle (proliferation)-associated genes (BUB1B, CENPA, NEK2, RACGAP1 and RRM2), the HOXB13:IL17BR (H:I) ratio which represents activation of the ER signalling pathway, and 4 housekeeping genes. The BCI assay uses RT-PCR and can be applied to FFPE tissues [70, 71]. The independent prognostic value of BCI in predicting the risk of distant metastasis in ER+/HER2- patients treated with endocrine therapy has been validated in several studies [72-74]. A recent study showed that BCI has more prognostic accuracy than Oncotype DX, indicating that additional subsets of patients with ER+ BC identified by BCI may be suitable candidates for extended endocrine therapy [75]. The St. Gallen Group and ASCO guidelines recommend that BCI may be used for

predicting outcome and aiding decision of chemotherapy for patients with ER+/HER2-negative BC [14, 54].

### ***EndoPredict (EP)***

The EndoPredict (EP) assay measures the gene expression profiles of eight BC-relevant genes (BIRC5, UBE2C, DHCR7, RBBP8, IL6ST, AZGP1, MGP and STC2) and 3 housekeeping genes in FFPE tissue sections by qRT-PCR in decentralised laboratories. The assay is useful for predicting the likelihood of DR in patients with ER+ HER2- BC treated with endocrine therapy [76]. The combination of EP with the two clinicopathological parameters, tumour size and nodal status, has resulted in an EPclin risk score, which can classify patients into low- and high-risk recurrence groups [77]. A study of 1702 postmenopausal ER+/HER2-negative BC patients from two adjuvant phase III trials (ABC SG6, ABC SG8) showed that both EP and EPclin are useful prognostic tools for the identification of early and late DR following 5 years of adjuvant endocrine therapy [78]. The EPclin assay provides more prognostic information on late DR (10 years) after endocrine therapy than Oncotype DX, partly because of its integration with molecular data and the clinicopathological factors [79]. However, further validation for predicting late recurrence following endocrine therapy is required.



**Table 1:** Summary of predictive and prognostics models and recommendations of their use in ER+ BC.

Assay	No. of genes	Sample type	Platform	Risk category	Clinical utility	Prediction of endocrine therapy efficacy	Recommendations
<b>Multigene signatures:</b>							
<b>PAM50 Prosigna</b>	50	FFPE	PCR-based PAM50 signature on the NanoString platform	Low, intermediate or high risk for DR	Prognostic	No	<ol style="list-style-type: none"> <li>1. For patient with ER/PR+, HER2-negative (node-negative) early stage BC, in conjunction with other clinicopathologic variables, to guide decisions on adjuvant systemic chemotherapy [54].</li> <li>2. St. Gallen group and EGTM do not differentiate between lymph node-negative and lymph node-positive disease [14, 68].</li> </ol>
<b>Oncotype DX</b>	21	FFPE	RT-PCR	Low, intermediate or high risk for DR	Prognostic/predictive	No	<ol style="list-style-type: none"> <li>1. For patient with ER/PR+, HER2-negative (node-negative) early stage BC, to guide decisions on adjuvant systemic chemotherapy [54].</li> <li>2. St. Gallen group and EGTM do not differentiate between lymph node-negative and lymph node-positive disease [14, 68].</li> </ol>
<b>MammaPrint</b>	70	Fresh tissue /FFPE	DNA microarray	Low or high risk for DR	Prognostic	No	<ol style="list-style-type: none"> <li>1. For patient with ER/PR+, HER2-negative (node-negative) early stage BC, in conjunction with other clinicopathologic variables, to guide decisions on adjuvant systemic chemotherapy [67].</li> <li>2. St. Gallen group and EGTM do not differentiate between lymph node-negative and lymph node-positive disease [14, 68].</li> </ol>
<b>Breast cancer index (BCI)</b>	11	FFPE	RT-PCR	Low or high risk for DR	Prognostic	No	<ol style="list-style-type: none"> <li>1. ASOC and EGTM for patient with ER/PR+, HER2-negative (node-negative) early stage BC, to guide decisions on adjuvant systemic chemotherapy [54, 68].</li> <li>2. St. Gallen group do not differentiate between lymph node-negative and lymph node-positive disease [14].</li> </ol>
<b>EndoPredict</b>							<ol style="list-style-type: none"> <li>1. For patient with ER/PR+, HER2-negative (node-negative) early stage BC, to guide decisions on adjuvant systemic chemotherapy [54].</li> <li>2. St. Gallen group and EGTM do not differentiate between lymph node-negative and lymph node-positive disease [14, 68].</li> </ol>

(EP)	11	FFPE	RT-PCR	Low or high risk for DR	Prognostic	No
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### Single biomarkers

<b>ER</b>	Level of expression	FFPE	IHC	Positive or negative	Prognostic Predictive	Yes	ASCO [54], St. Gallen Group [14] and EGTM [68] recommend ER measurement on all newly diagnosed primary invasive BC.
<b>PR</b>		FFPE	IHC	Positive or negative	Prognostic Predictive	Yes	ASCO [54], St. Gallen Group [14] and EGTM [68] recommend ER measurement on all newly diagnosed primary invasive BC.
<b>HER2</b>		FFPE	IHC FISH*	Low or high	Prognostic Predictive	No	ASCO [54], St. Gallen Group [14] and EGTM [68] recommend HER2 measurement to guide anti-HER2 therapy in patients with early or advanced BC.
<b>Ki-67</b>		FFPE	IHC	Low or high	Prognostic	No	St. Gallen Group [14] and EGTM [68] recommend Ki-67 measurement in combination with established clinical and pathological factors for determining prognosis in early BC. ASCO do not recommend its use because lack of reproducibility and standardisation [54].

\*FISH: Fluorescence in situ hybridization.

## IHC surrogates for molecular classification

With the limited early application of the molecular classes identified using GEP in clinical practice, robust alternatives including IHC biomarkers have been adopted as a surrogate for molecular profiling and prognostic stratification of patients.

Using standard IHC methods, BC can be divided into luminal, HER2-enriched and triple-negative subtypes (using ER, PR and HER2). Means of distinguishing between luminal-A and B subtypes include Ki67 [80] and PR expression to define luminal-B subtype [5].

Although IHC is considered the standard assay for Ki67 determination, significant variation between centres in assessment of Ki67 expression continues to limit its use in clinical management of BC [81, 82]. As a consequence, the St Gallen International Expert Consensus (2013) defined luminal-BC subtypes using IHC as luminal-A (ER+, HER2-, <14% Ki-67 and  $\geq 20\%$  PR), luminal-B, subdivided into luminal-B HER2-negative (ER+, HER2- and at least one of: Ki-67  $\geq 14\%$  or PR negative or low), luminal-B HER2-positive (ER+, HER2+ regardless of Ki67 or PR) [14].

In terms of benefit from endocrine therapy, the St Gallen consensus suggested that luminal-A should be treated at relapse with endocrine therapy as it is less responsive to chemotherapy, while luminal-B HER2-negative should be treated with endocrine therapy plus chemotherapy. However, luminal-A tumours could be treated with chemotherapy in addition to endocrine therapy based on risk assessment e.g. large tumour size or patient preference [14]. This demonstrates that there remains uncertainty regarding risk assessment and choice of therapy and more precise molecular classification of ER+ BC is required in order to better predict benefit from endocrine therapy.

### ***IHC4 Assay***

IHC4 is a prognostic tool that measures the immunohistochemical level of four key markers ER, PR, HER2 and Ki-67 to predict risk of recurrence for patients treated with endocrine therapy, illustrated in Fig. 1. The IHC4 assay is performed on FFPE tissue and a risk score for recurrence is calculated. The amount of prognostic information of this assay is similar to that provided by Oncotype DX, and has been validated in an independent data set [59]. The main limitation of the IHC4 assay remains the lack of standardised quantification of the biomarkers, particularly with respect to Ki67 [83]. Like the multigene assays, IHC4 is not able to predict which patients will benefit from endocrine therapy, nor pick out those patients with early BC who would benefit from a combination of endocrine therapy and other targeted treatments.

### ***Mammostrat***

Mammostrat is an IHC based assay that measures the levels of five biomarkers (P53, SLC7A5, NDRG1, CEACAM5 and HTF9C) to provide an assessment of features of tumour biology distinct from hormone receptor status, HER2, or proliferation. This assay applied to FFPE tissues of ER+ BC tamoxifen-treated patients is able to predict the relative risk of recurrence (high, moderate or low) [84] and has been subsequently validated [85-87]. In patients with ER+ HER2- tumours, this assay may be used for determining prognosis and guiding decision making with respect to the use of chemotherapy. There is no data as yet on the clinical utility of the Mammostat assay in predicting benefit from endocrine therapy.

### *Nottingham Prognostic Index Plus (NPI+)*

NPI+ is based on the well-established clinicopathological variables used in the traditional NPI but has been refined to integrate tumour biology with these clinicopathological factors. Primary invasive breast carcinoma are initially classified into seven distinct molecular classes using a panel of 10 biomarkers (ER, PR, CK 5/6, CK7/8, EGFR, HER2, HER3, HER4, p53 and Mucin 1) which constitutes three luminal classes characterised by high luminal Ck7/8 and hormone receptor expression [88], the classification is shown in decision tree format in Fig. 2. Luminal-A and Luminal-B classes show high expression of CK7/8, ER, HER3 and HER4 but are separated by lower levels of PR expression in Luminal-B. Luminal-N class shows differential expression of HER3 and HER4. The classes are followed by a second stage of stratification to incorporate clinicopathological variables, resulting in distinct prognostic groups within each molecular class [88].

Using the NPI+ formulae, the NPI+ distinctive classes of BC achieved an improved patient outcome stratification compared to the traditional NPI. Recently, the reproducibility and prognostic value of this tool was validated in an independent cohort of primary BC [89], and was suggested to be a useful tool in predicting the risk of metastases in primary BC [90]. However, despite the utility of this assay, it has limited literature to determine its ability to predict benefit from endocrine therapy.

### **Are the above discussed assays useful in predicting benefit from endocrine therapy?**

There is increasing concern that all the assays discussed above are not sufficient to predict which patients with early BC will benefit from endocrine therapy, as their prognostic values are in predicting risk of recurrence or metastases. Furthermore, despite the fact that IHC4 has clinical validity for selecting women with ER+ BC who might be spared extended endocrine

therapy, there remains a clinical need for further research to establish new biomarkers to predict the benefit of endocrine therapy in early stage ER+ BC.

The side effects of prolonged endocrine therapy are not inconsiderable. Debilitating non-agent specific side effects of endocrine therapy that impair quality of life include hot flushes, fatigue and myalgia. Additionally, there are potentially serious side effects including bone demineralisation and osteoporosis (with AIs), and life-threatening toxicities such as venous thromboembolism (with tamoxifen). A biomarker assay that could pick out people with BC that would not benefit from endocrine therapy could spare people these risks and improve quality of life.

On the other hand, assays such as those discussed above can pick out patients at higher risk of relapse, who should therefore be given more intense treatment with chemotherapy. In the future, biomarkers of endocrine therapy benefit may also pick out those at higher risk who would benefit from combinations of endocrine therapy with other targeted therapies such as MTOR or CDK4/6 inhibitors.

### **Conclusion and future directions**

At present, there are no biomarkers that can be used to reliably predict which patients will benefit from endocrine therapy. Prediction of benefit from endocrine therapy is important in order to treat only those that have a good chance to respond, and spare non-responders from the side effects of additional hormonal treatment.

Despite the clear advancement in multigene signatures in molecular classification or risk stratification of BC, none of these assays have found clinical utility in assessing endocrine therapy benefit in early stage ER+ BC, prior to treatment. Future studies that aim to identify

new predictive biomarkers may integrate detailed clinicopathological and histopathological data from large cohorts with full clinical annotation and long-term follow-up, and use multivariate model testing to establish the independence of potential biomarkers in addition to ER and PR.

To identify new molecular biomarkers predictive of benefit from endocrine therapy, a retrospective study could be conducted using clinically annotated gene expression data from large cohorts, comparing clinical outcome in those treated only with endocrine therapy. This could then be integrated with protein expression data to produce a combined multigene signature and IHC score specific for predicting benefit from endocrine therapy.

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**Figures legend:**

**Figure 1:** Illustration of IHC4 and the corresponding risk score that combines the IHC4 score and the clinical score providing prognostic information on the risk of DR. The IHC4 score is based on the assessment of four key proteins ER, PR, HER2 and Ki-67. The clinical score is based on the evaluation of clinical parameters such as nodal status, tumour size, age, grade and endocrine treatment.

**Figure 2:** Illustration of NPI+ assay and the use of panel of biomarkers to classify BC into seven distinct molecular classes.



