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Review

Clinical use of biomarkers in breast cancer: Updated guidelines from the European Group on Tumor Markers (EGTM)

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Abstract Biomarkers play an essential role in the management of patients with invasive breast cancer. For selecting patients likely to respond to endocrine therapy, both oestrogen receptors (ERs) and progesterone receptors (PRs) should be measured on all newly diagnosed invasive breast cancers. On the other hand, for selecting likely response to all forms of anti-HER2 therapy (trastuzumab, pertuzumab, lapatinib or ado-trastuzumab emtansine), determination of HER2 expression or gene copy number is mandatory. Where feasible, measurement of ER, PR and HER2 should be performed on recurrent lesions and the primary invasive tumour. Although methodological problems exist in the determination of Ki67, because of its clearly established clinical value, wide availability and low costs relative to the available multianalyte signatures, Ki67 may be used for determining prognosis, especially if values are low or high. In oestrogen receptor (ER)-positive, HER2-negative, lymph node–negative patients, multianalyte tests such as urokinase plasminogen activator (uPA)-PAI-1, Oncotype DX, MammaPrint, EndoPredict, Breast Cancer Index (BCI) and Prosigna (PAM50) may be used to predict outcome and aid adjunct therapy decision-making. Oncotype DX, MammaPrint, EndoPredict and Prosigna may be similarly used in patients with 1–3 metastatic lymph nodes. All laboratories measuring biomarkers for patient management should use analytically

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and clinically validated assays, participate in external quality assurance programs, have established assay acceptance and rejection criteria, perform regular audits and be accredited by an appropriate organisation.

Biomarkers currently play an indispensable role in the management of patients with breast cancer, especially in deciding the type of systemic therapy to be administered. In 2005, the European Group on Tumor Markers (EGTM) published guidelines on the use biomarkers in breast cancer [1]. However, since then, a number of important new developments have been reported, especially with tissue-based biomarkers. These include the use of multiparameter signatures for predicting patient outcome and the use of HER2 for the upfront identification of likely response to several different forms of anti-HER2 therapy. In addition, new recommendations have been published for performing a number of breast cancer biomarker assays such as oestrogen receptors (ERs), progesterone receptors (PRs) and HER2. The aim of this article is therefore to expand on, and update the 2005 guidelines, focussing on tissue-based biomarkers.

The main target groups of these guidelines include physicians, surgeons and nurses involved in the management of patients with breast cancer and laboratory professionals involved in the measurement of breast cancer biomarkers. The guidelines however, may also be of value for healthcare providers, health policy makers, breast cancer researchers, regulatory organisations, patients with breast cancer and companies involved in the manufacture/supply of biomarker assays and targeted drugs related to breast cancer.

To update these guidelines, the published literature (PubMed and the Cochrane Library) relevant to the use of tissue biomarker in breast cancer was reviewed. Apart from the American Association of Cancer Research (AACR) and the American Society of Clinical Oncology (ASCO) 2016 conferences, abstracts of meetings were not searched. The review covered the period from June 2005 until June 2016. As in previous guideline publications [2–4], particular emphasis was placed on studies involving the validation of biomarkers in prospective or prospective-retrospective trials, systematic reviews, pooled/meta-analyses of biomarker studies and relevant guidelines published by other expert panels. For each specific recommendation, we indicate the level of evidence (LOE) [5,6] and strength of recommendation (SOR) [7] for its clinical use. In addition to providing recommendations for clinical use, we also make suggestions for further research with the recommended biomarkers.

1. Oestrogen and progesterone receptor for predictive endocrine sensitivity

Oestrogen receptor (ER) exists in two main forms, ERα and ERβ. Currently, a validated clinical role has only been established for ERα which will be referred to as ER in this article. Whereas the original ligand-binding ER assays are likely to have detected both ERα and ERβ, the current immunohistochemistry (IHC) measurements detect only ERα. Similarly, progesterone receptor (PR) exists in two forms, dubbed PRA and PRB. Currently used IHC assays detect both these forms of PR.

The main clinical application of steroid hormone receptors, i.e. ERα and PR is in selecting patients with invasive breast cancer for treatment with endocrine therapy, i.e. administration of selective oestrogen receptor modulators (tamoxifen), third-generation aromatase inhibitors (anastrozole, letrozole or exemestane), LH-RH agonists (leuprolide, goserelin), pure oestrogen receptor downregulators (fulvestrant), oophorectomy and other endocrine therapies. As predictive markers for endocrine therapy, ER and PR are used in the neoadjuvant, adjuvant and advanced disease settings [8–11].

In early disease, a meta-analysis of individual data from 20 randomised clinical trials (n = 21,457) showed that treatment of ER-positive patients (i.e. $\geq$10 fmol/mg protein determined with a biochemical assay) for approximately 5 years with adjuvant tamoxifen significantly reduced the 15-year odds of disease recurrence by 39% and odds of breast cancer mortality by 30% [9]. In contrast to the findings with ER-positive disease, treatment of ER-negative patients (<10 fmol/mg protein) with tamoxifen had no significant effect on either breast cancer recurrence or mortality. In this meta-analysis, PR did not provide independent predictive information. While not predictive of the benefit from tamoxifen in this meta-analysis, high levels of PR levels, however, were reported to be independently correlated with an increased probability of response to tamoxifen, longer time to treatment failure and longer overall survival in patients with metastatic breast cancer [12,13]. It should be stated that most of the studies included in the above meta-analysis used the original biochemical assays (ligand-binding and ELISA) to measure ER and PR. These assays were not standardised and it is unclear if all the laboratories participated in quality assurance programs.

Although 5 years of adjuvant tamoxifen treatment was the standard form of endocrine treatment for steroid hormone receptor–positive patients for several years, administration of tamoxifen or an aromatase inhibitor (i.e. letrozole) for 10 years was recently shown to be superior to a 5-year course [14,15]. This enhanced benefit, however, should be weighed against the potential additional side-effects of the extended treatment. Ideally, therefore, biomarkers should be available to identify those patients who are at high risk of developing late recurrences, as these women may benefit from the extended therapy. Equally important, theses biomarkers should help to identify women at low risk of late relapse, as these could be spared the side-effects and costs of the extended treatment. Emerging data suggest that specific gene signatures (see below) may be able to differentiate between patients with respect to their risk for early or late relapses following endocrine therapy. These include Prosigna (PAM50 and Risk of Recurrence score) [16], EndoPredict [17], IHC4 [18], Breast Cancer Index (BCI) [18] and the HOXB13/IL17BR ratio [19].

Adjuvant treatment with third-generation aromatase inhibitors (AIs) in postmenopausal hormone receptor–positive patients was shown to be superior to a 5-year course of tamoxifen in reducing the risk of recurrence albeit with modest effect (1–2%) in extending overall survival. This increased efficacy was found whether AIs were initially administered or sequentially used following 2–3 years of tamoxifen treatment [20]. Most expert panels, including the American Society of Clinical Oncology (ASCO) [21], the National Comprehensive Cancer Network (NCCN) [22] and the European Society of Medical Oncology (ESMO) [23] recommend the incorporation of an aromatase inhibitor, either upfront or in sequence with tamoxifen, in the adjuvant treatment of postmenopausal patients. According to the 2015 St. Gallen Consensus Group [24], tamoxifen alone may be suitable for low-risk women. In contrast, for high-risk women, the group recommended that an aromatase inhibitor should be considered and administered initially.

For premenopausal receptor-positive patients, tamoxifen has been the standard treatment for several decades. However, based on recent findings from two clinical trials [25,26], the ASCO 2016 guidelines have recommended that high-risk patients should receive ovarian suppression in addition to standard adjuvant endocrine therapy, whereas low-risk patients should not have the additional ovarian suppression [27].

2. ER and PR for determining prognosis

While the primary value of steroid hormone receptors is as predictive biomarkers for endocrine therapy, the receptor status of a primary invasive breast cancer can also provide prognostic information. Indeed, several retrospective studies have shown that patients with ER or PR-containing tumours tend to have a better outcome than those lacking the receptors [28–36]. However, the favourably prognosis associated, at least with ER-positive tumours, mostly occurs during the first 5–7 years after initial diagnosis [28–30]. Thereafter, the risk of relapse tends to be greater in ER-positive than ER-negative tumours. Steroid hormone receptors alone thus cannot be used to identify women who may benefit from extended endocrine therapy.

Furthermore, the impact of therapy on the prognostic impact of steroid hormone receptors prognosis is difficult to exclude, as almost all receptor-positive patients are treated with endocrine therapy in either early or advanced disease. Indeed, in some studies, the improved outcome with receptor-positive tumours was only found in patients treated with endocrine therapy [29,30].

3. ER and PR: EGTM recommendation

- In agreement with previously published guidelines [1,3,21–24,36], the EGTM panel recommends that ER and PR be measured on all newly diagnosed primary invasive breast cancers **(for ER, LOE IA; SOR, A and for PR, LOE 1B; SOR, A/B).** If ER or PR is found to be negative in the core needle biopsy specimen from a primary tumour, we suggest to re-assay them in the corresponding surgical sample. This suggestion however, is not evidence based but is based on several studies showing a discordance in hormone receptor status between a core needle biopsy and a corresponding surgical specimen. A possible reason for negative findings on the core needle but positive findings on the surgical specimens is an error in sampling. This may occur, especially in heterogeneous tumours, where the core biopsy specimen is not representative of the whole tumour. On the other hand, negative findings on the surgical specimen but positive findings with the core needle biopsy could relate to fixation artifacts, caused by a delay in exposure of the center of a surgical specimen to formalin [37].
- ASCO guidelines state that when discordant results are found between the primary and metastatic site, it is preferable to use the receptor status of the metastatic tumour, provided it is supported by the clinical situation and in agreement with the patients' wishes [38]. In contrast, both the European School of Oncology (ESO)-ESMO Consensus Conference for Advanced Breast Cancer (ABC) group [39] and NCCN [22] recommend administering endocrine therapy if any biopsy is receptor positive. It is important to state that the recommendation to measure ER/PR on metastatic sites when treating recurrent disease is not evidence based but would appear to be prudent, because of the possibility of an alteration in receptor status as a result of tumour progression. Thus, based on a meta-analysis of 33 published studies containing a total of 4200 patients, Aurilio *et al.* [40] concluded that the discordance rates for ER between the primary and metastatic sites were 20% (95% confidenc interval [CI], 16–35%), with 24% of tumours converting from positive to negative and 14%

converting from negative to positive status. In this meta-analysis, the pooled discordance for PR status between primary and metastatic sites was 33% (95% CI, 29–38) [40]. With PR, 46% of the samples changed status from positive to negative, whereas 15% changed status in the opposite direction.
- Finally, both ER and PR should be measured by IHC using an analytically and clinically validated assay.

4. ER and PR: recommendation for further research

- Development of biomarkers for increasing the positive predictive value of ER.
- Identify biomarkers for selecting patients that preferentially benefit from an aromatase inhibitor vis-à-vis tamoxifen or vice versa.
- Validate biomarkers for selecting patients who do not need extended adjuvant endocrine therapy.
- Establish the optimum clinical cut-off points for both ER and PR, in particular to establish if these should be 1%, 10% or indeed a different percentage of positive cell nuclei staining [41].
- Develop and validate assays for ERβ with a view to ascertaining a potential clinical role for this form of ER in breast cancer [42].
- Establish the relative endocrine therapy predictive impact of the two forms of PR, i.e. PRA and PRB [33].
- Determine if ER mutations at recurrent sites have predictive ability. Recently, such mutations were found in approximately 20% of patients with metastatic breast cancer, most of whom were treated with an aromatase inhibitor [43]. It remains to be shown, however, if the presence of these mutations has a predictive impact.

5. HER2 for predicting the response to anti-HER2 therapies

The main clinical use of HER2 measurement is in predicting the response to anti-HER2 therapy in the neoadjuvant, adjuvant and advanced disease settings [44]. Currently, four anti-HER2 therapies are approved for the treatment of HER2-positive breast cancer, trastuzumab, lapatinib, pertuzumab and trastuzumab emtansine (T-DM1). Based on the available evidence, HER2 gene amplification/overexpression appears to be necessary but not sufficient for response to all of these anti-HER2 therapies. Recently, several expert panels, including ASCO [45], NCCN [22] and ABC [39], recommended that the first-line therapy for patients with HER2-positive advanced breast cancer should be trastuzumab, pertuzumab and a taxane, if not previously treated with trastuzumab. It was also suggested that this regimen is a treatment option for those who previously received trastuzumab. For those who progress during or after first-line anti-HER2 treatment, T-DM1 should be administered [45]. In the adjuvant setting, trastuzumab (in combination with chemotherapy) is the standard and still the only approved form of anti-HER2 therapy administered. For the neoadjuvant setting, dual treatment with trastuzumab and pertuzumab is approved both in Europe and in the USA. According to the NCCN guidelines, a pertuzumab-containing regimen may be administered in the neoadjuvant setting to HER2-positive patients who are lymph node–positive or have tumours ≥2 cm [22].

6. Measurement of HER2

Two main types of tests are available to measure HER2 gene amplification/protein overexpression, i.e. IHC and in situ hybridisation (ISH). ISH assays may use fluorescent ISH (FISH) or brightfield ISH. Guidelines for performing and interpreting HER2 results have been published by several expert panels including groups in the US (ASCO/CAP) [46], UK [47] and elsewhere [48].

7. HER2: EGTM recommendation

- HER2 gene amplification or overexpression should be determined on all patients with primary invasive breast cancer **(LOE, IA; SOR, A).** Where feasible, measurement should also be performed on any metastatic lesion. According to ASCO, if discordance exits between the two locations, the HER2 status of the metastatic site should be used in determining the management [40]. The ABC Consensus Guidelines and NCCN, however, state that if any biopsy is positive, the patients should receive anti-HER2 therapy [22,39]. As with ER and PR, the recommendation to measure HER2 on a metastatic lesion is not evidence based. However, like ER and PR, the HER2 status can vary between a primary and metastatic site. Thus, in the meta-analysis referred to above [40], 13% of cancers that were positive in the primary cancer were found to be negative in the metastatic lesion, whereas 5% that were negative in the primary lesion were positive in the metastatic specimen.
- As stated by the ASCO/CAP panel, measurement can be performed on either a core needle biopsy or on a surgical resection specimen [46]. As with ER/PR, if HER2 is found to be negative in the biopsy specimen from a primary tumour, it is recommended to re-assay it in the corresponding surgical sample (as tumour heterogeneity may have been responsible for the negative finding in the biopsy sample). Fine needle aspirates of primary cancers should not be used to measure HER2, as such samples do not allow reliable differentiation between invasive and in situ malignancy.
- Measurement of HER2 in DCIS should not be performed.
- HER2 measurement should be performed and positivity defined using the updated ASCO/CAP guidelines [46]. Ideally, an approved assay (e.g. by the FDA in the US or possessing the Conformité Européenne Mark in Europe) using IHC, brightfield ISH, or FISH assay should be used.

- If the HER2 test result is still equivocal, after reflex testing with an alternative assay, consideration should be given to the feasibility of testing a separate tumour specimen [46].
- Serum levels of soluble HER2 protein or tumour levels of HER2 mRNA should not be used for predicting the response to anti-HER2 therapy.

8. HER2: recommendations for further research

- Identify additional markers to increase the positive predictive value of HER2. This should focus on HER2-positive patients who do not benefit from trastuzumab or other forms of anti-HER2 therapy, as well as the identification of the small number of patients who derive long-term benefit from anti-HER2 therapy.
- Identify biomarkers for selecting the most appropriate form of anti-HER2 therapy for a given patient.
- Markers should be identified for selecting patients likely to particularly benefit from dual anti-HER2 therapies such as combined trastuzumab and either pertuzumab or lapatinib in the neoadjuvant setting or combined trastuzumab and pertuzumab in the advanced disease setting. The preliminary results suggesting that high levels of HER2 as measured by a quantitative HER2 assay (HERmark) predicts an enhanced response to dual anti-HER2 therapy [49] should be confirmed.
- Establish whether patients with equivocal scores should or should not receive anti-HER2 therapy. This question might be addressed by evaluating the potential predictive value of other assays for HER2 such as the use of ELISA for HER2 protein or RT-PCR for HER2 mRNA.
- Finally, the potential biomarker value of HER2 mutations [50] in predicting the response or resistance to specific anti-HER2 therapies should be explored, especially in patients with high-grade lobular cancer, where the frequency of HER2 mutations may reach 15–20% [51,52].

9. Ki67

A multiplicity of studies, including retrospective evaluation of randomised clinical trials and meta-analyses, have shown that elevated levels of Ki67 are independently associated with adverse outcome in patients with breast cancer (for review, see refs. [53–55]). In one of the largest studies, Petrelli *et al.* [54] performed a systematic review of the literature which was followed by a meta-analysis of the individual studies. In total, 41 studies encompassing 64,196 patients were identified. Although different cut-off points over the range 10 to >25% cell staining were investigated, the threshold displaying the strongest prognostic significance for overall survival was found to be >25% cell staining (hazard ratio, [HR] 2.05; 95% CI, 1.7–2.5; $p < 0.00001$).

The consistent relationship between high Ki67 values and poor outcome in patients with breast cancer has been found despite the reported poor interlaboratory precision for the assays employed, use of different methods to measure Ki67 and use of different cut-off points for differentiating between tumours with low and high Ki67 concentrations [56,57]. According to Denkert *et al.* [58], imprecision is particularly found at borderline or intermediate concentrations of Ki67. Indeed, these investigators suggested that clinical decision-making should not be based on Ki67 levels in the borderline range. However, in contrast to the poor precision at intermediate levels, a multicentre study carried out by the International Ki67 Working group concluded that good interlaboratory agreement was achievable using centrally stained core needle biopsies when Ki67 scores were higher or lower than intermediate scores (i.e. <10% or >20% cell staining) [59].

In addition to undergoing evaluation for prognostic value, Ki67 has also been investigated for potential therapy predictive use, especially in the neoadjuvant and adjuvant settings. In the neoadjuvant setting, most but not all reports found a significant association between high Ki67 levels and response to chemotherapy as measured by clinical or pathological response [58]. In the adjuvant setting, however, the relationship between Ki67 levels and the benefit from chemotherapy is less clear [58]. With endocrine therapy in the neoadjuvant setting, a number of studies have found that treatment-induced alterations in Ki67, even after short-term therapy, predict response and patient outcome [59–62]. Little data is available on the chemo or endocrine predictive value of Ki67 in the metastatic setting.

10. Ki67: EGTM recommendation

- Although methodological problems exist in the determination of Ki67, because of its clearly established clinical value, wide availability and low costs relative to the available multianalyte signatures, Ki67 may be used in combination with established prognostic factors for determining prognosis, especially if values are low (e.g. <10% cell staining) or high (e.g. >25% cell staining; **LOE, IB; SOR, B** for using high cut-off point). The higher cut-off value is based on the meta-analysis discussed above [54] which concluded that a threshold of >25% cell staining was associated with a greater risk of death compared with lower values. The lower cut-off point, however, is not evidence derived, but based on the expert opinion of the authors. Until a standardised assay becomes available, measurement of Ki67 should adhere to the previously published recommendations of the International Ki67 in Breast Cancer Working Group [56].

11. Ki67: recommendations for further research

- Improve interlaboratory variation with assay standardisation.
- Establish an optimum cut-off point or evaluate the use of Ki67 as a continuous variable.
- Establish if different cut-off points are necessary for prognosis and therapy prediction.
- Evaluate the potential of automated image analysis for reducing between-assay variability.

12. Multigene/multiprotein test

In the last decade, several multianalyte tests have been proposed for predicting outcome in patients with newly diagnosed primary invasive breast cancer (Tables 1 and 2). Some general points that apply to all or most of these multianalyte tests include the following (for review, see refs. [63–65]):

- All appear to provide prognostic information for relapse-free survival independent of the traditional prognostic factors such as tumour size, tumour grade and lymph node status.
- The majority were discovered and validated in ER-positive, HER2-negative, lymph node–negative patients between 40 and 65 years of age. Oncotype DX, MammaPrint, EndoPredict and Prosigna (see below), however, were also found to be prognostic in lymph node–positive patients (1–3 metastatic nodes), see below.
- Only urokinase plasminogen activator (uPA)/PAI-1 [66,67], Oncotype DX [68,69] and MammaPrint [70] have to-date been evaluated for clinical value as part of a randomised prospective trial. Prosigna [71], EndoPredict (NCT01805271) and Genomic Grade Index, (NCT01916837) however, are currently undergoing evaluation in such trials.
- Results from the prospective OPTIMA prelim trial [71] suggest that although the proportion of patients identified as being at low or high risk are largely similar irrespective of which test is used, major differences were found with respect to classification of individual patients. Thus, the proportion of patients classified as low/intermediate risk was 82.1% for Oncotype DX, 72.0% for IHC4, 65.6% for Prosigna and 61.4% for MammaPrint [71].

Table 1
Gene/protein signatures previously proposed for predicting outcome in patients with newly diagnosed breast cancer.

Test	Tissue required	Molecule measured	No. of analytes	Studied in prospective randomised trial
uPA/PAI-1	Fresh/frozen	Protein	2	Yes and ongoing
Oncotype Dx	FFPE	mRNA	21	Yes and ongoing
MammaPrint	Fresh/frozen/ FFPE	mRNA	70	Yes and ongoing
Prosigna/PAM50	FFPE	mRNA	50[a]	Ongoing
GGI	FFPE	mRNA	97	Ongoing
BCI	FFPE	mRNA	11	No
Mammostrat	FFPE	Protein	5	No
IHC4 score	FFPE	Protein	4	No
EndoPredict	FFPE	mRNA	11	Ongoing
Rotterdam signature	Fresh/frozen	mRNA	76	No
OncoMasTR	FFPE	mRNA	7	No
Curbest 95GC	FFPE	mRNA	95	No

FFPE, formalin-fixed and paraffin-embedded. GCI, Genomic Grade Index; BCI, Breast Cancer Index.
[a] In addition to 50 genes from the PAM50 panel, the test also contains eight control genes for normalisation, six positive controls and eight negative controls (Prosigna packet insert).

Table 2
Recommendations for the use of multianalyte tests in ER-positive, HER-negative breast cancer patients by different expert panels.

Test	ASCO	NCCN[a]	ESMO[b]	St. Gallen[b] group	EGTM[c]
uPA/PAI-1	LN–	NR	LN–, LN+	LN–, LN+	LN–
Oncotype DX	LN–	LN–, LN+	LN–, LN+	LN–, LN+	LN–, LN+
MammaPrint	NR	NR	LN–, LN+	LN–, LN+	LN–, LN+
Prosigna	LN–	NR	LN–, LN+	LN–, LN+	LN–, LN+
EndoPredict	LN–	NR	LN–, LN+	LN–, LN+	LN–, LN+
BCI	LN–	NR	NR	LN–, LN+	LN–

LN–, lymph node–negative; LN+, lymph node-positive (refers to 1–3 metastatic lymph nodes); NR, not recommended; BCI, Breast Cancer Index.
[a] NCCN guidelines discuss MammaPrint and Prosigna but do not specifically recommend either test.
[b] ESMO and the St. Gallen group do not differentiate between lymph node–negative and lymph node–positive disease.
[c] EGTM guidelines relate to data in this article.

- Most were developed and validated in European and North American patient populations.
- The most important genes in the multigene profiles for predicting patient outcome are those involved in cell proliferation.
- None can currently be recommended for predicting the response to a specific form of chemotherapy.
- Although relatively expensive to perform, use of some multigene signatures (uPA/PAI-1, Oncotype DX and MammaPrint) were shown to be cost-effective in lymph node–negative patients as they reduce the use of adjuvant chemotherapy [72–75].
- It is unclear whether the routine employment of multianalyte tests leads to a better outcome for patients.
- Several multianalyte tests are commercially available. These include uPA/PAI-1 (Femtelle), Oncotype DX, MammaPrint, Prosigna, EndoPredict, BCI and Genome Grade Index (MapQuant Dx). Some of the best validated signatures are discussed below.

13. Urokinase plasminogen activator and PAI-1 for determining prognosis and therapy response

Although not widely used, uPA and its inhibitor, PAI-1 are presently amongst the best validated prognostic biomarkers for breast cancer. Consistent with their ability to promote cancer progression, several retrospective and prospective studies have shown that high concentrations of uPA and PAI-1 protein are independent and potent predictors of adverse prognosis in patients with newly diagnosed invasive breast cancer [76]. Uniquely for breast cancer prognostic biomarkers, the clinical value of uPA/PAI-1 for predicting outcome in lymph node–negative breast cancer has been validated in two independent level of evidence-I studies (LOE I), i.e. in both a randomised prospective clinical trial in which uPA/PAI-1 evaluation was the main aim of the trial [66,67] and a large pooled analysis of individual patient-related data (n = 8377) from retrospective and prospective studies [77]. Both uPA and PAI-1 are thus

amongst the best validated prognostic biomarkers currently available for lymph node–negative breast cancer. As well as being prognostic, high levels of uPA and PAI-1 were also shown to be associated with benefit from adjuvant chemotherapy in patients with early breast cancer [66,67,78–81]. Furthermore, in addition to their extensive clinical validation, uPA/PAI-1 measurement by ELISA has undergone detailed analytical validation [82,83].

Currently, uPA and PAI-1 are undergoing further investigation in two randomised prospective trials, i.e. in the NNBC-3 and WSG-Plan B trials [84,85]. The NNBC-3 trial compares 5-FU, epirubicin and cyclophosphamide followed by docetaxel with 5-FU, epirubicin and cyclophosphamide as adjuvant therapy for high-risk lymph node–negative patients (NCT01222052) [84]. In this trial, the risk of recurrence was determined by clinicopathological criteria or by a combination of uPA/PAI-1 levels and clinicopathological factors. In contrast to the NNBC-3 trial, the WSG-Plan B trial is comparing an anthracycline and taxane-based adjuvant chemotherapy combination with an anthracycline-free taxane-based regimen in patients with HER2-negative breast cancer patients that are either high-risk node negative or node positive (NCT01049425) [85]. This trial also aims to compare the prognostic and predictive value of uPA/PAI-1 with that of Oncotype DX.

14. uPA and PAI-1: EGTM recommendation

- Levels of PA and PAI-1 protein levels may be combined with established factors for assessing prognosis and identifying ER-positive, HER2-negative and lymph node–-negative breast cancer patients that are unlikely to benefit from adjuvant chemotherapy **(LOE, IA; SOR, A).**
- For clinical use, uPA and PAI-1 should be measured by a validated ELISA (e.g. FEMTELLE, American Diagnostica/Sekisui) using extracts of fresh or freshly frozen breast tumour tissue, either from biopsy or surgical specimen.
- Currently, IHC or PCR should not be used when measuring uPA or PAI-1 for clinical purposes.

15. uPA and PAI-1: recommendation for further research

- Future research should aim to establish, validate and standardise a method for measuring uPA and PAI-1 by IHC or other techniques using formalin-fixed and paraffin-embedded tumour tissue.

16. Oncotype DX for determining prognosis and therapy response

The Oncotype DX test (Genomic Health Inc, Redwood City, CA, USA) involves measurement of the expression of 16 prognostic/predictive and five reference genes at the mRNA level by reverse transcriptase PCR in formalin-fixed and paraffin-embedded breast tumour tissue [86]. Based on the relative expression levels of these 16 genes to the average expression level of the five control genes, a recurrence score (RS) was developed that predicts the risk of distant disease recurrence at 10 years for lymph node–negative, ER-positive breast cancer patients, receiving adjuvant tamoxifen. The RS which ranges continuously from 0 to 100, was used to stratify newly diagnosed patients with invasive breast cancer into three different risk outcome groups, i.e. low risk of recurrence (RS, <18; 51% of patients), intermediate risk of recurrence (RS, 18–31; 22% of patients) and high risk of recurrence (RS, >31; 27% of patients) [86]. Outcome analysis showed that the formation of distant metastases at 10 years follow-up was 6.8% in the low-risk group, 14.3% in the intermediate-risk group and 31% in the high-risk group.

Recently, the prognostic value of a low Oncotype DX RS was evaluated in a prospective study carried out as part of TAILORx trial (NCI-2009-00707) [68]. In this trial, women with lymph node–negative, hormone receptor–positive and HER-negative disease with an RS of $\leq$10 received endocrine therapy alone, those with a RS $>$ 25 were treated with both endocrine therapy and chemotherapy, whereas those with an intermediate score (i.e. 11–25) were randomised to receive endocrine therapy with or without chemotherapy. Of the 10,253 eligible patients in the trial, 1626 (16%) exhibited an RS of <11. After a follow-up period of 5 years for this low-risk group, 93.8% were found to be free of invasive disease and 99.3% were free from recurrence at a distant site, while the overall survival was 98.0%.

In another further prospective trial (WSG Plan B) which enrolled 3198 HER2-negative patients with node-positive or high-risk lymph node–negative disease (T2, grade2/3, high uPA/PAI-1 or age $<$ 35 years), the 3-year disease-free survival for patients with low RS ($\leq$ 11) was found to be 98% [69]. This excellent outcome was achieved despite these high-risk patients not receiving adjuvant chemotherapy. Follow-up in this trial, however, was relatively short, the median being only 35 months.

Further confirmation of the prognostic impact of Oncotype DX in both lymph node–negative and lymph node–positive (1–3 positive nodes) patients was obtained in the recently reported SEER population-based study [87]. In this large prospective study, the breast cancer–specific mortality (BCSM) for node-negative patients (n = 38,568) was 0.4%, 1.4% and 4.4% for those with a RS of <18, 18–30 and 3, respectively ($P < 0.001$). For patients with lymph node–positive disease (micrometastasis and 1–3 positive nodes; n = 4691), the corresponding BCSMs were 1.0%, 2.3% and 14.3% ($P < 0.001$). It is important to point out, that as this study did not involve a randomised trial, biases and confounding factors may have been present. Thus, as an observation study, it had limitations such as

limited follow-up after 5 years, likely under-reporting of chemotherapy use and absence of data on recurrence. A further shortcoming was that it was unclear as to the number of node-positive patients who had only micrometastases. Despite these limitations, the study was prospective, involved in excess of 50,000 patients and importantly, was informative with respect to the use of Oncotype DX in clinical practice. The results of all the above studies when combined, suggest that node-negative or node-positive (1–3 positive nodes) patients with low RS patients have an excellent outcome and are unlikely to derive clinically significant benefit from adjuvant chemotherapy.

The value of Oncotype DX in predicting late distant recurrences is unclear. While some studies concluded that has little value in this setting [18], a recent retrospective analysis of two randomised controlled trials concluded that the RS was prognostic for late distant recurrences in patients with high-ER mRNA expression [88]. Risk of late recurrence was, however, relatively low for patients with low a RS. If confirmed, these results could lead to the use of extended endocrine therapy in patients with intermediate and high RS with high-ER mRNA [88].

In addition to its prognostic utility, retrospective analysis of two randomised trials (NSABP B-20 and SWOG-8814) suggest that the Oncotype DX RS may also be used to identify patients likely to benefit from adjuvant chemotherapy [89,90]. In these two trials, patients with a high RS ($\geq$31) benefited from the addition of chemotherapy to endocrine therapy. In contrast, those with a RS of $\leq$18 failed to benefit from the addition of the chemotherapy. Further validation of these findings, however, is needed from the randomised group of patients participating in both the TAILORx [68] and the ongoing RxPONDER trials (NCT01272037).

17. Oncotype DX: EGTM recommendation

- Oncotype DX RS may provide added value to established factors for determining prognosis and aiding decision-making with respect to administration of adjuvant chemotherapy in newly diagnosed breast cancer patients with lymph node–negative invasive disease that is ER-positive but HER2-negative **(LOE, IB; SOR, A)**. In addition, Oncotype DX may be considered for identifying HER2-negative, ER-positive patients with 1–3 involved lymph nodes for treatment with adjuvant chemotherapy **(LOE, IB; SOR, A)**.
- Before performing the test, any biopsy cavity in the cancer specimen should be removed by manual dissection.

18. Oncotype DX: recommendations for further research

- Two of the most important questions relating to the use of Oncotype DX are currently being addressed in prospective randomised trials, i.e. whether lymph node–negative ER-positive patients with intermediate RS benefit from adding adjuvant chemotherapy to endocrine therapy (TAILORx trial) and whether lymph node–positive (1–3 nodes positive), ER-positive patients with low to intermediate RS benefit from adjuvant chemotherapy (RxPONDER trial). In the RxPONDER trial, women with 1–3 positive lymph nodes who have hormone receptor–-positive but HER2-negative disease with RS $\leq$ 25 are randomised to receive endocrine therapy alone or endocrine therapy plus chemotherapy.
- Establish if Oncotype DX can predict response to specific forms of adjuvant chemotherapy.

19. MammaPrint

The prognostic value of MammaPrint (also known as the 70-gene signature) (Agendia, Amsterdam) for both lymph node–negative and lymph node–positive patients has been shown in several retrospective trials [91–95], a prospective trial [96] and in a randomised prospective trial [70]. In addition, a retrospective study showed that the addition of chemotherapy to endocrine treatment enhanced the outcome in patients identified as being at high-risk patients by the test. In contrast, patients identified as being at low risk failed to benefit from the addition of chemotherapy [97].

The clinical utility of MammaPrint was recently confirmed in a randomised prospective trial that involved 6692 newly diagnosed breast cancer patients with 0–3 metastatic lymph nodes (MINDACT; EORTC 10041/BIG 3-04, NCT000433589) [70]. In this trial, patients were deemed to be at low or high risk for disease recurrence based on both MammaPrint (designated G) and clinical-pathological criteria (designated C).

After a median follow-up of 5 years, the distant metastasis-free survival for patients identified to be at high risk based on the C criteria but at low risk based on the MammaPrint test was found to be 94.7% (well above the null hypothesis of 92%). This finding was obtained irrespective of whether or not the patients received adjuvant chemotherapy. Overall, there was an absolute 14% reduction in the use of adjuvant chemotherapy when using G rather than C criteria, for deciding on the treatment strategy. The use of MammaPrint test in all clinically high-risk patients, as defined by MINDACT, however, would result in a 46% reduction in the administration of chemotherapy. The results of this randomised prospective trial clearly showed that the use of MammaPrint test can alter the clinical practice by decreasing the frequency of administrating adjuvant chemotherapy to patients deemed to be at high risk based on traditional clinical and pathological factors, without impairing the long-term outcome.

20. MammaPrint: EGTM recommendations

- MammaPrint may be used for determining prognosis and guiding decision-making with respect to the administration of adjuvant chemotherapy in patients with newly diagnosed

invasive breast cancer that is lymph node negative or lymph node positive (1–3 metastatic nodes). Patients at high risk based on clinical and pathological criteria but at low risk based on MammaPrint may be the candidates for avoiding having to receive adjuvant chemotherapy **(LOE, IA; SOR, A).**

21. MammaPrint: recommendation for further research

- Further validation after longer follow-up.
- Investigate if MammaPrint can predict response to specific forms of systemic treatment.

22. Prosigna

The Prosigna test (previously known as PAM50) (NanoString Technologies Inc.) measures the expression of 50 discrimination genes at the mRNA level. Based on the relative expression of these genes, a risk of RS extending from 0 to 100 is calculated. Using this score, node-negative patients are classified as low risk (0–40), intermediate risk [41–60] or high risk [61–100]. Node-positive patients are classified as low risk (0–40) or high risk (41–100). The prognostic impact of Prosigna in ER-positive postmenopausal patients with either lymph node–negative or lymph node–positive disease treated with endocrine therapy has been validated in two independent prospective-retrospective studies [98,99]. Similarly, its prognostic impact in lymph node–positive patients treated with adjuvant chemotherapy has undergone validation in two prospective-retrospective trials [100,101]. Furthermore, combined analysis of two studies [98,99] confirmed the independent prognostic value of Prosigna in patients with 1–3 metastatic lymph nodes [102]. In addition, as mentioned above, Prosigna has been reported to predict the emergence of late recurrences following adjuvant endocrine therapy [16]. In this study, Prosigna was found to be superior to Oncotype DX or IHC4 in foretelling the formation of late recurrences.

23. Prosigna: EGTM recommendation

- In combination with established clinical and pathological factors, Prosigna may be used for predicting outcome and aiding adjuvant therapy decision-making in hormone receptor–positive, HER2-negative patients that are either lymph node–negative or lymph node–positive (1–3 metastatic nodes). **(LOE IB; LOR, A).**

24. Prosigna: recommendation for further research

- Validation in a prospective randomised trial. This is currently ongoing as part of the OPTIMA trial (71, ISRCTN42400492).
- Establish if Prosigna can predict benefit from adjuvant chemotherapy.
- Further validation for predicting late recurrences following adjuvant endocrine therapy.
- Further validation in premenopausal patients.

25. EndoPredict

The EndoPredict test detects the expression levels of 11 genes (eight test genes and three reference genes) (Sividon Diagnostics/Myriad). The expression levels of these genes have been combined with lymph node status and tumour size to generate a comprehensive risk score dubbed the EPclin score [103]. The independent prognostic impact of EndoPredict has been validated in several prospective-retrospective trials. These trials included HER2-negative, ER-positive patients treated with adjuvant endocrine therapy [103,104], as well as HER2-negative lymph node–positive patients receiving adjuvant chemotherapy [101,105]. Furthermore, EndoPredict has been shown to identify a subgroup of patients who have good long-term prognosis following 5 years of adjuvant endocrine therapy [17].

26. EndoPredict: EGTM recommendation

- In combination with established clinical and pathological factors, EndoPredict may be used for predicting outcome and aiding adjuvant therapy decision-making in hormone receptor–positive, HER2-negative patients that are either lymph node negative or lymph node positive (1–3 metastatic nodes). **(LOE IB; SOR, A).**

27. EndoPredict: recommendation for further research

- Validation in a prospective randomised trial. This is currently ongoing as part of the UNIRAD trial (NCT01805271).
- Establish if EndoPredict can predict benefit from adjuvant chemotherapy.
- Further validation for predicting late recurrences following adjuvant endocrine therapy.
- Further validation in premenopausal patients.

28. Breast Cancer Index

The Breast Cancer Index (BCI; bioTheranostics, San Diego, California) test measures the expression of 11 genes (7 test genes plus 4 reference genes) [106]. The test was developed from the algorithmic combination of two previously identified biomarker tests, i.e. the HOXB13:IL17BR ratio and the Molecular Grade Index. Most of the test genes measured in BCI are involved in proliferation or oestrogen signalling [106]. Several prospective-retrospective trials have demonstrated a prognostic value for BCI for detecting early and late recurrences in ER-positive, HER2-negative and lymph node–negative patients treated with adjuvant endocrine therapy [18,19,107,108]. BCI may thus be of value for the identification of ER-positive lymph node–negative patients that may not require extended endocrine therapy after 5 years of treatment. Indeed, the BCI test was found to be superior to Oncotype DX or IHC4 in

predicting late recurrence when all three tests were compared for long-term follow-up in the same trial [18]. To-date, however, the prognostic value of BCI for predicting late recurrences has not been directly compared with EndoPredict or MammaPrint.

29. BCI: EGTM recommendation

- In combination with established clinical and pathological factors, BCI may be used for predicting outcome and aiding adjuvant therapy decision-making in lymph node–negative, hormone receptor–positive and HER2-negative patients. **(LOE IB; LOR, A).**

30. BCI: recommendation for further research

- Validation in patients with lymph node–positive disease.
- Validation in a prospective randomised trial.
- Further validation for predicting late recurrences following adjuvant endocrine therapy [109,110].

31. Concordance and discordance in published guidelines on breast cancer biomarkers

Apart from ER, PR and HER2 which are universally endorsed, recommendations for the clinical use of other breast cancer biomarkers vary, depending on the specific expert panel. Likely reasons for these variations include different interpretations of published data, different grading systems of published data used in making recommendations, different inclusion and exclusion criteria used in reviewing the literature and timing of literature review, e.g. new findings can render a previously published recommendation obsolete.

Of the biomarkers discussed in this article, perhaps the greatest disagreement between different expert panels exists for the measurement of Ki67. Both ASCO [111] and NCCN [22] are opposed to the use of this biomarker because of analytical problems with its measurement and lack of standardisation. In contrast, both ESMO and the St. Gallen Consensus Panel recommend its measurement in specific situations [23,24]. Although analytical problems exist with the current Ki67 assays (see above), because of its wide availability and low cost, the EGTM panel cautiously recommends its measurement, especially in countries in which the more expensive multianalyte tests are not available.

In addition to Ki67, guidelines on the use of some multianalyte tests also tend to vary depending on the Expert Panel (Table 2). Thus, the recently published ASCO guidelines were opposed to the use of MammaPrint [109]. However, since the publication of these guidelines, Cardoso *et al.* [70] reported the primary endpoint results from the MINDACT prospective randomised trial (see above). Based on this LOE IA study, the EGTM panel states that MammaPrint may be used for determining prognosis and aiding clinical decision-

Table 3
Guidelines on the use of biomarkers in patients with invasive breast cancer. EGTM recommendations. BCI, Breast Cancer Index; LOE, (level of evidence) based on Ref. [6] SOR (strength of recommendation) based on Ref. [7].

Biomarker	Recommendation	LOE	SOR
ER	For predicting the response to endocrine therapy in patients with early or advanced breast cancer. Mandatory in all patients.	IA	A
PR	In combination with ER for predicting response to endocrine therapy in patients with early or advanced breast cancer. Mandatory in all patients.	IB	A/B
HER2	For predicting response to anti-HER2 therapy in patients with early or advanced breast cancer. Mandatory in all patients.	IA	A
Ki67	In combination with established clinical and pathological factors for determining prognosis in patients with newly diagnosed invasive breast cancer, especially if values are low or high.	IB	A/B
uPA/PAI-1	For determining prognosis and aiding decision-making for the administration of adjuvant chemotherapy to patients with ER-positive, HER2-negative, lymph node–negative disease.	IA	A
Oncotype DX	For determining prognosis and aiding decision-making for the administration of adjuvant chemotherapy in patients with ER-positive HER2-negative lymph, node–negative and lymph node –positive (1–3 nodes) disease.	IB	A
MammaPrint	For determining prognosis and aiding decision-making for the administration of adjuvant chemotherapy to patients with ER-positive, HER2-negative, lymph node–negative and lymph node–positive (1–3 nodes) disease.	IA	A
Prosigna	For determining prognosis and aiding decision-making for the administration of adjuvant chemotherapy to patients with ER-positive HER2-negative, lymph node–negative and lymph node –positive (1–3 nodes) disease.	IB	A
EndoPredict	For determining prognosis and aiding decision-making for the administration of adjuvant chemotherapy to patients with ER-positive HER2-negative lymph node–negative and lymph node –positive (1–3 nodes) disease.	IB	A
BCI	For determining prognosis and aiding decision-making for the administration of adjuvant chemotherapy in patients with ER-positive, HER2-negative, lymph node–negative disease.	IB	A

making with respect to the administration of adjuvant chemotherapy in patients with 0–3 metastatic lymph nodes.

Similarly, based on the recently published results [69] that were not available to the ASCO panel, the EGTM panel recommends the use of Oncotype DX in lymph node–positive (1–3 nodes involved) patients. Other multianalyte tests for which disagreement exists on their clinical use include Prosigna and EndoPredict in node-positive patients. As with Oncotype DX, the ASCO panel was opposed to the use of these tests in node-positive patients [108]. In contrast, both ESMO and the St. Gallen panel recommended their use but neither panel was specific as to the lymph node status of the patients [23,24]. Based on both Prosigna and EndoPredict having undergone validation in at least two retrospective-prospective trials also involving one to three lymph node–positive patients (see above), the EGTM panel recommends the use of these tests in this subgroup of patients.

32. Conclusion

Despite the massive investment of time and money into the development of new breast cancer biomarkers, there are still only three that are mandatory for all patients with diagnosed breast cancer, i.e. ER and PR for predicting the benefit from endocrine therapy and HER2 for predicting the benefit from anti-HER2 therapy. In addition to these mandatory biomarkers, multianalyte tests, such as uPA/PAI-1, Oncotype DX, MammaPrint, Prosigna, EndoPredict or BCI, may be performed in specific subgroups of breast cancer patients (Table 3).

For laboratories measuring breast cancer biomarkers for clinical use, it is essential to use analytically and clinically validated tests, perform regular internal quality control checks, have established assay acceptance and rejection criteria, participate in external quality assurance programs and be accredited by an appropriate organisation (e.g. based on ISO 15189 criteria or CLIA in the US). The external quality assurance should include clinical interpretation of results and the assessment of interlaboratory variation. Finally, it is recommended that laboratories carry out technical and clinical audits on an ongoing regular basis to establish that the biomarker assays are performing as expected and are being used in the originally intended setting [112].

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MJD is a clinical advisor to OncoMark and Atturos. NH has reported honoraria for lectures or consulting from Amgen, Celgene, Genomic Health, Nanostring, Novartis, Pfizer and Roche. ES has reported honoraria or consultation fees from: AstraZeneca, Celgene, Novartis, Pfizer, Roche and travel expenses from: AstraZeneca, Egis, Novartis, Roche. FC has reported advisory board participation for Astellas/Medivation, AstraZeneca, Celgene, Daiichi-Sankyo, Eisai, GE Oncology, Genentech, GlaxoSmithKline, Merck-Sharp, Merus BV, Novartis, Pfizer, Pierre-Fabre, Roche, Sanofi and Teva. MN, RM and AN report no conflict of interest. MJD, MN, RM and AN are members of the EGTM. NH, ES and FC are guest authors.

Disclaimer

Adherence to these guidelines is voluntary since the ultimate decision regarding biomarker use must be made by the treating clinician. It is also important to note that the present guidelines are intended for routine clinical use and do not necessarily apply to clinical trials, which may have a different remit.

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