

Emerging Biomarkers of the Future: Changing Clinical Practice for 2020

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Abstract Progress in biomarker development has greatly enhanced our ability to categorize breast cancer into several clinical subtypes and to deliver better personalized therapies. Technological advances in gene expression profiling, signaling pathways, proliferation markers and tumor monitoring through detection of circulating tumor cells and free DNA, and measurements of genomic instability and germline mutations are being vigorously pursued in breast cancer research. Their application in routine clinical practice is increasing and helping further development of precision medicine. Ongoing challenges include assessing the utility and feasibility of these tests, interpreting the large amounts of genomic data that are being generated, translating the information to clinical practice, and constructing clinical trials on molecularly driven approaches. In this article, we will review current and emerging promising biomarkers and their roles in the management of patients with breast cancer.

Keywords Circulating biomarkers · Tumor tissue markers · Breast cancer · Personalized medicine · Biomarkers

Introduction

Breast cancer is a group of heterogeneous diseases that have different molecular and genomic features. The workup in

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patients with breast cancer includes screening the tumor for established prognostic and predictive biomarkers, such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2/neu (HER2), which help guide endocrine, cytotoxic, and HER2-targeted systemic treatments [1]. The prognosis of patients is classically assessed by the anatomy of the disease (tumor size and lymph node status) as well as its histology and immunohistochemistry (type, grade, lymphovascular invasion, and receptors). In 2001, the pivotal studies by Perou and Sorlie [2, 3] classified breast cancer according to molecular subtypes (luminal-A, luminal-B, ERBB2/HER2+, and basal-like) thereby introducing a new concept to breast cancer classification and management. Further research and technological advances, particularly high-throughput gene expression profiling, have generated multigene signatures that provide further prognostic and predictive information and enable optimal selection of adjuvant therapy candidates. Proliferation markers and cellular pathways involved in tumor growth and invasion like p53, retinoblastoma (RB), P13K/AKT/mTOR, and RAS/MAPK are being identified, and therapies based on this new information are being evaluated in prospective clinical trials [4]. Mutations associated with genetic instability and epigenetic alterations, including histone methylation and acetylation, DNA methylation and microRNA expression, tumor microenvironment, and tumor-infiltrating lymphocytes (TIL), have become important [4]. Moreover, the emerging “liquid biopsy”, which is the term for detecting genetic material from tumor cells in an individual’s or patient’s blood, is a promising non-invasive test that may enable the detection of genetic material and biomarkers of breast cancer cells [5].

Despite advances in diagnostics and treatment strategies, breast cancer remains responsible for the deaths of around 90,800 women in Europe [6] and 40,290 women in the USA [7]

every year. Although the incidence of the disease is generally lower in developing countries, mortality is much higher, largely because of delays in diagnosis [8–10]. These numbers underscore the gaps in our knowledge of the pathophysiology of the disease and the lack of effective preventive interventions.

A World Health Organization (WHO) report has predicted a 50 % increase in the global incidence of cancer from 2000 to 2020 [11]. This should stimulate more efforts for prevention, early detection, and better management using more effective and less toxic drugs. The latter can be better achieved by further research into phenotypic and genomic biomarkers. By 2020, it is also anticipated that a number of tools will provide a thorough analysis of biomarkers, hopefully enabling better stratification of patients whose risk of recurrence cannot be established by clinicopathological criteria alone.

Biomarkers and Personalized Medicine

Personalized medicine is an emerging concept involving the addition of genomic information to classical clinical data in order to better predict and improve responses to therapy and to reduce toxicity, thereby advancing individual patient-centered care.

While tumor cell alterations have a great impact on drug activity and impose the detection of predictive biomarkers, it is the genetic makeup of the patient that chiefly determines whether the patient will experience severe toxicities [12]. Many factors are involved in this layout, including sets of genes, pathophysiological and epigenetic factors, as well as large inter- and intra-individual tumoral variability in space and time. The huge volumes of molecular and genetic data being produced by high-throughput techniques, namely next-generation sequencing (NGS) assays, are expediting breakthroughs in pharmacogenomics and will certainly make further contributions in the foreseeable future.

Challenges of Emerging Biomarkers

The rapid progress that has been made in understanding the molecular basis of breast cancer, and progress in management, has transformed the outcomes of women with breast cancer, from a 5-year overall survival (OS) of 63 % in the early 1960s to around 90 % today, and most of the improvement has taken place in the last two decades [13]. The major challenges in biomarker research have long included the complexity of tumors, shortfalls in the funding of basic and technologic research, disappointing results from studies with methodological shortcomings, inappropriate validation, and a lack of clinical applications [14]. By 2020, we foresee that the obstacles to finding early predictive and prognostic biomarkers will no longer be insurmountable [15], as the financial, intellectual, and technological investments of the last decade are expected to yield big rewards. A number of novel products and their

companion diagnostics are expected to be launched in the market by 2020. Additionally, assays for collecting and analyzing biological data are improving by many orders of magnitude and becoming much cheaper. In effect, by 2020, genetic testing may become part of mainstream medical practice.

Tumor Tissue Markers

Ki-67 Antigen

The Ki-67 antigen (Kiel University, clone 67), originally described in 1983 [16], is a nuclear protein present in proliferating cells and is expressed throughout phases of the cell cycle. This simple assay has been gaining popularity and controversy in the setting of hormone receptor-positive breast cancer, in order to be able to make clinical separation of cases as luminal-A and luminal-B as per molecular subtyping [17] and, for example, in order to better guide treatment decisions in early stage breast cancer. Using a cutoff of 14 % to define high-risk patients as per the St. Gallen's recommendations [18–20], high Ki-67 indicates poor prognosis and predicts potential benefit from chemotherapy. The 2015 St. Gallen's Expert Panel agreed that higher levels of Ki-67 (20–29 %) can be used to separate luminal-A and luminal-B cases [21].

Low Ki-67, especially <5 %, indicates good prognosis and low rate of recurrence, correlates with low recurrence scores, and indicates low benefit of adding chemotherapy to hormonal therapy in the adjuvant setting. In the neoadjuvant setting, Ki-67 level is increasingly used early to assess the degree of response to hormonal therapy and guide further therapy [22–24]. In primary breast cancer, high Ki-67 levels (cutoff of 50 %) predict pathological complete response after neoadjuvant therapy [25]. High Ki-67 levels predict benefit from adding chemotherapy to hormonal therapy [26].

As data on Ki-67 stem mainly from retrospective studies, recommendations for its routine use remain controversial [23]. While many recommend the use of Ki-67 as a prognostic marker, questions about the cutoff level for positivity and the optimal tumor area to be tested, as well as challenges concerning reproducibility, remain unresolved. In triple-negative breast cancer (TNBC), high Ki-67 levels and lack of androgen receptor (AR) expression were found to correlate with shorter overall survival and more aggressive disease [27]. However, in another study, Ki-67 expression was not found to be significantly associated with pathologic complete response in TNBC [28]. The National Comprehensive Cancer Network (NCCN) and the American Society of Clinical Oncology (ASCO) do not currently recommend routine testing for Ki-67 expression in breast cancer tissue [4]. In the setting of ER-positive tumors, analysis of the Ki-67 level helped to stratify patients for poor disease-specific and recurrence-free survival [29]. Given this potential role, methodological and analytical standardizations of Ki-67 have been implemented by the

International Ki-67 in Breast Cancer Working Group to facilitate integration of this testing into ongoing and future trials [30].

HER2: a Timeless Biomarker

The 1980s produced a major advance in the field of breast cancer biomarkers with the discovery of HER2 and its role as a prognostic marker of worse outcome and as a predictor of response to trastuzumab, an anti-HER2 humanized monoclonal antibody of the immunoglobulin G1 type directed against the extracellular portion of HER2 [31–33]. HER2 gene amplification/overexpression occurs in 15–25 % of patients with invasive breast cancer, leading to aggressive tumor behavior and reduced survival [31, 32, 34].

In the early 2000s, in metastatic disease, trastuzumab was reported to improve progression-free survival (PFS) and OS [35]. In the adjuvant setting, it produces benefits in both disease-free survival (up to 23.9 %) and long-term OS (up to 8.8 %) in the updates of the HERA, NSABP-B31, N9831, and BCIRG006 clinical trials [36–39].

Two trials reported that the addition of trastuzumab to chemotherapy in the neoadjuvant setting increased the pathologic complete response (pCR) rate from 25 % in patients on chemotherapy alone to 66.7 % when trastuzumab was added in one trial, and from 15.7 to 31.7 % in the other trial [40, 41]. In the NOAH trial, neoadjuvant chemotherapy with trastuzumab followed by adjuvant trastuzumab significantly improved 3-year event-free survival over neoadjuvant therapy without trastuzumab (71 vs. 56 %) [42].

Lapatinib is a HER2 tyrosine kinase inhibitor that is currently approved for use in combination with chemotherapy (capecitabine and paclitaxel, for example) [43, 44] or trastuzumab [45].

Moreover, around one third of HER2-positive tumors were found to co-express a truncated form of the HER2 receptor lacking the C-terminal fragment of the extracellular domain (called p95HER2), known as the binding site for trastuzumab [46]. This truncated fragment is highly tumorigenic and directly confers intrinsic resistance to trastuzumab-based therapy in the metastatic setting [47–49]. In contrast, p95 expression indicates response to neoadjuvant trastuzumab containing treatment in primary HER2-positive breast cancer but not resistance [50].

Furthermore, baseline genotyping analysis from the NeoALTTO trial suggests that activating mutations in PIK3CA (detected in 23 % of cases of HER2-positive breast tumors) significantly predict poorer pCR in patients with HER2-positive breast cancer receiving anti-HER2-based neoadjuvant therapies [51]. Consequently, the combination of anti-HER2 agents and PI3K inhibitors is being investigated (NCT01589861).

Pertuzumab (a humanized monoclonal antibody that prevents HER2/HER3 and HER2 dimerization) has shown significant positive effects in metastatic breast cancer (CLEOPATRA) [52]. In addition, T-DM1 is a drug conjugate of trastuzumab linked to the maytansinoid microtubule inhibitor emtansine and has been shown to improve survival in second-line therapy for metastatic HER2+ breast cancer (EMILIA trial) [53].

Further research into biomarkers is needed to determine which subset of patients may benefit from the combination of T-DM1 and pertuzumab, as the MARIANNE trial (NCT01120184) showed no overall better outcomes but reduced toxicity. Information is needed on subtyping and biomarkers for predicting which patients are resistant or which ones would develop resistance to trastuzumab (and pertuzumab). The KATHERINE trial (NCT01772472) is assessing the value of adding T-DM1 after surgery in patients who have residual disease following neoadjuvant chemotherapy.

A prospective randomized phase III trial of patients with operable HER2-positive breast cancer evaluated trastuzumab, lapatinib, and the combination with paclitaxel chemotherapy to determine how different gene expression subtypes in HER2-positive tumors affect pCR rates in the breast. Intrinsic HER2 subtype, ER, HER2 signaling, and immune cell signatures were significantly associated with pCR, indicating the clinical importance of the biologic heterogeneity within HER2-positive breast cancers [54].

Future investigations into circulating biomarkers and other techniques to understand the complexity of HER2-positive disease are expected to permit the de-escalation of therapy in suitable patients and improve outcomes [55].

The regulatory interactions between the HER2 and ER pathways in breast cancer cells are also under investigation. HER2 overexpression leads to the redistribution of ER to the cytoplasm. Trastuzumab restores ER in the nucleus by down-regulating HER2-mediated growth factor receptor signaling, and this might explain how the combined action of trastuzumab and antiestrogen therapy potentiates growth inhibition [56].

Parra-Palau et al. proposed that the subgroup of tumors positive for p95HER2 (a collection of carboxy-terminal receptor fragments) may benefit from a dual treatment with lapatinib and anti-ER therapies because p95HER2 exerts a potent, but reversible, downmodulation of ER expression, and it is likely that its inhibition results in the upregulation of ER [57].

The recent discovery that HER2-negative breast cancers might harbor HER2 stem cells indicates a potential clinical benefit of HER2-targeting agents in this population of patients [58]. An analysis of the Cancer Genome Atlas project found that 2 % of breast cancers carry HER2 somatic mutations [59], defined as activating mutations requiring DNA sequencing for their detection instead of the standard HER2 testing. At the

preclinical level, this subset of tumors appears to be sensitive to the special effect of the small molecule receptor tyrosine kinase inhibitor neratinib but resistant to established HER2-targeting agents [60]. However, available clinical data remain insufficient to endorse the predictive value of any of the above findings. One promising strategy currently under investigation is the use of circulating tumor cells (CTCs) as a predictive biomarker for HER2-targeting therapies [61].

Targeting Molecular Pathways

TP53 Tumor Suppressor Gene

Along with the known germline mutations, somatic mutations also play a key role in breast cancer outcomes. Wild-type TP53 gene is a chief gatekeeper of the genome and is of crucial significance for DNA integrity through its dual role in DNA repair in damaged cells and apoptosis when this repair is not feasible [62]. TP53 germline mutations account for less than 1 % of breast cancers while somatic mutations are detected in roughly 43 % of these cancers (13 % in luminal-A, 71 % in HER2-positive, and 80 % in basal-like subtypes) [3]. These mutations have been linked to worse disease outcomes, especially in ER-positive node-negative [63, 64] and basal-like tumors [3]. In fact, Dookeran et al. [65] reported that TP53 mutation remains an independent predictor of survival even after adjustments for the effects of age, stage, grade, and subtype. More recently, however, a number of reports have found that the prognostic value of TP53 expression is lost or diluted over time, with worse survival being limited to the first 2 to 5 years after diagnosis [66, 67].

Although testing for TP53 alterations has not been endorsed by ASCO as a routine prognostic indicator because of the lack of reproducibility of results [68], two clinical trials (NCT00004038 and NCT00044993) of TP53-targeting therapy alone or in combination with chemotherapy were recently completed and their results are awaited.

Cell Cycle-Related Proteins and the Retinoblastoma Pathway (CDK4/6 Inhibitors)

Cyclin D1 and cyclin E1 are nuclear proteins that bind to cyclin-dependent kinases (CDKs), promoting transition from the G1 to the S phase of the cell cycle [69, 70]. The RB protein is responsible for cellular G1-S checkpoint arrest following inhibition of the cyclin-CDK complexes by CDK inhibitors such as the p16, p18, p21, and p27 proteins. Cyclin D1 binds specifically to CDK4/6, and the latter's activity is instrumental in ER-positive breast cancers [71], whereby ER orchestrates the transcription of the gene encoding cyclin D1 (*CCND1*). Luminal-B tumors exhibit frequent (58 %) gains of the *CDK4* and *CCND1* genes compared to luminal-A (29 %) HER2-positive tumors (38 %), as well as loss of the genes encoding

p16 (*CDKN2A*) and p18 (*CDKN2C*), leading to major disturbances of the cell cycle [72].

Palbociclib is a selective inhibitor of CDK4/6 [73] that blocks the hyperphosphorylation of pRB and induces G1 cell cycle arrest in ER-positive cell lines and in HER2-amplified cell lines in combination with anti-estrogens or anti-HER2 respectively [74]. Palbociclib was also noted to synergistically inhibit growth activity when combined with the anti-estrogen drug tamoxifen, and it was found to be efficacious in a model of acquired tamoxifen resistance [74]. In a proof-of-concept randomized phase II study of postmenopausal women with advanced ER-positive and HER2-negative disease (PALOMA-1/TRIO-18), two cohorts of treatment-naive patients were enrolled to receive letrozole with palbociclib or letrozole alone, either on the basis of their ER-positive and HER2-negative biomarker status alone (cohort 1) or on the basis of additional amplification of cyclin D1 (*CCND1*), loss of p16 (*INK4A* or *CDKN2A*), or both. In cohort 1 ($n=66$), median PFS was 5.7 months (2.6–10.5) for the letrozole group and 26.1 months for the palbociclib plus letrozole group; in cohort 2 ($n=99$), median PFS was 11.1 months for the letrozole group and 18.1 months (13.1–27.5) for the palbociclib plus letrozole group (HR 0.508, 0.303–0.853; one-sided $p=0.0046$) [75].

Although 73 % of the study population were biomarker positive, neither amplification of cyclin D nor loss of p16 predicted for response to palbociclib nor the latter has been approved for an unselected marker population. In addition, the PALOMA-1 trial evaluated gene amplification and deletion only, while many other mechanisms for gene overexpression and loss of expression do exist. In February 2015, palbociclib became the first approved CDK4/6 inhibitor, when the FDA granted it accelerated approval for use in combination with letrozole to treat postmenopausal women with ER+/HER2 advanced breast cancer as initial endocrine therapy [76]. At the molecular level, further analysis and an optimal predictive biomarker are warranted in the postmarketing setting.

In a population with endocrine-resistant metastatic ER-positive breast cancer, the PALOMA-3 trial demonstrated a 5.4-month median PFS benefit of palbociclib in combination with fulvestrant (hazard ratio for disease progression or death, 0.42; 95 % CI, 0.32–0.56; $p<0.001$) [77], further demonstrating the efficacy of palbociclib. It continues to be the subject of investigation in the ongoing PALOMA-2 (NCT01740427), PALOMA-3 (NCT01942135), PALOMA-4 (NCT02297438), PEARL (NCT02297438), and PENELOPE-B (NCT01864746) trials. Another CDK4/6 inhibitor, ribociclib, is being investigated for the treatment of HR+/HER2 advanced breast cancer in the MONALEESA-2, -3, and -7 phase III clinical trials, and abemaciclib is also under investigation. In addition, attention is being turned to drugs with CDK2 inhibitory activity, as they may prove useful for the treatment of palbociclib-resistant ER-positive breast

cancers that re-enter the cell cycle via cyclin E-CDK2 activation [78]. Dinaciclib, which has CDK1, 2, 5, and 9 activity, is also undergoing clinical investigation [79].

Cyclin-dependent kinase inhibitors are the most refined cell cycle checkpoint therapeutics available to date. This promising area is currently the focus of a number of studies, the results of which will lead to better understanding of the molecular mechanisms involved in unrestrained cancer growth.

Circulating Biomarkers and Tumor Cells

The overall prognosis of breast cancer patients is mainly determined by the loop of circulating malignant cells between the site of origin and their new distant niche [80], as well as by the highly selective process of “cancer dormancy” [81, 82]. As this latter phenomenon is unpredictable, it is almost entirely dependent on the progressively acquired and unique genomic alterations of disseminated tumor cells compared to the malignant cells of origin [83, 84].

Upon disease progression or recurrence, biopsy of the new tumor site appears to be mandatory for advanced understanding of tumor biology [85, 86]. However, this technique is invasive and may also carry the risk of further tumor seeding [87]. Moreover, the complex intra-tumoral heterogeneity prevents a credible mapping of the genomic background of breast cancer [88]. Diagnostic and therapeutic clinical oncology has long awaited the availability of blood-based personalized biomarkers to predict and evaluate the response of a tumor to treatment, to estimate prognosis and risk of occult disease in its early stages and to monitor and foresee metastatic disease. Earlier research studied circulating glycosylated tumor-derived proteins as potential biomarkers, but current interest is in the value of liquid biopsy, namely CTCs, microRNAs, and, most importantly, cancer-derived cell-free DNA or circulating tumor-derived DNA (ctDNA).

Circulating Tumor Cells

CTCs are increasingly being recognized as markers of the epithelial-mesenchymal transition (EMT) pooled from primary tumor and different metastatic sites, and thus, they are useful in determining prognosis [89, 90]. CTCs provide real-time assessment of response or resistance to treatment [85, 91]. Many CTC platforms have been developed [92]. The CellSearchR technique is currently the only FDA-approved modality for dynamic prognostication in metastatic breast cancer. This approval was promoted by the prospective work of Cristofanilli et al. [93] and was more recently endorsed by a meta-analysis [94], as well as a pooled analysis in 1944 patients [95]. Five or more CTCs at any chosen baseline significantly predicted worse PFS and OS in metastatic breast cancer [96]. For early stage breast cancer, CTCs were also associated with lymph node involvement and shorter disease-free

survival [97–100]. The CTC detection threshold was lower in early compared to metastatic breast cancer, raising again the issue of the lack of standardization and inter-reader variability between multiple assays [101].

In HER2-positive breast cancer treated with targeted therapy, the survival benefit conferred by therapy has been found to mitigate, by far, the prognostic value of CTCs [102, 103], reflecting the fact that liquid biopsies have limited analytical validity, low sensitivity, and high false-negative rates.

The SWOG S0500 study [104] of 123 patients was the first published phase III trial to address the benefit of a preemptive early switch therapy strategy based on persistence of CTCs following chemotherapy. Although detectable CTCs were associated with poor prognosis, the early switch strategy did not improve outcomes partly because of failure of the second-line treatment.

As CTCs are detected in only 44 to 65 % of the population with metastatic disease [105, 106], efforts are being made to improve this tool beyond use of EpCAM, whether through enrichment with anti-epithelial antibodies [107–109] or through the addition of further markers of EMT [89, 106].

Phase III trials to determine how measurements of CTC protein expression, not only enumeration of CTCs, will affect clinical outcomes, and therapeutic decision making are ongoing. A study sponsored by the National Cancer Institute (NCT00382018) with women with treatment-naïve metastatic breast cancer is currently underway. In this trial, following the first round of treatment, patients are randomly assigned to either remain on their current therapy or modify their regimen at the treating oncologist's discretion, if they demonstrate persistently elevated CTC levels based on the CellSearch assay (five or more cells per 7.5 mL of blood). A European trial (NCT01349842) is enrolling patients with metastatic breast cancer with detectable CTCs but before they undergo the third line of treatment. After one cycle of therapy, these patients will be randomly assigned to continue therapy either based on standard radiographic responses or according to changes in CTC level, with an expected follow-up period of 4 years and a primary endpoint of OS.

The use of CTCs as a prognostic biomarker is well validated and robust in breast cancer. A growing body of evidence suggests that they also hold promise as a tumor tissue source to study DNA mutations, to study drug sensitivity by ex vivo cultures for NGS [110], and to analyze protein and RNA expression [91, 95, 111].

Nevertheless, against the background of clonal heterogeneity in cancer, every molecular analysis conducted on CTCs must be interpreted with caution, as reports of their use in this situation in the literature are contradictory [112–115].

Circulating-Free Tumor DNA

More recently, circulating-free tumor DNA (cfDNA) has been recognized as a more advanced modality of liquid biopsy [116], particularly for the detection of tumor-specific somatic mutations, microsatellite instability, and loss of heterozygosity (LOH) [117, 118].

This DNA is released at low levels into circulation following its degradation during apoptosis [119]. It correlates with tumor burden [120] and reflects the underlying tumor genome [121]. DNA fragments exist in various sizes, and the proportion of a given fragment compared to total DNA correlates well with tumor size and nodal involvement [122]. Earlier studies [123, 124] focused mostly on metastatic disease, where cfDNA levels are naturally very elevated but still considered to be useful for quantifying tumor burden and monitoring response to treatment [125]. This has been validated by a prospective study by Dawson et al. [120] of 30 women with metastatic breast cancer with genetic alterations; cfDNA was detected in 97 % of the participants. In the same study, Dawson et al. demonstrated that cfDNA levels are useful for estimating OS: 89 % of the participants who relapsed were found to have increased cfDNA levels by an average of a factor of 505 from the nadir. Interestingly, in 53 % of relapses, these levels increased an average of 5 months before documentation of radiological disease progression, leading to the conclusion that measurement of cfDNA may provide the earliest assessment of metastatic disease response currently available.

In a recent study by Olsson et al. [126], tumor samples from 20 patients with early breast cancer treated with surgery and adjuvant chemotherapy and/or radiation therapy were first sequenced to detect genetic defects; then, a specific digital droplet PCR (dd-PCR) assay was designed to record the same defects in plasma samples. With 93 % sensitivity and 100 % specificity, rising ctDNA levels predicted clinical evidence of metastasis with an average lead time of 11 months. Moreover, high ctDNA levels recorded before surgery were predictive of poor disease-free and overall survival. Although this study could be criticized for its retrospective nature, small population, and lack of power to fully estimate the predictive and prognostic values of the plasma ctDNA assay, it certainly forms a promising platform for larger clinical studies.

It also offers hope that in the foreseeable future, it may be possible to use ctDNA levels to determine which subset of patients with early breast cancer needs additional or intensified adjuvant treatment after primary surgery.

Unfortunately, assays for cfDNA detection lack general standardization, which has meant that it has not been possible to precisely determine their analytical validity. DNA can be extracted from plasma or serum, and genetic alterations can be analyzed by various techniques [127], but high-quality DNA is mandatory, especially when analyzing large sets of genetic

data using NGS. Additionally, these results appear to be less reproducible in women with early stages of breast cancer, because of an insufficient quantity of DNA [117], a low tumor burden [128], or high false-positive rates reported with some benign lesions [129].

Perspective on Liquid Biopsies

Liquid biopsies are blood tests that detect CTCs and tumor DNA fragments that are shed into the bloodstream [5]. It is expected that major technical and methodological refinements will optimize the sensitivity and specificity of these assays by 2020, especially given the number of ongoing trials (see Table 1). These refinements include serial peripheral blood sampling to allow subsequent systematic comparison of baseline plasma ctDNA concentrations to those obtained upon clinical progression of the disease, validated methods and kits for plasma DNA isolation, as well as a standardized bank of tumor-specific rearrangements with varying allele frequencies to account for evolutionary clonal/subclonal heterogeneity occurring throughout disease progression. It is expected that ctDNA assays will reach the point of being prognostic and/or predictive, leading to better tailoring of breast cancer therapy and perhaps improvements in patient survival. Ideally, such assays, even those using whole genome sequencing, should be feasible, inexpensive, rapid, validated, expanded, and extended to the whole population [130]. The near future looks promising, particularly given the significant decrease in sequencing costs in recent years.

Special Settings

ER-Positive Breast Cancer

Despite a reduction in recurrence rates of ER-positive breast cancer with current standard hormonal therapies, the risk of recurrence may resurge over a prolonged period of time [131]. A large number of multigene assays have therefore been developed in an attempt to predict late recurrences and the genetic markers of tumor dormancy [132]. Furthermore, as assays of CTCs and ctDNA have been developed to investigate minimal residual disease over long periods of time in many malignancies, they have also been used in this special setting of breast cancer. In one study, CTCs were detected in around one third of breast cancer patients 7–22 years after the initial diagnosis [133].

cfDNA assays were evaluated by Beaver et al. [134] in presurgery and postsurgery patients with early stage breast cancer, demonstrating their usefulness and applicability in this setting. Similarly, Shaw et al. [135] reported that cancer-related cfDNA could still be detected up to 12 years after initial diagnosis. In metastatic disease, a score combining CTC enumeration with ER, HER2, BCL-2, and Ki-67

Table 1 Summary of major ongoing clinical trials in breast cancer using liquid biopsy as adjunct to treatment decision-making

Study/NCT.gov identifier	Phase/ <i>N</i>	Population	Intervention	Primary endpoint	Status
CirCe01 (NCT01349842)	III/568	Refractory MBC with detectable CTCs prior to CT	Treatment decision based on CTCs vs. based on clinical and radiologic responses	OS	Recruiting
DETECT-III (NCT01619111)	III/120	HER2-negative MBC but with HER2-positive CTCs	Standard therapy (CT or ET) vs. physician choice + lapatinib	CTC clearance rate	Recruiting
STIC-CTC (NCT01710605)	III/1000	MBC, HR +, HER2-negative	Physician choice vs. CTC-based choice between ET and CT	PFS	Recruiting
TREAT-CTC (NCT01548677)	II/2175	HER2-negative early BC with detectable CTCs after (neo)adjuvant CT	Adjuvant trastuzumab vs. placebo	CTC detection at week 18	Recruiting
DETECT-IV (NCT02035813)	II/520	HER2-negative MBC with persistent CTCs	Everolimus + ET or eribulin	PFS	Recruiting
NCT01975142	II/480	HER2-negative MBC with HER2-positive CTCs	TDM-1	Response rate	Recruiting

N number of patients in each study, *BC* breast cancer, *MBC* metastatic breast cancer, *CT* chemotherapy, *CTCs* circulating tumor cells, *ET* endocrine therapy, *HR* hormone receptors, *OS* overall survival, *PFS* progression-free survival

expression was recently developed to predict endocrine resistance, and its clinical validity is currently being prospectively evaluated in clinical trials (NCT01701050 and NCT02137837) [136].

Assays evaluating CTCs and ctDNA in patients who are five or more years beyond their initial diagnosis remain an area of intense investigation. To date, longitudinal data are still lacking. Ongoing studies are focusing on validating quantitative minimal residual disease to accurately predict recurrence in a preset time frame.

Triple-Negative Breast Cancer

TNBC is a heterogeneous spectrum of breast cancers grossly characterized by lack of ER and PR expression and by the absence of HER2 overexpression. TNBC accounts for 15–20 % of all breast cancers and has been historically associated with poor patient outcomes [137].

Multiple signaling pathways and dynamic microenvironmental changes are the hallmarks of TNBC, and they remain poorly understood [138–141]. The available classifications of this disease are therefore based on immunohistochemical biomarkers and limited gene signatures, including PAM50 and Lehmann's system, reflecting striking heterogeneity [3, 142].

Despite the identification of promising biomarkers, a large number of molecularly enriched clinical trials have proven elusive in this setting, largely because of the heterogeneity of TNBC as well as the lack of consistent recurrent mutations [143]. Nevertheless, the preliminary results of SHIVA (NCT01771458) [144] and the M-PACT [145] and MATCH [146] pilot studies look promising.

As only a small number of driver or founding mutations have been discovered in TNBC, exceptional therapeutic strategies involving biomarkers are unlikely to be developed for this type of breast cancer in the foreseeable future [72]. p53

mutations occur in around 80 % of basal-like TNBC [147] and are currently being targeted in phase I clinical trials through a number of agents, including Gendicine (adenovirus mediated transfer of human wild-type p53), PRIMA-1MET (p53-binding molecule to restore p53 wild-type transcriptional properties), and MDM2 inhibitors (inhibitors of p53-negative regulators) [148]. Additionally, PARP inhibitors and PI3K/AKT/mTOR inhibitors are being tested for breast cancer patients with BRCA1/BRCA2 and PI3KCA mutations in ongoing clinical trials [149, 150].

Several TNBC subtypes have been identified, raising the possibility of offering targeted therapies, including anti-androgens, in the future [151].

In the near future, any personalized therapy for TNBC will probably entail biology-oriented approaches. Therefore, innovative trials including biomarker enrichment and adaptive designs need to be developed for this disease [152, 153] (see Table 2).

Immunogenicity and Immune Signatures in Breast Cancer

The immunogenicity of breast cancer has been recognized since an abundance of lymphocytic infiltrates was correlated with favorable outcomes in medullary carcinoma [154, 155].

Although immune signatures appear to predominate in TNBC [2, 156], they have been recognized in all breast cancer subtypes. In a number of studies, higher counts of TILs correlated significantly with pathologic response, as well as improved outcomes including risk of recurrence and death [37, 157]. In the Geparsixto trial [158], TILs also predicted that adding carboplatin to neoadjuvant chemotherapy would be beneficial in TNBC. Immune checkpoint inhibition is also emerging as a promising strategy for the treatment of TNBC. Prominent expression of PD-L1 was observed in BRCA1-

Table 2 Summary of major ongoing clinical trials using targeted therapy in TNBC

Targeted agent	Targeted pathway	Class	NCT.gov identifier
BMN673	PARP	PARP inhibitor	NCT01286987
Rucaparib	PARP	PARP inhibitor	NCT01482715
Olaparib + BKM120	PARP + PI3K	PARP inhibitor + PI3K inhibitor	NCT01623349
Veliparib	PARP	PARP inhibitor	NCT01149083
Veliparib	PARP	PARP inhibitor	NCT01506609
Dinaciclib	Cell cycle checkpoints	CDK inhibitor	NCT01624441
MK-1775	Cell cycle checkpoints	WEE1 inhibitor	NCT01748825
BKM120	Signaling pathway	PI3K/AKT/mTOR	NCT01790932
GDC-0941	Signaling pathway	PI3K/AKT/mTOR	NCT00960960
X-396	Signaling pathway	CMET and EGFR inhibitors	NCT01625234
AR0197	Signaling pathway	CMET and EGFR inhibitors	NCT01738438
Erlotinib	Signaling pathway	EGFR inhibitor	NCT00733408
Ruxolitinib	Signaling pathway	JAK-2 inhibitor	NCT01562873
Tivozanib	Angiogenesis	VEGF receptor inhibitor	NCT01745367

deficient/mutant patients as well as in androgen receptor-negative TNBC [159]. It was also suggested that genomic instability and DNA repair defects enhance the immunogenicity of tumor cells, leading to increased chemosensitivity. This suggestion has led to trials exploring the use of immunotherapy alone (NCT01848834 and NCT01191216) or in combination with chemotherapy (NCT01525602).

The use of CTCs to detect PD-L1 suggests that this is an auspicious predictive biomarker for immune checkpoint blockade therapies in future clinical trials, as recently reported by Mazel et al. [160] in the first study of its kind.

Era of Genomic Medicine

The benefits of the clinical information provided by ER, PR, and Her2 status in the adjuvant or metastatic settings are firmly established, and these indicators are not expected to be replaced despite the advent of a variety of new molecular-based diagnostic tests.

The last 10 years have seen the successful introduction and application of gene expression-based diagnostic assays such as the 21-gene recurrence score (Oncotype Dx) to guide treatment decisions regarding the utility and optimal benefits of adjuvant chemotherapy in early stage hormone receptor-positive breast cancer [161, 162].

Nevertheless, these tests, in their current form, are unlikely to supply all of the information needed to optimally manage breast cancer. The integration of multiple parameters, including established ones and new ones provided by molecular profiling, offers better biologic insights. Although commercially available tests overlap in very few genes, if any, the key factors are consistently represented, including ER and proliferation markers. Although the goal is for these assays to be objective and reproducible, they can at times be subjective and prone to inter-

observer variation, lacking correlation with morphological features.

The genomic landscape of breast cancer has more recently entered a new era with the introduction of NGS [142]. Mutational processes are variable and exist as either single “driver mutations” or multiple combinations of these mutations, including TP53, PIK3CA, and PTEN [163]. The current trend is to test for genomic alterations in an individual tumor, aiming for better individualized precision medicine. It remains a tremendous challenge to establish the real feasibility and utility of “targeted” therapies for such complex and quite rare patterns of recurrence.

Role of BRCA1 and BRCA2 Susceptibility Genes

The role of BRCA1 and BRCA2 germline mutations in the natural history of breast cancer was established 20 years ago [164], and potential clinical uses of these mutations as biomarkers for the stratification and management of breast cancers will no doubt continue to evolve. A number of proof-of-concept studies have been conducted that elegantly support the role of platinum-based compounds and PARP inhibitors in this setting [149, 165].

More recently, BRCA1 and BRCA2 genes have been implicated in the process of genetic scarring, through homologous recombination (HR) DNA repair defects, mostly in breast and ovarian cancers [166–168]. This type of genomic instability is permanently imprinted in the tumor and can be quantified through specific assays. For instance, high levels of such genome instability have predicted better response to platinum-based compounds in non-BRCA1/2 mutation-associated triple-negative breast cancer in some studies [157, 169, 170]. This clinical benefit is likely to evolve in the future, especially as our understanding of this concept improves and the cost of BRCA1 and BRCA2 germline testing decreases markedly.

Conclusion

A new avenue involving highly developed diagnostic and therapeutic technologies has opened in the daily practice of breast cancer management, which has led to a profound yet incomplete understanding of the biology of this cancer. Substantial progress has been made in recent decades, but significant challenges remain in the treatment of all subtypes of breast cancer, particularly TNBC, resistance in HER2-positive breast cancer, late recurrences of hormone-positive breast cancer, and de-escalation of therapy in low risk patients. With the flood of molecularly based tools in development, real opportunities for personalized breast cancer management are expected in the next few years.

Given the heterogeneity of breast cancer, we are eagerly awaiting results of ongoing research on multidimensional collective approaches, including studies on clinicopathologic criteria and individual genetic codes and prospective studies on molecularly enriched approaches.

Compliance with Ethical Standards

Conflict of Interest Hazem I. Assi, Rita E. Assi, and Nagi S. El Saghir declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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