Multigene Assays for Classification, Prognosis, and Prediction in Breast Cancer: a Critical Review on the Background and Clinical Utility

Multigenassays zur Beurteilung der Klassifikation, Prognose und Prädiktion beim Mammakarzinom. Ein kritischer Review zum Hintergrund und klinischen Nutzen

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Abstract
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Gene signatures which are based on multigene profiling assays have been developed for the purpose to better define the prognosis and prediction of therapy results in early-stage breast cancer. These assays were designed to be more specific than conventional clinico-pathologic parameters in the selection of patients for (neo-)adjuvant treatment and in effect help to avoid unnecessary cytotoxic treatment. In this review we describe molecular risk scores, for which tests are commercially available (PAM50®, MammaTyper®, MammaPrint®, Oncotype DX®, Endopredict®, Genomic Grade Index®) and IHC risk scores (Mammostrat® and IHC4), and discuss the current evidence of their clinical use.

Zusammenfassung
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In den letzten Jahren wurden eine Reihe verschiedener Gensignaturen, die auf Multigenassays basieren, für die Abschätzung der Prognose und Prädiktion beim frühen Mammakarzinom entwickelt. Die Zielsetzung dieser Assays ist die Verbesserung der Spezifität gegenüber konventionellen klinisch-pathologischen Parametern für die Therapieplanung, speziell für die Optimierung der Selektion von Patienten für die (neo-)adjuvante Therapie und zur Vermeidung überflüssiger zytotoxischer Therapien. In diesem Review beschreiben wir wichtige molekulare Risikoscores, für die Tests kommerziell angeboten werden (PAM50®, MammaTyper®, MammaPrint®, Oncotype DX®, Endopredict®, Genomic Grade Index®), sowie vergleichbare immunhistochemische (IHC) Risikoscores (Mammostrat®, IHC4), und diskutieren die wissenschaftliche Evidenz dieser Tests und deren klinischen Anwendungsbereich.

Introduction
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Historically, breast cancer was the first solid malignancy for which the determination of molecular treatment related factors was introduced. In particular, the analysis of hormone receptor expression for endocrine therapy, and the human epidermal growth factor receptor (HER2) for targeted treatment with specific antibodies such as trastuzumab, have an immediate impact on systemic treatment decisions in the (neo-)adjuvant setting. On the other hand, the indication for chemotherapy has traditionally been based on prognostic factors, such as histopathology, (p)TNM stage, and clinical tumor characteristics, as well as combined approaches, prognostic scores, and clinical algorithms, such as the St. Gallen Consensus [1]. It has repeatedly been shown that these prognosis-based approaches lead to an overtreat-ment of patients, and approximately 85% of patients do not benefit from (neo-)adjuvant cytotoxic chemotherapy regarding 10-year breast cancer specific survival [2]. About 20% of patients die of breast cancer within 8 years despite currently available adjuvant cytotoxic chemotherapies [2]. Therefore, classic prognostic and predictive criteria are not specific enough for decision making regarding adjuvant chemotherapy. In the last decade, a new conceptual approach to the biology of breast cancer has emerged, and provided us with new hope to better understanding the biology of the disease, and guiding of therapy. This approach was initially based on gene expression arrays, and has later been translated to quantitative real-time polymerase chain reaction (qRT-PCR) and other molecular methods.

From this molecular characterization of breast cancer not only a new phenotypic classification

of breast cancer has been derived, but also a variety of prognostic and predictive gene signatures were defined. Subsequently, it has become apparent that breast cancer is not a single type of tumor, but a group of different diseases with distinct molecular properties. Each of these molecularly different breast cancer types tends to respond differently (or not at all) to (neo-)adjuvant therapy. Therefore, it has become practice not to think of and treat breast cancer as one disease but according to its intrinsic subtype, for example as luminal A or triple-negative [1]. In addition to that, the promise of molecular probing of breast cancer is to provide more detailed and specific information about therapy-related tumor properties, which will ultimately lead to individualized or more precise treatment of breast cancer.

Today, we have only started to understand why a given breast cancer is behaving and responding to therapy in a given way [3]. But already, there are a number of commercially available molecular tests, namely gene expression assays, that were designed to provide a better guidance to (neo-)adjuvant chemotherapy decisions than clinico-pathological parameters alone. The purpose of this review is to describe the scientific background and the clinical evidence of these tests, and give some guidance as to their current usefulness in daily practice.

Intrinsic Subtyping
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Already in 2000, Perou et al. described the molecular heterogeneity of breast cancer in a way that is still valid today, and this classification has been confirmed by the Cancer Genome Atlas Network project only recently [4]. The term "molecular portraits" that was used in the initial publication accurately reflects the nature of this classification system, which is a phenotypic model based on the statistical analysis of unsupervised clustering of gene expression data. The term intrinsic subtypes was coined after the use of intrinsic genes (genes with minimal variation within a tumor sample, but maximal variation between different patients) to build the model. Four major clusters of gene expression were consistently found and named according to their major characteristics: luminal (divided into luminal-A and luminal-B), HER2-enriched, and basal-like [5]. The luminal-A subtype is characterized by high levels of ER and ER-related genes, while luminal B tumors have lower ER levels but higher expression of proliferation-associated genes. More recently it was shown that some of these subtypes are heterogeneous themselves, such as the basal-like cluster [6], and very likely are not a single phenotype but one group representing different molecular tumor types.

The intrinsic subtypes can be considered as a major classification framework of breast cancer. This approach has gained wide acceptance to both preclinical as well as clinical research for the further exploration of the biology of breast cancer. Initially, the intrinsic subtypes were identified by mRNA expression analysis of 1753 genes in 84 samples [7] and 427 genes in 78 samples [8] in fresh frozen tissue samples by mRNA expression analysis. This has delayed the introduction of the subtype classification into clinical practice, and in the last years the intrinsic subtype classification has been established by commercially available assays. This concerns the PAM50 assay (Nanostring technology) and the MammaTyper assay (qRT-PCR technology). Also intrinsic subtyping can be approximated by IHC alone, using four markers (ER, PR, HER2, Ki-67) [9]. With IHC, the numerical distribution of tumor types is similar to what would be expected from the distri-

bution of tumor types seen by multigene array profiling [10]. However, on an individual basis the concordance of intrinsic subtyping with conventional IHC is only moderate [11]. Only 77% of ER-negative/HER2-positive tumors by IHC were correctly identified by the PAM50 assay, and in triple-negative category by IHC 57% were basal-like, and 30% were classified as HER2-enriched by PAM50 [11]. Obviously, results obtained by gene array intrinsic subtyping are not immediately comparable with IHC.

The different intrinsic tumor types are associated with specific histological, clinical, epidemiological, and therapeutic characteristics [12]. The prognosis of the different categories of intrinsic tumor types differs both with respect to short-term and long-term survival, but one must be aware that (neo-)adjuvant therapy affects prognosis in a subtype-specific way [13]. When the natural history of breast cancer is analyzed [14], HER2-enriched and basal-like breast cancers show an aggressive course in the first two years after diagnosis. Thereafter, HER2-positive tumors not treated with trastuzumab continue developing recurrences, and after 6 years of follow up constitute the prognostically most unfavourable group. However, in the long run (after 10 and more years), luminal-B and HER2-enriched cancers are similarly unfavourable in prognosis, with basal-like cancers being intermediate, and luminal A tumors remained to show only little risk of relapse [14].

PAM50 classifier

PAM50 is a standardized gene set for intrinsic subtype classification, and was designed to improve the classification concordance reported by investigators [14]. The PAM50 assay is based on the Nanostring nCounter technology [15], and believed to be a robust assay with a high concordance between laboratories, provided that the data are normalized [16]. However, it has to be taken into account that the PAM50 gene set may classify some tumors as Luminal A or Luminal B, that are clinically HER2 positive according to standard HER2 (IHC and ISH) techniques. Conversely up to 30% of the HER2 enriched tumors are HER2 negative clinically [4]. The PAM50 classifier for intrinsic subtyping was recently validated in a clinical trial of 348 premenopausal patients receiving tamoxifen [17], and may be superior to IHC in this setting with respect to prognosis and prediction of endocrine response [17], but this observation lacks validation in an independent series of breast carcinomas. When comparing the PAM50 assay with the Oncotype DX® recurrence score, there was a reasonably good agreement between the Oncotype DX® and PAM50 assays for the high and low risk groups as defined by Oncotype DX® [18]. More patients were assigned to the low risk category by the PAM50 score being luminal A than by the Oncotype DX® recurrence score. The Oncotype DX® intermediate risk group was classified as luminal-A in 59% by the PAM50 score, as luminal-B in 33% and as HER2-enriched in 8% [18].

MammaTyper

The MammaTyper® IVD kit determines intrinsic subtypes based on quantitative measurement of ER, PR, HER2 and Ki67 on mRNA level instead of semiquantitative assessment of these markers by IHC. The rationale for this test is that although the IHC approximation of intrinsic subtypes, especially luminal and HER2 type breast cancers, is generally good [19], the distinction between Luminal A and B type breast cancers on the basis of Ki-67 and tumor grade often is discordant to molecular subtyping [19]. This is in part due to the fact that the IHC approximation suffers from the lack of reproducibility of proliferation assessment based on Ki-

i67 IHC in Grade 2 tumors [20], which is the most critical parameter for distinguishing luminal A from luminal B tumors with IHC. The MammaTyper® makes use of a cut-off definition at the 75% percentile for HER2 which showed high concordance with central IHC assessment in a clinical trial of 352 breast carcinomas [21]. By using this clinically validated cut-off, the HER2 status was quantitatively determined and results with regard to prediction of disease-free and overall survival were better than for HER2-IHC [21] and similar for the prediction of neoadjuvant chemotherapy [22]. With regard to the quantitative single parameter assessment of ESR1, mRNA may be a better quantifiable measure compared to ER-IHC for the prediction of tamoxifen benefit [23]. Therefore, the MammaTyper® not only provides subtype information but also provides continuous values of other parameters including ESR1 and Ki67 for therapy planning. The accuracy of subtyping results of MammaTyper has not yet been systematically compared with PAM50 or IHC, however.

Prognostic and Predictive Multigene Assays
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Multigene assays can be divided into those that have been validated on cohorts that allow for the evaluation of prognosis (such as Endopredict®) and assays that evaluate the benefit of adjuvant chemotherapy or both (such as Oncotype DX®). Early prognostic and predictive gene signatures were based on the assumption that breast cancer is one disease and used complex mathematical algorithms in a supervised approach to define risk of recurrence primarily in node negative disease. With these first generation assays, the mathematical models, which are used to calculate a risk score, were derived from only one or few clinical studies, and the patient population in this and in the validation studies determined what kind of risk was measured. For example, multigene assays derived from studies in which many but not all patients were treated with tamoxifen may not only measure prognosis, but also predict the response to tamoxifen.

The multigene assays, which will be described below include genes of related function, such as proliferation or estrogen receptor pathway, but differ with regard to the specific kind of genes measured. The common denominator of all assays is the proliferation genes, and it is believed that the group of proliferation-associated genes has the biggest impact on the measurement of prognosis [24]. Also, these multigene assays are mostly, if not exclusively applicable to luminal type breast cancers [25, 26].

Mammaprint

The first, and still widely distributed gene expression assay is the 70-gene classifier or Mammaprint® assay. In 2002, this gene signature was developed to distinguish patients with a high probability of metastasis-free survival from patients with risk to develop distant metastases within 5 years after diagnosis [27]. The functions of the 70 genes tested are related to the six hallmarks of cancer including apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, tissue invasion and metastasis, and sustained angiogenesis [28]. The test was established using 78 lymph node-negative breast cancers less than 5 cm in diameter from patients under 55 years of age at diagnosis from the Netherlands Cancer Institute (NKI) [27]. The first validation cohort included 295 consecutive women with both ER-positive and ER-negative breast cancers treated at the NKI (some of which also had been included in the original test set), with 151 of them being lymph node-negative

[29]. In this lymph node-negative cohort, the 10-years distant disease-free survival group of patients identified by the test as being "low risk" was 87 vs. 44% in the "high risk" group. In a second validation study for Mammaprint®, 307 patients without adjuvant therapy were studied with a follow-up of at least 10 years. In this study, distant-recurrence free survival was 88 vs. 71% in the low risk vs. the high risk group, respectively [30]. In both studies, the Mammaprint® classification was statistically independent from tumor stage and histopathologic factors such as tumor grading and the Nottingham prognostic index, as well as the Adjuvant! Online estimator. However, in both studies the effect of chemotherapy could not be tested because of the low numbers of patients who had received chemotherapy. Therefore the results concern the prognosis only. When compared to the Adjuvant! Online risk score, 34% of high risk patients according to Adjuvant! Online had a low risk MammaPrint profile, and conversely 14% of low risk patients according to Adjuvant! Online had a high risk MammaPrint® profile [30].

Further validation studies [31–34] did confirm the independent prognostic value of the 70-gene classifier in older patients and in lymph node positive patients, but with a lower predictive value than in the initial studies [32]. A common observation between these validation studies was that the MammaPrint risk is mostly related to recurrences occurring early in the course of disease which may be explained by the development strategy of the test itself [30]. This early relapse patient population might be well suited to receive adjuvant chemotherapy, and in fact, it was shown in a metaanalysis that MammaPrint® is also predictive to chemotherapy [35]. In the high risk group, the addition of chemotherapy improved the distant-recurrence free survival by 12% vs. hormonal therapy alone, but no such benefit was seen in the Mammaprint® low risk group. Therefore, Mammaprint® is regarded as both a prognostic and a predictive assay.

Oncotype DX

Second on the european market, and the most widely applied gene expression assay worldwide, was the 21-gene classifier, which is commercially available as the Oncotype DX® test. The 21 genes were selected by correlating the expression levels of 250 genes with relapse-free survival in three clinical trials with a total of 447 breast cancer patients [36–38]. The gene set contains the mRNA quantification of ER, PR, HER2 and tumor proliferation, but these genes are not included to substitute the IHC determination or provide subtype information. Clinical validation trials included the tamoxifen-treated patients of the NSABP B-14 trial [39], and the NSABP B-20 trial comparing tamoxifen-treated and tamoxifen plus CMF chemotherapy treated patients [40]. A high recurrence score was associated with a worse outcome in the first validation study [39], and with a benefit from CMF chemotherapy in the second study [40]. As part of the NSABP B-20 trial 2299 patients with estrogen receptor positive tumors (potentially including HER2 positive tumors) were randomly assigned to receive either tamoxifen alone or in combination with CMF or MF from 1988 to 1993. For the retrospective analysis FFPE tissue from 670 out of 2299 patients was available (29.1%). On the basis of these data, the Oncotype DX® is regarded both as a prognostic as well as a predictive test. A recent study indicates that Oncotype also is predictive for modern anthracycline containing chemotherapy in node-positive patients [41].

The 21 genes that are evaluated by the Oncotype DX® assay belong to the ER-related genes, proliferation genes, HER2, invasion-related genes, and others, including five genes to normalize

for RNA quantity [39]. The quantitative RNA expression levels of these genes are used to calculate a numerical score (recurrence score, RS) with a range from 0–100. In the original publication, a score below 18 has been regarded low risk, a score between 18–30 intermediate risk, and a score of 31 or above high risk. A high recurrence score indicates potential benefit from chemotherapy, compared to patients with low- and intermediate recurrence scores. Notably, these threshold values have been changed afterwards to below 11, 11–25 and above 25 for the low, intermediate and high risk group, respectively, in order not to deny chemotherapy to a patient who might benefit [42]. The randomized prospective TAILORx trial has been designed to test the treatment options for the intermediate risk group, while low-risk patients are treated with endocrine therapy alone and high-risk patients will receive chemotherapy additional to endocrine therapy in the TAILORx trial [42]).

A meta-analysis of 11 published decision-impact studies (1154 patients) concluded that patients can be spared adjuvant chemotherapy in a high percentage. After consideration of the recurrence score in only 404 (49%) patients out of 820 patients who had initially been assigned to chemo-endocrine therapy, chemotherapy was further recommended [43]. Additionally, 99 (16%) patients out of 632 patients with the initial recommendation of endocrine therapy alone were changed to chemotherapy plus endocrine therapy. In total, treatment recommendations changed in 515 (35%) out of 1457 patients. Similarly, in a recent German study, recommendations changed for 33% of patients after consideration of the Oncotype DX® risk score [44]. The change in treatment decision was similar for lymph node-negative and lymph node-positive patients [44]. Changes in treatment decisions with the net result of a reduction in chemotherapy were shown to be cost effective for the health system, considering the cost of therapy, and the cost of the Oncotype DX assay [45]. But, thus far, no impact on survival has been shown in these decision-impact studies, and no follow-up of these patients is planned. Therefore the decision impact studies have to be taken cautiously with regard to benefit for the patient.

Endopredict

More recently, the EndoPredict® (EP) assay was introduced as a novel multigene classifier to assess prognosis in ER-positive, HER2-negative breast cancer patients. EndoPredict is a 12-gene expression test that was developed using tumor tissues from different institutions in Germany and Austria. A total of 964 tissue samples were screened and 63 candidate genes were selected in the training sets by stepwise selection of gene expression data [46]. From this set, 8 genes were chosen for the EndoPredict® assay and 4 genes for serving as an internal control. Genes are related to tumor proliferation and to hormone receptor activity, but do not include ER, PR, or HER2 status. This is in contrast to the PAM50®, Oncotype DX® and MammaTyper® assays that also directly measure ER, PR, and HER2 receptors. The EP score ranges between 0 and 15 with a threshold of 5 to discriminate low and high risk. As with the MammaTyper® and PAM50 gene expression assays, the EndoPredict assay is designed to be performed decentrally [47].

For validation of the assay, paraffin tumor blocks from the clinical trials ABCSG-6 and ABCSG-8 [48] were selected and processed on an automated RNA extraction platform. The sample size for validation of the assay included 378 tumor blocks from the ABCSG-6 study (22%) and 1324 tumors from the ABCSG-8 study (41%). Both trials were randomized phase III trials involving endocrine

therapy only. In the multivariate cox model, the two most significant risk factors for distant disease recurrence (but excluding death) were nodal status (hazard ratio = 2.32) and the EndoPredict® score (hazard ratio = 1.27) [46]. However, 51.1% of these patients (870/1702) were determined to be "high risk" and may have been subjected to treatment with chemotherapy, if the EP score would have been taken as guidance for therapy selection. This high proportion of high risk cases in this cohort of clinically low risk patients with endocrine therapy only points to the fact that multigene assays not necessarily reflect the clinical risk estimation. Also, because these clinical trials did not include chemotherapy, the EP score has to be considered as a prognostic, not as a predictive score. In order to improve the prognostic significance of this multigene assay, the EndoPredict® score was combined with tumor size and nodal status in a linear model and called EP-clin score. Recently, the EPclin score was directly compared to purely clinical risk classifications (like St. Gallen, German S3, and NCCN) and found to be superior to these classifiers [49]. In another recent study, the EPclin score led to a change of therapy in 37.7% of patients (endocrine therapy alone in 25.4% or additional chemotherapy in 12.3%) in a clinical decision impact study [50], but the effect of this treatment change on survival has not yet been evaluated.

PAM50-derived risk scores

In order to translate the prognosis that is associated with the different intrinsic subtypes as determined by the PAM50 classifier into a clinically relevant prognostic score, Parker et al. have devised the PAM50 ROR score [14]. Consequently, the ROR score can be applied to all the subtypes of breast cancer, and not only to ER-positive tumors as in the MammaPrint®, Oncotype DX®, and EndoPredict® tests. It was used to divide node-negative and node-positive tamoxifen-treated patients into low and intermediate risk groups and was more prognostic than clinical factors and IHC in this sample [51]. The ROR classifier was also superior to the Oncotype DX® and IHC4 scores on a cohort of 1017 patients receiving tamoxifen or anastrozole in the ATAC trial [52]. Additionally, an eleven gene PAM50 proliferation score was described that predicted overall survival with a better accuracy than Ki-67 in the GEICAM/9906 trial (FEC treated patients), and – unexpectedly – was predictive for paclitaxel benefit in the low-proliferative group [53].

Genomic Grade Index

Histopathologic tumor grading has been regarded as the second most important conventional prognostic indicator, next to lymph node status [54, 55]. It is considered even more important nowadays as more and more primary breast cancers are node-negative. However, grading suffers from inter- and intraobserver variability [56], and the most common grading category (G2) may be of little help in the context of clinical decision making. With the purpose of making tumor grading more objective, a 97-gene signature (Genomic Grade Index, GGI) was identified using a discovery cohort of 189 breast cancers and a validation cohort of 597 tumors of different subtypes [57]. By applying the GGI it was possible to assign a two-tier grading to these tumors, and in effect reclassify intermediate grade, ER-positive breast cancers to a high or a low genomic grade category [58]. The GGI was shown to be predictive of recurrence in endocrine treated patients [59, 60], and prognostic in the neoadjuvant setting [61]. From the original 97-gene signature that requires microarray testing, an 8-gene signature (4 genes for the GGI and 4 reference genes) was derived

that can be used in clinical practice on fresh frozen and formalin fixed, paraffin embedded (FFPE) tissues by qRT-PCR [62]. As all first generation assays, the GGI is also only informative in luminal, non HER2 positive tumors [59].

Related IHC Scores
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In order to simplify the determination of tissue-based prognostic factors, and make it more economical, it was attempted to use protein-based prognostic scores that are determined by IHC as a surrogate for RNA-based gene signatures. This requires a standardized IHC procedure with several markers and semiquantitative or quantitative evaluation on the microscope. Advantages of IHC based scores may include not only that they are less expensive, but also that IHC is based on the evaluation of the tumor cells, not on the tumor tissue including the tumor stroma.

IHC4 score

The IHC4 score includes ER, PR, Ki-67, and HER2 and was developed on a retrospective cohort from the ATAC trial of 1125 ER-positive patients who did not receive adjuvant chemotherapy [63]. This 4-parameter IHC score was compared with the Oncotype DX recurrence score using distant metastasis as the primary end point [63]. A prognostic IHC4 model was calculated, and on a separate cohort of 786 patients it was shown that the IHC4 score not only provided prognostic information independent of classical clinico-pathologic variables, but also the IHC4 score was found to be similar in strength when compared with the recurrence score [63]. Only little was gained by combining the two scores. In order to make the IHC4 score more powerful, it was combined with a clinical score that included the pN and pT categories, tumor grade and the patients age. For the purpose of decision making, this IHC4+C score was found to be useful to downgrade about half of the patients deemed to have a high risk by Adjuvant! Online to an intermediate or low risk category, and thereby sparing chemotherapy to these patients [64]. However, it is yet unclear how the IHC4 score might perform with decentralized testing at local pathologies, considering the interobserver and interlaboratory variability of quantitative IHC assessment [20].

Mammostrat

The Mammostrat® test is a IHC signature which based on the IHC evaluation of the expression of five genes (p53, NDRG1, CEACAM5, SLC7A5, and HTF9C). Similar to RNA-based signatures, Mammostrat was developed to test the prediction of outcome in ER-positive breast cancer patients [65]. The five genes were selected from 700 gene targets in gene expression assays in three patient cohorts of 466, 299 and 344 patients after the validation of several IHC panels in 195/466 ER-positive, node-negative patients from the first training cohort [65]. A further validation study included a subset of 287 placebo and 550 tamoxifen-treated patients from the NSABP B-14 and a subset of 161 tamoxifen-treated patients and 296 tamoxifen plus chemotherapy treated patients from the NSABP B-20 trial [66]. The Mammostrat® test subdivides patients into low, moderate and high-risk groups in an age-specific way. With patients treated by tamoxifen there was 6, 8 and 22% risk of progression for low, intermediate and high risk patients 60 years and older, as compared to 20% risk of disease progression already in the low risk group for younger patients. Therefore, this test, which was developed in a predominantly postmenopausal cohort, may be specific for older patients,

but this age dependency still has to be confirmed. Recenty, Bartlett et al. confirmed the efficacy of the Mammostrat test in a tissue-microarray validation study on 3837 tumor samples from tamoxifen or exemestane treated, node-positive and node-negative patients from the TEAM trial [67]. Age was no significant parameter in this study.

Discussion
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Multigene assays have provided a new approach not only to breast cancer subtyping but also to prognostic and predictive tumor classification [9]. The molecular phenotypic classification scheme that divides breast cancer into four main classes (luminal A, luminal B, HER2-enriched, basal-like) was verified by different approaches, including gene expression analysis, genetic and epigenetic studies [4], and can be considered as established. Open questions remain regarding the reproducibility between different methodological approaches, the definition of each subtype, and the way in which heterogeneity within these subclasses should be addressed. Any further information and clinical usefulness above that is provided by the intrinsic subclassification, in addition to ER, PR, HER2 and proliferation as determined by IHC, has only been partially explored yet [68,69]. Specifically, the distinction between luminal A and B type breast cancers currently is under debate, and among the luminal ER-positive/HER2-negative breast cancers a high-risk group can be identified by loss of PR-expression or increased proliferation (e.g. Ki-67 > 20%) by IHC [70]. Similarly, triple-negative tumors can be subdivided into prognostically different subgroups by IHC [71] or by gene expression profiling [6]. In view of the striking genetic heterogeneity in the luminal-B or triple-negative subtype, and the probable prognostic, and (future) therapeutic implications of specific gene mutations such as PI3CA or PTEN affecting the mTOR-pathway, it is likely that this subtyping will become more specific in the next years. Also, intrinsic subtyping has not yet taken into account the characteristics of special histologic tumor types such as metaplastic or adenoid cystic breast cancer [72].

In contrast to gene profiles that are aimed at intrinsic tumor classification, the gene profiling assays that were designed by supervised approaches to define recurrence risk in luminal tumors were called first-generation multigene assays, and mainly identify the poor prognostic highly proliferative, ER-positive breast cancers [73]. These assays (e.g. Mammaprint®, Oncotype DX®) were created to reduce overtreatment and to guide treatment selection, especially the decision for or against chemotherapy in the (neo-)adjuvant setting in hormone-receptor positive patients, when conventional clinicopathologic features (i.e. tumor size, lymph node status, histologic grading) are indeterminate or would have suggested otherwise. Statistically, the higher the individual risk of recurrence, the greater the likelihood that a patient will benefit from chemotherapy. Therefore, the gene profiling tests were established as prognostic tests, measuring the risk of recurrence and death of disease. Patients with a low risk score have only a small absolute benefit from cytotoxic chemotherapy. However, even in a high risk situation, it remains unclear whether a given patient will benefit or not, and the use of a prognostic test is not necessarily predictive. Also, measuring the prognosis by molecular assays is not inherently better only because the tests are based on the molecular biology of the tumor, but it was shown repeatedly that the prognostic scores as measured gene profiling are independent from clinicopathologic parame-

Table 1 Tests available for intrinsic subtyping, and prognostic or predictive risk assessment.

Test	Category	Indication	Company	Test conduction	Tissue requirements	Technique	Output/score
PAM50®	classifier, prognostic test	invasive breast cancer, regardless of stage or ER status	NanoString Technologies, Inc.	central lab	formalin-fixed, paraffin-embedded	Nanostring nCounter (50 genes)	intrinsic subtype, risk of recurrence score (continuous)
MammaTyper®	classifier	invasive breast cancer, regardless of stage or ER status	BioNTech AG	local pathology	formalin-fixed, paraffin-embedded	qRT-PCR (4 genes)	intrinsic subtype
MammaPrint®	prognostic and predictive test	invasive breast cancer, pT 1–2, pN0, age < 61 years	Agendia BV	reference lab	fresh/frozen or formalin-fixed, paraffin-embedded	DNA microarrays (70 genes)	risk category (low risk vs. high risk)
Oncotype DX®	prognostic and predictive test	ER-positive, node-negative disease	Genomic Health Inc.	reference lab	formalin-fixed, paraffin-embedded	qRT-PCR (16 genes)	risk score and category (high vs. moderate vs. low risk)
Endopredict®	prognostic test	ER-positive, HER2-negative disease	Sividon Diagnostics GmbH	local pathology	formalin-fixed, paraffin-embedded	qRT-PCR (8 genes)	risk score and category (low risk vs. high risk)
Genomic Grade Index	prognostic test	any invasive, primary, ER-positive, grade 2 tumors	Ipsogen S. A., Marseille	central lab	formalin-fixed, paraffin-embedded	DNA microarrays (97 genes) or qRT-PCR (4 genes)	risk category (low risk vs. high risk)
IHC4	prognostic test	any invasive, primary, ER-positive tumors	none	local pathology	formalin-fixed, paraffin-embedded	IHC (4 IHC markers)	risk score and category (low risk vs. high risk)
Mammostrat®	prognostic test	ER-positive, lymph node-negative tumors treated with tamoxifen	Applied Genomics, Inc.	local pathology	formalin-fixed, paraffin-embedded	IHC (5 IHC markers)	risk category (high vs. moderate vs. low risk)

Classifier = Test for intrinsic subtype classification. Prognostic test = Prognostic significance in retrospective studies. Predictive test = Predictive significance for adjuvant chemotherapy in retrospective studies.

ters. Molecular tests therefore provide additional information, and can be combined to a molecular-clinical risk score (such as EPclin) to improve the prognostic assessment of a given patient. Whether this additional information provided by molecular tests can be substituted by IHC for ER, PR, HER2, and Ki-67 still is subject to debate, and the prognostic information of Oncotype DX® was equivalent to IHC in a large retrospective study [63].

Those tests, which can be applied on FFPE tissues have been validated retrospectively on samples from prospective studies [39, 48]. It has been proposed that data generated in such a setting provide level 1 evidence [74]. Although no minimal requirement can be stated as universally applicable, Simon and coworkers [74] suggested that samples from at least two-thirds of the patients be available for analysis. This is clearly not the case for most of the validation studies which have been performed with Oncotype DX® as well as Endopredict® (NSABP B-14: 26%, NSAPB B-20: 29%, SWOG 8814: 35%, ABCSG-6: 22%, ABCSG-8: 41%). In our point of view, there is currently no level 1 evidence for the predictive or prognostic value of multigene assays and the results of ongoing prospective studies have to be awaited.

It has been argued that multigene assays were more objective and therefore inherently better for the evaluation of prognosis as compared to the evaluation of clinicopathologic features. This may be true for the individual assay, but when directly comparing molecular assays for risk assessment, the concordance with regard to estimation of risk is only moderate [52, 75, 76]. The overall concordance of results regarding classification into risk groups was 76% when comparing the EndoPredict score with the Oncotype DX recurrence score [76]. However, in this study, there was a remarkable discrepancy for tumors identified as high risk cases, with 26% (9/34 cases) by Oncotype DX and 67% as high risk (23/34 cases) by Endopredict [76]. Since low and intermediate risk patients (Oncotype DX risk score) did not benefit from chemotherapy in the NSABP B-20 trial [40], the EndoPredict test seems to overestimate the potential benefit in the high risk group. Prat et al. concluded from a comparison of the PAM50-ROR, OncotypeDX, Mammaprint and SET signatures regarding survival that the assays should not be considered to be interchangeable, because the predictors of the different assays were statistically independent [77]. Therefore, the level of discordance of molecular tests is not dissimilar from the variability in the interpretation of histopathological factors such as tumor grading by pathologists [78], but concordance in pathology can be improved by measures such as training [56], or image analysis [79]. In an important study, Weigelt et al. have compared three different gene signatures used to define the intrinsic breast cancer subtypes in four different expression datasets from breast cancer series. While all signatures identified patient groups with similar survival rates, they did not reliably assign the same patients to the same molecular subtypes but instead produced a substantial variation of results, when subtyping is performed on microarray based hierarchical cluster analysis of fresh tissue samples [80]. However, it has to be taken into account that the classical microarray-based hierarchical clustering method is not suitable for assigning intrinsic subtypes to particular samples. Clearly, more robust, non-hierarchical clustering methods have to be applied for subtyping and single sample prediction.

Other issues that need to be addressed, before gene expression profiling can be put into routine clinical use, even as an ancillary test, concern the measurement of therapeutic targets in these

tests, such as ER, PR, or HER2, and practical issues such as sample collection [81]. The agreement of hormone receptor testing in qRT-PCR-based assays and IHC is generally good [82–84], and in fact, the determination of ER and PR status by qRT-PCR may be superior to IHC testing [85]. The contrary has been shown for the HER2 results that are measured by the Oncotype DX® assay [86], because the Oncotype DX® assay has not initially been designed to precisely determine the HER2 status. The multigene assays also differ with regard to sample collection. The use of fresh frozen tissue is required by the MammaPrint® assay, as compared to FFPE tissue as required by most other assays (**○ Table 1**).

It can be said that, as of today, no signature can replace the classical clinico-pathologic parameters, but molecular assays may add additional information when there is no clear indication for chemotherapy otherwise [87]. Additionally to all points discussed above, standardized and reproducible assessments of clinico-pathological parameter and ER, PR, HER2, Ki-67 status in routine pathologic diagnostics are needed in order to more reliably identify patients' subpopulations whose samples should subsequently undergo further multigene assays. Taken together, the present data are insufficient to recommend the routine use of first generation gene expression assays, and although gene-expression profiling clearly has a great potential to improve breast cancer management, the benefit of molecular tests for adjuvant systemic treatments has yet to be defined better [88]. This is the primary endpoint of large ongoing prospective clinical trials in patients with lymph node-negative early breast cancer (MINDACT [89] and TAILORx [42]) which will provide level I evidence about the relevance of applying gene signatures to the daily management of breast cancer patients.

Conflict of Interest
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References
1 *Goldhirsch A, Wood WC, Coates AS et al.* Strategies for subtypes – dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the primary therapy of early breast cancer 2011. Ann Oncol 2011; 22: 1736–1747
2 *Peto R, Davies C, Godwin J et al.* Comparisons between different polychemotherapy regimens for early breast cancer: meta-analyses of long-term outcome among 100,000 women in 123 randomised trials. Lancet 2012; 379: 432–444
3 *Kreipe HH, Ahrens P, Christgen M et al.* Jenseits von Staging, Typing und Grading. Herausforderungen und Perspektiven für die Tumorpathologie der Mamma. Pathologe 2010; 31: 54–59
4 *Cancer Genome Atlas Network.* Comprehensive molecular portraits of human breast tumours. Nature 2012; 490: 61–70
5 *Weigelt B, Baehner FL, Reis-Filho JS.* The contribution of gene expression profiling to breast cancer classification, prognostication and prediction: a retrospective of the last decade. J Pathol 2010; 220: 263–280
6 *Lehmann BD, Bauer JA, Chen X et al.* Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin Invest 2011; 121: 2750–2767
7 *Perou CM, Sorlie T, Eisen MB et al.* Molecular portraits of human breast tumours. Nature 2000; 406: 747–752
8 *Sorlie T, Perou CM, Tibshirani R et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001; 98: 10869–10874
9 *Sotiriou C, Pusztai L.* Gene-expression signatures in breast cancer. N Engl J Med 2009; 360: 790–800
10 *Blows FM, Driver KE, Schmidt MK et al.* Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies. PLoS Med 2010; 7: e1000279
11 *Bastien RR, Rodriguez-Lescure A, Ebbert MT et al.* PAM50 breast cancer subtyping by RT-qPCR and concordance with standard clinical molecular markers. BMC Med Genomics 2012; 5: 44
12 *Weigelt B, Horlings HM, Kreike B et al.* Refinement of breast cancer classification by molecular characterization of histological special types. J Pathol 2008; 216: 141–150
13 *Cheang MC, Voduc KD, Tu D et al.* Responsiveness of intrinsic subtypes to adjuvant anthracycline substitution in the NCIC.CTG MA.5 randomized trial. Clin Cancer Res 2012; 18: 2402–2412
14 *Parker JS, Mullins M, Cheang MC et al.* Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol 2009; 27: 1160–1167
15 *Reis PP, Waldron L, Goswami RS et al.* mRNA transcript quantification in archival samples using multiplexed, color-coded probes. BMC Biotechnol 2011; 11: 46
16 *Perou CM, Parker JS, Prat A et al.* Clinical implementation of the intrinsic subtypes of breast cancer. Lancet Oncol 2010; 11: 718–719; author reply 720-711
17 *Chia SK, Bramwell VH, Tu D et al.* A 50-gene intrinsic subtype classifier for prognosis and prediction of benefit from adjuvant tamoxifen. Clin Cancer Res 2012; 18: 4465–4472
18 *Kelly CM, Bernard PS, Krishnamurthy S et al.* Agreement in risk prediction between the 21-gene recurrence score assay (Oncotype DX(R)) and the PAM50 breast cancer intrinsic Classifier in early-stage estrogen receptor-positive breast cancer. Oncologist 2012; 17: 492–498
19 *Nguyen B, Cusumano PG, Deck K et al.* Comparison of molecular subtyping with BluePrint, MammaPrint, and TargetPrint to local clinical subtyping in breast cancer patients. Ann Surg Oncol 2012; 19: 3257–3263
20 *Varga Z, Diebold J, Dommann-Scherrer C et al.* How reliable is Ki-67 immunohistochemistry in grade 2 breast carcinomas? A QA study of the Swiss Working Group of Breast- and Gynecopathologists. PLoS ONE 2012; 7: e37379
21 *Fountzilas G, Valavanis C, Kotoula V et al.* HER2 and TOP2A in high-risk early breast cancer patients treated with adjuvant epirubicin-based dose-dense sequential chemotherapy. J Transl Med 2012; 10: 10
22 *Noske A, Loibl S, Darb-Esfahani S et al.* Comparison of different approaches for assessment of HER2 expression on protein and mRNA level: prediction of chemotherapy response in the neoadjuvant GeparTrio trial (NCT00544765). Breast Cancer Res Treat 2011; 126: 109–117
23 *Kim C, Tang G, Pogue-Geile KL et al.* Estrogen receptor (ESR1) mRNA expression and benefit from tamoxifen in the treatment and prevention of estrogen receptor-positive breast cancer. J Clin Oncol 2011; 29: 4160–4167
24 *Goldhirsch A, Ingle J, Gelber R et al.* Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the primary therapy of early breast cancer 2009. Ann Oncol 2009; 20: 1319–1329
25 *Desmedt C, Haibe-Kains B, Wirapati P et al.* Biological processes associated with breast cancer clinical outcome depend on the molecular subtypes. Clin Cancer Res 2008; 14: 5158–5165
26 *Wirapati P, Sotiriou C, Kunkel S et al.* Meta-analysis of gene expression profiles in breast cancer: toward a unified understanding of breast cancer subtyping and prognosis signatures. Breast Cancer Res 2008; 10: R65
27 *van't Veer L, Dai H, van de Vijver M et al.* Gene expression profiling predicts clinical outcome of breast cancer. Nature 2002; 415: 530–536
28 *Tian S, Roepman P, van't Veer LJ et al.* Biological functions of the genes in the mammaprint breast cancer profile reflect the hallmarks of cancer. Biomark Insights 2010; 5: 129–138
29 *van de Vijver M, He Y, van't Veer L et al.* A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002; 347: 1999–2009

30 *Buyse M, Loi S, van't Veer L et al.* Validation and clinical utility of a 70-gene prognostic signature for women with node-negative breast cancer. J Natl Cancer Inst 2006; 98: 1183–1192

31 *Bueno-de-Mesquita JM, Linn SC, Keijzer R et al.* Validation of 70-gene prognosis signature in node-negative breast cancer. Breast Cancer Res Treat 2009; 117: 483–495

32 *Wittner BS, Sgroi DC, Ryan PD et al.* Analysis of the MammaPrint breast cancer assay in a predominantly postmenopausal cohort. Clin Cancer Res 2008; 14: 2988–2993

33 *Mook S, Schmidt MK, Viale G et al.* The 70-gene prognosis-signature predicts disease outcome in breast cancer patients with 1–3 positive lymph nodes in an independent validation study. Breast Cancer Res Treat 2009; 116: 295–302

34 *Mook S, Schmidt MK, Weigelt B et al.* The 70-gene prognosis signature predicts early metastasis in breast cancer patients between 55 and 70 years of age. Ann Oncol 2010; 21: 717–722

35 *Knauer M, Mook S, Rutgers EJ et al.* The predictive value of the 70-gene signature for adjuvant chemotherapy in early breast cancer. Breast Cancer Res Treat 2010; 120: 655–661

36 *Esteban J, Baker J, Cronin M et al.* Tumor Gene Expression and Prognosis in Breast Cancer: Multi-Gene RT-PCR Assay of Paraffin-embedded Tissue. Abstract #3416. Chicago, IL: ASCO Annual Meeting; 2003

37 *Cobleigh MA, Bitterman P, Baker J et al.* Tumor Gene Expression predicts distant disease-free Survival (DDFS) in Breast Cancer Patients with 10 or more positive Nodes: high throughput RT-PCR Assay of Paraffin-embedded Tumor Tissues. Abstract #3466. Chicago, IL: ASCO Annual Meeting; 2003

38 *Paik S, Shak S, Tang G et al.* Multi-gene RT-PCR assay for predicting recurrence in node negative breast cancer patients – NSABP studies B-20 and B-14. Breast Cancer Res Treat 2003; 82 (Suppl.): S10–S11

39 *Paik S, Shak S, Tang G et al.* A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N Engl J Med 2004; 351: 2817–2826

40 *Paik S, Tang G, Shak S et al.* Gene expression and benefit of chemotherapy in women with node-negative, estrogen-receptor-positive breast cancer. J Clin Oncol 2006; 24: 3726–3734

41 *Albain KS, Barlow WE, Shak S et al.* Prognostic and predictive value of the 21-gene recurrence score assay in postmenopausal women with node-positive, oestrogen-receptor-positive breast cancer on chemotherapy: a retrospective analysis of a randomised trial. Lancet Oncol 2010; 11: 55–65

42 *Sparano JA, Paik S.* Development of the 21-gene assay and its application in clinical practice and clinical trials. J Clin Oncol 2008; 26: 721–728

43 *Markopoulos C.* Overview of the use of Oncotype DX((R)) as an additional treatment decision tool in early breast cancer. Expert Rev Anticancer Ther 2013; 13: 179–194

44 *Eiermann W, Rezai M, Kummel S et al.* The 21-gene recurrence score assay impacts adjuvant therapy recommendations for ER-positive, node-negative and node-positive early breast cancer resulting in a risk-adapted change in chemotherapy use. Ann Oncol 2013; 24: 618–624

45 *Hornberger J, Chien R, Krebs K et al.* US insurance program's experience with a multigene assay for early-stage breast cancer. J Oncol Pract 2011; 7: e38s–e45s

46 *Filipits M, Rudas M, Jakesz R et al.* A new molecular predictor of distant recurrence in ER-positive, HER2-negative breast cancer adds independent information to conventional clinical risk factors. Clin Cancer Res 2011; 17: 6012–6020

47 *Denkert C, Kronenwett R, Schlake W et al.* Decentral gene expression analysis for ER+/Her2− breast cancer: results of a proficiency testing program for the EndoPredict assay. Virchows Arch 2012; 460: 251–259

48 *Dubsky PC, Jakesz R, Mlineritsch B et al.* Tamoxifen and anastrozole as a sequencing strategy: a randomized controlled trial in postmenopausal patients with endocrine-responsive early breast cancer from the Austrian Breast and Colorectal Cancer Study Group. J Clin Oncol 2012; 30: 722–728

49 *Dubsky P, Filipits M, Jakesz R et al.* EndoPredict improves the prognostic classification derived from common clinical guidelines in ER-positive, HER2-negative early breast cancer. Ann Oncol 2013; 24: 640–647

50 *Muller BM, Keil E, Lehmann A et al.* The EndoPredict gene-expression assay in clinical practice – performance and impact on clinical decisions. PLoS ONE 2013; 8: e68252

51 *Nielsen TO, Parker JS, Leung S et al.* A comparison of PAM50 intrinsic subtyping with immunohistochemistry and clinical prognostic factors in tamoxifen-treated estrogen receptor-positive breast cancer. Clin Cancer Res 2010; 16: 5222–5232

52 *Dowsett M, Sestak I, Lopez-Knowles E et al.* Comparison of PAM50 risk of recurrence score with Oncotype DX and IHC4 for predicting risk of distant recurrence after endocrine therapy. J Clin Oncol 2013; 31: 2783–2790

53 *Martin M, Prat A, Rodriguez-Lescure A et al.* PAM50 proliferation score as a predictor of weekly paclitaxel benefit in breast cancer. Breast Cancer Res Treat 2013; 138: 457–466

54 *Rakha EA, El-Sayed ME, Lee AH et al.* Prognostic significance of Nottingham histologic grade in invasive breast carcinoma. J Clin Oncol 2008; 26: 3153–3158

55 *Rakha EA, Reis-Filho JS, Baehner F et al.* Breast cancer prognostic classification in the molecular era: the role of histological grade. Breast Cancer Res 2010; 12: 207

56 *Paradiso A, Ellis IO, Zito FA et al.* Short- and long-term effects of a training session on pathologists' performance: the INQAT experience for histological grading in breast cancer. J Clin Pathol 2009; 62: 279–281

57 *Sotiriou C, Wirapati P, Loi S et al.* Gene expression profiling in breast cancer: understanding the molecular basis of histologic grade to improve prognosis. J Natl Cancer Inst 2006; 98: 262–272

58 *Loi S, Haibe-Kains B, Desmedt C et al.* Definition of clinically distinct molecular subtypes in estrogen receptor-positive breast carcinomas through genomic grade. J Clin Oncol 2007; 25: 1239–1246

59 *Desmedt C, Giobbie-Hurder A, Neven P et al.* The Gene expression Grade Index: a potential predictor of relapse for endocrine-treated breast cancer patients in the BIG 1-98 trial. BMC Med Genomics 2009; 2: 40

60 *Naoi Y, Kishi K, Tanei T et al.* High genomic grade index associated with poor prognosis for lymph node-negative and estrogen receptor-positive breast cancers and with good response to chemotherapy. Cancer 2011; 117: 472–479

61 *Liedtke C, Hatzis C, Symmans WF et al.* Genomic grade index is associated with response to chemotherapy in patients with breast cancer. J Clin Oncol 2009; 27: 3185–3191

62 *Toussaint J, Sieuwerts AM, Haibe-Kains B et al.* Improvement of the clinical applicability of the Genomic Grade Index through a qRT-PCR test performed on frozen and formalin-fixed paraffin-embedded tissues. BMC Genomics 2009; 10: 424

63 *Cuzick J, Dowsett M, Pineda S et al.* Prognostic value of a combined estrogen receptor, progesterone receptor, Ki-67, and human epidermal growth factor receptor 2 immunohistochemical score and comparison with the Genomic Health recurrence score in early breast cancer. J Clin Oncol 2011; 29: 4273–4278

64 *Barton S, Zabaglo L, A'Hern R et al.* Assessment of the contribution of the IHC4+C score to decision making in clinical practice in early breast cancer. Br J Cancer 2012; 106: 1760–1765

65 *Ring BZ, Seitz RS, Beck R et al.* Novel prognostic immunohistochemical biomarker panel for estrogen receptor-positive breast cancer. J Clin Oncol 2006; 24: 3039–3047

66 *Ross DT, Kim CY, Tang G et al.* Chemosensitivity and stratification by a five monoclonal antibody immunohistochemistry test in the NSABP B14 and B20 trials. Clin Cancer Res 2008; 14: 6602–6609

67 *Bartlett JM, Bloom KJ, Piper T et al.* Mammostrat as an immunohistochemical multigene assay for prediction of early relapse risk in the tamoxifen versus exemestane adjuvant multicenter trial pathology study. J Clin Oncol 2012; 30: 4477–4484

68 *Weigelt B, Pusztai L, Ashworth A et al.* Challenges translating breast cancer gene signatures into the clinic. Nat Rev Clin Oncol 2012; 9: 58–64

69 *Weigelt B, Reis-Filho JS.* Molecular profiling currently offers no more than tumour morphology and basic immunohistochemistry. Breast Cancer Res 2010; 12 (Suppl. 4): S5

70 *Braun L, Mietzsch F, Seibold P et al.* Intrinsic breast cancer subtypes defined by estrogen receptor signalling-prognostic relevance of progesterone receptor loss. Mod Pathol 2013; DOI: 10.1038/modpathol.2013.60

71 *Elsawaf Z, Sinn HP, Rom J et al.* Biological subtypes of triple-negative breast cancer are associated with distinct morphological changes and clinical behaviour. Breast 2013; DOI: 10.1016/j.breast.2013.05.012

72 *Weigelt B, Reis-Filho JS.* Histological and molecular types of breast cancer: is there a unifying taxonomy? Nat Rev Clin Oncol 2009; 6: 718–730

73 *Rakha EA, Reis-Filho JS, Ellis IO.* Combinatorial biomarker expression in breast cancer. Breast Cancer Res Treat 2010; 120: 293–308

74 *Simon RM, Paik S, Hayes DF.* Use of archived specimens in evaluation of prognostic and predictive biomarkers. J Natl Cancer Inst 2009; 101: 1446–1452

75 *Fan C, Oh DS, Wessels L et al.* Concordance among gene-expression-based predictors for breast cancer. N Engl J Med 2006; 355: 560–569

76 *Varga Z, Sinn P, Fritzsche F et al.* Comparison of EndoPredict and Oncotype DX test results in hormone receptor positive invasive breast cancer. PLoS ONE 2013; 8: e58483

77 *Prat A, Parker JS, Fan C et al.* Concordance among gene expression-based predictors for ER-positive breast cancer treated with adjuvant tamoxifen. Ann Oncol 2012; 23: 2866–2873

78 *Robbins P, Pinder S, de Klerk N et al.* Histological grading of breast carcinomas: a study of interobserver agreement. Hum Pathol 1995; 26: 873–879

79 *Gudlaugsson E, Skaland I, Janssen EA et al.* Comparison of the effect of different techniques for measurement of Ki67 proliferation on reproducibility and prognosis prediction accuracy in breast cancer. Histopathology 2012; 61: 1134–1144

80 *Weigelt B, Mackay A, A'Hern R et al.* Breast cancer molecular profiling with single sample predictors: a retrospective analysis. Lancet Oncol 2010; 11: 339–349

81 *Geyer FC, Marchio C, Reis-Filho JS.* The role of molecular analysis in breast cancer. Pathology 2009; 41: 77–88

82 *Chang JC, Makris A, Gutierrez MC et al.* Gene expression patterns in formalin-fixed, paraffin-embedded core biopsies predict docetaxel chemosensitivity in breast cancer patients. Breast Cancer Res Treat 2008; 108: 233–240

83 *Esteva F, Sahin A, Cristofanilli M et al.* Prognostic role of a multigene reverse transcriptase-PCR assay in patients with node-negative breast cancer not receiving adjuvant systemic therapy. Clin Cancer Res 2005; 11: 3315–3319

84 *Denkert C, Loibl S, Kronenwett R et al.* RNA-based determination of ESR1 and HER2 expression and response to neoadjuvant chemotherapy. Ann Oncol 2013; 24: 632–639

85 *Symmans WF, Hatzis C, Sotiriou C et al.* Genomic index of sensitivity to endocrine therapy for breast cancer. J Clin Oncol 2010; 28: 4111–4119

86 *Bhargava R, Dabbs DJ.* Oncotype DX test on unequivocally HER2-positive cases: potential for harm. J Clin Oncol 2012; 30: 570–571

87 *Azim Jr. HA, Michiels S, Zagouri F et al.* Utility of prognostic genomic tests in breast cancer practice: the IMPAKT 2012 Working Group consensus statement. Ann Oncol 2013; 24: 647–654

88 *Scharl A.* Mammakarzinom-Therapie: Der routinemäßige Einsatz von Gentests ist derzeit nicht sinnvoll. Dtsch Arztebl International 2012; 109: 2085–2086

89 *Cardoso F, van't Veer L, Rutgers E et al.* Clinical application of the 70-gene profile: the MINDACT trial. J Clin Oncol 2008; 26: 729–735