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Bioclinical markers in breast cancer: updates and perspectives

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1. ABSTRACT

Molecular studies have definitely changed our knowledge of the biology of cancers, and breast cancer's tremendous social impact has stimulated a large mass of research. Classic markers have opened a road, but their usefulness appears limited to prognosis or follow up, while several new markers, both genetic and molecular, are assuming different, yet still controversial, importance: they may play a major role in the surveillance of subjects at risk,

in detecting primary or recurrent cancers, and in predicting the need of adjuvant therapy, or the response to therapy.

Nevertheless, the mandatory routine markers out of trials are not really modified when compared to the 2007 guidelines, essentially due to a lack of appropriate levels of evidence. For this reason we can only recommend to include as many women as possible in specific trials, in order to reach the evidence level that we need to substantially improve our understanding of cancer and eventually the outcome for women with breast cancer.

2. INTRODUCTION

In the last 50 years many advances have been made in the understanding of cancer and in the development of humoral and tissue tumor markers. Although the first tumor marker was identified in the Bence-Jones protein, this definition came into common use after the discovery of CEA. This discovery led to hypothesizing the presence of proteins and other substances in the blood, body fluids, and tissues, allowing for an early diagnosis of cancer and its recurrences. Unfortunately, the sensitivity and specificity of these markers was later found to be too low to achieve these objectives. Nevertheless, studies on cancer expression as circulating or tissutal proteins have flourished since then, delineating ever more complex and heterogeneous patterns for many cancers, especially breast cancer. Globocan 2000 estimated that 1,150,000 women worldwide are diagnosed with breast cancer every year, and over 400,000 die from this cancer (1). The enormous impact of this easily explains the large number of studies focused on earlier identification of the disease, and finding new targets for therapies and new prognostic markers, in the hope of modifying the prognosis of this cancer.

Unfortunately, until now the only effective way to reduce mortality from breast cancer has been early diagnosis by mammography, but the best results have been obtained in postmenopausal women. Younger women, not covered by screening programs and with denser breasts, would probably benefit from screening programs based on MRI, as shown by the encouraging results in early diagnosis in young women at moderate- risk levels (2-5). Since the cost of MRI is still too high to be used indiscriminately for screening purposes, we need to pre-select high risk women for this type of screening. A tumor marker sensitive and specific enough to identify early breast cancer, or at least to identify a high risk woman to whom a personalized program of screening or prevention could be applied, would be the perfect answer to the problem.

Unfortunately, the typical serum tumor markers are not useful in the early detection of cancer, and epidemiological and anamnestic studies are still the most effective way to select high risk women. In the future, it is likely that a biomarker panel will be able to detect breast cancer in asymptomatic patients, even in women with normal physical examinations and mammograms (6). Genomics, proteomics and molecular studies have actually produced a large number of possible markers, and some have become targets for therapies, changing many of our opinions about breast cancer, and even the classification of the disease itself. These new modalities will probably modify the algorithm of follow up, since imaging studies such as CT, positron emission tomography, bone scans, laboratory studies, and classical tumor markers have failed to demonstrate an improvement in cost-effectiveness, survival, or quality of life in asymptomatic patients (7).

A careful review of the most recent literature on this subject has been performed by Medline, Ovid and Scopus using the key words “breast cancer”, “tumor markers”, “bioclinical markers”, with the aim of summarizing actual and prospective indications of the use of markers in breast cancer, from screening to therapy and follow up.

3. SCREENING

Prevention of cancer can be almost solely based on early diagnosis. The difficulty of early diagnosis in younger groups of women has stimulated research toward the selection of high risk groups on which to perform more aggressive screening programs, using MRI or even preventive surgery.

A better knowledge of cancerogenesis mechanisms is needed in order to reliably identify high risk groups. In this effort, biological, genetic and molecular studies have the role of formulating and proving a theoretical basis to support clinical evaluation. Actually, various mechanisms have already found validation, and some hypotheses seem very promising.

3.1. Genetic Markers

The use of markers in breast cancer screening is currently limited to the genetic testing of women with familial breast cancer (FBC), which account for about 20-30 % of all cases.

Approximately 40% of FBC is sustained by either BRCA1 or BRCA2 mutations in equal parts. Mutations in these two genes determine a lifetime risk of BC that can reach up to 85 %, making these genes the strongest risk factor for breast cancer (2). Recent evidence supports the hypothesis that the BRCA1 gene involved in hereditary breast cancer plays a role in breast stem cell function (8, 9).

An Australian study showed that the incidence of BRCA 1or 2 reaches 50% in women with at least two relatives affected by BC and/or ovarian cancer, while falling to 0.055% in those with only one relative affected (10). This finding lead Olopade *et al.* to conclude that the inherited susceptibility to breast cancer is multifactorial, and multiple genetic variants, still unknown, can be responsible for its development (2). The stratification of the risk of developing BC is more difficult in small families, or when the genes BRCA 1 and 2 are inherited via paternal transmission.

Furthermore, not all the different race extractions present the same prevalence of mutations for these genes. In fact, in a recent demographic study on Northern Californian women with breast cancer, John *et al.* confirmed the previous observation regarding the more prominent presence of BRCA mutations in Ashkenazi Jewish women (8.3%), followed in descending order by Hispanic women (3.5%), non-Hispanic white women (2.2%), African-American women (1.3%), and Asian-American women with a prevalence of 0.5 % (11).

Another gene carrying a high risk is TP53 (a nuclear phosphoprotein with a tumor suppressor role, involved in apoptosis), whose mutations cause Li-Fraumeni syndrome with high risk of juvenile breast cancer, but in very small incidence (less than 1% of FBC) (2) . The mutation can be inherited but can occur in a single cell in sporadic cancerogenesis. Lu *et al.* proposed the possibility of utilizing autoantibodies as an early marker of cancer, and reported that antibody response to p53 was detected in 11.6 % of patients with DCIS (12). This could prove that these antibodies occur early in the course of cancer and can predict undetected malignancy. The limit of p53 is its lack of specificity for a particular type of cancer. The addition of p53 serum antibody to conventional markers (CA15-3 for breast cancer) increased the diagnostic sensitivity by 8% without decreasing specificity (13).

Many other genes have been identified, and others are suspected to be associated with cancer by a variety of mechanisms. Actually, some authors (2, 13) believe that susceptibility to cancer is due to a combination of multiple low -penetrance variants that alone can be frequently found in the population.

A study by Closas *et al.* on American populations of European origin showed 8 common low-penetrance genetic variations : 5 genes and 3 genomic regions. Less than 5 % of FBC are due to these mutations: FGFR2, TNRC9, MAP 3K1, LSP1, 2q35, 5p12, 8q24. Sixty percent of European women with sporadic breast cancer carry a sporadic mutation of these genes. More recently CASP8 has been added to this group (13).

Based on this evidence the deCODE Breast Cancer Gene Test has been developed and commercialized in USA as a test for susceptibility to cancer. The test is based on the seven original genes of the first study, not including CASP8. These genes could even influence the biological behavior of the cancer, five of these mutations (FGFR2, TNRC9, 2q25, 5p12, 8q24) being related to the expression of estrogen receptors in postmenopausal women.

PTEN, ATM, STK11,/LKB1, MSH, MLH1, BRIP1, PALb2, RAD50, NBS1 account for an additional 1% of FBC, while mutations in CHEK 2 are responsible for 5% of FBC (14).

Although 50% of genetic alterations of FBC is still unknown and under investigation (2), genetic counseling remains of fundamental importance in women with FBC.

3.2. Estrogens

The known relationship between estrogens and breast cancer has stimulated a specific line of research aimed at identifying a new risk marker. The carcinogenic potential of estrogens is due to the formation of catechol estrogen quinones, which react with DNA to form specific depurinating estrogen-DNA adducts (15). Gaikwad *et al.* investigated these metabolites in the urine samples from 46 healthy control women, 12 high-risk women and 17 women with breast cancer (r16). The levels of depurinating DNA adducts and their respective estrogen metabolites and conjugates ratios were significantly higher in high-risk women ($p < 0.001$) and women with breast cancer ($p < 0.001$) than in control subjects. The high-risk and breast cancer groups were not significantly different ($p = 0.62$). Furthermore, the significance of these values was not reduced by the patients characteristics. So Gaikwad concluded that the depurinating estrogen-DNA adducts are possible biomarkers for early detection of breast cancer risk and response to preventive treatment (16).

3.3. Cytokines

The role of cytokines in cancer is as varied as the number of cytokines discovered. Some have a protective effect by stimulating the immune-response, while others inhibit immunity and promote cancer (17). Cytokines are growing in importance as a method of diagnosing cancer. Lyon *et al.* showed that the levels of cytokines and their patterns were markedly different in women with and without breast cancer (18). They compared cytokine levels of 35 women who had been recently diagnosed with breast cancer with 24 women with a suspicious breast mass that was later demonstrated to be benign. They found significantly higher systemic cytokine values in women with cancer for all cytokines measured, with the exception of granulocyte colony-stimulating factor and interferon-gamma. Only interleukin-8 and macrophage inflammatory protein-1 beta increased as age increased in women with negative biopsies. Particularly interesting was the behavior of 3 cytokines (granulocyte colony-stimulating factor, interleukin-6, and interleukin-17) that resulted very specifically related to the presence of cancer, and allowed for a differentiation between the breast cancer and no-cancer groups (18). In spite of these important discoveries, none of these candidate markers can be recommended for routine use in the detection of breast cancer, and further studies are needed to evaluate their possible role.

4. PATHOLOGICAL CLASSIFICATION AND BIOMARKERS

Until now nodal status has been the principal prognostic factor in breast cancer staging. ER status and cErb-B2 assessments have added some accuracy to the effectiveness of staging. Molecular studies and genomic have been the protagonists of a new way to classify breast cancer: molecular classification. This in fact appears to be the best approach to prognosis and targeted therapies.

Epithelial cells of the mammary gland are of two distinct types: basal (myoepithelial), deeper and closer to basement membrane, and luminal, composing the upper and more differentiate layer. These two types of cells can be easily distinguished by histochemical staining for cytokeratins. The basal type expresses reactivity to the cytokeratin 5/6 antibody, while the luminal type is positive for CK 8/18. The positivity for specific CK is preserved even after transformation into cancer and is the basis for the new classification. The two types correlate to epigenetic expression of receptors and as a result, other classifications with 3 or 5 categories have been proposed .

luminal A (ER+ and/or progesterone receptor positive [PR+], HER2-),
luminal B (ER+ and/or PR+, HER2+),
basal-like (ER-, PR-, HER2-, cytokeratin 5/6 positive, and/or HER1+),
HER2+/ER- (ER-, PR-, and HER2+),
and unclassified (negative for all 5 markers) (19)

The same classification can be applied to Ductal Carcinoma *In situ* (DCIS), but the distribution appears to be quite different. In fact DCIS is more likely to be of the luminal B and HER2 phenotypes than invasive tumors. HER2 and basal-like phenotypes seem to be common among both high-grade DCIS and high-grade invasive lesions (20).

Other authors disagree with the molecular classification, and instead classify BC as LUMA or LUMB based on the degree of ER expression, excluding HER positivity from these groups. When reaction for HER is present they suggest a more complex indication, such as LUMA HER2 or LUMB Her 2 hybrid (21). A normal - like is described by some authors (2), while not included in others (22).

Triple Negative Breast Cancers (TNBCs) represents the group defined by negativity for hormone receptors and HER2. Although this group can be identified with Basal cancer, and the receptor expression is the easiest way to characterize the different groups, a molecular diagnosis based on staining for cytokeratins 5/6 and other specific markers, such as P-cadherin and epidermal growth factor receptor, is considered more reliable than the histochemical method (23, 24).

The clinical characteristics of the triple negative cancer are aggressive clinical behavior, lack of effective targeted therapies, and usual resistance to standard chemotherapeutic regimens. These tumors tend to occur in premenopausal women and members of specific ethnic groups (25).

The Carolina Breast Cancer Study has showed that Basal-like breast tumors occurred with a higher prevalence among premenopausal African American patients, compared to postmenopausal African American and non–African American patients (19).

A subset of these patients is associated with heritable BRCA1 mutations. Indeed, Basal-like is the most frequent phenotype associated with BRCA1 carcinomas, and these are characterized by the expression of basal markers such as basal keratins, P-cadherin and epidermal growth factor receptor. Furthermore, BRCA1 carcinomas frequently carry p53 mutations. BRCA1 has been associated with the development and progression of cancer even in sporadic TNBC (25).

BRCA2 carcinomas are more frequently estrogen and progesterone receptor positive, and only occasionally show a basal phenotype. Both BRCA1 and BRCA2 have a low frequency of HER2 expression/amplification. Hereditary carcinomas that are not attributable to BRCA1/2 mutations are heterogeneous, and have phenotypic similarities to BRCA2 tumors. A small group of cases are secondary to mutations in other breast cancer susceptibility genes, such as p53, PTEN or CDH1. As a result of the low frequency of breast carcinomas attributable to the mutations in these genes, it is very difficult to establish a specific phenotype for each genotype, other than the association of lobular carcinomas with CDH1 germline mutations (26).

5. THE PROGNOSIS

5.1 St. Gallen' s classification

In 2007 the St. Gallen Consensus Conference established the most reliable prognostic parameters and risk categories for breast cancer, excluding molecular evaluations and giving, aside from nodal status, a specific importance to grade of hormonal receptor expression and to presence of HER2 positivity.

Breast cancers were classified in (1) **Highly endocrine responsive** : tumors express high levels of both steroid hormone receptors in the majority of cells (identified with proper immuno-histological methods). (2) **Incompletely endocrine responsive** : some expression of steroid hormone receptors, but at lower levels or lacking of either ER or PgR. (3) **Endocrine non-responsive:** tumors having no detectable expression of steroid hormone receptors. This group is clearly defined in terms of lack of responsiveness to endocrine therapies, but includes tumors of diverse phenotypes. The total absence of steroid hormone receptors and the amplification or over-expression of HER2 were each considered sufficient to exclude from the low-risk class, except for rare tumors such as medullary or apocrine carcinoma (which usually lack of any of these receptors) (27). (Table 1).

HER2-positivity, assessed by either strong IHC staining (3+) of >30% of the tumor cells, or, alternatively, by determination of gene amplification by FISH (fluorescence *in situ* hybridization: ratio of HER2 gene copies to chromosome 17 centromers > 2.2) or CISH (chromogenic *in situ* hybridization: more than six HER2 signals per cell) is sufficient in itself to exclude from the low-risk group (28).

The degree of HER-2 positivity has an impact on both overall survival (OS) and disease free survival (DFS) in a large study (28). In fact, ER2 3+ status was associated

Table 1. Definition of risk categories for patients with operated breast cancer

Risk categories	Nodal status	Other features
Low risk	**Node negative**	AND all of the following features: pT < o = 2 cm, AND Grade 1, AND Absence of extensive peritumoral vascular invasion, AND ER and/or PgR expressed, AND HER2/neu gene neither over-expressed nor amplified, AND Age = o >35 years
Intermediate risk	**Node negative**	AND at least one of the following features: pT >2 cm, OR Grade 2-3, OR Presence of extensive peritumoral vascular invasion, OR ER and PgR absent, OR HER2/neu gene over-expressed or amplified, OR Age <35 years
	Node positive (1-3 involved nodes)	AND ER and/or PgR expressed, AND HER2/neu gene neither over-expressed nor amplified
High risk	**Node positive (1-3 involved nodes)**	AND ER and PgR absent, OR HER2/neu gene over-expressed or amplified
	Node positive (4 or more involved nodes)	

Modified from A. Goldhirsch *et al.* (27)

with higher relapse rates in node-positive and node-negative subgroups, whereas HER2 2+ lead to higher recurrences only in node-positive patients. The analysis of the relapses according to the type of therapy provided evidence of responsiveness of HER2-positive tumors to chemotherapy, especially taxanes. The prognostic significance of HER2 seemed correlated to receptor expression level and could lead to consider HER2 2+ and HER2 3+ tumors as distinct diseases with different outcomes and specific features (29).

In some studies, the over-expression of HER-2 has been associated with a higher occurrence of CNS metastasis (30-32), while patients with triple negative disease have the worst prognosis, showing a median survival of 4.0 months after diagnosis of Brain Metastasis, compared with 11.2 months for all other patients (with BM) (33).

Molecular and genetic testing were excluded from this evaluation, since they were still being validated. What we could expect from these tests is a refinement in the identification of high and low risk. Their use is specially targeted to women with early breast cancer (LNN), in which it is difficult to assign an adjuvant therapy, since some of them do not need any, and some others will not benefit from it (34). In other words, are there patients with small, LNN ER/PgR + HER- tumors who can benefit from adjuvant therapy? Conversely, are there patients with T1-T2 LNN or 1-3 LN+ ER- HER + cancers who may be spared from CHT (22)?

Some evidence of effectiveness of these tests is forthcoming from various well-conceived studies, and it may be useful to quickly review the better known ones.

5.2. The Rotterdam 76-gene set

The Rotterdam 76-Gene set was designed to predict the prognosis of patients with node-negative breast cancer (35). Its high sensitivity for distant metastasis (93%) and its specificity of 48% in the first cohort of 280 patients, determined enough interest to further confirming the good performance of the gene set in two additional studies (36, 37).

The first study conducted on 180 LNN women showed good sensitivity (90%) in predicting relapses within the first 5 years, but only a 47% specificity (36). The second study on 198 LNN patients of less than 61 years of age followed for more than 10 years confirmed the good performance of the gene test at the 5 year follow up, but a lower ability to predict long term recurrences (37).

5.3. Invasive gene signature

186 genes associated with tumorigenic breast "stem" cells were used to stratify patients with early stage breast cancer at high risk of metastasis or death into good or poor prognostic groups. The 10-year follow-up showed 81 % of survival in the good prognosis group and 57% in the poor prognosis one, demonstrating a correlation between IGS and clinical outcome (22).

5.4. The wound response indicator

The wound response indicator was developed from genes whose expression changes after activation of cultured fibroblast with serum. This indicator has been validated in early stages breast cancers, and it appears to be an independent predictor of death in a multivariate analysis (22, 38).

5.5. Onco*type* DX™

Onco*type* DX™ Recurrence Score™. This 21-gene indicator is composed of 16 cancer-related genes and

5 reference genes. An algorithm developed from the expression levels of the 16 cancer related genes was the basis for a Recurrence Score correlated with recurrence rates at 10 years. NSABP clinical trial B-14 evaluated this score by outcome in LNN patients on Tamoxifen with a good correspondence. Trial Assigning Individualized Options for Treatment (TAILORx) is assigning women with ER+ LNN breast cancer to three different arms based on RS, with cut off different from previous studies ((TAILOR)). The first group has RS < 11, the second is comprised between 11 and 25, the third >25. The groups are respectively assigned to only hormonal therapy, randomized to CHT and Ht or only HT, the third is assigned to CHT and HT. The results of this study, which is still open, will be very important for the evaluation of reliability of Oncotype and RS as a prognostic index. (22, 37). ASCO guidelines in 2007 validated the Oncotype giving an evidence level of 1 to studies on this gene signature (38).

5.6. Mammaprint ™

Mammaprint or 70-gene prognosis-signature, or Amsterdam gene set, (22) has been validated also on a sample of 241 T1-T3 with 1-3 Node positive. The study found origin in the observation that many women with less than 4 positive lymph nodes have a good outcome. The study showed that women with good prognosis as result of the test had 91% relapse-free survival, and 96% overall survival at 10 years. On this basis some authors consider it possible to spare adjuvant CHT in these patients (40). Mammaprint is under validation in the MINDACT trial not only on LNN but even on 1-3 LNP .

Although two large randomized clinical trials - TAYLORx in North America and Mindact in Europe - are validating on a large scale the two tests currently marketed (OncotypeDX and MammaPrint respectively), the groups of high- and low-risk cancers detected by gene signatures appear largely to overlap the classes identified by the St Gallen criteria. Mammaprint has been compared with Adjuvant online, showing instead a better performance (39). This is easily understandable because the software does not take into account HER, or vascular invasion. So it is possible that IHC tests are still the most cost-effective way to assess the risk in low- and intermediate-risk women (41).

5.7. The HER family

The families of genes usually related to classical histochemical tumor expression gave origin to a large series of research aimed at identifying the tissue markers in body fluids and blood (i.e. HER -neu in extracellular domain), or genes of the same family, like Her -3 or HER 4.

The first human samples in which HER was identified were pleural effusion and serum of patient-bearing advanced breast cancer (42). Subsequent studies showed that about 25% of BC patient present serum ECD HER, and the majority of these patients expressed HER in the primary tumor (38). So the utility of this assay could be limited to patients in which the primitive cancer has neither been studied for HER nor is available for the examination (38). In these cases ECD HER has the same prognostic value as tissutal HER, and can be used to predict the benefit from antracycline regimen or response to Trastuzumab or as a marker for recurrence.

A recent study from Italy confirmed the value of search for ECD HER on 256 consecutive stage I-III breast cancer patients, finding high values (>or=15 ng/ml) in 23 patients (9.0%) while HER2-positive status in tumor tissue was observed in 42 patients (16.4%) with a concordance of 87.1% (42). High HER2 ECD levels were significantly associated with high histological grade (P = 0.003), stage III (P = 0.008), lymph node involvement (P = 0.035) and negativity related to both estrogen (P = 0.016) and progesterone (P = 0.007) receptors. In multivariate analysis, high serum HER2 ECD levels were a significant independent prognostic factor of worse DFS (P = 0.009) (43).

The HER (Human EGFR -Epidermal Growth Factor -Receptor) family includes three other genes, HER 1, HER 3 and HER 4, with structural and functional homology, whose ability to interact with HER 2 has been demonstrated in several studies. HER 1 is exceedingly rare, while HER3 is frequently over-expressed in breast cancer. In addition, co-expression of HER 1 and 2 or 2 and 3 are strong indicators of poor prognosis (43). ErbB3 has also been implicated in the development of resistance to anti-estrogens such as tamoxifen and ErbB tyrosine kinase inhibitors such as gefitinib. Persistent activation of the AKT pathway has been postulated to contribute to ErbB3-mediated resistance to these therapies. This activation may be due in part to the inappropriate production of the ErbB3 ligand heregulin (44-46). HER 4 on the contrary has showed a positive impact on outcome (44, 47).

5.8. The BCL 2 family

The BCL 2 family of genes is variously involved in apoptosis either as promoter or as inhibitor, and has been investigated for many years, but its role as marker has been postulated more recently without reaching any real evidence (48). A recent meta-analysis by Callagy *et al.* strongly supports the prognostic role of BCL2 as assessed by immuno-histo-chemistry in breast cancer, and shows in multi-variate analysis that this effect is independent of lymph node status, tumor size or tumor grade, as well as a range of other biological variables (49).

5.9. uPA /PAI-1

The usefulness of uPA /PAI-1 (Plasminogen Activator Inhibitor) is well known, and the reliability of the ELISA test is considered superior to the IHC for its efficacy. uPA- PAI-1 has been shown experimentally to be involved in invasion, angiogenesis and metastasis (Harris), and high levels of these markers are an indication for CHT even in early stages cancers.

A recent French multi-centered study has confirmed the role as a marker of Plasminogen Activator inhibitor, demonstrating an interesting correlation between

PAI 1 Levels and the risk for metastasis in both pN0 and pN+ patients . PAI 1 is an independent prognostic factor, in particular in pN0 breast ductal carcinoma. The same study investigated uPA that did not result in any adjunctive value (50).

5.10. TIMP

Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) has been suggested as a marker of both prognosis and response to treatment. Several studies have demonstrated the association between TIMP-1 and prognosis in breast cancer (38). New studies have focused on the possibility of using blood samples or paraffin embedded tissue instead of tumor tissue extracts for measurements of TIMP-1(ref). This could lead to a better standardization of assays and to a future larger use, even though this marker cannot be indicated for routine use (51).

5.11. Chemokines

Chemokines have also been largely investigated. A recent study on Chemokine expression CXCR4 in patients receiving neo-adjuvant chemotherapy has shown that the relative risks for recurrence and death in the high CXCR4 group were 27.3- (95% CI:6.2-120.8; P = 0.001) and 4.8-fold (95% CI: 1.5-15.0; P = 0.0076) higher, respectively than those in the low CXCR4 group. High CXCR4 over-expression in specimens from LABC patients was predictive of cancer outcome (52).

A great number of studies has been recently published on genes involved in cancer progression that could, in the future, reveal a role as tumor marker, even if this role remains to be demonstrated.

5.12. NHERF1/EBP50

The adaptor protein NHERF1/EBP50 (Na/H exchanger regulatory factor 1/ezrin-radixin-moesin-binding phosphoprotein 50) emerged recently as an important player in breast cancer progression. It consists of two tandem PDZ domains linked to a carboxyl-terminal ezrin-binding region. NHERF1 assembles macromolecular complexes at the apical membrane of epithelial cells in many epithelial tissues, including the mammary gland. NHERF1 couples molecules involved in cell growth, such as the platelet-derived growth factor receptor (PDGFR) and PTEN (phosphatase and tensin homolog deleted on chromosome 10) (53).

5.13. p 27 and Skp2

Other genes like p 27 and Skp2 did not show a real predictive value. Low p27 and high Skp2 were associated with unfavorable prognostic factors including larger size and higher grade tumors, absence of estrogen and progesterone receptor, human epidermal growth factor receptor 2 over-expression and high Ki-67 (each $P < 0.05$). They did not correlate with disease-free survival ($P = 0.42$ and $P = 0.48$, respectively) nor with response to chemo-endocrine or endocrine therapy, so the authors of the study did not recommend a routine use of these determinations (54).

5.14. Circulating tumor cells

Data on the prognostic value of circulating tumor cell monitored by the CellSearch system are now available on patients with measurable metastatic breast cancer receiving chemotherapy, whereas no such data is yet available in adjuvant or neo-adjuvant settings. The detection of cytokeratin 19 mRNA-positive cells before the initiation of adjuvant chemotherapy was shown to be an independent prognostic factor for worse clinical outcome in patients with early breast cancer. Interestingly, this was mainly observed in patients with triple-negative and HER2-positive, but not estrogen receptor-positive/HER2-negative, early breast cancer. Finally, gene-expression profiling of single cells was reported to be feasible with important implications for eliminating circulating tumor cells (55).

5.15. Proliferative activity

Since the proliferative activity has been used as an index of the biological behavior of the cancer for at least 20 years, is there still a role for these traditional measurements?

A large number of studies have been conducted, aimed at exploring the validity of Ki-67, a nuclear protein, mitotic index (MI), proliferating cell nuclear antigen (PCNA) and thymidine or bromodeoxyuridine labeling index (LI)) with respect to in early breast cancer survival. Stuart- Harris *et al.* have analyzed 85 studies involving 32,825 patients. Ki-67 (43 studies, 15,790 patients), MI (20 studies, 7021 patients), and LI (11 studies, 7337 patients) were associated with significantly shorter overall survival and disease-free survival, using results from univariate and multivariate analyses from the individual studies. PCNA (11 studies, 2677 patients) was associated with shorter overall survival by multivariate analysis only, because of a lack of data (56). Nevertheless it is impossible to establish whether these markers add any information, other than that actually available by IHC or molecular analysis. Other studies not included in this review modify this conclusion (57, 58).

Other proliferative index is given by MIB 1 (percentage of cycling cells).

To improve its predictive value, some authors have added the duration of the cell cycle (assessed by argyrophilic nucleolar organizer regions proteins [AgNORs] measurement). The study by Abboud *et al.* included 90 patients with invasive node-negative breast cancer. None of the patients received chemotherapy. With the help of a double-staining technique, a proliferation index (PI) was determined by multiplying the percentage of MIB1-positive cells by the mean area of the AgNORs present in those MIB1-positive cells. Determination of OS and DFS showed a statistically significative relationship between PI and OS that could add some prognostic information in node-negative patients (59).

6. BREAST CANCER AND THERAPY

An alternative classification of breast cancer - aside from and in addition to TNM - that takes into consideration IHC and molecular characteristics has a direct consequence on the therapeutic approach.

There are three possible ways to define the relationship between breast cancer markers and treatment.

- defining target
- predicting response
- monitoring response

The most important and clearly defined markers are hormonal receptors (ER and PR) and human epidermal growth factor receptor 2 (Her 2). While the first are a perfect target for hormonal therapies (from Tamoxifen to Aromatase inhibitors), the latter are a target for specific antibodies (Trastuzumab) and for the orally available HER1- and HER2-targeted tyrosine kinase inhibitor Lapatinib (60).

Nevertheless, some patients with early cancer (LNN) will experience a failure even after an adjuvant treatment, while others could do well even without undergoing such treatments. The identification of molecular biomarkers with the potential to predict treatment outcomes is essential in selecting patients to receive the most beneficial therapy, and in the future may drive stratification in clinical trials (61).

Analyzing each marker individually, we will try to summarize the possible interactions which explain and predict failure of adjuvant therapies

6.1. ER and PR

Positivity of ER and /or PR is a strong indicator for responsiveness to tamoxifen and aromatase inhibitors, while negativity is an indicator for non-response and actually contraindicates hormonal therapies. How can we predict a failure to respond to such therapies in ER+ patients?

One possible approach is based on the nature of ER itself. SERMs (Selective estrogen-receptor modulators) are effective on Er alpha, which are the ER expressed in mammary glands. The presence of ER beta could explain a lower effectiveness of SERMs. Furthermore, it is possible that ER beta are expressed even in ER alpha negative patients. Available data gives evidence that the role of ER-beta is different when co-expressed with ER -alpha or when expressed alone. Skliris *et al.* summarize the literature on this issue and hypothesize that ER beta may be a therapeutic target in these tumors (62). A variant of Er-beta (Er beta cx) has been associated with better response to endocrine therapies and longer OS. However, cohort size and numbers of independent studies are small to date, and more studies are needed with better standardization of antibodies and protocols (62).

Regarding response to aromatase- inhibitors, some ER+ cancer are less (or not at all) responsive to such drugs. Analysis of the type of receptor can explain this, improving predictability of histochemical markers. In the study of Generali *et al.* on 114 women with T2-4 N0-1v estrogen receptor (ER) alpha-positive tumors, randomly assigned to neo-adjuvant letrozole or letrozole plus metronomic cyclophosphamide, increased p44/42 MAPK and HIF-1alpha were significant factors for treatment resistance . Activated ERalpha form can be considered an independent factor for sensitivity to chemo-endocrine treatment, whereas HIF-1alpha and p44/42 MAPK were independent factors for resistance. Although further confirmatory analyses are needed, these findings have clear potential implications for future strategies in the management of clinical trials with aromatase inhibitors in breast cancer (63).

Few markers are available that can predict response to tamoxifen treatment in estrogen receptor (ER)-positive breast cancers. An immuno-histo-chemical study was conducted on the tissue of NSBP B14 and B20 trials comprising 1001 LNN ER+ patients, using five monoclonal antibodies targeting p53, NDRG1, SLC7A5, CEACAM5, and HTF9C. There were 711 patients in the tamoxifen treated-B14 -B20 trials, and 296 in the tamoxifen - chemotherapy-treated B20 trial. They were classified as high, mild and low-risk by classical criteria that were significantly associated with clinical outcome. The test showed an evident relationship between the presence of antibodies and benefit from chemotherapy, both in high and low-risk patients. The test may be able to identify patients who have greater absolute benefit from adjuvant chemotherapy compared to unstratified patient populations, particularly in LNN ER+ postmenopausal women (64).

In a study aimed at identifying markers associated with failure of endocrine therapies, Vendrell *et al.* found that a 2 genes BCL2/FOS signature was interestingly associated with prognosis and prediction of response to therapies, being a low expression of the 2 genes significant in earlier relapse after primary or adjuvant Tamoxifen (65). BCL 2 has been investigated since 1984 (66), and the expression of this anti-apoptotic gene has been associated with good prognosis in N0 or N1 ER + HER- BC (67). FOS, first identified in retroviral DNA (68), is involved in transcription regulation but its prognostic value has remained obscure.

Other factors have been investigated as a possible marker for patients who may be treated by tamoxifen alone. Among these, PITX2 (paired-like homeo-domain transcription factor 2) methylation can be reliably assessed by real-time PCR technology in FFPE tissue. Multi-centric studies bring substantial evidence that the determination of PITX2 methylation can become of routine clinical use in predicting the outcome in node-negative, tamoxifen-treated breast cancer, a lower value being associated with a significantly better outcome than a higher one (85 % vs 69 % respectively, 10 years DFS) (69, 70).

ER negative patients are usually candidate to antracycline regimen whether HER is positive or not. So, aside from HER, other panels of possible markers have been investigated for prognostic value. MAPK expression is a significant prognostic factor for non-metastatic patients with hormone receptor-negative breast cancer. A lower level of staining is shown to be associated with antracycline resistance and overall survival, whereas a higher expression level is correlated with shorter survival following initial relapse. This suggests a possible role of different molecular

mechanisms pertaining to tumor progression once recurrence occurs (71).

6.2. HER 2

The presence of HER 2 is a strong indicator of poor prognosis. This calls for anthracycline-based adjuvant chemotherapy even in early breast cancer and a target for specific therapy.

6.2.1. HER 2 and endocrine therapies

Approximately half of breast cancers that over-express human epidermal growth actor receptor 2 (HER2) also express hormone receptors (HR) (72). The expression of HER2 has been related to the failure of response to anti-estrogens (73-75). Interactions and cross-signaling from the HER2 receptor to other growth factor receptors such as insulin–like growth factor receptor may potentially contribute to both primary and acquired therapeutic resistance (76-77).

Neo-adjuvant studies suggest that HER2+ cancers may derive greater benefit from AIs than from tamoxifen, however, a recent analysis of the BIG-I-98 trial, while suggesting that HER2 was indeed predictive of early relapse, failed to substantiate the interaction between HER2 and treatment with AIs (78), because of the low rate of HER2 positivity (about 5%) and the low event rate to date (3.7%) Therefore, it is actually impossible to predict response to AI only on the basis of co-presence of HER2. Faratian *et al.* raise the need for further trial on AI (79, 80).
6.2.2

6.2.2. Her 2 and Chemotherapy

A large review aimed at analyzing efficacy of anthracyclines in early breast cancer has been performed on eight studies involving 6564 randomly assigned patients, of whom 5354 had HER2 status information available. The results showed that the added benefits of adjuvant chemotherapy with anthracyclines appear to be confined to women who have over-expressed HER2 or amplified breast tumors. In HER2-positive disease (n = 1536 patients), anthracyclines were superior to non-anthracycline-based regimens in terms of disease-free (pooled HR of relapse = 0.71; 95% confidence interval [CI] = 0.61 to 0.83; P < .001) and overall (pooled HR of death from any cause = 0.73; 95% CI = 0.62 to 0.85; P < .001) survival. In HER2-negative disease (n = 3818 patients), anthracyclines did not improve disease-free survival (HR = 1.00; 95% CI = 0.90 to 1.11; P = .75) or overall (HR = 1.03; 95% CI = 0.92 to 1.16; P = .60). The test for treatment by HER2 status interaction yielded statistically significant results: for disease-free survival, the chi-square statistic for interaction was 13.7 (P < .001), and for overall survival, it was 12.6 (P < .001) (81).

6.2.3. HER2 as a target for specific therapies

Trastuzumab, a recombinant monoclonal antibody against the Her2 receptor, is the only FDA-approved targeted agent for treatment of Her2-over-expressing breast cancer. Many women will either not respond or eventually progress despite trastuzumab treatment. As a result, significant efforts have been applied to find other therapies besides trastuzumab for the treatment of Her2-positive breast cancer. Research goals have been directed to trying to elucidate the exact mechanism of resistance to trastuzumab, and identifying ways to overcome it. Also additional goals are aimed at increasing the efficacy of trastuzumab by combining it with other therapeutic agents, and at investigating other novel agents (82-84).

In order to predict which patient will benefit from trastuzumab,, a correct analysis is needed. FISH is actually considered the gold standard for confirming or excluding HER2 amplification (85).

Nevertheless some patients present resistance by expressing amplification of HER 2, leading to further evaluation of possible interactions between HER and other genes. Bender and Nahta reviewed the mechanisms of resistance to the HER2-targeted antibody trastuzumab, including signaling from other members of the HER family, increased signaling through the PI3-kinase pathway, and cross talk from the insulin-like growth factor-I receptor to HER2 (76).

Other genes involved in trastuzumab resistance in breast cancer have been studied. Among them PTEN and an oncogenic mutation of PIK3CA conferred resistance to trastuzumab in cell culture. Both oncogenes may provide a biomarker to identify patients unlikely to respond to trastuzumab-based therapy (86, 87). Other parameters, such as topoisomerase-II alpha and c-myc co-amplifications, have also been identified as potentially useful predictors of response to trastuzumab-based chemotherapy regimen (88). Patients with HER2-positive breast cancer whose disease has become resistant to the anti-HER2 monoclonal antibody trastuzumab can benefit from lapatinib, a dual epidermal growth factor receptor/HER2 tyrosine kinase (TK) inhibitor (89-92).

Other authors directed their focus of research to ECD HER. In fact, evaluating the presence of HER 2 in the serum could provide an accurate monitor of the response to therapy with trastuzumab both in HER + and HER -. In an analysis of 307 patients with MBC, individuals who did not achieve a significant decline (>or=20%) in serum HER-2/neu levels had decreased benefit from trastuzumab-based therapy, and these patients should be considered for clinical trials evaluating additional HER-2/neo-targeted interventions (93).

Because HER-2 extracellular domain (ECD) levels have been correlated with disease progression in the metastatic setting, some authors demonstrated a certain response in women with recurrent HER2- breast cancers using trastuzumab: in total, 13 (59.1%) patients obtained a biochemical response. In this study, patients with conventional HER-2-negative disease but with expression of HER-2 ECD above the normal limit (> or =15 ng/ml) displayed a rapid response, both biochemically and clinically, to the trastuzumab-taxane combination. The study by Ardvanis is the first to assess anti-HER-2-based treatment in HER-2-negative advanced breast cancer

according to HER-2 ECD positivity; if these results are confirmed, additional patients with "hidden" HER-2-positive breast cancer might benefit from anti-HER-2 treatment (94).

6.3. VEGF

Anti-vascular endothelial growth factor therapy (VEGF) is an important new treatment modality in oncology. Various selective inhibitors have been introduced in therapy showing efficacy and safety in refractory breast cancer (as bevacizumab and sunitinib) with response even in patients treated with trastuzumab. A specific marker for response should be of great interest, and assays for anti-VEGF have been tested.

A randomized trial designed to determine the efficacy and safety of the humanized monoclonal anti-VEGF antibody bevacizumab, and vinorelbine as treatment for refractory breast cancer, and to explore the role of plasma VEGF as a predictor of treatment outcome, showed that lower levels of baseline VEGF were associated with longer time to progression.

Plasma VEGF is surely worth further evaluation as a prognostic marker for treatment outcome in advanced breast cancer patients receiving anti-VEGF therapy as Sunitinib (95).

6.4. Triple negative or basal-like breast cancer

Triple-negative breast cancer is an important subgroup of breast cancer with a distinct outcome and therapeutic approach. The basal-like cancer as a distinct class of tumors which share a specific molecular profile characterized by positivity for specific cytokeratine (5/6), are characterized by high proliferative activity and express specific markers. This type of tumor is a good candidate for the development of specific targeted therapy (96-99).

6.5. Generic markers of treatment response

6.5.1. Circulating tumor cells

These have been studied for long time, but although their role has been proposed for almost every possible use from screening to follow up, some evidence exists only for establishing prognosis and monitoring effects of therapies in metastatic breast cancer (100). With regard to prognosis, it has been demonstrated that the detection of cytokeratin 19 mRNA-positive cells before the initiation of adjuvant chemotherapy is an independent prognostic factor for worse clinical outcome in patients with early breast cancer at high to moderate risk.

6.5.2. TOP 2A (TOPOISOMERASI 2 ALFA)

The DBCG trial 89D studied aberrations of Topoisomerases 2 Alfa on 980 Danish women, and suggested benefit from adjuvant chemotherapy with epirubicin in patients with primary breast cancer having TOP2A amplifications, and perhaps deletions. Additional studies are needed to clarify the exact importance of TOP2A deletions on outcome, but deletions have proven to be associated with a very poor prognosis (101). A thorough review of literature has confirmed the value of TOPA2 in predicting response to aggressive chemotherapy (102).

6.5.3. Class III beta-tubulin isotype

This has been tested as a possible marker of response on a sample of 173 patients with locally advanced or metastatic breast cancer who participated in the TAX-303 phase III trial in which patients were randomly assigned to receive docetaxel or doxorubicin. Patients with "high" expression of class III beta-tubulin isotype had a higher probability of response to docetaxel than to doxorubicin treatment (odds ratio, 1.9; 95% confidence interval, 1.01-3.7; P = 0.05). No difference was observed in terms of time to progression or in terms of overall survival (103).

6.5.4.Thymidine phosphorylase (TP)

Preclinical data have indicated a synergistic interaction between docetaxel and capecitabine by means of taxane-induced up-regulation of thymidine phosphorylase (TP). On the basis of such premises, a phase II trial explored the relationship between TP tumor expression and benefit from this regimen. A significantly higher TTP was observed in patients with TP-positive tumors A subgroup analysis confirmed this TTP benefit in patients with TP-positive tumors obtaining a tumor response (log-rank test, P = 0.03), whereas the statistical significance was lost in non-responders (log-rank test, P = 0.3). TP expression may be a predictive marker for therapeutic benefit (104).

6.5.5. HIF-1 (hypoxia-inducible factor-1)

Hypoxia occurs in breast cancer and in other solid tumors due to the tumor outgrowing the existing vasculature. Hypoxia leads to an adaptive response orchestrated by HIF-1 (hypoxia-inducible factor-1)**,** crucial for tumor progression and therapy resistance and is responsible for poor patient outcome. In several studies, downstream targets of HIF-1alpha were considered as hypoxia markers. The recent data suggests that treatment outcome depends on individual genetic features and that the hypoxia signature is a significant prognostic factor (61, 105).

6.5.6. betaIII-tubulin isoform

Over-expression of the betaIII-tubulin isoform is associated with taxane resistance in cell lines. Some clinical studies support a relationship between poor response to taxanes and over-expression of betaIII-tubulin. BetaIII-tubulin over-expression seems not to affect sensitivity to ixabepilone. Estrogen receptor negativity, low expression of microtubule-associated protein tau, and perhaps HER2 amplification may define a subset of patients with higher than average sensitivity to paclitaxel (22, 106).

6.5.7. Other

Since spindle microtubules are the primary drug targets for taxanes, important SAC proteins such as **MAD2, BUBR1, Synuclein-gamma and Aurora A** have emerged as potentially important predictive markers of taxane resistance, as have specific checkpoint proteins such as BRCA1. Moreover, over-expression of the drug efflux pump MDR-1/P-gp, altered expression of microtubule-associated proteins (MAPs) including tau, stathmin and

MAP4 may help to identify those patients who are most at risk of recurrence, and those patients most likely to benefit from taxane treatment (107).

7. FOLLOW UP AND DETECTION OF RECURRENCE

Detecting the recurrences of cancer has been the first duty of classic tumor markers. CA15-3 and CA 27-9 are simple hematologic tests aimed at identifying MUC-1 antigen. CA 15-3, CA 27-9 and CEA actually have no role in screening and diagnosis, while probably having some prognostic value (108, 38). In fact, ASCO guidelines excluded their use from the decision-making tree of adjuvant or neoadjuvant therapies. Ca 15-3 and CA 27-9 present definite usefulness in follow up, having been reported to anticipate the clinical evidence of distant metastasis by 5-6 months. Also the most recent studies, in particular the one by Mariani conducted on 900 women, confirmed the efficacy of the determination of CA 15-3 in association with CEA for early detection of distant metastasis. The test failed to identify local or regional recurrence. In this study, as in previous studies, the main criteria for predicting recurrence have been either the presence of values of at least one marker higher than the cut-off, or the increase in values between two consecutive determinations (109).

The major issue remains, whether an early detection of asymptomatic metastasis is useful or not, because there is no prospective randomized clinical trial (38) that addresses the impact of the treatment of occult or asymptomatic metastasis on survival, quality of life or cost effectiveness.

CEA , carcinoembryonic antigen, is the most classical tumor marker, but its utility in breast cancer seems limited to those cases of metastatic breast cancer in which MUC-1 antigens (CA 15-3 or CA 27-9) are negative, with the aim -together with imaging- to monitor the effects of anticancer treatment. It has no role at all in screening, diagnosis staging or follow up after primary therapies (38).

8. CONCLUSION

Every month scientific literature on breast cancer is enriched by a large volume of interesting and well-conducted studies, to which genetic and proteomic give a particular input. Nevertheless the mandatory routine markers out of trials are not really modified when compared to 2007 guidelines, essentially because of the lack of appropriate levels of evidence. This is the reason why we can only recommend including as many women is possible in specific trials, so that we can reach the evidence level needed to substantially improve our understanding of cancer, of the mechanisms that regulate response to therapies, and eventually the outcome of women with breast cancer.

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Abbreviations: BC= Breast cancer; FBC= Familial breast cancer; LABC = Locally advanced breast cancer ; OS= Overall survival; DFS= Disease free survival; LNN= Lymph node negative; LNP= Lymph node positive; ER= Estrogen receptor; PR= Progesterone receptors; TNBC= Triple negative breast cancer; PI = Proliferation index; AI = Aromatase inhibitor; PAI .1= Plasminogen activator inhibitor type 1; TIMP.1= Tissue inhibitor of metalloproteinases-1; UPA= urokinase-type plasminogen activator

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