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Review

Prognostic and predictive biomarkers in breast cancer: Past, present and future

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ABSTRACT

Following a diagnosis of breast cancer, the most immediate challenges in patient management are the determination of prognosis and identification of the most appropriate adjuvant systemic therapy. Determining prognosis can best be addressed with a combination of traditional clinicopathological prognostic factors, biomarkers such as HER2/*neu* and specific multigene genes tests. Amongst the best validated prognostic multigene tests are uPA/PAI1, Oncotype DX and MammaPrint. Oncotype DX and MammaPrint, may be used for predicting outcome and aiding adjunct therapy decision making in patients with ER-positive, HER2-negative breast cancers that are either lymph node-negative or node positive (1–3 metastatic nodes), while uPA/PAI-1 may be similarly used in ER-positive, lymph node-negative patients. For selecting likely response to endocrine therapy, both estrogen receptors (ER) and progesterone receptors (PR) should be measured. On the other hand, for identifying likely response to anti-HER2 therapy, determination of HER2 gene amplification or overexpression is necessary. To identify new prognostic and predictive biomarkers for breast cancer, current research is focusing on tumor and circulating DNA (ctDNA) and RNA (e.g., micro RNAs) and circulating tumor cells. A promising ctDNA biomarker is the mutational status of ER (*ESR1*) for predicting the emergence of resistance to aromatase inhibitors. Challenges for future research include the identification of biomarkers for predicting response to radiotherapy and specific forms of chemotherapy.

1. Introduction

Following a diagnosis of invasive breast cancer, one of the most immediate challenges is to identify who should or should not receive adjuvant treatment, especially adjuvant chemotherapy. If the decision is to administer adjuvant therapy, the next challenge is to identify the most suitable therapy or combination of therapies for a given patient. Addressing the first challenge can be aided by prognostic factors/biomarkers while addressing the second challenge can be helped with predictive biomarkers. The aim of this article is to discuss how prognostic and predictive biomarkers help optimize therapy decision making in patients with newly diagnosed breast cancer. First however, we briefly review the contribution of the traditional prognostic factors for the management of patients with early breast cancer.

2. Traditional prognostic factors

With so much emphasis being placed on molecular prognostic tests in recent years, the important contribution of the traditional clinical/pathological factors (Table 1) tends to be forgotten. Of the factors listed in Table 1, the most widely used are the number of regional lymph nodes with metastases, tumor size and tumor grade [1,2]. Despite the availability of multiple molecular tests in recent years, these factors continue to be mandatory for determining prognosis and aiding therapy decision making in patients with newly diagnosed breast cancer.

2.1. Lymph node metastasis

The presence and number of axillary node metastases has been and remains the single most important prognostic factor in breast cancer. Indeed, a direct relationship exists between the number of metastatic axillary lymph nodes and risk of metastasis. This relationship is independent of tumor size [3]. Most of the studies that were related to lymph node metastases, as well as the other which widely used histological prognostic factors for predicting patient outcome, were performed several decades ago and thus prior to the availability of modern

Abbreviations: uPA, urokinase plasminogen activator; PAI-1, plasminogen activator inhibitor 1; ctDNA, circulating tumor DNA; CTC, circulating tumor cells; PFS, progression-free survival; pCR, pathological complete response; SNVs, single nucleotide variants; CNVs, copy number variants

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Table 1
Principal conventional and investigational prognostic and predictive biomarkers.

Biomarker category	Prognosis		Prediction of	
	Worse	Better	Response	Resistance
Conventional; in tissue				
Lymph-nodes (N)	[a]Positive	[a]Negative	NA	NA
Tumor size (T)	[b] > 2 cm	[b] ≤ 2 cm	NA	NA
Tumor grade (G) (Nottingham score)	2–3	1	NA	NA
ER	Negative	Positive	[c]Positive	[c]Negative
PR	Negative	Positive	[c]Positive	[c]Negative
Ki67	> 25%	< 10%	NA	NA
HER2/neu	Positive	Negative	[d]Positive	[d]Negative
Oncotype DX RS	> 30	< 18	[e] > 30 (CT)	[e] < 18 (CT)
Mammaprint	High risk	Low risk	[e]High risk (CT)	[e]Low risk (CT)
uPA ans PAI test	High	Low	NA	NA
Investigational, in tissue and in circulation				
miRNAs (see Table 4a–Table 4b)				
CTCs	[f] ≥ 1/7.5 mL [g] ≥ 5/7.5 mL	[f] < 1/7.5 mL [g] < 5/7.5 mL	NA (ongoing trials)	NA (ongoing trials)
ctDNA (and ESR1 mutational status)	present	absent	NA (ongoing trials)	NA (ongoing trials)

Also see text. RS: recurrence score, low risk < 18, intermediate risk 18–30, high risk > 30; NA: not applicable; CT: chemotherapy; CTCs: circulating tumor cells; ctDNA: circulating tumor DNA.

[a] Direct correlation with the number of the involved lymph-nodes.
[b] In spite of the current ASCO and NCCI guideline this cut-off value is reported in a recent large study.
[c] To antiestrogens.
[d] To anti-HER2 mAbs/TKIs.
[e] In adjuvant setting, high-risk scored patients.
[f] More than 1 tumor cell per 7.5 mL of blood in adjuvant setting.
[g] More than 5 tumor cells per 7.5 mL in metastatic setting.

treatments for breast cancer. However, in a recently published multicenter investigation carried out in Canada, lymph node status (node-negative vs node-positive/number of nodes dissected) was found to be a significant predictor of local recurrence, regional recurrence, distant metastases, breast cancer-specific survival and overall survival [4]. In this study, the prognostic benefit of lymph node status was independent of tumor size, tumor grade, type of surgery performed, chemotherapy administered and patient age.

As a prognostic factor however, the extent of axillary node involvement is not ideal. This is because almost 50% of patients with nodal metastases are cured by local therapy and approximately 30% of untreated patients without nodal involvement will develop recurrent/metastatic disease by 10 years [5].

2.2. Tumor size

As with the number of axillary node metastases, measurement of tumor size is also mandatory in assessing prognosis for breast cancer, as the likelihood of the formation of metastases increases with increasing tumor size, regardless of the number of lymph node metastases [3]. Thus, patients with a tumor size < 1 cm were reported to have a 5 year overall survival close to 100%, compared to 89% for patients with a tumor size 1–3 cm and 86% for those with a tumor size 3–5 cm (3). In the recently published Canadian multicenter study referred to above [4], tumor size (≤ 2 cm vs > 2 cm), like lymph node status, was also an independent prognostic factor for local recurrence, regional recurrence, distant metastases, breast cancer-specific survival and overall survival [3].

2.3. Tumor grade

Like lymph node metastases and tumor size, tumor grade is widely used for determining prognosis in patients with breast cancer [1,2]. The grading of a breast cancer is based on the microscopic similarity of breast cancer cells to normal breast tissue. One of the most widely used and best validated grading systems is the Nottingham system [6–9]. This system utilizes 3 microscopic features to assign grade to a tumor, nuclear pleomorphism, gland or tubule formation and number of dividing cells. Each of these factors is assigned a score from 1 to 3 (with 1 being the closest to normal breast and 3 the least close). These scores are then added together. If the combined tumor score is between 3 and 5, it is assigned to be grade 1. If the combined score is 6 or 7, the tumor is designated as grade 2 and if the combined score is 8 or 9, it is designated as grade 3. In 2017, the Nottingham tumor grading system was incorporated into the American Joint Committee on Cancer for breast cancer staging [10].

Although grade is widely used for assessing prognosis in patients with breast cancer, it has 2 main limitations. The first of these is lack of reproducibility in grading among pathologists. The Nottingham grading system however, has been reported to more reproducible than some of the older systems [11–13]. The second limitation is that the vast majority of tumors are classified as grade 2 which is a highly heterogeneous group with respect to outcome [14].

3. Molecular prognostic biomarkers

Although the number of lymph node metastases, tumor size and tumor grade all supply independent prognostic information in patients with newly diagnosed breast cancer, it is widely accepted that these factors alone are inadequate for optimum patient management, especially as we move towards the era of personalized treatment [15,16]. Consequently, in recent years, a substantial amount of research has been devoted to the development and validation of molecular biomarkers that can provide not only prognostic information but perhaps more importantly, can predict response to therapy. In the last decade therefore, several new prognostic tests have been proposed for breast cancer [15,16]. Rather than measuring single analytes, most of these tests detect multiple analytes, especially mRNA species (Table 2). They are thus frequently referred to as multi-gene, multi-analyte or multi-parameter tests. Several of these tests are now recommended by expert

Table 2
Gene/protein signatures previously proposed for predicting outcome in patients with newly diagnosed breast cancer. FFPE, formalin-fixed and paraffin-embedded. GCI, Genomic Grade Index; BCI, Breast Cancer Index. *EGTM guidelines relate to data in this article. **In addition to 50 genes from the PAM50 panel, the test also contains 8 control genes for normalization, 6 positive controls and 8 negative controls (Prosigna packet insert). Reproduced from Ref. [19].

Test	Tissue required	Molecule measured	No of analytes	Studied in prospective randomized 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	FFPE	mRNA	50**	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	12	Ongoing
Rotterdam Signature	Fresh/ frozen	mRNA	76	No
OncoMasTR	FFPE	mRNA	7	No
Curbest 95GC	FFPE	mRNA	95	No

Table 3
Gene/protein signatures proposed for predicting outcome in patients with newly diagnosed breast cancer, together with their recommendations by international expert panels. ASCO, American Society of American Oncology; NCCN, National Comprehensive Cancer Network; EGTM, European Group on Tumor Markers; AJCC, American Joint Committee on Cancer. *Relates to lymph node-negative patients only.

Test	Recommended in ASCO guidelines	Recommended in NCCN guidelines	Recommended in EGTM guidelines	Included in AJCC staging
uPA/PAI-1	Yes*	No	Yes	No
Oncotype Dx	Yes*	Yes	Yes	Yes*
MammaPrint	No	No	Yes	Yes*
Prosigna	Yes*	No	Yes	Yes*
BCI	Yes*	No	Yes*	Yes*
GGI	No	No	No	No
Mammostrat	No	No	No	No
IHC4 score	No	No	No	No
EndoPredict	No	No	No	No
Rotterdam Signature	No	No	No	No
OncoMasTR	No	No	No	No
Curbest 95GC	No	No	No	No

panels (Table 3) [17–20] and are thus being increasingly used in clinical practice. Amongst the best-validated of these tests are Oncotype DX and MammaPrint and uPA/PAI-1 (Femtelle). These tests are briefly discussed below.

3.1. Oncotype DX

Oncotype DX is one of best-validated and most widely used multigene signature for predicting outcome in breast cancer. Using RT-PCR, the test measures the expression of 21 genes at the mRNA level. Of these 21 genes, 16 are linked to cancer and 5 are used as reference or control genes. Based on the relative expression of the 16 cancer-related genes to the 5 reference genes, a score known as the recurrence score (RS) is calculated. RS is a continuous variable that divides patients into 3 subgroups indicating different risks of disease recurrence, i.e., low risk (< 18), intermediate risk (18–30) and high risk (≥ 30) [21].

Oncotype DX has 2 main uses in breast cancer, predicting the probability of disease recurrence and identifying those who are likely to benefit from adjuvant chemotherapy [22]. For predicting the probability of disease recurrence, the test has been extensively validated, including validation in large population-based studies, prospective-retrospective trials as well as in 2 prospective trials [for review, see Ref. [22]. In one of these prospective trials, i.e., the TAILORx trial (NCT00310180), Sparano et al., [23] found that endocrine-treated patients who were lymph node-negative, ER-positive and HER2-negative disease with a RS of < 11, had an extremely low risk of developing recurrence. Thus, after 5 years of follow-up, 93.8% of patients were free of invasive disease, 99.3% were free from recurrence at a distant site, while the overall survival was 98.0%. In another large prospective trial, which included HER2-negative patients with node-positive or high-risk lymph node-negative disease, the 3-year disease-free survival for patients with low RS (≤ 11) was found to be 98% [24]. This excellent outcome was achieved despite these high-risk patients not receiving adjuvant chemotherapy. Although still with limited follow-up, the results of these prospective studies suggest that node-negative or node-positive (1–3 positive nodes) patients with low Oncotype DX RS have an excellent prognosis and consequently are unlikely to derive a clinically significant benefit from receiving adjuvant chemotherapy.

Although less widely investigated than for prognosis, Oncotype DX has also been reported to predict the likelihood of benefit from adding adjuvant chemotherapy to endocrine therapy. Using archival samples from 2 clinical trials (NSABP B20 and SWOG 8814) involving ER-positive and HER2-negative patients, those with high RS were found to benefit from adjuvant chemotherapy. In contrast, those with low RS appeared to derive little if any benefit from the chemotherapy [25,26]. In both these trials, it was unclear if patients with an intermediate RS obtained any benefit from chemotherapy. For ER-positive lymph node-negative patients, this question is currently being evaluated in the TAILORx trial. On the other hand, the RxPONDER trial (NCT01272037) is addressing if adjuvant chemotherapy is beneficial in ER-positive, HER2-negative, node-positive (1–3 positive nodes) patients with RS $\leq$ 25.

Multiple studies have shown that use of Oncotype DX can alter patient management, especially in ER-positive, HER2-negative, lymph node-negative patients [22,27]. Thus, in a meta-analysis of 4 prospective studies from different European countries, performance of the Oncotype DX test led to changed treatment recommendations in 32% of patients investigated [28]. Overall, recommendation for use of chemotherapy decreased from 55% to 34%. The largest management changes occurred in patients originally recommended to receive chemotherapy and in those with grade II tumors.

Consistent with its ability to reduce the administration of adjuvant chemotherapy, use of Oncotype DX has been shown to be cost effective and in some healthcare systems to be cost saving. Following a systematic review of the literature, Rouzier et al., [27] identified 18 studies that evaluated the cost-effectiveness of Oncotype DX in ER-positive, HER2-negative early breast cancer patients. In all the studies identified, performance of the test was shown to be cost-effective based on the widely-accepted thresholds for cost-effectiveness. The cost-effectiveness was shown for lymph-node-negative, lymph node-positive as well as in cohorts consisting of a mixture of both node-negative and node-positive patients. In the US, Oncotype DX was found to be cost-saving, probably because chemotherapy is both more expensive and more widely used in that country.

Since the Oncotype DX test has been extensively validated, shown to reduce the use of adjuvant chemotherapy and to be cost-effective/cost saving, it is widely recommended for clinical use in Western countries [19]. Thus, the European Group on Tumor Markers (EGTM) state that “the Oncotype DX test 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” [19]. In addition, the panel states that “Oncotype DX may be considered for identifying HER2-negative, ER-positive patients with 1–3 involved lymph nodes for treatment with adjuvant chemotherapy”. Essentially, similar guidelines have been published by the American Society of Clinical Oncology (ASCO) for lymph node-negative patients [17]. According to the ASCO [17] guidelines, if a lymph node-negative patient has hormone receptor-positive, HER2-negative breast cancer, Oncotype DX may be used to guide decisions on adjuvant systemic chemotherapy. However, in contrast to the EGTM guidelines [19], ASCO were opposed to the use of the RS in lymph node-positive patients.

Although recommended for clinical application and widely used, Oncotype DX has limitations. These limitations include its lack of validation in ER-negative patients as well as lack of validation for long-term follow-up. Furthermore, as mentioned above, it is still unclear if lymph node-negative ER-positive patients with intermediate RS benefit from adding adjuvant chemotherapy to endocrine therapy and whether lymph node-positive (1–3 positive nodes) ER-positive patients with low to intermediate RS benefit from adjuvant chemotherapy. Hopefully, answers to the last 2 questions will become available in the next few years.

3.2. MammaPrint

MammaPrint, like Oncotype DX, has been extensively validated for predicting the probability of disease recurrence and guiding treatment

decision making in patients with breast cancer [29–35]. This test uses microarray to measure the expression of 70 genes that are implicated in all the key hallmarks of cancer. Based on the expression levels of these genes, breast cancer patients are separated into 2 groups, i.e., low or high risk for disease recurrence. The clinical benefit of MammaPrint was recently validated in a prospective randomized trial, known as the MINDACT study [36]. This trial which enrolled 6693 patients with early breast cancer who were either lymph node-negative or had 1–3 metastatic axillary lymph nodes, showed that patients classified as being at low risk of recurrence according to MammaPrint but at high risk based on clinicopathological criteria had an excellent outcome, with a 5-year distant metastasis-free survival of 94.7%. The trial also showed that there was a 14% reduction in the use of adjuvant chemotherapy when MammaPrint rather than the traditional criteria was used in deciding on adjuvant treatment. In the clinical high risk patients however, use of MammaPrint would result in a 46% reduction in the administration of chemotherapy.

The results of this randomized prospective trial clearly showed that use of MammaPrint can alter breast cancer patient management by providing high level evidence for the reduced administration of adjuvant chemotherapy to patients deemed to be at high-risk as assessed by clinical and pathological criteria, without negatively impacting on outcome.

Although less widely investigated than Oncotype DX, performance of MammaPrint test has also been shown to impact on the administration of adjuvant chemotherapy [37–40] and to be cost effective [27]. Thus, in a recent prospective observational multicenter study in ER-positive, HER2-negative patients, under 70 years of age, availability of the MammaPrint result changed the intended recommendation of the physician to administer chemotherapy in approximately half of the patients included in the study [34]. As with Oncotype DX, use of MammaPrint has been shown to be cost-effective in several different health care systems [27].

Performance of MammPrint is now recommended by several expert panels [19]. According to the EGTM guidelines, the MammaPrint test "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 candidates for avoiding having to receive adjuvant chemotherapy" [19]. Again, in contrast to EGTM, ASCO were opposed to the routine use of MammaPrint for treatment decision making in patients with breast cancer [17]. The ASCO guidelines however were published prior to publication of the MINDACT trial data [36]. Although ASCO were opposed to the use of MammaPrint, the test has been cleared by the US Food and Drug Administration (FDA) for breast cancer lymph node-negative patients with stage I or stage 11 disease and a tumor size of ≥ 5.0 cm. The FDA also added that MammaPrint as a prognostic biomarker should only be used in combination with clinic-pathological factors proposed.

3.3. uPA and PAI-1

In contrast to Oncotype DX and MammaPrint, the measurement of uPA and PAI-1 is a simpler and less expensive test. This test involves the measurement of 2 proteins by ELISA in extracts of fresh or freshly-frozen breast cancer [35]. Patients with high levels of these 2 proteins have a significantly worse outcome than those with low levels. For patients with lymph node-negative disease, the uPA/PAI-1 test has been validated using both a multi-center prospective randomized trial and a pooled analysis of individual patient data from 18 different datasets that contained a total of 8377 patients [41–44]. As with Oncotype DX and MammaPrint, measurement of uPA and PAI-1 has been shown to decrease the administration of adjuvant chemotherapy and to be cost-effective [45].

Measurement of uPA and PAI-1 is now widely recommended for determining prognosis and therapy decision making, especially in lymph node-negative breast cancer patients [17,19]. Thus, the EGTM guidelines state that "levels of PA and PAI-1 protein 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" [19]. Similarly, the ASCO guidelines recommend that if a node-negative patient has ER/PR-positive, HER2-negative breast cancer, uPA/PAI-1 may be used to guide decisions on adjuvant systemic therapy [17].

3.4. Other prognostic biomarkers

Although multigene signatures such as those discussed above are finding increasing use in the day-to-day management of patients with early breast cancer, these tests are expensive and indeed prohibitively expensive in many countries. Considerable effort has therefore been devoted to the development and validation of inexpensive and simple prognostic biomarker tests. One of the most widely used of these inexpensive biomarkers is Ki67 [46]. Although methodological problems exist in the determination of Ki67 (poor inter laboratory precision, lack of a validated cut-off point) [47]), multiple studies including retrospective evaluations of randomized clinical trials and meta-analyses, have shown that elevated levels are independently associated with adverse outcome in patients with breast cancer [46].

Although having methodological problems, because of its clearly established clinical value, wide availability and low costs relative to the available multianalyte signatures, Ki67 is widely used in many countries. Indeed, the EGTM expert panel state that "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)' [19]. Other expert panels such as ASCO [17] and National Comprehensive Cancer Network (NCCN) [18] however, are opposed to the use of Ki67 as a prognostic biomarker for breast cancer.

In addition to Ki67, other potential inexpensive biomarker prognostic tests for breast cancer include IHC4 [48,49], which involves the detection of ER, PR, HER2 and Ki67; a risk score including ER, PR, grade and tumor size [50] or a risk score including ER, PR, Ki67, tumor size and the Nottingham Index [51]. All these inexpensive multiparametric tests however, require further validation before they can be recommended for clinical use.

All the prognostic biomarkers discussed above require tumor tissue for their determination. Clearly, it would be desirable to have strong and clinically validated circulating prognostic biomarkers. Although elevated circulating biomarkers such as CA 15-3, CEA and TPS have all been shown to predict poor outcome in patients with breast cancer, they are not widely used for prognostic determination in clinical practice [52–58]. Possible reasons for their limited clinical use in aiding prognosis include their lack of validation in clinical trials and the absence of studies showing that their determination can alter patient management. However, since these biomarkers are simple and relatively inexpensive to measure, they should be further studied, including investigation in clinical trials.

4. Predictive biomarkers

In contrast to prognostic biomarkers which predict the risk of disease recurrence, predictive biomarkers help identify upfront those patients that are likely to respond or be resistant to specific therapies. Of all the common cancers, breast cancer has led the way in the use of therapy predictive biomarkers. For example, the measurement of estrogen and progesterone receptors (ER, PR) to predict response to endocrine therapy entered clinical use over 40 years ago while measurement of HER2 for predicting benefit from trastuzumab (Herceptin) became routine over 15 years ago. The clinical utility of these 3

biomarkers is discussed below.

4.1. Estrogen and progesterone receptors

Although the oldest, ER i.e., ER-alpha, is the most important biomarker currently available for breast cancer. Indeed, measurement of ER is mandatory in all newly diagnosed cases of breast cancer and where feasible, also in recurrent/metastatic lesions. Although the ER can provide both prognostic and therapy predictive information, its most important clinical use is as a predictive biomarker for endocrine therapy. If patients are positive for ER, endocrine should be administered or at least considered for administration. On the other hand, if ER is absent, endocrine should be withheld. As a predictive marker for endocrine therapy, ER is used in the neoadjuvant, adjuvant and advanced disease settings [59–61].

The rationale for using ER as a predictive marker for endocrine therapy is based on the observations made several decades ago that the growth of at least some breast cancers was dependent on estrogens, especially estradiol. Estrogens are believed to stimulate breast cancer cell growth by associating with regulatory elements in the genome, thereby enhancing the transcription of genes such as *MYC* and cyclin D (*CCND1*) [62]. Since estrogens stimulate tumor growth via binding to and activating ER, it was hypothesised that levels of this receptor in breast cancers would correlate with benefit from anti-estrogenic therapy [61]. Support for this hypothesis first emerged in the early 1970s when it was shown that approximately 50% of ER-positive patients with advanced breast cancer underwent objective regression when treated with the then available endocrine therapies, i.e., ovariectomy, adrenalectomy, hypophysectomy [61]. In contrast, patients with tumors lacking ER rarely experienced tumor regression with these therapies [61].

Although still used to predict response to endocrine therapy in patients with advanced breast cancer, the current main use of ER is in identifying patients with early breast cancer for adjuvant treatment with drugs such as selective estrogen receptor modulators (tamoxifen), aromatase inhibitors (AI) (anastrozole, letrozole or exemestane), LH-RH agonists (leuprolide, goserelin), pure selective estrogen receptor downregulators (SERDS) (fulvestrant) and oophorectomy. All these therapies ultimately target ER, preventing it from stimulating breast cancer proliferation. Consequently, their use is restricted to patients with ER-positive cancers. However, since they act via different mechanisms, resistance to a specific drug does not necessary result in resistance to related compounds [62]. Thus, different classes of endocrine therapy may be used sequentially for the treatment of ER-positive breast cancers.

The progesterone receptor (PR) is usually measured simultaneously with ER. The original rationale for measuring PR alongside ER was based on the observation that PR was induced by estrogen [63]. The presence of PR was thus proposed as a biomarker for a functional ER [63]. While ER can stimulate the expression of PR, PR in the presence of progesterone has been shown to interact with and alter the location at which ER binds to chromatin [64]. The altered chromatin binding of ER result in a switch from regulating genes implicated in proliferation to modulating genes associated with cell cycle arrest, apoptosis and differentiation [65,66]. This finding together with the 'functional' receptor hypothesis may explain why PR in ER-positive breast cancer is associated with good outcome, see below.

Consistent with the above findings, several studies in patients with early or advanced breast cancer found that those with ER-positive and PR-positive tumors were more likely to respond to endocrine therapy than women with ER-positive/PR-negative tumors [66–69]. Other studies, especially in the adjuvant setting however, concluded that the presence of PR provided no additional predictive value to ER [69,70]. Possible reasons for these conflicting findings may relate to the cut-off value used for defining PR positivity, length of patient follow-up and whether or not adjuvant chemotherapy was also administered.

Although it is unclear if PR has independent predictive value for adjuvant endocrine therapy [60,66–70], several studies in ER-positive patients have reported that it provides independent prognostic information for predicting the risk of recurrence [71–74]. Expert panels therefore recommend measurement of both ER and PR in all newly diagnosed cases of breast cancer [17–19]. Furthermore, most also recommend measurement of these receptors in recurrent/metastatic lesions when feasible.

4.2. Mutational status of ER (ESR1) as a prognostic and predictive biomarker

Although the presence of wild-type ER is predictive of benefit from endocrine treatment, specific mutations in the ER (*ESR1*) gene appear to be associated with acquired resistance to endocrine therapy. Such mutations are rare in primary breast cancers but are found in 10–40% of recurrent/metastatic lesions, especially after long-term endocrine treatment that includes aromatase inhibitors [75,76]. Most of the mutations are found in the ER ligand-binding domain, especially at residues D538G, Y537S, E380Q, Y537C, and Y537N [75–77]. Functional *in vitro* studies show that at least some of these mutations resulted in ligand-independent constitutive activation of ER, resistance to estrogen deprivation therapy with aromatase inhibitors and relative resistance to tamoxifen and fulvestrant [75,76]. However, the extent of ligand-independent activation depends on the specific mutation, being maximal with the Y537S mutation [77]. Consistent with this relatively strong ligand-independent activation, the presence of the Y537S mutation required higher doses of fulvestrant for inhibition than did other mutant forms of ER (e.g., D538G, Y537C) or wild-type ER [77]. In contrast to fulvestrant, a new SERD, known as AZD9496, which is currently undergoing clinical trials (NCT02248090) was to found to effectively inhibit the Y537S mutant form of ER [77]. The potential clinical significance of *ESR1* mutations is currently being assessed in multiple prospective-retrospective clinical trials. To avoid having to biopsy metastatic lesions, many of these studies are using liquid biopsies in which mutations are being detected in circulating tumor DNA (ctDNA) (see below).

4.3. HER2

As with ER, measurement of HER2 is now mandatory on all new cases of invasive breast cancer and when feasible, also on recurrent/metastatic lesions. Overexpression of HER2 drives tumor growth by constitutive activating the MAPK and PI3K/AKT signaling pathways, which in turn enhances cell proliferation, invasion and metastasis [78]. A further mechanism by which HER2 overexpression may promote oncogenesis or tumor progression is by deforming cell membranes [79]. According to Chung et al. [79], this deformation can result in cells becoming less attached to their surrounding extracellular matrix/neighboring cells and thus more likely to acquire an invasive phenotype.

While ER is present in approximately 80% of breast cancer, the HER2 gene is amplified or overexpressed in only 15–20% of invasive cases. Overall, HER2-positive tumors are found more frequently in ER-negative than in ER-positive tumors. Thus, approximately 30% of ER-negative samples but only 12% of ER-positive tumors are positive for HER2 gene amplification/overexpression [80]. Indeed, a significant inverse relationship exists between HER2 and both ER and PR levels in breast cancer [81].

Although HER2 was originally proposed as a prognostic biomarker for breast, its current utility is mainly for predicting response to anti-HER2 therapy in the neoadjuvant, adjuvant and advanced disease settings. Currently, 4 form of anti-HER2 therapy are approved for administration in the US and Europe, i.e., trastuzumab (Herceptin), lapatinib, pertuzumab and trastuzumab emtansine (TDM-1) [82]. Based on available evidence, HER2 gene amplification/overexpression

appears to be necessary for response to all these treatments. Thus, at present, only patients that are HER2-positve can receive anti-HER2 therapies.

Of the available anti-HER2 treatments, trastuzumab has been investigated in most detail and is approved for treatment of breast cancer in the neoadjuvant, adjuvant, and advanced disease settings. In the neoadjuvant setting, administration of trastuzumab plus chemotherapy to HER2-positive patients was found to induce pathological complete response in 26–65% of HER2-positive patients compared to a 19–27% response rate with chemotherapy alone [83].

In adjuvant setting, administration of trastuzumab in combination with chemotherapeutic drugs decreases recurrence rates by approximately 50% and mortality by about 30% [86]. Overall, survival rates at 8–10 years of follow-up are 70–80%. In the metastatic setting, combined administration of trastuzumab plus chemotherapy resulted in response rates of 30–85%, median times to progression periods of 5–18 months and overall survival periods of 11–39 months [84]. Ten to 15% of HER2-positive breast cancer patients with advanced disease treated with trastuzumab and chemotherapy derive long-term benefit with progression-free survival periods > 3 years [85].

To further enhance response, a number of trials with dual anti-HER2 therapy have been performed, especially in the neoadjuvant and advanced disease settings. In both these settings, administration of dual anti-HER2 therapies resulted in superior anti-tumor activity compared to single agent therapy [86,87]. Thus, a meta-analysis of randomized phase II/III trials found that combined treatment with trastuzumab, lapatinib and chemotherapy led to a superior pathological complete response rate (pCR) than trastuzumab and chemotherapy (55.8% vs 38.4%, $p = 0.0007$) [86]. Similarly, combined treatment with trastuzumab, pertuzumab and chemotherapy led to a significantly better pCR (45.8%) compared to those receiving trastuzumab and chemotherapy (29%) or those receiving pertuzumab and chemotherapy (24%) ($p = 0.01$) [87].

In the metastatic setting, combined treatment with trastuzumab and pertuzumab has also has been shown to be superior to single agent treatment. In the CLEOPATRA trial, administration of trastuzumab, pertuzumab and docetaxel resulted in a median overall survival of 56.5 months, in contrast to only 40.8 months in the group without pertuzumab (HR, 0.68; $P < 0.001$). Unlike the situation in the neoadjuvant and advanced disease settings, combined anti-HER2 treatment (i.e., trastuzumab and lapatinib) was not shown to be superior to trastuzumab the adjuvant setting [88].

Since dual anti-HER2 therapy has been shown to be superior to single agent anti-HER2 blockage in the neoadjuvant and advanced disease settings, it is important to be able to identify those patients that particularly benefit from the combined drug approach. Equally important, it would be desirable to identify those patients that do not require the dual anti-HER2 treatment. Recently, Llombart-Cussac et al., [89] reported that HER2-postivive early stage patients that had a HER2-enriched subtype, as determined by the Prosigna gene signature, were more likely to achieve a pCR than patients with other molecular subtypes of breast cancer. In this study, 41% of 101 patients with the HER2-enriched subtype achieved pCR with dual trastuzumab-lapatinib treatment, in contrast to only 5% in patients lacking the HER2-enriched form.

4.4. Attempts to identify additional predictive biomarkers for anti-HER2 therapies

Although HER2 gene overexpression appears to be a prerequisite for response to anti-HER2 therapy, most HER2-positive patients fail to respond to trastuzumab, at least in the metastatic setting [90]. Furthermore, in the adjuvant setting, approximately 30% of HER2-positive patients develop resistance to trastuzumab after 11 years of follow-up [91]. Clearly, additional biomarkers are necessary to more accurately predict response to trastuzumab as well as to other anti-HER2 therapies. Attempts to identify additional or secondary biomarkers for predicting benefit form anti-HER2 therapies are discussed below.

4.4.1. PIK3CA mutational status

Since anti-HER2 therapies at least partially exert their anti-cancer activity via inhibition of the PI3K signaling pathway, it might be expected that constitutively acting mutations in this gene or its pathway would give rise to resistance. Indeed, several cell lines and animal model studies show that specific mutation in the p110 alpha-catalytic subunit of *PI3K*, i.e., *PIK3CA*, results in resistance to trastuzumab or lapatinib [92,93].

In order to evaluate the clinical relevance of these preclinical findings, several studies, especially in the neoadjuvant setting, have related the mutational status of *PIK3CA* with response to combined treatment with anti-HER2 compounds and chemotherapy [94,95]. Although a trend for a decreased pCR in patients with *PIK3CA* mutations was found in many of the trials, the difference was not statistically different in most. However, a meta-analysis of 5 of these trials concluded that the presence of *PIK3CA* mutations were significantly associated with a lower response rate than in those with the wild-type gene. This difference in pCR has not yet however, been shown to be accompanied by a difference in patient outcome.

Similarly, multiple trials have related the mutational status of *PIK3CA* with response to dual anti-HER2 therapy. In the NeoSphere neoadjuvant trial which randomized patients into one of four different treatment groups: trastuzumab plus docetaxel; pertuzumab and trastuzumab plus docetaxel; pertuzumab and trastuzumab; or pertuzumab plus docetaxel, mutations in *PIK3CA* were not significantly related with pCR in any of the treatment groups. However, mutations in exon 9 but not in exon 20 of *PIK3CA* were associated with lack of response in the pooled group [96].

In another neoadjuvant trial, i.e., neo-ALLTO, which compared response to: lapatinib plus paclitaxel; trastuzumab plus paclitaxel or concomitant lapatinib and trastuzumab plus paclitaxel, mutations in *PIK3CA* failed to correlate with response [97]. However, mutations in the PIK3CA pathway were associated with resistance to trastuzumab but not to lapatinib. Although mutations in *PIK3CA* or its pathway were unrelated to response to lapatinib, mutations in the RhoA pathway were associated with pathological response to this agent [97]. Finally, the mutational status of *PIK3CA* has not to date been shown to be predictive of benefit from anti-HER2 in the adjuvant or advanced disease settings [98,99].

Clearly, based on available evidence, the mutational status of *PIK3CA* cannot currently be used as an adjunct to HER2 in anti-HER2 therapy decision making.

4.4.2. Tumor infiltrating lymphocytes

Tumor infiltrating lymphocytes (TILs) are mostly composed of T cells but can also include B cells, NK cells, dendritic cells and macrophages [100]. However, for clinical studies at present, only mononuclear cells such as lymphocytes and plasma cells should be scored [100]. Several adjuvant retrospective studies have shown that increased levels of TILs predict favorable patient outcome, especially in triple-negative and HER2-positive breast cancer, irrespective of the therapy administered [101,102]. Although pCR response rates to neoadjuvant anti-HER2 therapy were found to correlate with high levels of TILs in several studies [103–105], disagreement exists as to whether a linear relationship exists between levels of TILs and response rates [103–106]. Thus, in the neoadjuvant NeoALTTO trial, levels of TILs greater than 5% were associated with higher pCR rates independent of treatment received. However, overall, the relationship between levels of TILs and pCR was non-linear. Although levels on TILs in this study were not linearly related to pCR rates, for every 1% increase in TILs, there was an associated with a 3% decrease in the rate of an event across all treatment groups [104]. Similarly, in the metastatic setting, increasing levels of TILs were associated with overall survival in patients treated

with trastuzumab, pertuzumab and chemotherapy [107].

Thus, the majority of studies conclude that high levels of TILs are associated with increased benefit from anti-HER2 therapy. The challenge for the future is to identify an optimum cut-off level, if such a level exists. For the present however, TIL should be assessed as a continuous score [100]. Further research should also establish if additional prognostic/predictive information might be obtained by molecular classification of the TILs or by including additional subsets of lymphocytes (macrophages, dendritic cells or myeloid-derive suppressor cells).

4.4.3. ER status

As mentioned above, an inverse relationship exists between HER2 and levels of both ER and PR. Thus, since ER-negative tumors tend to have higher concentrations of HER2, they might be expected to preferentially benefit from anti-HER2 therapy. Consistent with this hypothesis, 3 neoadjuvant trials involving chemotherapy with either single agent or dual agent anti-HER2 therapy showed that pCR rates were significantly higher in patients with receptor-negative than in those with receptor-positive tumors [108]. These differences in pCR were particularly striking in patients treated with trastuzumab, pertuzumab and chemotherapy or with the 2 monoclonal antibodies without chemotherapy. Thus, in patients receiving the triple therapy, pCR rates were 63.2% in the receptor-negative but only 20% in the receptor-positive patients. In the patients receiving the dual monoclonal therapy without the chemotherapy, response rates in the receptor-negative population were 29.1% as opposed to only 5.9% in the receptor-positive group [108]. Despite these differences in response rates, hormone receptor status, like TILs and the mutational status of *PIK3CA* should not be used in anti-HER2 therapy decision making in breast cancer.

5. The need for development of an advanced technology: DNA and RNA genotyping techniques

Due to the genetic and epigenetic origin of cancer the need for more rapidly and accurately sequencing DNA and RNA and to better investigate the transcriptional machinery has strongly emerged. In a relatively short time huge progress in the available techniques as well as the related computational biology has been made. All these techniques which have been applied to samples from tissue and recently from peripheral blood (PB) samples as well have had a great impact in discovering at genetic and epigenetic level several new potential biomarkers. These techniques, the main characteristics or variants and their evolution are here briefly summarized.

5.1. First and second generation sequencing

Dideoxy-sequencing of each exonic region of interest (frequently mutated exons) has been carried out for many years (first generation sequencing) to detect point mutation and small insertion and deletions. However, most mutations are heterozygous and often the contamination with non-cancerous material strongly decreases the mutational signal. This technique has not the necessary sensitivity to identify in a reliable manner this weak signal. In addition, usually fluorescence in situ hybridization (FISH)-based microscopy must be performed in parallel for detection of somatic copy number alterations (SCNAS) and genomic rearrangements/fusions. Further limitation is that both approaches are time consuming and that an high amount of tumor tissue is required [109].

The successive generation (second generation sequencing) has been developed to contemporaneously analyze with high sensitivity in each tumor sample many genes called “the mutational signatures”. Mass-spectrometric genotyping, real-time PCR and multiplexed PCR-based parallel “next generation sequencing” were some principal of them. All these techniques have high efficiency and sensitivity and allow examination of a large number of genes; furthermore, they provide results more quickly, detect somatic mutations at low allele frequency and additionally lower amount of tissue sample is necessary. However, with mass-spectrometric genotyping and real-time PCR any individual mutation must be pre-specified to be detected and they cannot be used to localize novel and rare mutations. This is overcome by PCR amplification of the regions to be examined followed by massively parallel next-generation sequencing in which any base of interest is interrogated many times, typically several 100-fold. The number of sequencing reads is termed “coverage” which is the measure of sequencing depth and thus of sensitivity. Also, the problem of tumor impurity due to tumor contamination is solved due to a statistically significant representation of all mutants and wild-type alleles in a sample. However, with PCR-based multiplex sequencing, as for all techniques relying on high-level PCR amplification, for different reasons false positive and falsely negative results may occur [110]. Moreover, FISH analysis to detect SCNAS and genomic rearrangements must be performed in parallel. This analysis that requires amplification and manual inspection reduces the amount of tumor DNA available for PCR [109].

5.2. Next generation sequencing/massively parallel sequencing and more

These techniques instead of relying on PCR amplification uses small amount of tumor material to enrich the genomic regions of interest (“hybrid-capture-based enrichment”). This is followed by massively parallel next-generation sequencing and subsequent computational analysis. Three different methods for massively parallel enrichment of the template to be sequenced have been described: the microarray capture, the multiplex amplification or the hybrid selection with ultra-long oligonucleotides. The last solution seems to combine simplicity with robust performance and to overcome the weakness of previous methods [111]. Basing on this technique all relevant genomic alterations (point mutations, small insertions and deletions, copy number alterations, genomic rearrangements/fusions) can be detected in a single assay saving precious tumor material and time [112].

The main advantages are a) the necessary amount of the input DNA material commonly is < 100 ng and it is independent of the number of genes to be detected; b) large size regions (from several 100 kilobases to megabases) can be sequenced; c) the high sequencing coverage allow to detect all types of genome alterations even those at very low allele frequency thus with high sensitivity; d) it can similarly be carried out in blood samples [113]. On the other side, complex computational algorithms are necessary to process a great deal of information in a reliable way. Therefore, much more sophisticate infrastructure (IT) needs for such fast at high performance computation data handling.

To assure the quality of next generation sequencing in clinical laboratory practice a summary on the suggested procedures including validation and quality control has been published [109,114]. As an application of last generation genotyping, the whole exome (WES) or the whole genome (WGS) sequencing have been also proposed. Unlike the WES the WGS permits to reliably detect gene fusions. WGS mainly occurs in non-coding regions with high risk to detect unhelpful passenger mutations and to erroneously consider them potentially important for clinical practice. Accordingly, the great deal of sequenced bases accounts for possible fixation artifacts, false mutations and not reliable control over the coverage of all the sequenced regions. WES determines the variations of coding regions that is the protein coding fraction which represents about 1% of the human genome with more favorable cost-effectiveness ratio than WGS. However, both sequencing procedures cannot be yet implemented in the current clinical practice as they generate a huge amount of data which require for analysis an expensive computational IT [15].

Immuno-histochemistry concomitant with immunofluorescence staining and western blotting includes preparation of tumor tissue microarrays [115] that are immune-histochemically stained to detect previously selected histone marks and other markers [116]. Chromatin immune-precipitation (ChIP)-based assays needs DNA PCR

amplification and map protein-DNA interactions in vivo to a specific region of the genoma. In the context of the endocrine signaling this technique allows targeted and genome-wide in vivo analyses. Some variants as ChIP-chip that combines ChIP with DNA microarray or ChIP-seq that combines ChIP with DNA deep sequencing have been adapted to genome-wide scale investigation [117]. ChIP-DNA selection and ligation technique (DSL) [118] has been adapted to analyze ER binding along with histone acetylation and methylation following hormone treatment of MCF7 cells on promoter and enhancer tiling arrays.

6. Investigational prognostic and predictive biomarkers

In the last years, many investigations using the new techniques have been carried out. In tissue the expression of single or multiple (genetic signature) genes or single or multiple (cluster, micro-RNA signature) micro-RNAs (mi-RNAs) and in peripheral blood (PB) mi-RNAs, circulating tumor cells (CTCs) and circulating tumor DNA (ct DNA) have been detected. Moreover, in serum/plasma miRNAs benefit from the advantage to be highly stable [119]. In some instances, databases have been a helpful tool to discover the novel biomarkers. Following these and other ongoing researches our armamentarium of prognostic and predictive biomarkers is continuously increasing.

6.1. Databases a helpful tool

The Cancer Genome Atlas (TGCA), Gene Expression Omnibus (GEO), Surveillance, Epidemiology and End Results (SEER) and Embase have been among the most used databases to obtain bio-information. FromTGCA data, Piwi-interacting RNAs and PIWI genes [120], miR-574-3p and miR-660-5p [121] RNA-protein complex, namely Musashi RNA-binding protein 2 [122] and tristetrapolin (TTP ZFP36) [123] were found to be prognostic markers, while constitutive pSTAT3 [124] and KLK10 [125] expression was found associated with trastuzumab resistance. From the GEO database, down-regulated miR-126/miR-126(*) were reported to correlate with poor metastasis-free survival [126]. SEER is a regularly up-dated database that collects cancer incidence and survival data from population-based cancer registries. A study used this source to report on correlation in N positive patients between the over-expression of eukaryotic initiation factor 4E and higher risk for systemic spread [127]. Embase is a biomedical and pharmacological database mainly referring to drug and pharmaceutical research. A study using Embase [128] showed close relationship between Her-2 expression and response to endocrine treatment. Moreover, in four meta-analysis from the same database [129–132] miR-21 overexpression correlated with poor prognosis and in a further one [133] miR-155 was found in HER-2 positive or lymph-node metastasis positive or p53 mutant type of breast cancer.

6.2. The tissue microRNAs (mi-RNAs)

mi-RNAs are small non-coding RNAs molecules of around 20–25 nucleotides that at post-trascriptional level take part to regulation of gene expression involved in key cellular processes such as proliferation, differentiation, angiogenesis, migration and apoptosis [134]. Each mi-RNA can regulate the expression of many genes and many signaling pathways. In cancer mi-RNAs act as oncogenes (oncomirs) or tumor suppressor genes, pro-metastatic (metastamiRs) or metastasis suppressor genes and mi-RNAs play a significant role in resistance to various therapies [135].The principal determinant for mi-RNA binding to mRNA is a 6–8 nucleotide sequence placed at the 5′ end of the mi-RNA and named “seed” sequence. Any complementary sequence between the loaded mi-RNA and the seed region provokes a significant decrease in the target mRNA expression. Depending on the degree of homology mi-RNAs can induce the translational repression or degradation of mRNAs [136–138]. In addition to this “canonical” mechanism different “non-canonical” mechanisms can increase or reduce the translation of a target mRNA [139]

6.2.1. Prognostic mi-RNAs

Many different mi-RNA signatures and individual mi-RNAs have been proposed as candidate biomarkers for information on clinical course. In a study [140] miR-148 and miR-210 were found to be associated with shorter relapse-free-survival (RFS). Successively [141] prognostic mi-RNAs signatures for poor time to metastasis and overall survival (OS) were identified; namely miR-127-3p, miR-210, −185, −143* and let-7b at univariate and multivariate analysis were significantly associated with time to metastasis while miR210, −21, −221 and-652 were reported among those correlated with OS; miR-210, −21,106b*, −197 and let-7i were common to both prognostic signatures. The Kaplan-Meier curves showed earlier time to metastasis and shorter OS in patients over-expressing miR-210.

Other findings [142–145] confirmed that miR-21 over-expression in addition of being driver of metastasis correlated with poor OS and DFS. Similarly, other data [146] and two more recent systematic review and meta-analyses reported on miR210 associated with tumor aggressiveness and on miR-210 over-expression as poor predictor of DFS and RFS [147,148]. Foekens and co-workers [149] reported on miR-210 associated with metastatic capability in ER negative and triple negative breast cancer (TPNBC) patients.

In another report [150] let-7b and miR205 in luminal subtype were found to be positively associated with survival. For let-7b the findings were confirmed by Ma and co-workers [151] who in a heterogeneous cohort observed poor prognosis and shorter OS associated with low levels of let-7b expression. Conversely Markou and co-workers [144] in a heterogeneous cohort showed an association between miRNA-205 down-regulation and longer DFS. Farazi et al. [152] in 97 triple negative (n = 48) or hormone receptor negative HER2-positive (n = 49) patients who developed metastasis, found that sf-miR423-3p/sf-miR-423-5p and miR375/sf-miR-375 clusters were significantly increased and cluster miR-184/sf-miR-184 decreased (p values < 0.001). Kaplan–Mejer analysis confirmed the findings for cluster-miR-423 ($p < 0.013$) and cluster sf-miR-184/miR-184 ($p < 0.041$) in HER2-positive patients. In addition, at univariate and multivariate Cox regression, cluster miR-423 was an independent predictor of outcome in presence of other conventional factors. Decreased miR30a expression predicts decreased relapse-free-survival (RFS) and DFS [153]. The positive correlation of miR30 with prognosis was confirmed in a further study [154].

In TNBC Gasparini and co-workers [155] identified up-regulated miR-155 and miR-493 as “protective” and correlating with better outcome and down-regulated miR-30e and miR-27a as “risk-associated” and correlating with worse outcome. For miR-30e, the finding was confirmed by D’Aiuto et al. [156]. In this study, in treated and untreated N- ER+/HER2- and in HER2+ subtypes, miR-30e over-expression was associated with a good prognosis. Conversely, miR-155 over-expression in tumor tissue sections was found associated with poor prognosis by Song C and co-workers [157]. Leivonen and co-workers [158] have reported on better OS in patients with higher miR-342-5p expression likely by inhibiting HER2 and cell growth.

Similarly, in another study [159], a better prognosis has been observed associated with miR-497 overexpression. Shen and co-workers [160] reported on miR-27b-3p as independent poor predictor of distant metastasis-free-survival and validated this finding in a subset (n = 41) of TPNBC. Zhou and co-workers [161] showed a significantly lower expression of miR-9 in patients without local recurrence (LR). Moreover, in ER positive patients miR-9 over-expression was associated with lower 10-year LR-free survival. miR-187 and miR-155 have been reported to be predictors of poor outcome; in particular, miR-187 over-expression has been found associated with decreased breast cancer-specific survival (BCSS) [162] and miR-155 with OS [163]. Let-7c and miR-125b have been reported [164] as potential biomarker of good

outcome in luminal A patients. Tuomarila and co-workers [165] found that miR200c overexpression directly correlated with outcome in PR negative and inversely in PR positive patients. miR-9 [166], miR-10b [167], miR-29a [168], miR-155 [169], miR-520c and miR-373 [170], miR-301 [171], miR-548j [172] are additional metastasis promoters mi-RNAs; miR-17/20 [173], miR-34 [174], miR-29b [175], miR-708 [176], miR-126 [177], miR-193b [178], miR-206 [179], miR-335 [180], miR-448 [181], miR-601 [182], miR-138 [183], miR-515-5p [184], miR-203 [185], miR-200 family and miR-205 [186] are additional metastasis suppressors miRNAs.

6.2.2. Predictive mi-RNAs

These mi-RNAs are candidate to drive the decision-making process in the context of personalized medicine. Sensitizing effect on tamoxifen response of miR-375 [187], miR-342 [188,189], miR-26a [190,191], miR-30c [192], miR-10a and −126 [193] and a positive correlation of miR-375 with DFS in tamoxifen-treated patients [187] were reported. ID4 was identified as putative target of miR-342 [194].

In another study [164], similar positive correlation between miR-125b, miR99a, Let-7c cluster expression and response to antiestrogens was found. A direct correlation of response to letrozole and Let-7f expression in a further study occurred [195]. Conversely miR-221-222 cluster [196], miR-519a [197], miR-155 [198] and miR-301 [171] have been found to be involved in resistance to tamoxifen with miR-221-222 cluster [199,200] also involved in the resistance to ER down-regulator fulvestrant while miR-181a has been reported to be involved in resistance to letrozole [201]. miR-21 [202] and miR-221 over-expression [203] have been reported to mediate resistance to trastuzumab therapy. Climent and co-workers [204] found miR-125b deletion on chromosome 11q associated with clinical benefit from anthracyclines. Sensitizing effect of miR-451 [205], miR-326 [206], miR-200c [207], miR-134 [208], miR-34a [209] and miR-128 [210] on doxorubicin, miR-30c [211] on doxorubicin and paclitaxel, miR-100 (luminal A cells) and miR-16 on paclitaxel [212,213] and miR-621 on paclitaxel-carboplatin combination [214] have been described. Induction of resistance to paclitaxel for miR-125b [215] and miR-21 [216], to doxorubicin for miR-21 [217], to taxane-anthracycline based therapies for miR-141, miR-125b [218] and miR-128 [219] has been reported. The role of miR-181a in regulating chemosensitivity to adryamicin by targeting BCL2 has been reported by Zhu and co-workers [220]. Induction of resistance to cisplatin for miR-203 has been described in an experimental study where knockdown of miR-203 sensitized MCF-7 breast cancer cells to cisplatin-mediated apoptotic cell death [221]. Stankevicins L and co-workers found low levels of miR-34a associated to breast cancer cells more resistant to radiotherapy [222]. Tables 4a and 4b summarizes the main results of the shown mi-RNAs tissue biomarkers. For most of them different researches confirmed the principal findings although in few cases, as for miR-128, miR-155 and miR-205, they were controversial.

6.3. Biomarkers in circulation: the “liquid biopsy”

The use of PB as “liquid biopsy” has been for a long time an appealing expectation. Liquid biopsy unlike tissue biopsies provides a non-invasive easily accessible tool to better stratify patients and to drive drug selection in the adjuvant setting or improve management of metastatic disease through a serial analysis of contemporary tumor samples. In addition to mi-RNAs, CTCs and ctDNA, some studies focused on exosomes which are endosome-derived 50–100 nm sized vesicles secreted from many cell types that include cancer cells and encapsulate mi-RNAs indicative of their cell of origin. However, exosomes are not here discussed because most data on exosomes-derived mi-RNAs support them as an additional diagnostic tool for an early diagnosis of breast cancer [223].

6.3.1. Prognostic mi-RNAs

Wu and co–workers [224], using deep-sequencing methods on blood of patients with stage II or III breast cancer, observed a direct correlation of miR-375 and an inverse correlation of miR-122 levels with the clinical outcome. Sahlberg and co-workers [225] found that serum miR-18b, −103, −107, −652 signature was predictive of tumor relapse and survival in TPNBC. Madhavan and co-workers [226] reported on plasmatic miR-200b as surrogate markers for CTCs and prognostic marker in metastatic patients. Particularly it was associated with worse PFS and OS. In other studies serum miR-202 correlated with decreased OS [227] and miR-155 levels with poor outcome [163]. In a further one [228] miR-16, −25, −222 and 324-3p were found to be increased in high risk patients. Chen W and co-workers [229] have reported on circulating level of miR-10b and miR-373 as potential biomarkers of lymph node metastasis while in another investigation [230] miR-214 showed to promote metastasis by PTEN targeting.

6.3.2. Predictive mi-RNAs

In a study by Jung and co-workers [231] elevated plasma miR-210 baseline levels correlated with resistance to trastuzumab-included chemotherapy. Wang and co-workers [232] found high circulating miR-125b to predict resistance to various chemotherapies including 5-FU and FEC polychemotherapy. Zhao and co-workers [233] have reported on plasma miR-221 as a predictive biomarker for chemo-resistance in patients having previously received neoadjuvant chemotherapy. Finally, Sun and co-workers [234] described serum miR-155 as a potential biomarker correlating with the treatment course.

6.4. CTCs

Commonly tumor cells from primary tumor site spread to peripheral blood (PB) as intact cells to move to secondary homing sites. Well known is the prognostic clinical relevance of these disseminate tumor cells (DTC) also termed “minimal residual disease” (MRD). Initially and for a long time most studies focused on bone marrow and in 2005 a pooled analysis of bone marrow aspirates confirmed a significant correlation of DTC with shorter DFS and OS [235]. The ease access and recent detection availability account for most studies having thereafter shifted their interest on liquid biopsies and particularly some among them on determination of CTCs in peripheral blood (PB).

A few studies focused on detection of the synchronous presence of CTCs in PB and bone marrow; concordance rates ranged from 66% to 94% [236–239]. CTCs in PB occur as single CTC but more often as homotypic or heterotypic CTC clusters. CellSearch [240], qRT-PCR [238], RT-PCR [241] and AdnaTest [242] have been the most used methods for CTC detection in PB. Moreover, a comprehensive appropriately referenced list of the used CTC detection/captures platforms has been recently reported [243]

6.4.1. Prognostic role

A few studies conducted in early breast cancer have reported on the unfavorable prognostic significance of the presence (detecting ≥ CTC) of CTCs in the PB. These clinical trials have largely documented worse outcome, particularly DFS, distant disease-free survival (DDFS), breast cancer specific survival (BCSS) and OS in SUCCESS trial [244,245] or DFS and OS [241,246] or DDFS and BCSS [247] or DDFS and OS [248] or metastasis-free survival (MFS) and BCSS [238] or MFS and OS [249]. However, while these results have been confirmed for high-risk patients there is lower evidence in low-risk patients with small tumors and negative lymph-nodes. In addition, so far, not enough evidence of the prognostic relevance of CTCs there is with regard to the specific subtypes [250]. These findings made the mere enumeration of CTCs less helpful than expected and efforts are ongoing to characterize their phenotype [251,252].

As to metastatic disease, the prognostic value of CTCs detection has been first examined in 2004 by the seminal study of Cristofanilli et al. [240]. In the study, a threshold of > 5 CTC/7.5 mL assessed at baseline and few weeks following the treatment distinguished patients with

Table 4a
Prognostic and predictive tissue miRNAs.

miRNA overexpression	Prognosis		Prediction of		References
	Worse	Better	Response	Resistance	
miR-148	RFS	–	–	–	[140]
miR-210, miR-21, miR-221, miR-652 signature	OS	–	–	–	[141]
miR-210	Metastasis promoter RFS, DFS, OS	–	–	–	[140–141,146–149]
miR-375, miR-342, miR-26a, miR-30-c, miR-10a, miR-126	–	–	TAM	–	[187–193]
miR-375	–	DFS (TAM treated patients)	–	–	[187]
miR-342-5p	–	OS	–	–	[158]
miR-21, miR-205	Metastasis promoter DFS, OS	–	–	H, PTX, DOX	[129–132,142–145,186,202,217]
let-7f	–	–	Letrozole	–	[195]
miR-125b, miR-21	–	–	–	PTX	[215,216]
miR-125b, miR-99a, let-7c cluster, miR-125 (deletion)	–	Outcome	Antiestrogens, Anthracyclines	–	[164,204]
let-7b, miR-205, let-7c	–	Outcome, RFS, OS	–	–	[150–151,164]
miR-221–222 cluster	–	–	–	TAM and FV	[196,199–200,233]
miR-221	–	–	–	H	[203]
miR-519a, miR-301, miR-155	–	–	–	TAM	[171,197–198]
miR-621	–	–	PTX, CBDCA	–	[214]
miR-155, miR-493, miR-30e, miR-27a signature	–	Outcome (TNBC)	–	Taxanes	[155]
miR-181a	–	–	–	LTR, DOX	[201,220]
miR-34a	–	–	Radiotherapy	–	[222]
miR-27b-3p	Outcome (TNBC)	–	–	–	[160]

short from those with long progression-free survival (PFS). This finding has been confirmed ten years later in a pooled analysis of 1944 patients. Moreover, in this study the superiority of CTC detection over serum CEA and CA.15.3 was demonstrated [253]. In another trial considering the CTC count at baseline and after one cycle of treatment (high or low) in metastatic patients, four different PFS profiles were found with the worse prognosis referring to those having elevated CTCs at both times points [254].

6.4.2. Predictive role

In some clinical trials, CTCs as monitoring tool of response to neoadjuvant therapy were assessed. In these studies, the detection rate

Table 4b
Prognostic and predictive tissue miRNAs.

miRNA overexpression	Prognosis		Prediction of		References
	Worse	Better	Response	Resistance	
miR-30a	–	Outcome, DFS, RFS	–	–	[153,154]
miR-100, miR-16	–	–	PTX	–	[212,213]
miR-30e	–	Outcome	–	–	[156]
miR30c, miR-128	–	–	DOX, PTX	–	[210,211]
miR-141, miR-125b, miR-128	–	–	–	Taxane-anthracycline-based therapies	[218,219]
miR-497	–	DFS, OS	–	–	[159]
miR-451, miR-326, miR-200c, miR-134, miR-34a	–	–	DOX	–	[205–209]
miR-200c	RFS (in PR negative patients)	–	–	–	[165]
miR-9	10-yrs LRFS (in ER +)	–	–	–	[161]
miR-187, miR-155	Outcome, BCSS, OS	–	–	TAM	[157,162–163,198]
miR-203	–	–	–	Cisplatin	[221]
miR-574-3p	–	OS	–	–	[121]
miR-660-5p	OS	–	–	–	[121]
miR-127-3p, miR-210, miR-185, miR-143*, let-7b signature, sf-miR-423-3p/sf-miR-423-5p and miR-375/sf-miR-375 clusters, miR-9, miR-10b, miR-29a, miR-155, miR-520c, miR-373, miR-301, miR-548i	Metastasis promoter	–	–	–	[141,152,166–172]
miR-184/sf-miR-184 cluster, miR-170/20, miR-34, miR-29b, miR-708, miR-126, miR-126*, miR-193b, miR-206, miR-335, miR-448, miR-601, miR-138, miR-515-5p, miR-203, miR-200 family, miR-205	–	Metastasis suppressor	–		[126,152,173–186]

Also see text. DFS: disease free survival; RFS: relapse free survival; OS: overall survival; LRFS: local recurrence free survival; BCSS: breast cancer specific survival; TNBC: triple negative breast cancer; PR: progesterone receptor; TAM: tamoxifen; H: trastuzumab; PTX: paclitaxel; DOX: doxorubicin; FV: fulvestrant; CBDCA: carboplatin; AptomiRs: miRNAs that play an important role in promoting (pro-AptomiRs) or inhibiting (anti-AptomiRs) apoptosis (about 40% of the AptomiRs targets regulate apoptosis by the p53 network or Bcl2 family proteins); Prognostic AptomiRs: miR-155, miR-497; Predictive AptomiRs: miR-26a, miR-34a, miR-125b, miR-203, miR-181a, miR-221-222; Prognostic and predictive AptomiRs: miR-21, miR-210, miR-342.

was ≥1 CTC/7.5 mL and whole blood samples were collected before the beginning and during and/or after neo-adjuvant therapy. The prognostic impact on DFS and/or OS worsening of the persistence of CTCs was controversial. However, no significant association occurred with the pathological complete response in the Gepar Quinto and Gepar Quattro [255,256] in the Beverly-2 [257] and in a pooled analysis of Beverly-1 and Beverly-2 trials [258]. Thus, currently in the adjuvant setting treatment decisions are not dependent on presence of MRD but only on the principal characteristics of the primary tumor. However, a liquid biopsy-based large trial [259] is ongoing and early results are expected. In this EORTC 90091-10093 Treat CTC trial, patients with persistence of CTCs following adjuvant chemotherapy are randomized to receive six cycles of trastuzumab vs observation. In this study, although HER 2 CTCs status is assessed, the randomization is only based on the presence of CTCs. Regarding metastatic disease, in 2015 the ASCO clinical practice guidelines for CTC count considered a reasonable option for clinical oncologists do not use CTCs count to drive treatment of metastatic patients [260]. This followed the negative results of the SWOG S0500 trial [261] where patients whose CTC count after the first cycle of the first line chemotherapy was higher than 5CTC/7.5 mL of blood had been randomized to an early switch to second line chemotherapy.

Nevertheless, some criticisms to the study design have been raised to explain the negative results and therapy choices driven by CTCs count is an issue by far from being abandoned. In the CirCe01 study (NCT01349842), a multicentre randomized phase III trial, patients are recruited before the beginning of third line chemotherapy and they are monitored with the CTC count during the successive lines of treatment; besides the choice is decided based on conventional clinical and radiological criteria vs CTC guided treatment according to whether there is or not CTC persistence under chemotherapy. In the STIC CTC trial (NCT01710605) the choice between first-line hormone therapy or chemotherapy for relapsing ER positive Her2/neu negative patients is decided by the clinical oncologist or by the baseline CTC count. In the DETECT III (NCT01619111) and DETECT IV (NCT02035813) trials HER2-negative metastatic breast cancer patients with HER2-positive or negative CTCs respectively are recruited. In the DETECT III trial Her-2 negative patients with ≥ 1CTC are randomized to receive standard therapy (endocrine therapy or chemotherapy) of clinical oncologist's choice +- lapatinib and CTC disappearance is the primary endpoint. In the DETECT IV trial Her2 negative metastatic breast cancer patients with Her2 negative ≥1 CTC/7.5 mL are recruited. ER positive tumors receive hormone therapy plus everolimus (DETECT IVa) while ER positive tumors indicated for chemotherapy and TPNBCs are given eribulin (DETECT IVb). In the DETECT trials, in addition to CTC count, CTC characterization of the HER 2 status is inclusion criterion. A score formed with CTC count and CTC characterization for ER, Bcl-2, Her 2, and Ki 67 is currently being tested in the COMETI phase II study (NCT01701050). This study recruited ER positive/Her2 negative patients progressing after at least one line of endocrine therapy. In this study, CTCs count and score are used to rapidly identify (within 3 months) progressing patients to move them to another line of endocrine therapy. RNA expression analysis and evaluating DNA aberration in CTCs are further expanded application of CTC assay and the feasibility of detecting specific somatic mutation or other genetic alterations in clusters of CTCs or single CTC has been already shown [262,263].

Analyzing whole genome copy number aberrations or whole exome sequencing of single CTC by array comparative genomic hybridization (aCGH) and NGS techniques are non-targeted variants under examination. Limiting false positive and false negative results and defining the number of CTCs to be analyzed are the main challenges of this last application [264,265]. These and other investigations conducted with CTCs in vitro and in vivo models [266–268] are expected to provide a definitive answer as to the relevance of CTCs in predicting response and in guidance to select the treatment.

6.5. Circulating tumor DNA (ct DNA)

Unlike CTCs, ctDNA follows nucleosomal pattern of DNA fragmentation from apoptotic tumoral cells and the amount of ctDNA likely depends on their rate. Nucleosome is the core unit of chromatin formed by an octamer of the four highly conserved core histone proteins (H3, H4, H2A, H2B) connected by a linker histone H1 to 146 base pairs of DNA [269]. In PB nucleosomes are mainly carried out as oligonucleosomes or mononucleosomes. ctDNA is a small proportion of total circulating DNA and distinguishing ctDNA from cell-free DNA (cfDNA) has proven a challenge. Unlike CTCs where potential analyses include DNA, RNA and proteins for ctDNA they are limited to DNA.

6.5.1. Main methodological aspects

Usually the length of nucleosomal fragments is 160–180 bp however has been found that a high amount of ctDNA is < 100 bp [270,271]. Therefore, the developed assay methods have to detect a nucleosome-type size but considering that an optimal detection could require the use of primers targeting amplicons < 100 bp. Enzyme linked immunosorbent assays (ELISA) [272], PCR [273], beads, emulsion, amplification and magnetic sequencing (BEAMing) [274] and NGS [275] are techniques commonly used for DNA detection and analysis in serum and in plasma. However, there is a huge diversity as to technology/techniques selection used by researchers in their labs. Although this can help in identifying the limits of individual platform and consequently to improve next-generation protocols concomitantly it makes difficult to interpret and compare findings across laboratories. Thus, one major difficulty for integrating ctDNA detection into routine clinical practice is the absence of pre-analytical and analytical standard operating procedures (SOPs), assay validation and validation and identification of suitable prognostic and/or predictive read-out. While the specificity of ctDNA is elevated being based on the presence of pathognomonic or previously characterized molecular alterations in corresponding tumor tissue (single nucleotide (SNV), copy number (CNV), structural, methylation variants) whether sensitivity, clinical utility and cost/benefit ratio meet the necessary requirements is to be fully proven. Spatial and temporal tumor heterogeneities are important source of variability and further potential limitation is the cells from which ctDNA derives (apoptotic/necrotic CTCs or actively shedding tumor cells either in circulation or in primary/metastatic sites). Overall it is currently highlighted the need to move beyond the study of single gene mutation and to develop ctDNA platforms having wide genomic coverage for a broad applicability and to capture a more comprehensive snapshot of the tumor genome. These platforms should permit to identify tumor with multiple molecular markers and to anticipate molecular alterations expected with tumor evolution.

6.5.2. Clinical applications of ctDNA

Detection and ctDNA measurement have been suggested as early indicator of tumor response. This could allow to optimize neo-adjuvant therapy in noninvasive disease and to avoid unnecessary toxicity during the treatment of recurred disease. Detectable ctDNA levels after adjuvant treatment and during post-operative follow-up also could permit to early diagnose a MRD [276] thus better driving the decision making process. Discordance of molecular profile between ctDNA and tissue specimens or the appearance of new molecular alterations in the serial ctDNA assessment during treatment could be accounted by arising of resistance and potentially predict for different therapies. Similarly, quantification of circulating nucleosomes has been investigated and the monitoring of their changes has been found to correlate with tumor response. In fact, more elevated levels of nucleosomes in patients who did not respond to neo-adjuvant therapy occurred compared to those who did [277]. Detection and quantification of both histones and the concomitant PTMs as circulating tumor biomarkers is a proposed attractive option; in addition, it has been supposed that combining circulating miRNAs with nucleosome quantification and nucleosome

PTMs could define a more specific and sensitive bio-signature.

6.5.2.1. ctDNA ESR1 mutational status. The mutational status of ER as a prognostic and predictive biomarker is currently being assessed in clinical trials. In one of these trials (SoFEA), patients with ER-positive advanced breast cancer who had ctDNA positive for *ESR1* mutations, were found to exhibit improved progression-free survival (PFS) when treated with fulvestrant compared with exemestane [278]. In contrast, patients with wild-type *ESR1* had similar PFS following either treatment. In a further trial (PALOMA-3), which compared fulvestrant plus placebo to fulvestrant plus the CDK 4/6 inhibitor, palbociclib, in patients progressing on prior endocrine therapy, administration of the dual treatment was found to improve PFS compared with the single agent treatment in *ESR1* mutant and *ESR1* wild-type patients [278]. This preliminary finding suggests that the efficacy of palbociclib was unaffected by the mutational status of *ESR1*. In another study using ctDNA, Chandarlapaty et al. [279] reported that adding the mTOR inhibitor, everolimus to exemestane, significantly increased progression-free survival in patients with D538G mutations but failed to do so in those with Y537S mutations. Thus, the beneficial impact of combining everolimus with exemestane appears to depend on mutational status of *ESR1*.

Assuming that these early clinical findings can be confirmed in further trials, determining the mutational status of plasma *ESR1* is likely to become routine for selecting the most appropriate endocrine therapy in patients with endocrine-resistant advanced breast cancer. Furthermore, by serially assessing the mutational status of plasma *ESR1* while patients are receiving an AI, it should be possible to detect the early emergence of resistance. In such a scenario, changing treatment to a different form of endocrine therapy or administering chemotherapy might be expected to improve outcome.

7. Challenges for future research

Although substantial progress has been made in the identification and validation of prognostic and predictive biomarkers for breast, several major challenges remain. These include identification and validation of biomarkers for:

- Predicting response to specific forms of chemotherapy.
- Identifying patients likely to develop severe chemotherapy-related toxicity.
- Predicting response to radiotherapy.
- Enhancing the positive predictive value of ER.
- Selecting patients that preferentially benefit from an aromatase inhibitor vis-à-vis tamoxifen or vice-versa.
- Selecting patients who do not need extended adjuvant endocrine therapy, i.e., more than 5 years.
- Increasing the positive predictive value of HER2.
- Selecting patients likely to particularly benefit from dual anti-HER2 as opposed to single-agent anti-HER2 therapy.

In addition to above, research should focus on establishing a possible clinical utility for the mutational status of plasma *ESR1*. Addressing the above challenges should now be a priority for biomarker research in breast cancer.

Although multiple challenges remain, the increasing use of prognostic and predictive biomarkers is accelerating the move towards personalized treatment for breast cancer. Traditionally, treatment was based exclusively on histological and clinical factors. Today, these factors can be complemented with biomarkers such as ER, PR, HER2 and specific gene expression profiles. The key advantage of the biomarkers over the traditional factors is that they can provide therapy predictive information, in addition to providing prognostic value. One of the challenges for the future will be the identification of the early emergence of resistance to ongoing therapies. In this context, the use of ctDNA for detecting mutations in target genes is likely to be of value (e.g. use of ctDNA mutant ESR for predicting resistance to aromatase inhibitors).

8. Conclusion

Breast cancer has led the way in the introduction of prognostic and predictive biomarkers for cancer patients. Over 40 years ago, ER was first introduced for predicting response to endocrine therapy. Twenty years later, HER2 became available for identifying patients likely to benefit from trastuzumab and later to other forms of anti-HER2 therapy. In the last decade, several multigene signatures have been proposed for identifying patients with early breast cancer whose prognosis is so good, that they are unlikely to benefit from adjuvant chemotherapy. Currently, a considerable amount of research is focusing on DNA mutation testing (including measurement of ctDNA), miRNAs and CTCs, with the aim of identifying new prognostic and predictive biomarkers. These emerging biomarkers however, will have to undergo both analytical and clinical validation prior to entering clinical use. By combining established prognostic factors with validated prognostic/predictive biomarkers, we can begin the journey towards personalized treatment for every newly diagnosed patient with breast cancer.

Conflicts of interest

MJD is a clinical advisor to OncoMark and Atturos.

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