

Biomolecular markers of breast cancer

Andrea Nicolini¹, Angelo Carpi², and Giulio Tarro³

¹ Department of Internal Medicine, ² Department of Reproduction and Aging, University of Pisa, ³ Department of Diagnostics, Division of Virology, D Cotugno Hospital, Naples, Italy

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Tissue markers
 - 3.1. Intracellular receptors (estrogen and progesterone receptors)
 - 3.1.1. The estrogen receptors (ER-alpha, ER-beta)
 - 3.1.1.1. Structure
 - 3.1.1.2. Function
 - 3.1.1.3. Clinical utility
 - 3.1.2. The progesterone receptors (PR-A, PR-B)
 - 3.1.2.1. Structure and function
 - 3.1.2.2. Gene regulation
 - 3.1.2.3. Pathologic and clinical considerations
 - 3.2. HER2/neu (erbB2)
 - 3.2.1. Structure
 - 3.2.2. Function
 - 3.2.3. Clinical utility
 - 3.3. p53, gene and protein
 - 3.3.1. Structure and function
 - 3.3.2. Clinical utility
 - 3.4. Ki-67/MIB-1
 - 3.4.1. Structure and function
 - 3.4.2. Clinical utility
 - 3.5. Vascular endothelial growth factor (VEGF)
 - 3.5.1. Structure and function
 - 3.5.2. Clinical utility
4. Circulating tumor markers
 - 4.1. Tumor associated antigens (TAAs): general aspects
 - 4.1.1. Carcinoembryonic antigen (CEA)
 - 4.1.2. CA15.3
 - 4.1.3. Tissue polypeptide antigen (TPA)
 - 4.1.4. Mucin-like carcinoma associated antigen (MCA)
 - 4.1.5. CA549
 - 4.2. Clinical role of circulating TAAs
 - 4.3. Other circulating markers
 - 4.3.1. HER2/neu
 - 4.3.2. p53 autoantibodies
 - 4.3.3. Cytokeratin-positive cells
 - 4.3.4. Autoantibodies to nucleophosmin (NPM)
 - 4.3.5. Serum soluble vascular cell adhesion molecules: intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin
5. Summary and perspective
6. References

1. ABSTRACT

Here, the structure, function, biological and pathological significance and clinical utility of the principal biomolecular markers of breast cancer is reviewed. Each marker was scored for clinical utility using a recently developed *tumor* marker utility grading system (TMUGS). Among the tissue markers, ERs and PRs are important prognostic/predictive factors and the only tissue markers routinely determined. ER cross-talks with other growth

factors while co-regulatory factors enhance (co-activators) or decrease (co-repressors) its transcriptional activity. C-erbB-2 and Ki67/MIB-1 select for adjuvant chemotherapy a subgroup of lymph-node negative patients at a high risk of relapse. Monoclonal antibodies (trastuzumab, gefitinib, erlotinib and bevacizumab) targeting tissue markers and involved in *tumor* growth and metastasization (EGFR, C-erbB-2, VEGF) have been developed; they showed

Table 1. Main tissue molecular markers of breast cancer and their clinical outcome according to a Tumor Marker Utility Grading System (TMUGS) (203)

Marker	Structure	Function	Utility scale of clinical outcome			
			Gene amplification and/or mutation	Expression	Relapse (prognostic)	Response to therapy (predictive)
ER	protein	nuclear receptor	no	increased	++	++
ER-alpha	phosphoprotein	nuclear receptor	no	Increased	+/-	+/-
ER-beta	protein	nuclear receptor	no	increased	+/-	+/-
PR	protein	nuclear receptor	no	increased	++	++
PR-A	protein	nuclear receptor	no	increased	+/-	NA
PR-B	protein	nuclear receptor	no	increased	+/-	NA
c-erbB-2	proto-oncogene	encodes for HER2/neu	yes	-	+	+
HER2/neu	glycoprotein	membrane receptor	-	increased	+	+
p53 gene	tumor suppressor gene	encodes for wild type p53	yes	-	+/-	+/-
wild type p53	nuclear phosphoprotein	transcription factor	yes	increased	+/-	+/-
Ki67/MIB-1	nuclear protein	proliferation-associated nuclear antigen	-	increased	+	+
VEGF 121, 145, 165, 189, 206 (5 isoforms)	protein	growth factor	-	increased	+/-	+/-

See text for the details. Prognostic factors are those predicting relapse independent of future treatment effects; predictor factors are those predicting response or resistance to a specific therapy. Brief explanation of the used utility scale: 0 = the marker should not be ordered for that clinical use; NA = data are not available; +/- = data are suggestive for a contribution but they are preliminary, thus the marker is still considered highly investigational; + = sufficient data are available to demonstrate a contribution; however, the marker is still considered investigational; ++ = marker supplies information not otherwise available for other measures and it should be considered standard practice in selected situations (203). ¹VEGF 165 is the predominant isoform.

therapeutical single agent activity as well as potent synergy with chemotherapy agents in metastatic cancer. Among circulating markers, some are potentially useful in the early detection and monitoring of metastatic disease; nevertheless, none is routinely recommended. To suspect distant metastases, CEA-TPA-CA15.3 panel attained accuracy of about 90%. ECD HER2-neu, p53 and nucleophosmin antibodies seem suitable candidates for different associations. Preliminary observations suggest that an early detection with tumor markers and successive treatment of relapses significantly prolongs disease-free and overall survival in selected patients. In conclusion, biomolecular markers are improving understanding of biology and management of breast cancer.

2. INTRODUCTION

In the last decade the knowledge of biomolecular bases and the clinical use of breast cancer markers has been evolving greatly and new data are emerging. This accounts for an insight on the issue. Criteria other than that adopted in this review as structure, function and clinical role can be used for grouping the clinically relevant biomolecular breast cancer markers. We grouped them into tissue or biological fluid (blood) markers according to the principal site where they are evaluated for clinical use. Tissue markers include different categories such as intracellular or membrane receptors, oncogenes and tumor suppressor genes, nuclear antigens and growth factors, while circulating markers include the wide category of tumor associated antigens (TAA) and others.

3. TISSUE MARKERS

The tissue markers considered are listed in Table 1.

3.1. Intracellular receptors (Estrogen and Progesterone receptors)

3.1.1. The estrogen receptors (ER-alpha, ER-beta)

3.1.1.1. Structure

The estrogen receptor (ER) is a 65 kDa estrogen-binding phosphoprotein of 595 amino acids expressed by up to 70% of human breast cancers. It is a

member of a large superfamily of nuclear receptors which includes the receptors for other steroids, as well as nonsteroid hormones such as thyroid hormone, vitamin D and retinoic acid (1). Similar to other steroid hormone receptors, ER has a modular structure consisting of distinct domains labelled A-F starting from the N-terminus. To each domain important functions have been attributed (2-5).

Of the ERs, ER-alpha, discovered in 1988, mediates the effects of estrogens on target tissues. ER-beta more recently identified in rat, human and mouse, is a protein of 485-530 amino acids (6-8). ER-beta does not contain the A-B domain, the hinge region and the F domain. It binds estrogens with a similar affinity to ER-alpha and activates the expression of reporter genes containing EREs in an estrogen-dependent manner (6, 9-10).

3.1.1.2. Function

Target genes that are thought to mediate the clinical effects of ERs are those coding for proteins involved in transcription regulation, signal transduction, cell growth, tumor invasion, apoptosis, stress response, cell-cell adhesion and immune response. Some of them are likely to be involved in breast cancer and others are interferon-inducible (11-13). So far, three main different ways of ER function have been described, called classical, non classical and non genomic action (Figure 1). In the classical action, binding to ERs triggers specific conformational changes in the receptor (14) and receptor dimerization. Conformational change facilitates binding of coregulatory proteins, that modify and activate its transcriptional activity in the promoter region of target genes. After hormone binding and dimerization, ERs bind to specific EREs with high affinity through their DNA binding activity (DBA).

The non classical action is the ER regulation of gene expression without directly interacting with DNA, via tethering to other transcription factors such as AP-1 (15), SP-1 (16) and others (17). In the model described for the ER/AP-1 interactions (18),

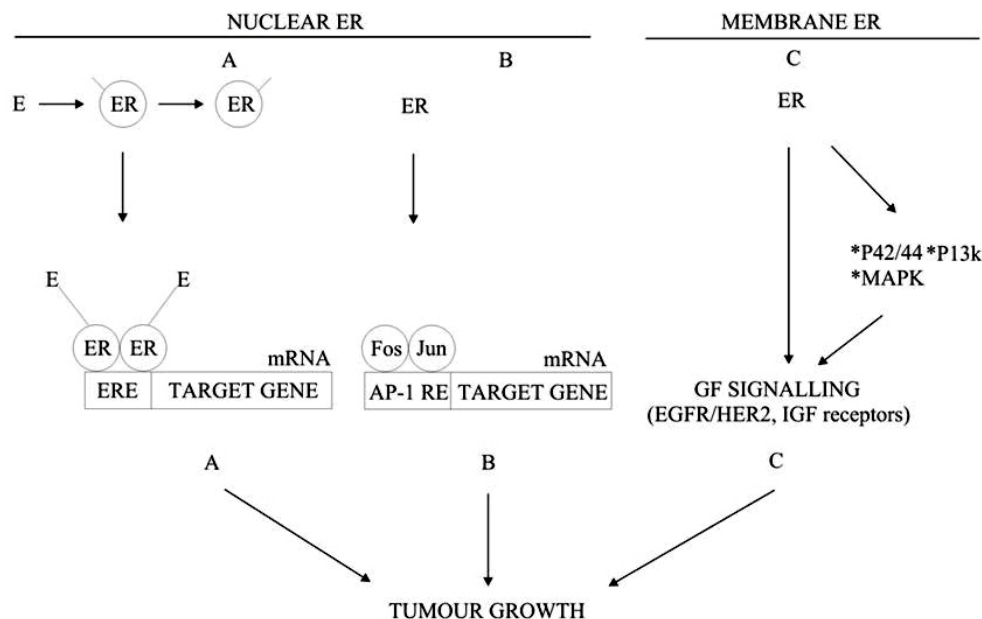


Figure 1. Estrogen receptor function. A) classical and B) non-classical actions; C) non-genomic action; *Signalling kinases (also see text); ERE = estrogen response element; AP-1 RE = AP-1 response element; Fos, Jun = transcription factors.

AP-1 is bound to both its response element and ER proteins. The transcriptional response from an ER/AP-1 complex is dependent on the ER and its ligand. In general, ligands elicit opposing effects through AP-1/ER-beta, compared with AP-1/ER-alpha (19). AP-1 transcription complex comprises either c-jun homodimers, c-jun/c-fos heterodimers or heterodimers among other members of these families (20). Expression and activation of AP-1 are regulated by many extracellular (growth factors and steroid hormones) and intracellular (those initiated by PKC, cAMP, MAPK and Janus kinases) signals. However, the consequences of AP-1 activation appear cell-context dependent. The non genomic action is cross-talk with other growth factor signalling pathways. This action is probably mediated by the receptors' activity close to the cytoplasmic membrane (21-22). Many studies suggest that in breast tumors stimulated ERs promote the expression and/or activation of other receptors (such as EGFR/HER2 family and IGF-I receptor), autocrine growth factors (TGF-alpha and IGF-II) and signalling intermediates (downstream kinases and signalling molecules). However, cross-talk of GF signalling with ER operates in both directions (23). Thus molecular communication from several GF or intracellular kinase signalling pathways to ER pathway can alter its function. Diverse signalling pathways and various agents that raise intracellular kinase activities induce ER activation in the presence of tamoxifen or in the absence of any activating ligand (24).

However, at the moment, the majority of the cellular effects of estrogens in the breast seems to be mediated through ER functioning as a transcription factor (classical action). Furthermore, many tissue-specific effects of ER may be dependent on the cellular pool of other factors that influence the final transcriptional activity and

are therefore defined co-activators or co-repressors (Figure 2) (25-27). The net agonist/antagonist activity of ER ligands depends on ligand induced conformational changes of the receptor and the receptor isoform as well as the particular ensemble of coregulatory proteins and promoter sequences that give functional specificity of the receptor down to the gene level (Figure 3).

3.1.1.3. Clinical utility

ER determination followed the use of the H222 and H226 antibodies (28) to identify different epitopes on the ER, the ligand binding domain (LBD) and the DBA respectively. In particular the H222 antibody forms the basis for two commercially available ER assay kits – an enzyme quantitative immunoassay (EIA) and a semiquantitative immunocytochemistry assay (ICA). Most recent clinical data on ER determination have been obtained using either these commercial kits, that cannot distinguish between ER-alpha and ER-beta isoforms. Nevertheless, it has also been reported that unlike in normal breast tissue, where ER-beta predominates, in most breast tumors ER-alpha is expressed either alone or in combination with ER-beta (29-30). Therefore, it is assumed that most available clinical data mainly reflects ER-alpha function. Overwhelming findings show that in breast cancer, positive ER predicts benefit from antiestrogen therapy and joins with less aggressive biology of tumor (31-33). Moreover, the concentration of ER in the tumor is known to correlate with responsiveness to endocrine manipulations (32, 34-35). Recent results provide support for a role of ER-beta as a poor prognostic factor in breast cancer. In fact, it has been reported that among 60 breast cancers examined, those tumors that coexpressed ER-alpha and ER-beta were significantly node positive and tended to be of higher grade (29). More controversial is the role in

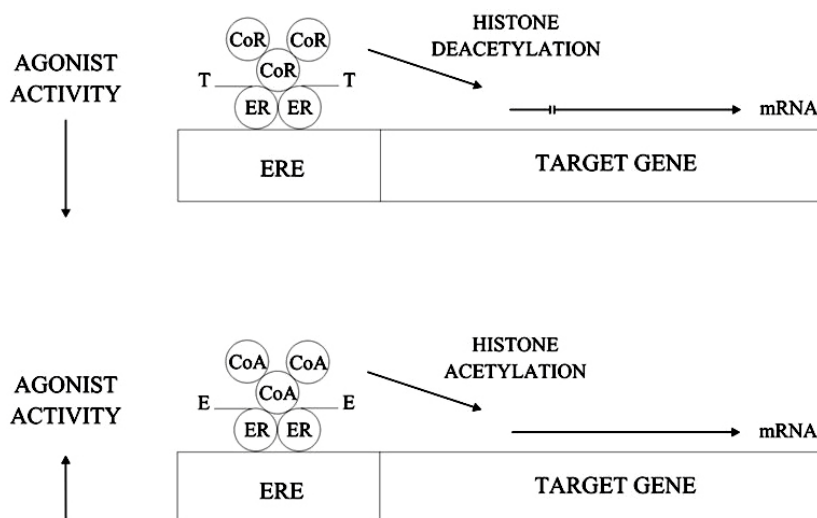


Figure 2. Function of estrogen receptors: role of co-regulatory proteins (activators or repressors) in ER transcriptional activity (see text). T = tamoxifen; E = estradiol; CoR = co-repressor; CoA = co-activator; ERE = estrogen response element.

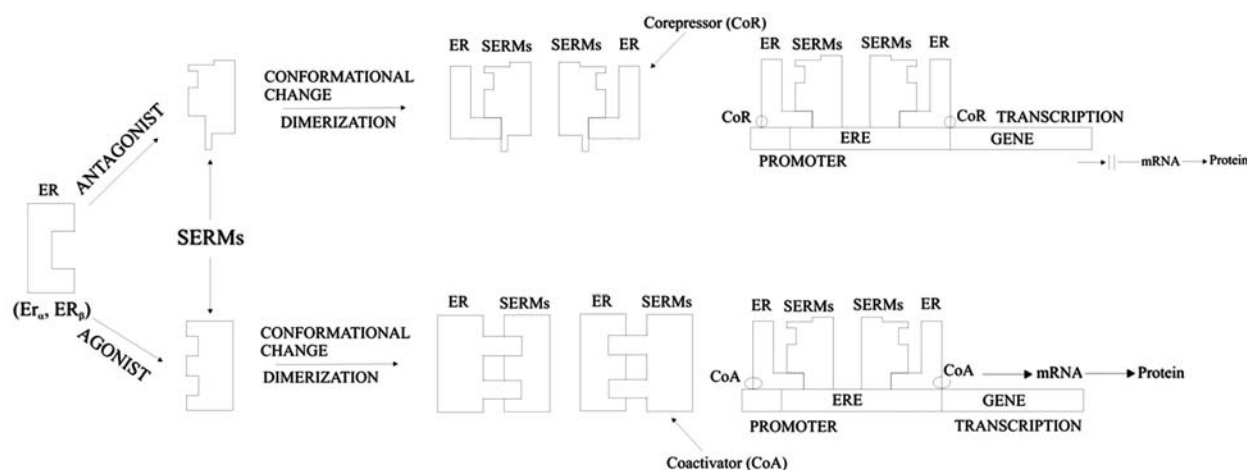


Figure 3. SERMs mediated estrogen receptor action. On binding an agonist or antagonist, the estrogen receptor (ER, the alpha or beta isoform) undergoes a conformational change followed by spontaneous dimerization and interaction of the dimer with estrogen response elements (EREs) located with target genes. Estrogen facilitates the interaction of the ER with co-activators. An antagonist-activated ER, on the other hand, interacts preferentially with a co-repressor protein. Co-regulators: CoR = co-repressor; CoA = co-activator.

the tamoxifen-resistant patients. In fact, up-regulated ER-beta mRNA has been reported to be significantly higher in patients who recurred while receiving tamoxifen than in those who did not (30). However, in another study on 50 ER positive primary breast cancer patients all treated with tamoxifen as the only adjuvant therapy, a low level of total ER-beta was significantly associated with tamoxifen resistance (36).

Therefore, while ER determination has been standard practice for a few years for the decision-making-process of selected situations, ER-alpha and ER-beta must still be considered highly investigational markers (Table 1).

ER plays an important role in breast cancer response and resistance to antiestrogen therapy. Usually, ER is involved in determining the estrogen response through genomic (classical and non classical) action. The precise mechanisms of de novo or acquired resistance to antiestrogens is unknown. However, the most important mechanism driving de novo resistance is lack of ER expression, because > 90% of ER negative tumors will not respond to antiestrogens and patients with ER positive tumors have a significantly higher response rate to antiestrogens than patients with ER poor/ER negative tumors (33). In general, approximately 70% of patients with ER positive/PgR positive tumors will respond to

tamoxifen, whereas 34% or 45% response rates are seen in ER positive but PgR negative or ER negative/PgR positive tumors (33, 37). The predictive power of PgR expression is likely related to the ability of estrogens to induce its expression thus reflecting the existence of an at least partially functional ER signalling pathway (38). For ER positive tumors, it seems likely that no single mechanism predominates for either *de novo* or acquired resistance. Indeed, each tumor, or each subpopulation within a tumor, may utilise a different resistance mechanism (genomic and/or non genomic). Nevertheless, some critical events driving response and resistance to tamoxifen are related to activities regulated, at least initially, through the ER signalling pathway(s). This may explain why so few ER negative tumors respond to antiestrogens and why a majority of initially responsive tumors acquiring resistance continue to express ER.

The ability of cells to acquire an estrogen independent phenotype without concurrently acquiring antiestrogen resistance could reflect the differential regulation of interrelated and/or interdependent gene networks (39-40).

One example of a putative resistance gene is Bcar1/p130Cas which has been identified by insertion of a retrovirus into tamoxifen-responsive cells (41).

Activation of AP-1 results in a down regulation of ER expression (42) and might be expected to antagonise ER function and produce antiestrogen resistance. These latter observations may partially explain the association of an up-regulation of AP-1, a down-regulation of ER and the TAM-stimulated but ICI 182,780 cross resistant phenotype of the MCF-WES cells (43). Consistent with these observations is the ability of transfection with c-jun to down-regulate ER, producing the consequent TAM resistant phenotype (44). AP-1 is an important molecule in signalling to both proliferation and apoptosis, and it is likely that perturbations in its gene regulation activities may explain some antiestrogen resistant phenotypes. One possible mechanism is through AP-1's inhibition of ER expression (42, 45). Conversion to ER negativity is not a particularly common form of acquired resistance (46). However, lack of ER expression is clearly a major *de novo* resistance mechanism. Perhaps the most important contribution of AP-1 is as one of the mechanisms that either initiate and/or maintain the *de novo*, ER negative, resistance phenotype. Nevertheless, further studies are necessary to better define the role of AP-1 in antiestrogen responsiveness/resistance as in many cell systems AP-1 protein expression and DNA binding activity are poor predictors of its transcriptional activity.

A consistent inverse relationship between ER and EGF-R expression has been widely reported in breast cancer cell lines and tumors. Primary breast tumors that have either low ER content, or lost the ability to express ER, frequently express high levels of EGF-R (47-48). This partly explains the association of high EGF-R expression and poor response to tamoxifen. Also there is some evidence that poor response rate to tamoxifen is seen in ER positive tumors that also express EGF-R (49). These and many other experimental data support the hypothesis that

another mechanism of tamoxifen resistance involves cross-talk between ER and growth factor and/or stress kinase signalling pathways (23). The transcriptional (nuclear) and the newly recognised rapid membrane effects of ER can activate growth factor signalling pathways. Furthermore, the kinase cascade generated by the insulin-like growth factor (IGF) and the epidermal growth factor receptor (EGF-R) families can activate ER and its co-regulatory proteins and initiate estrogenic signalling in the absence of estrogens. This causes a vicious cycle of cross talk that leads to enhanced tumor cell survival and proliferation (50). However, the precise contribution of non-genomic action to tamoxifen inhibitory effects remains controversial for the moment.

Immunologic mechanisms of tamoxifen resistance also have been hypothesised. The immunosuppressive activities of estrogens have been known for many years. Some of these effects are likely to be ER mediated, since expression of steroid hormone receptors is widely reported among some lympho-reticular cells. Some authors suggest that tamoxifen resistance derives from loss of responsiveness to tamoxifen-induced NK cell activation and/or changes in the susceptibility of the tumor cell to tamoxifen-induced lysis (33). In experimental breast cancer cells, genes inhibiting innate and adaptive cell-mediated immune system have been reported (51). Thus, other authors (52) hypothesise that tumor cells being in G0-G1 state during clinical benefit from antiestrogen salvage therapy do not inhibit the immune system. At the progression of metastatic disease, they perhaps recover their constitutive function and inhibit the immune system thus contributing to the occurrence of antiestrogen resistance.

3.1.2. The progesterone receptors (PR-A, PR-B)

3.1.2.1. Structure and function

Progesterone receptors (PRs) are ligand-dependent members of the nuclear receptor family of transcription factors and allow progesterone to exert its effects. Two PR isoforms A and B as alternate initiation of translation from the same mRNA or transcribed from two promoters on a single gene (53) exist in progesterone target tissues. Functional domains of both isoforms include the two transactivation domains: activation function-1 (AF1) located in a 90- amino acid segment (aminoterminal) and AF2, located within the carboxyl terminus (54).

3.1.2.2. Gene regulation

Known target genes that are regulated by PRs are those coding for proteins involved in membrane effects (cell adhesion or cytoskeletal interactions, secreted molecules, cytokines/cytokines receptors and chemokines, membrane-bound molecules), metabolism, cell cycling and apoptosis, transcription factors and nucleic acid and protein processing (55). Ninety four genes are regulated by progesterone; 82 of them are upregulated and the remaining 12 are downregulated. Among the upregulated genes, 59 are uniquely upregulated by PR-B, 4 uniquely by PR-A and only 19 by both. Among downregulated genes, 6 are targets of both PR isoforms, and 6 unique for PR-B. Interestingly, none are uniquely downregulated by PR-A (55). The low

number of genes regulated by both receptors is surprising and suggests that the two PR isoforms serve different functions.

Liganded PR-B homodimers regulate many more genes than PR-A, through the unique activation AF-3 function although the specific mechanism of AF-3 function is still unclear (56). With regard to the small number of genes that are uniquely regulated by PR-A, it is likely that only PR-A homodimers can bind their promoters. Many of the downregulated genes encode immune system proteins, suggesting a role of progesterone in protecting the fetus from immune attack.

Nuclear receptors are a class of transcription factors that can act as both activators and repressors of transcription. PR-A can inhibit PR-B isoform. Unlike hPR-B, hPR-A is not transcriptionally activated by progesterone antagonists. When the two receptor forms are present, the hPR-A phenotype is dominant (57). In summary, a series of cellular functions are activated by the two PR isoforms and the resulting combination of their effects determines the PR transcriptional activity.

In vivo the two PR isoforms are usually coexpressed in normal cells but their ratio varies in different tissues, physiological states and in disease (58).

3.1.2.3. Pathologic and clinical considerations

Because PR-A and PR-B isoforms have markedly different transcriptional effects, the function of PR-A/PR-B heterodimers may differ from that of the homodimers and the ratio of PR-A to PR-B is likely to control the final response of PR receptors. PR-A overexpression in transgenic mice results in extensive mammary epithelial cell hyperplasia, excessive ductal branching and a disorganised basement membrane, all features associated with malignancy (59). In contrast, PR-B overexpression leads to an under-differentiated state, that is to premature ductal growth arrest and inappropriate lobulo-alveolar development (60). When PR-A are selectively knocked-out, leaving only PR-B, early mammary gland development appears to be normal (61). In the breast equal expression of the 2 PRs is necessary for normal development and differentiation while PR-A to PR-B ratio is extensively dysregulated in breast cancers. An analysis of 202 PR positive breast cancers by immunoblotting showed that expression levels of PR-A were higher than PR-B in 59% of tumors and were 4 fold or greater in 25% of tumors (62). In another study of 32 PR positive breast cancers, excess PR-B correlated with the absence of HER2/neu indicating a good prognosis, while excess PR-A correlated with a poorly differentiated phenotype and higher tumor grade (63). These *in vivo* and other *in vitro* data (64) suggest that an excess of PR-A is particularly harmful in the breast.

Current immunohistochemical clinical PR assays do not distinguish between the two isoforms. In fact, recently it has been found that several clinically used anti-PR antibodies fail to detect PR-B by immunohistochemistry (65).

PRs subdivide ER-positives breast cancers (66-68). In fact, ER-positive/PR-positive tumors are much more likely to respond to hormone therapies than tumors with only one of the receptors. Additionally, independent of ERs, PRs are prognostic markers whose presence indicates reduced aggressiveness and a favourable disease-free survival profile and current clinical data suggest that PRs are superior to ERs as overall prognostic markers (56, 69).

PR as ER determination is standard practice in selected situations, while PR-A and PR-B must still be considered highly investigational markers (Table 1).

3.2. HER2/neu (erbB2)

3.2.1. Structure

The HER2 gene (c-erbB2 proto-oncogene) is located on chromosome 17 (17q11.2-q12) and encodes a 185 KDa transmembrane glycoprotein with tyrosine kinase activity that is a member of the erbB family of receptors (also known as type 1 receptor TKs). This receptor family, that plays a major role in promoting breast cancer cell proliferation and malignant growth (70) is comprised of four homologous receptors: the EGFR (erbB1/HER1), erbB2 (HER2/neu), erbB3 (HER3) and erbB4 (HER4). These receptors are composed of an extracellular binding domain, a transmembrane lipophilic segment and an intracellular protein TK domain with a regulatory COOH-terminal segment. HER3, however, is different from the other members in that it has a deficient TK domain (71).

A cross-talk among the erbB family also regulates the cellular effects mediated by these receptors. At least six different ligands (EGF, TGF- α , amphiregulin, heparin-binding EGF, betacellulin and epiregulin, known as EGF-like ligands) bind to EGFR (72). Another class of ligands, collectively termed heregulins, binds directly to HER3 and/or HER4 (73). Unlike the other erbB receptors, a soluble ligand of HER2 has not yet been identified (71).

3.2.2. Function

After ligand binding, the erbB receptors become activated by homodimerization or heterodimerization. No cognate ligand for HER2 has been found. However, HER2 is known to be the preferred co-receptor for the EGFR, HER3 and HER4 (72). Therefore, HER2 signals by the heterodimerization with the erbB receptor family (74, 75). Studies with breast cancer cell lines and human tumors have also demonstrated constitutive phosphorylation of HER2 (76) although the biochemical basis is not clear (77). After receptor dimerization, and tyrosine kinase phosphorylation, recruitment and phosphorylation of several intracellular substrates, as well as the binding of docking and adaptor molecules to specific phosphotyrosine sites on receptor molecules occur. Under physiological conditions, two important downstream signalling routes of the erbB family have been described. One is via the Ras-Raf-MAP kinase pathway (78), that through activation of MAPKs, ERK1 and ERK2 regulates transcription of molecules that are linked to cell proliferation, survival and transformation in laboratory studies (79). The other is via

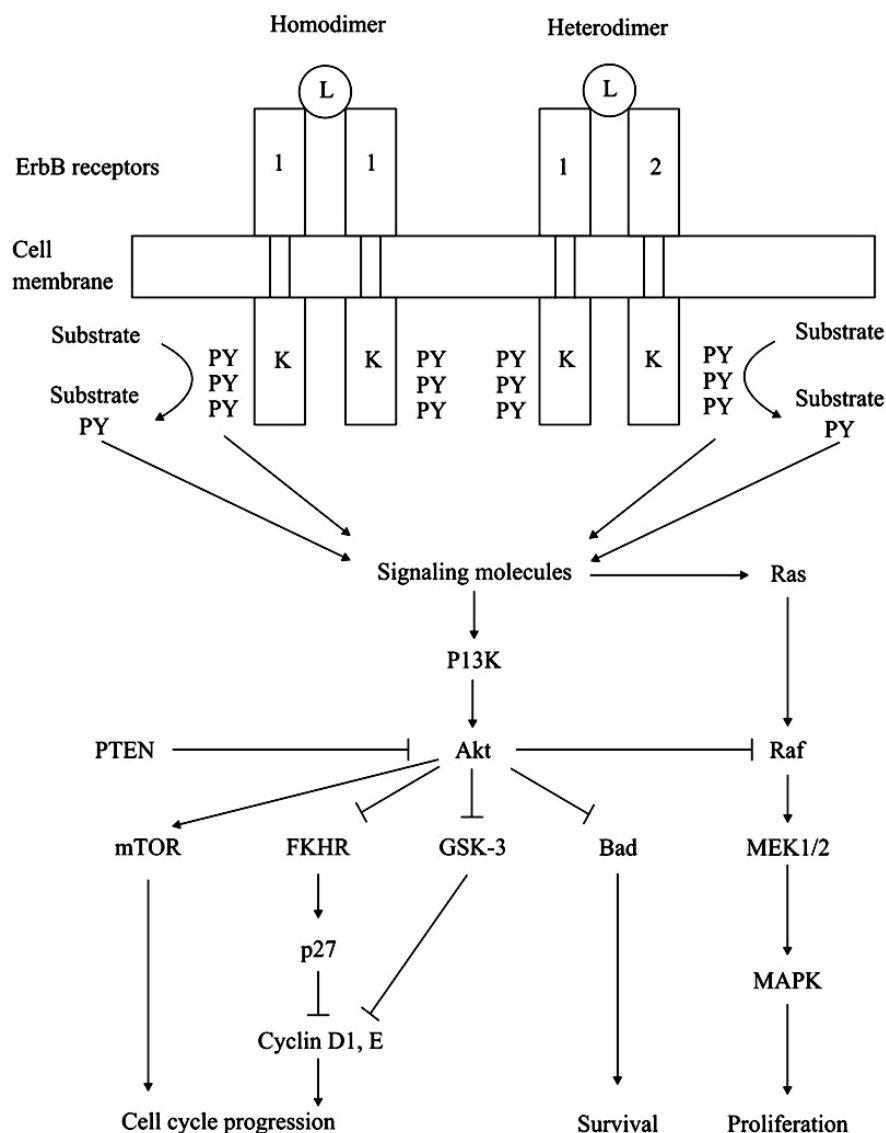


Figure 4. Activation and signalling mechanisms of epidermal growth factor receptors. L = ligand; 1 = erbB-1 extracellular binding domain; 2 = erbB-2 extracellular binding domain; K = tyrosine kinase domain; PY = phosphorylation.

phosphatidylinositol-3-kinase (PI3K) and the downstream protein-serine/threonine kinase AKT (80, 81). AKT transduces signals that trigger a cascade of responses from cell growth and proliferation to survival and motility (Figure 4) (80). Another route for signalling is via the stress-activated protein kinase pathway, involving protein kinase C and Jak/Stat. However, in tumor cells these receptors can be activated by additional mechanisms. In fact, ligand-independent mechanisms of receptor activation have been described with receptor overexpression, with mutant forms of receptor from gene rearrangements or via the urokinase plasminogen receptor (82-83).

HER2/neu activity was evaluated either by measurement of gene amplification or overexpression of its product, the 185 KDa glycoprotein. For measuring gene amplification southern blot (SB) or

polymerase chain reaction (PCR) were used, while methods to determine p185 overexpression included northern blot (NB), fluorescent *in situ* hybridization (FISH), western blot (WB) and immunohistochemistry (IHC). For the immunohistochemical staining different monoclonal and polyclonal antibodies and paraffin-embedded archived material are used. Molecular analysis of frozen breast cancer specimens indicates that amplification is closely associated with overexpression (84-85). However, in human breast cancer erbB2 positivity varies with respect to the method used for evaluation and different accuracy among different methods has been found (86). Nevertheless, when large series were considered, erbB2 positivity appeared to be very similar with the various methods (20-23.6%) and the overall rate of positivity in 22616 cases examined was 21.4% (87).

3.2.3. Clinical utility

The most consistent observation is that erbB2 positivity is strongly and inversely related to ERs and PRs, being related to a worse grade of malignancy and being related to a higher frequency of aneuploidism (87). Several studies also found a close association between erbB2 positivity and a high proliferation rate (87-88) and a correlation between HER2 overexpression and shorter disease-free (DFS) and overall survival (OS). This correlation was observed when the whole population of axillary lymph-node positive and negative patients was considered and univariate analysis was applied (87). When the multivariate analysis was used in the same studies, the correlation was significant only in a few of them (87). In lymph-node positive patients most reports confirmed a shorter DFS and OS both by uni and multivariate analysis, while in lymph-node negative patients they did not (87-88). However, usually erbB2 does not change the risk of node positive patients so much to affect the clinical decision on adjuvant therapy. In node negative patients HER2 expression in association with proliferative activity identifies a subgroup of patients with the worst prognosis who are candidates for specific intensive adjuvant therapy (89). Moreover, breast tumors that co-overexpress EGFR and HER2 exhibited a worse outcome than tumors that overexpressed either receptor alone (90).

So far conflicting data have been reported as to whether HER2/neu overexpression is associated with hormone and chemo-resistance both in the adjuvant and in the metastatic setting. In fact some have suggested that HER2/neu overexpression is associated with hormone (87, 91-92) and chemo-resistance (87), whereas others have found no such association (87, 93, 94).

Recently, a humanized monoclonal antibody directed against the extracellular domain of 185 KDa HER2 protein and called trastuzumab was constructed for therapy. Metastatic breast cancer women with HER2 overexpressing (+3 by IHC) and/or gene amplification (by FISH) tumors are candidates for trastuzumab. In these patients, compared with chemotherapy alone, chemotherapy with trastuzumab showed to improve clinical outcome, including response rate, time to progression and overall survival (95). In addition, experimental data confirm that HER2 transmodulation via EGFR signalling implies that specific EGFR tyrosine kinase inhibitors will be effective against breast tumor cells overexpressing HER2 as well as EGFR. This suggests their use in combination with HER2 antibodies against mammary carcinomas with high levels of the HER2 proto-oncogene (71, 96).

3.3. p53, gene and protein

3.3.1. Structure and function

The human p53 tumor suppressor gene has been mapped to chromosome 17p53, contains 11 exons and highly conserved regions (97-98). The p53 gene encodes for a 53KDa nuclear phosphoprotein called wild-type p53. This nuclear phosphoprotein has been implicated in controlling cell-cycle regulation, cell differentiation and the surveillance of genomic integrity (99-100). The p53 protein

is functionally divided into 3 domains: a central DNA-binding domain, an amino-terminal transactivation domain and a carboxy-terminal oligomerization domain. Since the DNA binding domain contains most of the p53 gene mutations identified in tumors, the ability of p53 protein to bind DNA and function as a transcription factor is probably a major component of its tumor-suppressor activity. The p53 protein transcriptionally regulates numerous genes as part of signal transduction pathways initiated by cellular stresses. Following a cellular stress, such as DNA damage, hypoxia or the activation of an oncogene, p53 protein levels increase as a result of increased synthesis and a longer half-life, then p53 protein activates transcription of p53-regulated genes and results in cell-cycle arrest or apoptosis (101). Wild-type p53 has two major functions: a) regulation of the checkpoints G1 to S and G2 to M of the cell cycle; b) induction of apoptosis after genotoxic damage. As to the first function, activated p53 causes a G1 cell-cycle arrest by increasing the transcription of the cyclin-dependent kinase (cdk) inhibitor p21 (102). The cdk inhibitor p21 blocks cdk4 activity and thereby inhibits entry into S-phase. In fact, cdk4 activity through retinoblastoma protein increases the transcription of S-phase genes. Activated p53 may also contribute to a G2 arrest by increasing the transcription of 14-3-3 σ . 14-3-3 σ sequesters the Cdc25C phosphates in the cytoplasm preventing it from entering the nucleus and activating cdc2, the cdk that promotes G2/M transition. As to the second function, p53 initiates apoptosis by inducing transcription of genes that regulate programmed cell death. p53 induction of bax can cause cytochrome C release from mitochondria which activates caspases, able to kill the cell. p53 can also induce the death receptors DR5 and FAS, which can activate caspases after binding to their respective ligands, TRAIL or FAS ligand. p53 also increases the transcription of IGF-BP3 which binds to the insulin-like growth factor-1 protein, preventing it from sending signals that inhibit caspase activation. Cellular oxidation genes are also induced by p53 and may cause apoptosis through redox mechanisms. Cell type, cellular environment and genetic context are important to determine whether a cell responds to a cellular stress with p53-induced cell-cycle arrest or with p53-induced apoptosis (101). The p53 mutations in breast tumors vary from 20 to 40% (103), although the timing of the involvement of these mutations in breast carcinogenesis remains unclear. In a few studies p53 mutations have been found in pre-invasive breast lesions as ductal carcinoma *in situ* (DCIS) and atypical hyperplasia (104-106). Besides, a significantly higher frequency of p53 mutations has been reported in high-grade than in intermediate-grade and low-grade DCIS (40 vs 4% and 0% respectively) (103). These findings suggest that when p53 gene mutation occurs, it represents an early event in breast carcinogenesis and may be a useful marker for an increased risk of progression to invasive and more aggressive breast cancers (103). Recently, in cancers with a wild-type p53 gene status, overexpression of a novel and critical inhibitor of p53, COP1, was correlated with a strong decrease in steady state p53 protein levels and attenuation of the downstream targeted gene, p21. This contributes to an accelerated degradation of p53 protein and attenuates the tumor suppressor function of p53 (107).

3.3.2. Clinical utility

Many studies showed that alterations in p53 are associated with poor prognosis. In fact, mutated p53 was found in a higher percentage of patients with inflammatory breast cancer (108) and p53 overexpression was associated with a worse outcome in high risk primary breast cancer patients (102, 109-110). Nevertheless others have not detected a similar significant association (102, 109-110). The lack of agreement may be due to different methods of detection of p53 alterations (111). In fact, while most investigators used immunohistochemical detection of mutant forms of p53 overexpression, others conducted more complete molecular analysis with determination of specific DNA alterations (112, 113). Controversial data also have been reported on predictive value in response to chemotherapy (102, 108, 114). It has been suggested that, depending on which p53-modulated activity predominates in a particular cell line, different observations might be expected after treatment of cells with p53 mutations (115). So, in cells in which the control of S phase entry predominates, the loss of wild-type p53 would sensitize the cell to DNA damage. Conversely, if apoptosis induction predominates, loss of functioning p53 would confer resistance.

3.4. Ki-67/MIB-1

3.4.1. Structure and function

Ki-67 is a non-histone nuclear protein that is closely linked to the cell cycle. It is expressed in proliferating cells during mid G1 phase, increases in level through S and G2 and peaks in the M phase of the cell cycle. It is rapidly catabolized at the end of the M phase, and it is undetectable in resting (G0 and early G1) cells. The function of Ki-67 is poorly understood. It was first isolated from a Hodgkin's disease cell line and it is located in the nucleus, possibly associated with the nucleolus and/or fibrillar components (116).

Measurement of cell proliferation has prognostic value for human breast cancer (117-118), and is one of the innumerable factors that have been proposed to supplement pathologic stage. A variety of methods have been used to assess cell proliferation. Proliferating cells can be identified with a monoclonal antibody specific for bromodeoxyuridine (BrdUrd), that is a thymidine analog. A brief *in vivo* infusion of BrdUrd just before tumor removal labels cells in S-phase similarly to those submitted to *in vitro* 3H-thymidine exposure (119). Other methods include S-phase fraction (SPF) measurement by flow cytometry and mitotic index established using large core needle biopsy specimens (120). However, the advent of antibodies, which recognise antigens related to cell cycle proteins and can be used for immunostaining archival material, has facilitated routine assessment. MIB-1 is an antibody that recognises the Ki-67 epitope in paraffin-embedded tissue. Among antibodies that have been raised against cell cycle specific antigens, MIB-1 is being used increasingly (121). The MIB-1 staining is commonly expressed as a percentage of staining cancer cells to the total population. As expected, BrdUrd labeling index (LI) correlated with a Ki67 LI based on MIB-1 antibody. Nevertheless, BrdUrd, mitotic index and SPF measurement were found to be more effective

prognostic and predictive factors than Ki67 (120, 122). Moreover, the appropriate cut-off values that distinguish between high and low proliferative activity using Ki67 has not been universally standardized (123). The relationship of Ki67 protein expression with gene expression profiles has not been fully investigated (124).

3.4.2. Clinical utility

Increased percentages of Ki-67 positive cells have been described as an independent negative prognostic factor for relapse and overall survival in some (122, 125-127) but not all studies (128-129). In a recent report, MIB-1 was determined in skeletal metastases of breast cancer patients operated on for pathological fracture. No correlation was observed between MIB-1 in skeletal metastases and post-operative survival or survival from diagnosis of breast cancer (130). Proliferation markers have been found associated with tumor sensitivity to chemotherapy; a positive correlation between pathological response to neoadjuvant chemotherapy and cell proliferation as assessed by Ki-67 expression (120, 131-132) has been reported.

3.5. Vascular endothelial growth factor (VEGF)

3.5.1. Structure and function

The human VEGF gene is located to chromosome 6p21.3 and consists of 8 exons and 7 introns (133). The molecular mechanisms of the increase in VEGF mRNA and VEGF protein production are not yet understood, although insulin, insulin-like growth factor-1, corticotropin, thyrotropin and steroid hormones have been reported to affect VEGF mRNA production. VEGF protein, also referred to as vascular permeability factor (VPF), is the most commonly studied vascular growth factor, specific mitogen and survival factor for endothelial cells and key promoter of angiogenesis. At least five isoforms of the VEGF protein, composed of 121, 145, 165, 189 and 206 amino-acids respectively can be translated, because of alternative VEGF mRNA splicing (134). The predominant isoform VEGF165 is a heparin-binding basic homodimer of 45 KDa that remains partly bound to the cell surface and the extracellular matrix (135).

VEGF protein acts primarily in a paracrine way and binds with high affinity to two receptors of the basal membranes of the endothelium. They are tyrosin kinase receptors, the FMS-like kinase (Flt-1, VEGFR-1) and the kinase domain receptor (KDR, VEGFR-2), which are produced predominantly by endothelial cells. Recently, a new form of VEGF receptor, neuropilin-1 has been identified with an important role in vasculogenesis (136). Binding of VEGF causes receptor dimerization and autophosphorylation for signalling. The antiapoptotic and mitotic functions of VEGF are mediated by KDR. Tumor cells tend to overexpress VEGF constitutively and hypoxia increases the production of Flt-1 and KDR although this increase is smaller than that of VEGF.

3.5.2. Clinical utility

VEGF has been reported to be an independent unfavourable prognostic factor for relapse and survival in a few studies (137-141). In some of them, this occurred only

Table 2. Main circulating markers of breast cancer and their clinical outcome according to a Tumor Marker Utility Grading System (TMUGS) (203)

Marker	Structure	Function	Prognostic or predictive value-clinical outcome				
			Relapse (P)			Monitor course (M)	
			Sensitivity % (range)	Specificity % (range)	Utility scale	Low, high correspondence	Utility scale
<i>TAA5</i>							
CEA	glycoprotein	cell-cell interaction	7-50% ^{149, 152, 155}	88-100% ^{149, 152}	+	low to high ^{149, 182}	+/-
CA15.3	glycoprotein	mucins	37-67% ^{152, 155}	92-100% ^{149, 152}	+	high ^{149, 182}	+
TPA	protein	cytokeratines	51-86% ^{155, 169-171}	48-72% ^{169, 165}	0	high ^{182, 184}	0
MCA	glycoprotein	mucins	43-84% ^{165, 177-178, 181}	43-89% ^{165, 177-178, 181}	0	high ^{162, 185}	0
CA549	glycoprotein	mucins	50-70% ^{179, 181}	79-98% ^{179, 181}	0	low to high ^{166, 182-183}	0
<i>Others</i>							
ECD HER2/neu	protein	antigen	31-45% ^{152, 190}	100% ¹⁵²	+/-	unclear ^{86, 193}	+/-
p53 antibodies	immunoglobulins	antibodies	0-46% ²⁰¹⁻²⁰²	>81% ¹⁹⁹⁻²⁰⁰	+/-	unclear ²⁰¹⁻²⁰²	+/-
Nucleophosmin antibodies	immunoglobulins	antibodies	NA	NA	+	NA	+/-
Cytokeratin-positive cells	protein	cytokeratines	NA	NA	+ ^{2a}	NA	+/- ^{2b}
ICAM-1	glycoprotein	adhesion molecule	NA	NA	+/- ^{2a}	NA	+/- ^{2b}
VCAM-1	glycoprotein	adhesion molecule	NA	NA	+/- ^{2a}	NA	+/- ^{2b}
E-selectin	glycoprotein	adhesion molecule	NA	NA	+/- ^{2a}	NA	+/- ^{2b}

See text for the details. For explanation of utility scale see Table 1. For TAAs, ECD HER2/neu, p53 and nucleophosmin antibodies prognostic value derives from rising level predicting an impending relapse; for predictive value see text. P = primary cancer; M = metastatic cancer; ECD = extracellular domain. ¹Referred to progression of disease, or response to therapy (complete or partial), or no change: high > 60%; low < 60%. Although they are investigational (+) or highly investigational (+/-) data are available as prognostic factor (^{2a}) or predictor factor (^{2b}). Data are not available (NA).

in subsets such as N- (134, 138, 142), N+ (137) or ER+ (143) patients. In a recent report on whole breast cancer population VEGF immunostaining was not a significant (univariate analysis) predictor of relapse. By multivariate analysis it was a weaker unfavourable prognostic factor than conventional clinical histopathological and other common biomolecular markers. Moreover it was one of the prognostic indicators included in the logistic regression, that showed the highest sensitivity and specificity (144). In the same study VEGF immunostaining was a significant unfavourable factor in N+ but not in N- subsets, as recently confirmed by others (145). As to its use as a predictive factor, so far in no relevant clinical study has it been reported to predict response to chemo and/or endocrine therapy. Recently, recombinant humanized monoclonal antibodies to VEGF have been prepared and in a phase III study in patients with refractory metastatic breast cancer doubled the response rate to capecitabine, however, survival was not affected (146).

4. CIRCULATING TUMOR MARKERS

4.1. Tumor associated antigens (TAAs): general aspects

Many human tumors produce substances that pass into the blood and may serve as tumor markers. More precisely, circulating tumor markers may either be 1) tumor-derived, i. e. produced by the tumor itself, or 2) tumor-associated, i.e. produced by other tissues in response to the presence of the tumor. Among different types of circulating tumor markers, TAAs are more widely investigated. They are tumor-derived proteins or glycoproteins and are detected in serum usually by immunological methods. Several monoclonal antibodies (MABs) have been described that are reactive with human mammary carcinomas (147, 148). In general, they are classified into three groups based on the immunogen used to generate the MAB. The immunogen can derive from breast tumor cell lines, milk fat globule membrane or membrane-enriched extracts of breast carcinoma metastases. Each MAB is characterised with respect to either percentage of reactive mammary tumors, percentage

of reactive cells within the tumor, location of reactive antigen within the tumor cell, or degree of reactivity with non mammary tumors as well as normal tissues (147, 148). This antigenic variability accounts for a different pattern of staining (focal staining, diffuse cytoplasmic staining, membrane staining and apical staining) with a given MAB within the same tumor. Antigenic reactivity can change in different areas probably because it may reflect, in part, cells in different phases of growth. In fact some MABs are most reactive with tumoral cells during the S-phase of the cell cycle. It also can change at different times; in fact, *in vitro*, some clones of tumoral cells showed a dramatic change in antigenic phenotype through-out the observation period. The antigenic variability is an important problem in the development and optimization of immunodiagnostic procedures for the management of breast cancer.

Many authors reported on the usefulness of TAAs in breast cancer patients to detect post-operatively relapses and monitor response to treatment (149). Among them, only CEA and CA15.3 are considered clinically relevant (149) (Table 2). However, the last updated guidelines of the American Society of Clinical Oncology (ASCO) (44) do not recommend the use of any circulating tumor marker post-operatively as they are not considered sufficiently accurate to be used routinely. In the last decade we and others (150-153) have defined suitable criteria for the use of circulating TAAs. Consequently, we reported a high accuracy of CEA-CA15.3-TPA association in the post-operative follow-up of breast cancer patients and identified clinically important benefits from their use (153-155). Therefore, these three markers are considered as follows. In addition, two other TAAs which appeared promising in the past or at the present time, are taken into account.

4.1.1. Carcinoembryonic antigen (CEA)

First described by Gold and Freedman (156), CEA is a serum glycoprotein. CEA is one of at least 19 related molecules that are members of the immunoglobulin gene superfamily. The functions of this family, thought to

be different, have as a common trait the fact that they are well characterised and that they serve cell-cell interaction, recognition or adhesion. In a study (157) it was postulated that CEA works as an adhesion factor for circulating tumor cells thus facilitating the formation of liver metastases. Since various members of the CEA gene family are found on the surface of white blood cells, e. g. monocytes, macrophages, and granulocytes, as well as on the surface of normal colonic mucosa cells, it was also suggested that these antigens play a role in the bacterial recognition of, and adhesion to, the mentioned cells. It is quite evident that further studies are needed to fully elucidate the true role (or roles) of CEA, which is an embryonic antigen before becoming re-expressed as a tumor antigen. The liver is the major site for clearance of CEA. CEA half life is 3-11 days (158).

Current CEA test uses immunoradiometric (RIA) or immunoenzymatic (EIA) assays. RIA assay uses a double monoclonal or monoclonal-polyclonal antibody format. Their interassay coefficient of variation ranges from 10% to 12% for mean values ranging from 3 to 5 ng/mL, and from 6% to 10% for CEA concentrations of 10 to 100 ng/mL.

All CEA test methods give a normal range of less than 3.0 ng/mL for non-smokers and less than 5.0 ng/mL for smokers.

4.1.2. CA15.3

CA15.3 is a mucin-like membrane glycoprotein with a molecular weight of 290 KDa, which is shed from tumor cells into the bloodstream. The antigen is present in high levels on apical borders of differentiated secretory mammary epithelial cells and in the cytosol of less differentiated cells. Prognostically favourable breast carcinomas as *in situ* carcinomas or tubular carcinomas and benign breast lesions show predominantly apical reactivity using a murine monoclonal antibody designated DF3 prepared against a membrane-enriched fraction of a human breast carcinoma (147). These lesions are organised around a lumen and have a cytoplasm differentiated into apical and basal regions. As the mammary epithelium progresses to infiltrating ductal carcinoma, a further loss of morphogenic capability and an increase in cytoplasmic staining with MAB DF3 occur.

MAB 115-D8 (159), developed at the Cancer Institute in Amsterdam (Holland) using as immunogen human milk fat globules, is utilised as “catcher” and MAB DF3 as “tracer” in a sequential double-determinant radioimmunoassay (RIA) to monitor circulating DF3 antigen. With this method the value higher than 150 U/mL is considered suspect. However, with a double determinant enzyme-linked immunoassay (EIA) a value higher than 30 U/mL is considered suspect (160).

4.1.3. Tissue polypeptide antigen (TPA)

TPA from cancer tissue consists of 4 different fragments A, B1, B2, C, with a molecular weight ranging from 20,000 to 40,000 Da. The B1 fragment with a molecular weight of 43,000 Da is an unbranched peptide that possesses antigenic and immunogenic properties and

takes part of the cytoskeleton. Proliferating cells of tumor or normal origin produce and release TPA. In cell culture (HeLa) perinuclear formation of TPA is seen early in the S-phase, followed by the development of a network of TPA throughout cytoplasm. Coinciding with the end of the S-phase, most TPA is localised close to and in the plasma membrane. After cell division, TPA is externalised leaving the cell free of visible TPA. At this stage, a rise of TPA in the cell culture medium is observed. Also, it has been hypothesised that TPA represents a degradation product resulting from cell death. In fact, solubilized break down products from intermediate filaments of the cytoskeleton, such as cytokeratins, appear in serum following decomposition of epithelial tissue. In particular, cytokeratins 8, 18 and 19 have been mentioned as a source of break down products that react in serum with certain antibodies to intact cellular keratins. Some markers such as TPA are claimed to represent related parts of cytokeratins 8, 18, 19 or 8 and 18. Therefore, tests for constituents of cytokeratins should provide information about cell destruction. Cell destruction can reflect the (total) cellular turnover and therefore the size of the tumor (161).

Currently, radioimmuno (RIA) and immunoenzymatic (EIA) assays are in use for determination of TPA. The RIA assay is based on sequential saturation of anti-TPA antibodies. The cut-off limit is 85 U/L. The ELISA assay is a solid phase sandwich assay based on immunochemical technique. A value higher than 95 U/L is considered suspect.

4.1.4. Mucin-like carcinoma associated antigen (MCA)

MCA is a molecule with antigenic and immunogenic properties reactive with MABs b-8, b-12 and b-15 (all IgG), raised against a mixture of different human breast carcinoma cell lines (ZR-75.1, MCF-7, HsO578T, SK-BR-3) (162). This molecule is a glycoprotein belonging to the heterogeneous family of mucins. Mucins are produced by specialised epithelial cells present in many exocrine tissues of different apparatuses (respiratory, genitourinary and gastrointestinal tracts) and by mammary, sweat and salivary glands. Mucins are also expressed and detectable in the serum of cancer patients. Mucins act primarily as a biological barrier to protect epithelia against osmotic and pH gradients, physical injuries, and viral and bacterial infections. Mucins consist of a protein backbone which is a non globular polypeptide to which oligosaccharides are attached (163). MCA takes part of type-1 mucin that is predominantly produced by mammary glands. It has a unique N-terminus and C-terminus and repeating sequence of 20 amino-acids (162). MCA was previously purified from ZR-75.1 and MCF-7 cell line extracts and supernatants and then from human milk by immunoaffinity chromatography with the monoclonal antibody b-12. MCA from human milk carries the epitopes defined by the MABs b-8, b-12 and b-15, but has a greater molecular weight (450 KDa) and it is present in this biological fluid in high concentrations (164). The antibody b-12 was used in a sandwich enzyme immunoassay to measure MCA concentration in biological fluids and in serum. The most commonly used cut-off values are 11 or 15 U/mL (162, 165).

4.1.5. CA549

CA549 is a circulating breast cancer associated antigen, that reacts with the murine monoclonal antibody BC4E 549 raised against a human breast cancer cell line. It is an acidic glycoprotein of apparent molecular weights of 400,000 and 512,000 Da (166).

A double monoclonal immunoradiometric assay is used. It employs the murine BC4N 154 raised against human milk fat globule membranes (HMFGM) as the capture antibody and BC4E 549 as the labelled antibody. The cut-off limit above which the serum level is considered positive is 10-12 U/mL (166).

4.2. Clinical role of circulating TAAs

No relevant prognostic role of their serum baseline levels has been observed (149, 167). Following the report of the ASCO in 1996, measurement of their circulating levels in post-operative management of breast cancer is not routinely recommended because previous data showed a low accuracy in predicting metastatic disease (149). Nevertheless, enough data are available which document high CEA and CA15.3 specificity and their contribution in the early detection of metastatic disease. This occurs in some patients in whom metastatic cells express these antigens (149, 152, 155).

In the last two decades the experience gained with the use of serum TAAs has suggested that although post-operatively none of them alone has enough diagnostic accuracy this could be attained when the following interventions and criteria have been adopted: a) association of more suitable tumor markers, b) dynamic evaluation of their concentration values, c) accurate patient's history and clinical examination.

To "early" detect breast cancer relapses, sensitivity of CEA or CA15.3 ranges from 7% to 50% or from 37% to 67% respectively (149, 152, 155, 168). Sensitivity of TPA has been reported to range from 51% to 86% (198, 155, 169, 170, 171). The association of more suitable markers, commonly 2 or 3, increases sensitivity (151, 152) but reduces specificity mainly because concomitant acute or chronic benign pathology is responsible for the falsely positive results (149, 155, 168). In particular, transient and chronic liver failure, diabetes and/or hepatic steatosis, acute joint or upper airways inflammations are more often the probable reason responsible for tumor marker increase in non-relapsed patients (155, 168). However, dynamic evaluation of their values and an accurate patient's history at each control visit allow to decrease considerably the number of the patients falsely suspected of a pending relapse (155, 168).

Dynamic evaluation, i.e. not the serum value itself but the time related changes of the tumor marker, is obtained by serial re-testing 2 to 4 weeks after a high value of one or more tumor markers of the association. This permits to distinguish three different kinds of tumor marker increase with different predictive values: isolated elevated value (IEV), constant elevation (CE) and progressive increase (PI). If the initially elevated tumor marker

decreases to a normal level, this is an IEV. It was reported that the IEV is commonly due to a concomitant transient benign pathology or acute worsening of a chronic benign pathology (155). Therefore in this case the predictive value for a pending relapse is very low and this patient does not need further radiological evaluation. We define as progressive the increase which is >30% higher in the sample following the initially elevated value, otherwise we define as CE two similarly high values. CE or PI in patients without a concomitant benign pathology considering an accurate history showed to have unfavourable predictive value. Only these should be strongly suspected as relapsing patients and submitted to instrumental examinations to localise the relapse.

Using these criteria, diagnostic accuracy of CEA-CA15.3-TPA association was about 90% with a sensitivity ranging from 80% to 91% and specificity ranging from 91% to 100% (153-155, 172-173). CEA-CA15.3-TPA association allows to post-operatively monitor breast cancer patients with a great saving of radiological examinations. In fact, disease-free patients can be followed-up at regular intervals of 4 or 6 months (consistent with the high or low risk of relapse) with only physical examination, routine chemistry and serum CEA-CA15.3-TPA panel. Conventional radiological examinations (chest x-ray, liver echography, bone scintigraphy) can be limited to symptomatic patients or patients suspected of a pending relapse with CEA-CA15.3-TPA panel. In these selected patients conventional and more sophisticated imaging techniques (CT and/or MRI) are necessary to localise the site of relapse. As 4.3 +/- 4.1 months is the mean time from the increase in one or more markers of the CEA-CA15.3-TPA association to the appearance of clinical and/or instrumental signs of relapse (155), hormone treatment can be started at this relatively early stage in probably hormone responsive patients. With this strategy, more prolonged disease-free, and overall survivals, were observed (174-175). Recently, also a five year relapse free survival of 52% has been reported in patients with limited metastatic disease. Most of these patients with low tumor burden were rendered free of disease by excision of their lesions before submission to high-dose chemotherapy with autologous stem-cell transplantation (176). All these findings suggest that a follow-up strategy based on the reported tumor marker panel should be considered.

Among the many other TAAs investigated, MCA and CA549 appeared very promising in the "early" detection of breast cancer relapses because of the relatively high sensitivity and specificity reported in a few studies (177-180). Nevertheless, this finding was not confirmed by others, and both markers showed an accuracy similar or lower than that of TPA (165, 181).

Another use of circulating TAAs is the monitoring of metastatic disease. A good general correlation between CA15.3, TPA, MCA serum levels and the time course of disease has been reported (149, 182-185). However, the data cannot be considered sufficient for a decision-making-process concerning treatment in the absence of clinical and/or instrumental confirmation (149).

4.3. Other circulating markers

Autoantibodies to serum extracellular domain (ECD) of HER2/neu oncogene, p53, nucleophosmin (NPM), cytokeratin positive cells and serum soluble vascular cell adhesion molecules are among the most interesting circulating tumor markers investigated in recent years.

4.3.1. HER-2/neu

ECD of the HER-2/neu oncoprotein is shed from cancer cells into the circulation and is measurable by enzyme immunoassays. In primary cancer increased ECD has been reported as poor indicator of overall survival and disease-free survival (186-188). Also in metastatic cancer elevated serum HER-2 value have been found independent unfavourable prognostic marker for median survival after relapse and relative risk of progression by some (189-192) although not by all authors (193). Moreover, increased ECD Her-2/neu levels predict a poor response to hormonotherapy and some chemotherapy regimens but can predict improved response to combination of Herceptin and chemotherapy (186-187, 194). In another recent study where HER-2/neu tissue results were correlated with the serum HER-2/neu levels at the time of metastatic spread, the number of patients with stage IV breast cancer and elevated serum HER-2/neu level was much higher than HER-2/neu positivity in tissue. Therefore, authors recommend that patients with an elevated serum HER-2/neu level and no tissue overexpression should be considered for retesting of tissue or a new biopsy (195).

ECD HER-2/neu specificity in the detection of relapse is reported as high as 100%, but sensitivity varies from 31% to 45% (152, 190). Its correspondence with clinical behaviour in the monitoring of metastatic disease is still controversial, and low to high accuracy has been reported (186, 193, 196).

4.3.2. p53 autoantibodies

Autoantibodies to p53 are detected in a relatively small proportion of breast cancer patients (197-199). It has been shown that p53 antibodies tend to develop in patients with tumor p53 accumulation, but p53 accumulation is neither sufficient nor necessary for the generation of the immune response. In fact, p53 antibody-positive patients do not have higher frequency of p53 gene mutations than p53 antibody-negative patients, but the former patient group is associated with a Tyr substitution in the protein product (200). Although strong correlation between the presence of p53 autoantibodies and lymph-node status or proliferation-associated antigen Ki-67 has been reported (199), it seems they have little independent predictive/prognostic value (198, 201). While specificity in the detection of relapse has been reported to be higher than 80%, sensitivity varies from 0% to 46% (202-203) and correspondence with clinical behaviour in the monitoring of metastatic disease is still highly investigational (201-202).

4.3.3. Cytokeratin-positive cells

In breast cancer using immunocytochemistry, several investigators have shown that epithelial cells can be identified in the peripheral blood of otherwise metastases-

free patients with stage I and II breast cancer (204-205). The presence of tumor cells outside the primary tumor could be clinically useful: 1) as unambiguous evidence for an early occult spread of tumor cells; 2) as a relevant risk factor for subsequent metastasis and, thus, a poor prognosis; 3) as a marker for monitoring treatment susceptibility. Several markers have been used to detect occult tumor cells in patients with breast cancer. The intermediate filament cytokeratin 19 (CK-19) which is stably and abundantly expressed in the majority of epithelial tumor cells, is one of the most frequently used markers. The detection of occult tumor cells may be improved by the use of the reverse transcription polymerase chain reaction (RT-PCR) amplification technique, which can identify cell-specific mRNA. This method can detect up to one tumor cell in 10^7 normal peripheral blood cells (206-208). The higher sensitivity of RT-PCR for the detection of occult tumor cells was demonstrated by Schoenfeld *et al* (207).

A recently reported trial (209) clearly showed that in metastatic breast cancer patients circulating tumor cells (CTCs) permit prediction of progression-free and overall survival as well as response to treatment. Prediction included the complete study group and most of the investigated subgroups. Interestingly, in this study a newly developed method was used. The system is based on the enumeration of epithelial cells, which are separated from the blood by antibody-coated magnetic beads and identified using fluorescently labelled antibodies against cytokeratin and with a fluorescent nuclear stain and fluorescent cytokeratin antibodies (209). Other studies confirmed that detection of cytokeratin-positive cells in the peripheral blood is an independent factor that identifies breast cancer patients with poor prognosis both as to progression free and overall survivals (210-211). It has also been reported that the number of circulating epithelial cells in patients with clinically active disease concurred or preceded changes in the disease activity (212).

4.3.4. Autoantibodies to nucleophosmin (NPM)

NPM is a nucleolar phosphoprotein, a substrate for phosphorylation by p34 cdc-2 kinase, protein kinase-C, and casein kinase II; and a repressor of the transcriptional regulating activities of the YY1 and IFN regulatory factor-1 transcription factors (213). NPM is induced by estrogen in MCF-7 cells and is upregulated in estrogen independent cells (214). In a recent report, NPM gene has been found upregulated in MCF-7/LCC9 compared to MCF-7/LCC1 cells the former being antiestrogen-resistant and the latter antiestrogen-responsive variants of the MCF-7 human breast cancer cell line (13). NPM provokes an autoimmune response in breast cancer patients, the magnitude of which is associated with tamoxifen therapy. The levels of anti-NPM autoantibodies increase 6 months before recurrence and they are significantly reduced in patients receiving tamoxifen, consistent with the antiestrogenic regulation of the antigen (213). It has been postulated that monitoring anti-NPM levels could be a useful biomarker for assessing tamoxifen responses and failures (33).

4.3.5. Serum soluble vascular cell adhesion molecules: intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin

Adhesion molecules are serum glycoproteins important in cell-cell and cell-basement interactions. They are intimately involved in inflammatory reactions, play a role in cell adhesion to the vascular endothelium and may have a role in tumor cell dissemination and progression. Particularly, in the last years three of them have been investigated: ICAM-1, VCAM-1 and E-selectin.

The circulating soluble form of ICAM-1 is elevated in breast cancer patients and associated with tumor progression (215). Nevertheless only occasionally could a poor prognostic value be established (216).

Serum levels of soluble VCAM-1 have been found to correlate closely with microvessel density in tumors ($r = 65$; $p < .001$), and women who developed early recurrence had higher pre-operative levels than those who remained disease-free (217). In the same study, serum VCAM-1 levels rose in women with advanced disease whose disease progressed, and remained unchanged, or fell in women with advanced disease whose disease remained stable or showed a partial response to hormonal therapy. In other studies elevated VCAM-1 levels in patients with stage II disease were predictive of decreased survival even when corrected for T and N status (218) or significantly correlated with circulating endothelial cells (216). Furthermore, high expression and high level of VCAM-1 has been reported concomitant with neovascularisation induced by malignant breast cancer cells (198). These data point out VCAM-1 as a surrogate marker of angiogenesis.

Increasing evidence suggests that soluble E-selectin contributes to tumor growth and metastasis, possibly stimulating angiogenesis and the adhesion of tumor cells to endothelial cells or distant sites. E-selectin has been found significantly higher in the serum of breast cancer patients than in controls and in benign breast disease (218-220). It has also been suggested that E-selectin serum levels might reflect the severity of invasive breast cancer (220) and that they are associated with the clinical course of liver metastases (221).

5. SUMMARY AND PERSPECTIVE

This review summarises the main advances in the study of biomolecular markers of breast cancer and has attempted to score their clinical utility using a recently developed grading system (Tables 1-2). Many efforts are ongoing to define either tissue or circulating biomolecular markers that independently, or in addition to the conventional pathological findings (T and N), better select patient management. In the adjuvant setting c-erbB2/HER2 neu positive tumors with concomitant high value of Ki67/MIB-1 defines a subgroup of lymph-node negative patients with higher risk of relapse. Cytokeratin-positive cells in the peripheral blood or high KI67/MIB-1 value proved to be an independent negative prognostic factor. Cytotoxic chemotherapy is an important therapy for breast cancer patients, but it is limited by toxicity, non-specificity

and inevitable development of resistance. The above mentioned new markers can help in the decision-making process on the use of anticyclines and/or taxanes in place of CMF. However, an effective therapy has to target cellular pathways involved in growth regulation. The term “targeted therapy” refers to a known therapeutic target that is important in the biology of the cancer cell and indicates a specific agent that acts by modifying the expression or activity of the target in the growth and progression of the cancer. With this approach, only patients with the likelihood of benefit are treated, so hopefully the therapeutic index will be improved. Tissue biomolecular markers, besides being prognostic and predictor factors, undoubtedly play a central role in targeted therapies that are among the most promising directions of clinical research. The discovery of the ER provided the first example of targeted therapy in breast cancer. In fact, tamoxifen selectively binds ER and competitively inhibits the activity of estrogens. Progress in the understanding of the biology of this receptor pathway, led to further targeted agents able to modulate the activity of this pathway (SERMs) or inhibitors of the production of the ligand (aromatase inhibitors) and improved management of the localised and advanced breast cancer expressing the ER. Using the same strategy, the recombinant humanized monoclonal antibody trastuzumab was developed as a therapy targeting the ECD of HER2 which is overexpressed in roughly one fourth of patients with invasive breast cancer. In properly selected patients with overexpression of the HER2 protein and/or amplification of HER2 gene in tumor tissue, trastuzumab showed single agent activity as well as potent synergy with many chemotherapy agents in metastatic breast cancer (222, 223).

Two main classes of agents have been developed that specifically target the EGFR. Gefitinib and erlotinib are small molecule inhibitors of the EGFR TK, and cetuximab is among a group of monoclonal antibodies that target EGFR.

Several other compounds have been developed that have broad anti-HER activity such as lapatinib and pertuzumab and more HER signalling inhibitors as gefitinib and trastuzumab have been combined.

Inhibiting multiple HER receptors has the potential of improving therapy by intensifying and broadening the inhibition of multiple and redundant cell pathways. Preliminary data from clinical trials with these new classes of agents have already been reported although clinical benefit was less than expected (224-227).

VEGF is another tissue biomolecular marker against which a recombinant humanized antibody has been developed. The antibody blocks binding of VEGF to its receptors and endothelial cells. Some trials with this antibody, called bevacizumab, have already been conducted (228, 229).

As to circulating markers, so far most data have been collected on the more common TAAs. Some of them have been investigated and have never, or sporadically,

been introduced into clinical practice. Others such as those reported in this review have been utilised in many clinical trials but none of them can be considered as standard practice (Table 2). While their preoperative levels did not show relevant prognostic or predictive value, their clinical use in the early detection and monitoring of relapses is widely documented. However, for some of them such as TPA, MCA and CA549 the high number of falsely positive results, and for others such as CEA and CA15.3 and the more recently evaluated ECD-HER2/neu and p53 antibodies, the high number of falsely negative results is the main obstacle to routine clinical utilisation. A progress in the field is the ongoing investigation to attain high accuracy with the association of more markers and the adoption of suitable clinical and laboratory criteria. CEA-TPA-CA15.3 could be one of these panels; however, ECD-HER2/neu and p53 antibodies, because of their high specificity, and nucleophosmin antibodies could be suitable candidates for different combinations. In a pilot study with an intensive post-operative follow-up with CEA-TPA-CA15.3, this tumor marker panel allowed to “early” detect distant metastases and an “early” treatment with antiestrogens significantly prolonged disease-free and overall survivals of responsive relapsed patients (174). If randomised clinical trials confirm these results, the new more effective therapies (new antiestrogens, targeted drugs) can be anticipated and further options can be considered in selected metastatic patients. Soluble vascular cell adhesion molecules are still highly investigational markers and overall seem to be useful as prognostic/predictor factors rather than factors in the early detection and monitoring of relapses (Table 2).

In conclusion, the study of biomolecular markers is a continuously expanding field that offers important opportunities to improve the understanding of biology and management of breast cancer.

6. REFERENCES

- Petkovich M, N.J. Brand, A. Krust & P. Chambon: A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* 330, 444-450 (1987)
- Tora L, J. White, C. Brou, D. Tasset, N. Webster, E. Scheer & P. Chambon: The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* 59, 477-487 (1989)
- Tzukerman M.T, A. Esty, D. Santiso-Mere, P. Danielian, M.G. Parker, R.B. Stein, J.W. Pike & D.P. McDonnell: Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. *Mol Endocrinol* 8, 21-30 (1994)
- Ham J. & M.G. Parker: Regulation of gene expression by nuclear hormone receptors. *Curr Opin Cell Biol* 1, 503-511 (1989)
- Montano M.M, V. Muller, A. Trobaugh & B.S. Katzenellenbogen: The carboxy-terminal F domain of the human estrogen receptor: role in the transcriptional activity of the receptor and the effectiveness of antiestrogens as estrogen antagonists. *Mol Endocrinol* 9(7), 814-825 (1995)
- Kuiper G.G, E. Enmark, M. Peltö-Huikko, S. Nilsson & J.A. Gustafsson: Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 93, 5925-5930 (1996)
- Mosselman S, J. Polman & R. Dijkema: ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett* 392, 49-53 (1996)
- Ogawa S, S. Inoue, T. Watanabe, H. Hiroi, A. Orimo, T. Hosoi, Y. Ouchi & M. Muramatsu: The complete primary structure of human estrogen receptor beta (hER beta) and its heterodimerization with ER alpha *in vivo* and *in vitro*. *Biochem Biophys Res Commun* 243, 122-126 (1998)
- Tremblay G.B, A. Tremblay, N.G. Copeland, D.J. Gilbert, N.A. Jenkins, F. Labrie & V. Giguere: Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. *Mol Endocrinol* 11, 353-365 (1997)
- Kuiper G.G, B. Carlsson, K. Grandien, E. Enmark, J. Haggblad, S. Nilsson & J.A. Gustafsson: Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* 138, 863-870 (1997)
- Fichtner I, M. Becker, R. Zeisig, & A. Sommer: *In vivo* models for endocrine-dependent breast carcinomas: special considerations of clinical relevance. *Eur J Cancer* 40, 845-851 (2004)
- Osborne C.K, H. Zhao & S.A. Fuqua: Selective estrogen receptor modulators: structure, function, and clinical use. *J Clin Oncol* 18, 3172-3186 (2000)
- Gu Z, R.Y. Lee, T.C. Skaar, K.B. Bouker, J.N. Welch, J. Lu, A. Liu, Y. Zhu, N. Davis, F. Leonessa, N. Brunner, Y. Wang & R. Clarke: Association of interferon regulatory factor-1, nucleophosmin, nuclear factor-kappaB, and cyclic AMP response element binding with acquired resistance to Faslodex (ICI 182,780). *Cancer Res* 62, 3428-3437 (2002)
- Jensen E.V: Overview of the nuclear receptor family. In: Nuclear hormone receptors: molecular mechanisms, cellular functions, clinical abnormalities. Ed: Parker M.G. Academic Press, London, UK (1991)
- Kushner P.J, D.A. Agard, G.L. Greene, T.S. Scanlan, A.K. Shiau, R.M. Uht & P. Webb: Estrogen receptor pathways to AP-1. *J Steroid Biochem Mol Biol* 74, 311-317 (2000)
- Saville B, M. Wormke, F. Wang, T. Nguyen, E. Enmark, G. Kuiper, J.A. Gustafsson & S. Safe: Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements. *J Biol Chem* 275, 5379-5387 (2000)

17. Altucci L, R. Addeo, L. Cicatiello, S. Dauvois, M.G. Parker, M. Truss, M. Beato, V. Sica, F. Bresciani & A. Weisz: 17beta-Estradiol induces cyclin D1 gene transcription, p36D1-p34cdk4 complex activation and p105Rb phosphorylation during mitogenic stimulation of G(1)-arrested human breast cancer cells. *Oncogene* 12, 2315-2324 (1996)
18. Webb P, G.N. Lopez, R.M. Uht & P.J. Kushner: Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. *Mol Endocrinol* 9, 443-456 (1995)
19. Paech K, P. Webb, G.G. Kuiper, S. Nilsson, J. Gustafsson, P.J. Kushner & T.S. Scanlan: Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* 277, 1508-1510 (1997)
20. Angel P. & M. Karin: The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta* 1072, 129-157 (1991)
21. Duval D, S. Durant, F. Homo-Delarche: Non-genomic effects of steroids. Interactions of steroid molecules with membrane structures and functions. *Biochim Biophys Acta* 737, 409-442 (1983)
22. Kelly M.J. & E.R. Levin: Rapid actions of plasma membrane estrogen receptors. *Trends Endocrinol Metab* 12, 152-156 (2001)
23. Schiff R, S. Massarweh, J. Shou & C.K. Osborne: Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response. *Clin Cancer Res* 9, 447S-454S (2003)
24. Lavinsky R.M, K. Jepsen, T. Heinzel, J. Torchia, T.M. Mullen, R. Schiff, A.L. Del-Rio, M. Ricote, S. Ngo, J. Gensch, S.G. Hilsenbeck, C.K. Osborne, C.K. Glass, M.G. Rosenfeld & D.W. Rose: Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proc Natl Acad Sci USA* 95, 2920-2925 (1998)
25. Kamei Y, L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S.C. Lin, R.A. Heyman, D.W. Rose, C.K. Glass & M.G. Rosenfeld: A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* 85, 403-414 (1996)
26. McKenna N.J, R.B. Lanz & B.W. O'Malley: Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev.* 20, 321-344 (1999)
27. McKenna N.J & B.W. O'Malley: From ligand to response: generating diversity in nuclear receptor coregulator function. *J Steroid Biochem Mol Biol* 74, 351-356 (2000)
28. Greene G.L & E.V. Jensen: Monoclonal antibodies as probes for estrogen receptor detection and characteristics. *J Steroid Biochem* 16, 353-359 (1992)
29. Speirs V, C. Malone, D.S. Walton, M.J. Kerin & S.L. Atkin: Increased expression of estrogen receptor beta mRNA in tamoxifen-resistant breast cancer patients. *Cancer Res* 59, 5421-5424 (1999)
30. Speirs V., A.T. Parkes, M.J. Kerin, D.S. Walton, P.J. Carleton, J.N. Fox & S.L. Atkin: Coexpression of estrogen receptor alpha and beta: poor prognostic factors in human breast cancer? *Cancer Res* 59, 525-528 (1999)
31. Sharma A.K, D. Grimshaw, K. Horgan, G.T. Neades, J.M.W. Gee, A.G. Douglas-Jones, R.E. Mansel & R.I. Nicholson: Analysis of the genes for oestrogen and epidermal growth factor receptors in human breast cancer. *The breast* 5, 344-350 (1996)
32. Robertson J.F: Oestrogen receptor: a stable phenotype in breast cancer. *Br J Cancer* 73, 5-12 (1996)
33. Clarke R, F. Leonessa, J.N. Welch & T.C. Skaar: Cellular and molecular pharmacology of antiestrogen action and resistance. *Pharmacol Rev* 53, 25-71 (2001)
34. Spataro V, K. Price, A. Goldhirsch, F. Cavalli, E. Simoncini, M. Castiglione, C.M. Rudenstam, J. Collins, J. Lindtner & R.D. Gelber: Sequential estrogen receptor determinations from primary breast cancer and at relapse: prognostic and therapeutic relevance. The International Breast Cancer Study Group (formerly Ludwig Group). *Ann Oncol* 3, 733-740 (1992)
35. Harvey J.M, G.M. Clark, C.K. Osborne & D.C. Allred: Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J Clin Oncol* 17, 1474-1481 (1999)
36. Esslimani-Sahla M, J. Simony-Lafontaine, A. Kramar, R. Lavaill, C. Mollevi, M. Warner, J.A. Gustafsson & H. Rochefort: Estrogen receptor beta (ER beta) level but not its ER beta cx variant helps to predict tamoxifen resistance in breast cancer. *Clin Cancer Res* 10, 5769-5776 (2004)
37. Honig S.F: Treatment of metastatic disease. In: Diseases of the breast. Eds: Harris J.R, Lippman M.E, Morrow M, Hellmann S, Lippincott-Raven, Philadelphia, PA (1996)
38. Horwitz K.B, W.L. McGuire & O.H. Pearson: Predicting response to endocrine therapy in human breast cancer: a hypothesis. *Science* 189: 726 (1975)
39. Clarke R. & N. Brunner: Cross resistance and molecular mechanisms in antiestrogen resistance. *Endocr Related Cancer* 2, 59-72 (1995)
40. Clarke R. & M.E. Lippman: Mechanism of resistance to antiestrogens and their implications for cross-resistance. In: Tamoxifen: beyond the antiestrogen. Ed: Kellen J.A, Birkhauser, Boston, MA (1996)
41. Van der Flier S, C.M. Chan, A. Brinkman, M. Smid,

- S.R. Johnston, L.C. Dorssers & M. Dowsett: BCAR1/p130Cas expression in untreated and acquired tamoxifen-resistant human breast carcinomas. *Int J Cancer* 89, 465-468 (2000)
42. Martin M.B, P. Garcia-Morales, A. Stoica, H.B. Solomon, M. Pierce, D. Katz, S. Zhang, M. Danielsen & M. Saceda: Effects of 12-O-tetradecanoylphorbol-13-acetate on estrogen receptor activity in MCF-7 cells. *J Biol Chem* 270, 25244-25251 (1995)
43. Dumont J.A, A.J. Bitonti, C.D. Wallace, R.J. Baumann, E.A. Cashman & D.E. Cross-Doersen: Progression of MCF-7 breast cancer cells to antiestrogen-resistant phenotype is accompanied by elevated levels of AP-1 DNA-binding activity. *Cell Growth Differ* 7, 351-359 (1996)
44. Smith T.J, N.E. Davidson, D.V. Schapira, E. Grunfeld, H.B. Muss, V.G. 3rd Vogel & M.R. Somerfield: American Society of Clinical Oncology 1998 update of recommended breast cancer surveillance guidelines. *J Clin Oncol* 17, 1080-1082 (1999)
45. Doucas V, G. Spyrou & M. Yaniv: Unregulated expression of c-Jun or c-Fos proteins but not Jun D inhibits oestrogen receptor activity in human breast cancer derived cells. *EMBO J* 10, 2237-2245 (1991)
46. Johnston S.R.D, G. Saccanti-Jotti, I.E. Smith, J. Newby & M. Dowsett: Change in estrogen receptor expression and function in tamoxifen-resistant breast cancer. *Endocr Related Cancer* 2, 105-110 (1995)
47. Davidson N.E, E.P. Gelmann, M.E. Lippman & R.B. Dickson: Epidermal growth factor receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. *Mol Endocrinol* 1, 216-223 (1987)
48. Cattoretti G, S. Andreola, C. Clemente, L. D'Amato & F. Rilke: Vimentin and p53 expression in epidermal growth factor receptor-positive estrogen receptor-negative breast carcinomas. *Br J Cancer* 57, 353-357 (1988)
49. Nicholson R.I, R.A. McClelland, J.M. Gee, D.L. Manning, P. Cannon, J.F. Robertson, I.O. Ellis & R.W. Blamey: Epidermal growth factor receptor expression in breast cancer: association with response to endocrine therapy. *Breast Cancer Res Treat* 29, 117-125 (1994)
50. Gutierrez M.C, S. Detre, S. Johnston, S.K. Mohsin, J. Shou, D.C. Allred, R. Schiff, C.K. Osborne & M. Dowsett: Molecular changes in tamoxifen-resistant breast cancer: relationship between estrogen receptor, HER-2, and p38 mitogen-activated protein kinase. *J Clin Oncol* 23, 2469-2476 (2005)
51. Wang T, G. Niu, M. Kortylewski, L. Burdelya, K. Shain, S. Zhang, R. Bhattacharya, D. Gabrilovich, R. Heller, D. Coppola, W. Dalton, R. Jove, D. Pardoll & H. Yu: Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. *Nat Med* 10, 48-54 (2004)
52. Nicolini A., A. Carpi & G. Rossi: An immunotherapy schedule in endocrine-dependent metastatic breast cancer: correlation between clinical course and immunologic parameters. *J Immunother* 28, 276-279 (2005)
53. Kastner P, A. Krust, B. Turcotte, U. Stropp, L. Tora, H. Gronemeyer & P. Chambon: Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. *EMBO J* 9, 1603-1614 (1990)
54. Sartorius C.A, M.Y. Melville, A.R. Hovland, L. Tung, G.S. Takimoto & K.B. Horwitz: A third transactivation function (AF3) of human progesterone receptors located in the unique N-terminal segment of the B-isoform. *Mol Endocrinol* 8, 1347-1360 (1994)
55. Richer J.K., B.M. Jacobsen, N.G. Manning, M.G. Abel, D.M. Wolf & K.B. Horwitz: Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. *J Biol Chem* 277, 5209-5218 (2002)
56. Jacobsen B.M, J.K. Richer, C.A. Sartorius, K.B. Horwitz: Expression profiling of human breast cancers and gene regulation by progesterone receptors. *J Mammary Gland Biol Neoplasia* 8, 257-268 (2003)
57. Tung L., M.K. Mohamed, J.P. Hoeffler, G.S. Takimoto & K.B. Horwitz: Antagonist-occupied human progesterone B-receptors activate transcription without binding to progesterone response elements and are dominantly inhibited by A-receptors. *Mol Endocrinol* 7, 1256-1265 (1993)
58. Kato J, S. Hirata, A. Nozawa & N. Mouri: The ontogeny of gene expression of progestin receptors in the female rat brain. *J Steroid Biochem Mol Biol* 47, 173-182 (1993)
59. Shyamala G, X. Yang, G. Silberstein, M.H. Barcellos-Hoff & E. Dale: Transgenic mice carrying an imbalance in the native ratio of A to B forms of progesterone receptor exhibit developmental abnormalities in mammary glands. *Proc Natl Acad Sci USA* 95, 696-701 (1998)
60. Shyamala G, X. Yang, R.D. Cardiff & E. Dale: Impact of progesterone receptor on cell-fate decisions during mammary gland development. *Proc Natl Acad Sci USA* 97, 3044-3049 (2000)
61. Mulac-Jericevic B, R.A. Mullinax, F.J. DeMayo, J.P. Lydon & O.M. Conneely: Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. *Science* 289, 1751-1754 (2000)
62. Graham J.D, C. Yeates, R.L. Balleine, S.S. Harvey, J.S. Milliken, A.M. Bilous & C.L. Clarke: Characterization of progesterone receptor A and B expression in human breast

cancer. *Cancer Res* 55, 5063-5068 (1995)

63. Bamberger A.M, K. Milde-Langosch, H.M. Schulte & T. Loning: Progesterone receptor isoforms, PR-B and PR-A, in breast cancer: correlations with clinicopathologic tumor parameters and expression of AP-1 factors. *Horm Res* 54, 32-37 (2000)

64. McGowan E.M & C.L. Clarke: Effect of overexpression of progesterone receptor A on endogenous progestin-sensitive endpoints in breast cancer cells. *Mol Endocrinol* 13, 1657-1671 (1999)

65. Mote P.A, J.F. Johnston, T. Manninen, P. Tuohimaa & C.L. Clarke: Detection of progesterone receptor forms A and B by immunohistochemical analysis. *J Clin Pathol* 54, 624-630 (2001)

66. Horwitz K.B & W.L. McGuire: Estrogen control of progesterone receptor in human breast cancer. Correlation with nuclear processing of estrogen receptor. *J Biol Chem* 253, 2223-2228 (1978)

67. Horwitz K.B, L.L. Wei, S.M. Sedlacek & C.N. d'Arville: Progestin action and progesterone receptor structure in human breast cancer: a review. *Recent Prog Horm Res* 41, 249-316 (1985)

68. McGuire W.L: Hormone receptors: their role in predicting prognosis and response to endocrine therapy. *Semin Oncol* 5, 428-433 (1978)

69. Costa S.D, S. Lange, K. Klinga, E. Merkle & M. Kaufmann: Factors influencing the prognostic role of oestrogen and progesterone receptor levels in breast cancer-results of the analysis of 670 patients with 11 years of follow-up. *Eur J Cancer* 38, 1329-1334 (2002)

70. Yarden Y. & M.X. Sliwkowski: Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2, 127-137 (2001)

71. Anido J, P. Matar, J. Albanell, M. Guzman, F. Rojo, J. Arribas, S. Averbuch, J. Baselga: ZD1839, a specific epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, induces the formation of inactive EGFR/HER2 and EGFR/HER3 heterodimers and prevents heregulin signaling in HER2-overexpressing breast cancer cells. *Clin Cancer Res* 9, 1274-1283 (2003)

72. Klapper L.N, S. Glathe, N. Vaisman, N.E. Hynes, G.C. Andrews, M. Sela & Y. Yarden: The ErbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors. *Proc Natl Acad Sci USA* 96, 4995-5000 (1999)

73. Klapper L.N, N. Vaisman, E. Hurwitz, R. Pinkas-Kramarski, Y. Yarden & M. Sela: A subclass of tumor-inhibitory monoclonal antibodies to ErbB-2/HER2 blocks crosstalk with growth factor receptors. *Oncogene* 14, 2099-2109 (1997)

74. Wada T, X.L. Qian & M.I. Greene: Intermolecular association of the p185neu protein and EGF receptor modulates EGF receptor function. *Cell* 61, 1339-1347 (1990)

75. Goldman R, R.B. Levy, E. Peles & Y. Yarden: Heterodimerization of the erbB-1 and erbB-2 receptors in human breast carcinoma cells: a mechanism for receptor transregulation. *Biochemistry* 29, 11024-11028 (1990)

76. Alimandi M, A. Romano, M.C. Curia, R. Muraro, P. Fedi, S.A. Aaronson, P.P. Di Fiore & M.H. Kraus: Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. *Oncogene* 10, 1813-1821 (1995)

77. Samanta A, C.M. LeVeae, W.C. Dougall, X. Qian & M.I. Greene: Ligand and p185c-neu density govern receptor interactions and tyrosine kinase activation. *Proc Natl Acad Sci USA* 91, 1711-1715 (1994)

78. Alroy I. & Y. Yarden: The ErbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand-receptor interactions. *FEBS Lett* 410, 83-86 (1997)

79. Lewis T.S, P.S. Shapiro & N.G. Ahn: Signal transduction through MAP kinase cascades. *Adv Cancer Res* 74, 49-139 (1998)

80. Vivanco I & C.L. Sawyers: The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* 2, 489-501 (2002)

81. Chan T.O, S.E. Rittenhouse & P.N. Tsichlis: AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu Rev Biochem* 68, 965-1014 (1999)

82. Kuan C.T, C.J. Wikstrand & D.D. Bigner: EGF mutant receptor vIII as a molecular target in cancer therapy. *Endocr Relat Cancer* 8, 83-96 (2001)

83. Liu D, J. Aguirre Ghiso, Y. Estrada & L. Ossowski: EGFR is a transducer of the urokinase receptor initiated signal that is required for *in vivo* growth of a human carcinoma. *Cancer Cell* 1, 445-457 (2002)

84. Slamon D, M. Press & W. Godolphin: Studies of the HER2/neu proto-oncogene in human breast cancer. *Cancer Cells* 7, 371-380 (1989)

85. Slamon D, W. Godolphin, L.A. Jones, J.A. Holt, S.G. Wong, D.E. Keith, W.J. Levin, S.G. Stuart, J. Udove & A. Ullrich: Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 244, 707-712 (1989)

86. Press M.F, D.J. Slamon, K.J. Flom, J. Park, J.Y. Zhou & L. Bernstein: Evaluation of HER-2/neu gene amplification and overexpression: comparison of frequently used assay methods in a molecularly characterized cohort

of breast cancer specimens. *J Clin Oncol* 20, 3095-3105 (2002)

87. Revillion F, J. Bonnetterre & J.P. Peyrat: ERBB2 oncogene in human breast cancer and its clinical significance. *Eur J Cancer* 34, 91-808 (1998)

88. Volpi A, O. Nanni, F. De Paola, A.M. Granato, A. Mangia, F. Monti, F. Schittulli, M. De Lena, E. Scarpi, P. Rosetti, M. Monti, L. Gianni, D. Amadori & A. Paradiso: HER-2 expression and cell proliferation: prognostic markers in patients with node-negative breast cancer. *J Clin Oncol* 21, 2708-2712 (2003)

89. Mirza A.N, N.Q. Mirza, G. Vlastos & S.E. Singletary: Prognostic factors in node-negative breast cancer: a review of studies with sample size more than 200 and follow-up more than 5 years. *Ann Surg* 235, 10-26 (2002)

90. Harris A.L, S. Nicholson, J.R. Sainsbury, J. Farndon & C. Wright: Epidermal growth factor receptors in breast cancer: association with early relapse and death, poor response to hormones and interactions with neu. *J Steroid Biochem* 34, 123-131 (1989)

91. Yamauchi H, A. O'Neill, R. Gelman, W. Carney, D.Y. Tenney, S. Hosch & D.F. Hayes: Prediction of response to antiestrogen therapy in advanced breast cancer patients by pretreatment circulating levels of extracellular domain of the HER-2/c-neu protein. *J Clin Oncol* 15, 2518-2525 (1997)

92. Stal O, M. Ferno & A. Borg: ERBB2 expression and benefit from 5 versus 2 years of adjuvant tamoxifen for postmenopausal stage II breast cancer patients. *Breast Cancer Res Treat* 46: 32 (1997)

93. Berry D.A, H.B. Muss, A.D. Thor, L. Dressler, E.T. Liu, G. Broadwater, D.R. Budman, I.C. Henderson, M. Barcos, D. Hayes & L. Norton: HER-2/neu and p53 expression versus tamoxifen resistance in estrogen receptor-positive, node-positive breast cancer. *J Clin Oncol* 118, 3471-3479 (2000)

94. Elledge R.M, S. Green, D. Ciocca, R. Pugh, D.C. Allred, G.M. Clark, J. Hill, P. Ravdin, J. O'Sullivan, S. Martino & C.K. Osborne: HER-2 expression and response to tamoxifen in estrogen receptor-positive breast cancer: a Southwest Oncology Group Study. *Clin Cancer Res* 4, 7-12 (1998)

95. Burstein H.J, L.N. Harris, P.K. Marcom, R. Lambert-Falls, K. Havlin, B. Overmoyer, R.J. Jr Friedlander, J. Gargiulo, R. Strenger, C.L. Vogel, P.D. Ryan, M.J. Ellis, R.A. Nunes, C.A. Bunnell, S.M. Campos, M. Hallor, R. Gelman & E.P. Winer: Trastuzumab and vinorelbine as first-line therapy for HER2-overexpressing metastatic breast cancer: multicenter phase II trial with clinical outcomes, analysis of serum tumor markers as predictive factors, and cardiac surveillance algorithm. *J Clin Oncol* 21, 2889-2895 (2003)

96. Moulder S.L, F.M. Yakes, S.K. Muthuswamy, R. Bianco, J.F. Simpson & C.L. Arteaga: Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/neu (erbB2)-overexpressing breast cancer cells *in vitro* and *in vivo*. *Cancer Res* 61, 8887-8895 (2001)

97. McBride O.W, D. Merry & D. Givol: The gene for human p53 cellular tumor antigen is located on chromosome 17 short arm (17p13). *Proc Natl Acad Sci USA* 83, 130-134 (1986)

98. Soussi T, C. Caron de Fromental & P. May: Structural aspects of the p53 protein in relation to gene evolution. *Oncogene* 5, 945-952 (1990)

99. Vogelstein B & K.W. Kinzler: p53 function and dysfunction. *Cell* 70, 523-526 (1992)

100. Lane D.P: Cancer. p53, guardian of the genome. *Nature* 358, 15-16 (1992)

101. Kirsch D.G & M.B. Kastan: Tumor-suppressor p53: implications for tumor development and prognosis. *J Clin Oncol* 16, 3158-3168 (1998)

102. Nieto Y, P.J. Cagnoni, S. Nawaz, E.J. Shpall, R. Yerushalmi, B. Cook, P. Russell, J. McDermit, J. Murphy, S.I. Bearman & R.B. Jones: Evaluation of the predictive value of Her-2/neu overexpression and p53 mutations in high-risk primary breast cancer patients treated with high-dose chemotherapy and autologous stem-cell transplantation. *J Clin Oncol* 18, 2070-2080 (2000)

103. Keohavong P, W.M. Gao, H.H. Mady, A. Kanbour-Shakir & M.F. Melhem: Analysis of p53 mutations in cells taken from paraffin-embedded tissue sections of ductal carcinoma *in situ* and atypical ductal hyperplasia of the breast. *Cancer Lett* 212, 121-130 (2004)

104. Kang J.H, S.J. Kim, D.Y. Noh, K.J. Choe, E.S. Lee & H.S. Kang: The timing and characterization of p53 mutations in progression from atypical ductal hyperplasia to invasive lesions in the breast cancer. *J Mol Med* 79, 648-655 (2001)

105. Walker R.A, S.J. Dearing, D.P. Lane & J.M. Varley: Expression of p53 protein in infiltrating and in-situ breast carcinomas. *J Pathol* 165, 203-211 (1991)

106. Rajan P.N, D.J. Scott, R.H. Perry & C.D. Griffith: p53 protein expression in ductal carcinoma *in situ* (DCIS) of the breast. *Breast Cancer Res Treat* 42, 283-90 (1997)

107. Dornan D, S. Bheddah, K. Newton, W. Ince, G.D. Frantz, P. Dowd, H. Koeppen, V.M. Dixit & D.M. French: COP1, the negative regulator of p53, is overexpressed in breast and ovarian adenocarcinomas. *Cancer Res* 15, 7226-7230 (2004)

108. Somlo G, P. Frankel, W. Chow, L. Leong, K. Margolin, R. Jr Morgan, S. Shibata, P. Chu, S. Forman, D.

- Lim, P. Twardowski, J. Weitzel, J. Alvarnas, N. Kogut, J. Schriber, E. Fermin, Y. Yen, L. Damon & J.H. Doroshow: Prognostic indicators and survival in patients with stage IIIB inflammatory breast carcinoma after dose-intense chemotherapy. *J Clin Oncol* 22, 1839-1848 (2004)
109. Aziz S.A, S. Pervez, S. Khan, N. Kayani, S.I. Azam & M.H. Rahbar: Case control study of prognostic markers and disease outcome in inflammatory carcinoma breast: a unique clinical experience. *Breast J* 7, 398-404 (2001)
110. Somlo G, J.F. Simpson, P. Frankel, W. Chow, L. Leong, K. Margolin, R. Jr Morgan, J. Raschko, S. Shibata, S. Forman, N. Kogut, M. McNamara, A. Molina, E. Somlo & J.H. Doroshow: Predictors of long-term outcome following high-dose chemotherapy in high-risk primary breast cancer. *Br J Cancer* 87, 281-288 (2002)
111. Hartmann A, H. Blaszyk, J.S. Kovach & S.S. Sommer: The molecular epidemiology of p53 gene mutations in human breast cancer. *Trends Genet* 13, 27-33 (1997)
112. Elledge R.M & D.C. Allred: Prognostic and predictive value of p53 and p21 in breast cancer. *Breast Cancer Res Treat* 52, 79-98 (1998)
113. Pharoah P.D, N.E. Day & C. Caldas: Somatic mutations in the p53 gene and prognosis in breast cancer: a meta-analysis. *Br J Cancer* 80, 1968-1973 (1999)
114. Thor A.D, D.A. Berry, D.R. Budman, H.B. Muss, T. Kute, I.C. Henderson, M. Barcos, C. Cirincione, S. Edgerton, C. Allred, L. Norton & E.T. Liu: erbB-2, p53, and efficacy of adjuvant therapy in lymph node-positive breast cancer. *J Natl Cancer Inst* 90, 1346-1360 (1998)
115. McGill G & D.E. Fisher: p53 and cancer therapy: a double-edged sword. *J Clin Invest* 104, 223-225 (1999)
116. Brown D.C & K.C. Gatter: Monoclonal antibody Ki-67: its use in histopathology. *Histopathology* 17, 489-503 (1990)
117. Silvestrini R, M.G. Daidone, G. Di Fronzo, A. Morabito, P. Valagussa, G. Bonadonna: Prognostic implication of labeling index versus estrogen receptors and tumor size in node-negative breast cancer. *Breast Cancer Res Treat* 7, 161-169 (1986)
118. Silvestrini R, M.G. Daidone, M. Mastore, G. Di Fronzo, D. Coradini, P. Boracchi, P. Squicciarini, B. Salvadori & U. Veronesi: Cell kinetics as a predictive factor in node-positive breast cancer treated with adjuvant hormone therapy. *J Clin Oncol* 11, 1150-1155 (1993)
119. Waldman F.M, K. Chew, B.M. Ljung, W. Goodson, J. Hom, L.A. Duarte, H.S. Smith & B. Mayall: A comparison between bromodeoxyuridine and 3H thymidine labeling in human breast tumors. *Mod Pathol* 4, 718-722 (1991)
120. Vincent-Salomon A, A. Rousseau, M. Jouve, P. Beuzeboc, B. Sigal-Zafrani, P. Freneaux, C. Rosty, C. Nos, F. Campana, J. Kljanienco, A. Al Ghuzlan & X. Sastre-Garau, Breast Cancer Study Group: Proliferation markers predictive of the pathological response and disease outcome of patients with breast carcinomas treated by anthracycline-based preoperative chemotherapy. *Eur J Cancer* 40, 1502-1508 (2004)
121. Levack P.A, A.P.M. Forrest & E.P. Miller: MIB-1 in relation to tumor response and survival in patients with breast cancer treated with primary systemic therapy. *Breast* 8, 77-83 (1999)
122. Goodson W.H. 3rd, D.H. 2nd Moore, B.M. Ljung, K. Chew, B. Mayall, H.S. Smith & F.M. Waldman: The prognostic value of proliferation indices: a study with *in vivo* bromodeoxyuridine and Ki-67. *Breast Cancer Res Treat* 59, 113-123 (2000)
123. Spyrtos F, M. Ferrero-Pous, M. Trassard, K. Hacene, E. Phillips, M. Tubiana-Hulin & V. Le Doussal: Correlation between MIB-1 and other proliferation markers: clinical implications of the MIB-1 cutoff value. *Cancer* 15, 2151-2159 (2002)
124. Tan P.H, B.H. Bay, G. Yip, S. Selvarajan, P. Tan, J. Wu, C.H. Lee & K.B. Li: Immunohistochemical detection of Ki67 in breast cancer correlates with transcriptional regulation of genes related to apoptosis and cell death. *Mod Pathol* 17, 1-8 (2004)
125. Molino A, R. Micciolo, M. Turazza, F. Bonetti, Q. Piubello, A. Bonetti, R. Nortilli, G. Pelosi & GL Cetto: Ki-67 immunostaining in 322 primary breast cancers: associations with clinical and pathological variables and prognosis. *Int J Cancer* 74, 433-437 (1997)
126. Brown R.W, C.D. Allred, G.M. Clark, C.K. Osborne & S.G. Hilsenbeck: Prognostic value of Ki-67 compared to S-phase fraction in axillary node-negative breast cancer. *Clin Cancer Res* 2, 585-592 (1996)
127. Kushlinskii N.E, M.B. Orinovskii, L.E. Gurevich, I.A. Kazantseva, S.Z. Talaeva, V.P. Shirokii, V.D. Ermilova, E.K. Dvorova & A.S. Ozherelev: Expression of biomolecular markers (Ki-67, PCNA, Bcl-2, BAX, BclX, VEGF) in breast tumors. *Bull Exp Biol Med* 137, 182-185 (2004)
128. Rudas M, M.F. Gnani, M. Mittlböck, R. Neumayer, A. Kummer, R. Jakesz, G. Reiner & A. Reiner: Thymidine labeling index and Ki-67 growth fraction in breast cancer: comparison and correlation with prognosis. *Breast Cancer Res Treat* 32, 165-175 (1994)
129. Wintzer H.O, I. Zipfel, J. Schulte-Monting, U. Hellerich & S. von Kleist: Ki-67 immunostaining in human breast tumors and its relationship to prognosis. *Cancer* 67, 421-428 (1991)
130. Wedin R, L. Skoog & H.C. Bauer: Proliferation rate, hormone receptor status and p53 expression in skeletal

metastasis of breast carcinoma. *Acta Oncol* 43, 460-466 (2004)

131. Colleoni M, E. Orvieto, F. Nole, L. Orlando, I. Minchella, G. Viale, G. Peruzzotti, C. Robertson, C. Noberasco, V. Galimberti, V. Sacchini, P. Veronesi, S. Zurrida, R. Orecchia & A. Goldhirsch: Prediction of response to primary chemotherapy for operable breast cancer. *Eur J Cancer* 35, 574-579 (1999)

132. Faneyte I.F, J.G. Schrama, J.L. Peterse, P.L. Remijnse, S. Rodenhuis, M.J. van de Vijver: Breast cancer response to neoadjuvant chemotherapy: predictive markers and relation with outcome. *Br J Cancer* 88, 406-412 (2003)

133. Vincenti V, C. Cassano, M. Rocchi & G. Persico: Assignment of the vascular endothelial growth factor gene to human chromosome 6p21.3. *Circulation* 93, 1493-1495 (1996)

134. Linderholm B, B. Tavelin, K. Grankvist & R. Henriksson: Vascular endothelial growth factor is of high prognostic value in node-negative breast carcinoma. *J Clin Oncol* 16, 3121-3128 (1998)

135. Ferrara N, K. Houck, L. Jakeman & D.W. Leung: Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr Rev* 13, 18-32 (1992)

136. Neufeld G, T. Cohen, S. Gengrinovitch & Z. Poltorak: Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J* 13, 9-22 (1999)

137. Linderholm B, B. Lindh, B. Tavelin, K. Grankvist & R. Henriksson: p53 and vascular-endothelial-growth-factor (VEGF) expression predicts outcome in 833 patients with primary breast carcinoma. *Int J Cancer* 89, 51-62 (2000)

138. Gasparini G, M. Toi, M. Gion, P. Verderio, R. Dittadi, M. Hanatani, I. Matsubara, O. Vinante, E. Bonoldi, P. Boracchi, C. Gatti, H. Suzuki & T. Tominaga: Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma. *J Natl Cancer Inst* 89, 139-147 (1997)

139. Toi M, K. Inada, H. Suzuki & T. Tominaga: Tumor angiogenesis in breast cancer: its importance as a prognostic indicator and the association with vascular endothelial growth factor expression. *Breast Cancer Res Treat* 36, 193-204 (1995)

140. Van der Auwera I, S.J. Van Laere, G.G. Van den Eynden, I. Benoy, P. van Dam, C.G. Colpaert, S.B. Fox, H. Turley, A.L. Harris, E.A. Van Marck, P.B. Vermeulen & L.Y. Dirix: Increased angiogenesis and lymphangiogenesis in inflammatory versus noninflammatory breast cancer by real-time reverse transcriptase-PCR gene expression quantification. *Clin Cancer Res* 10, 7965-7971 (2004)

141. Chelouche-Lev D, C.P. Miller, C. Tellez, M. Ruiz, M. Bar-Eli & J.E. Price: Different signalling pathways regulate

VEGF and IL-8 expression in breast cancer: implications for therapy. *Eur J Cancer* 40, 2509-2518 (2004)

142. Eppenberger U, W. Kueng, J.M. Schlaeppli, J.L. Roesel, C. Benz, H. Mueller, A. Matter, M. Zuber, K. Luescher, M. Litschgi, M. Schmitt, J.A. Foekens & S. Eppenberger-Castori: Markers of tumor angiogenesis and proteolysis independently define high- and low-risk subsets of node-negative breast cancer patients. *J Clin Oncol* 16, 3129-3136 (1998)

143. Berns E.M, J.G. Klijn, M.P. Look, N. Grebenchtchikov, R. Vossen, H. Peters, A. Geurts-Moespot, H. Portengen, I.L. van Staveren, M.E. Meijer-van Gelder, B. Bakker, F.C. Sweep & J.A. Foekens: Combined vascular endothelial growth factor and TP53 status predicts poor response to tamoxifen therapy in estrogen receptor-positive advanced breast cancer. *Clin Cancer Res* 9, 1253-1258 (2003)

144. Nicolini A, D. Campani, P. Miccoli, C. Spinelli, A. Carpi, M. Menicagli, P. Ferrari, G. Gadducci, G. Rossi, M. Fini, G. Giavaresi, V. Bonazzi & R. Giardino: Vascular endothelial growth factor (VEGF) and other common tissue prognostic indicators in breast cancer: a case-control study. *Int J Biol Markers* 19, 275-281 (2004)

145. Meo S, R. Dittadi, L. Peloso & M. Gion: The prognostic value of vascular endothelial growth factor, urokinase plasminogen activator and plasminogen activator inhibitor-1 in node-negative breast cancer. *Int J Biol Markers* 19, 282-288 (2004)

146. Ignoffo R.J: Overview of bevacizumab: a new cancer therapeutic strategy targeting vascular endothelial growth factor. *Am J Health Syst Pharm* 61, S21-26 (2004)

147. Kufe D, G. Inghirami, M. Abe, D. Hayes, H. Just-Wheeler & J. Schlom: Differential reactivity of a novel monoclonal antibody (DF3) with human malignant versus benign breast tumors. *Hybridoma* 3, 223-232 (1994)

148. Schlom J, J. Greiner, P.H. Hand, D. Colcher, G. Inghirami, M. Weeks, S. Pestka, P.B. Fisher, P. Noguchi & D. Kufe: Monoclonal antibodies to breast cancer-associated antigens as potential reagents in the management of breast cancer. *Cancer* 54, 2777-2794 (1984)

149. ASCO Special Article: Clinical practice guidelines for the use of tumor markers in breast and colorectal cancer. *J Clin Oncol* 14, 2843-2877 (1996)

150. Neville A.M, S. Patel, M. Capp, D.J. Laurence, E.H. Cooper, C. Turberville & R.C. Coombes: The monitoring role of plasma CEA alone and in association with other tumor markers in colorectal and mammary carcinoma. *Cancer* 42, 1448-1451 (1978)

151. Schlegel G, M. Luthgens, G. Eklund & B. Bjorklund: Correlation between activity in breast cancer and CEA, TPA, and eighteen common laboratory procedures and the improvement by the combined use of CEA and TPA.

Tumor Diagnostik 2, 6-11 (1981)

152. Molina R, J. Jo, G. Zanon, X. Filella, B. Farrus, M. Munoz, M.L. Latre, J. Pahisa, M. Velasco, P. Fernandez, J. Estape & A.M. Ballesta: Utility of C-erbB-2 in tissue and in serum in the early diagnosis of recurrence in breast cancer patients: comparison with carcinoembryonic antigen and CA 15.3. *Br J Cancer* 74, 1126-1131 (1996)

153. Nicolini A, A. Carpi, P. Ferrari & L. Pieri: Utility of a serum tumor marker panel in the post-operative follow-up of breast cancer patients with equivocal conventional radiological examinations. *Tumor Biol* 24, 275-280 (2003)

154. Nicolini A, P. Ferrari, A. Sagripanti & A. Carpi: The role of tumor markers in predicting skeletal metastases in breast cancer patients with equivocal bone scintigraphy. *Br J Cancer* 79, 1443-1447 (1999)

155. Nicolini A & A. Carpi: Postoperative follow-up of breast cancer patients: overview and progress in the use of tumor markers. *Tumor Biol* 21, 235-248 (2000)

156. Gold P & S.O. Freedman: Specific carcinoembryonic antigens of the human digestive system. *J Exp Med* 122, 467-481 (1965)

157. Hostetter R.B, L.B. Augustus, R. Mankarious, K.F. Chi, D. Fan, C. Toth, P. Thomas & J.M. Jessup: Carcinoembryonic antigen as a selective enhancer of colorectal cancer metastasis. *J Natl Cancer Inst* 82, 380-385 (1990)

158. Verhelst J, E. Vanden Broecke, J. Van Meerbeeck, W. De Backer, P. Blockx & P. Vermeire: Calculation of half-life of carcinoembryonic antigen after lung tumor resection: a case report. *Eur Respir J* 4, 374-376 (1991)

159. Hayes D.F, H. Sekine, T. Ohno, M. Abe, K. Keefe & D.W. Kufe: Use of a murine monoclonal antibody for detection of circulating plasma DF3 antigen levels in breast cancer patients. *J Clin Invest* 75, 1671-1678 (1985)

160. Hilkens J, J. Hilgers & F. Buijs: Monoclonal antibodies against human milk fat globule membranes useful in carcinoma research. *Prot Biol Fluids* 31, 1013-1016 (1983)

161. Bjorklund B: Tumor markers TPA, TPA-S and cytokeratins. A working hypothesis. *Tumor Diagnostik & Therapie* 13, 78-80 (1992)

162. Cocciolo M.G, D. Giani, C. Grassi, B. Pizzorno & A. Maurer: Mucin-like carcinoma associated antigen (MCA) in breast cancer. In: Updating in tumor markers in tissues and in biological fluids. Eds: Ballesta A.M, Torre G.C, Bombardieri E, Gion M, Molina R, Minerva Medica, Torino, Italy (1993)

163. Bombardieri E, E. Seregini, F. Crippa & G.L. Buraggi: An update of the biology of mucins as tumor markers. In: Updating in tumor markers in tissues and in biological

fluids. Eds: Ballesta A.M, Torre G.C, Bombardieri E, Gion M, Molina R, Minerva, Torino, Italy (1993)

164. Stahli C, M. Caravatti, M. Aeschbacher, C. Kocyba, B. Takacs & H. Carmann: Mucin-like carcinoma-associated antigen defined by three monoclonal antibodies against different epitopes. *Cancer Res.* 1, 6799-6802 (1988)

165. Nicolini A, G. Tartarelli, M.R. Metelli, P. Ferrari, L. Anselmi, A. Carpi, M. Conte & P. Miccoli: Intensive post-operative follow-up of breast cancer patients with tumor markers: CEA, TPA or CA15.3 vs MCA and MCA-CA15.3 vs CEA-TPA-CA15.3 panel in the early detection of relapse (unpublished data).

166. Cooper E.H: CA549: a marker for breast cancer. In: Updating in tumor markers in tissues and in biological fluids. Eds: Ballesta A.M, Torre G.C, Bombardieri E, Gion M, Molina R, Minervaa Medica, Torino, Italy (1993)

167. Chigira M, T. Shinozaki, T. Shimizu & K. Noda: No correlation between tumor markers and prognosis under polygenic control systems. *Med Hypotheses* 32: 245-247 (1990)

168. Nicolini A, C. Colombini, L. Luciani, A. Carpi & L. Giuliani: Evaluation of serum CA15-3 determination with CEA and TPA in the post-operative follow-up of breast cancer patients. *Br J Cancer* 64, 154-158 (1991)

169. Madeddu G, A. Farris, A.R. Casu, M.A. Saiu, R. Eriu, C. Costanza, M.L. Arras & S. Campus.: Serum tissue polypeptide antigen in breast cancer. *Cancer Detect Prev* 6, 617 (1983)

170. Shinozaki T, M. Chigira & K. Kato: Multivariate analysis of serum tumor markers for diagnosis of skeletal metastases. *Cancer* 69, 108-112 (1992)

171. Nicolini A, A. Carpi, G. Di Marco, L. Giuliani, R. Giordani & S. Palla: A rational postoperative follow-up with carcinoembryonic antigen, tissue polypeptide antigen, and urinary hydroxyproline in breast cancer patients. *Cancer* 63, 2037-2046 (1989)

172. Nicolini A, A. Carpi, P. Ferrari, L. Anselmi, C. Spinelli, M. Conte & P. Miccoli: The role of tumor markers in improving the accuracy of conventional chest X-ray and liver echography in the post-operative detection of thoracic and liver metastases from breast cancer. *Br J Cancer* 83, 1412-1417 (2000)

173. Nicolini A, A. Carpi & C. Tibaldi: The post-operative management of breast cancer patients: new concepts. In: Oncology. Eds: Carpi A, Sagripanti A, Mittermayer C Symposium, Munchen, Germany (1992)

174. Nicolini A, L. Anselmi, C. Michelassi & A. Carpi: Prolonged survival by 'early' salvage treatment of breast cancer patients: a retrospective 6-year study. *Br J Cancer* 76, 1106-1111 (1997)

175. Nicolini A, A. Carpi, C. Michelassi, C. Spinelli, M. Conte, P. Miccoli, M. Fini & R. Giardino: "Tumor marker guided" salvage treatment prolongs survival of breast cancer patients: final report of a 7-year study. *Biomed Pharmacother* 57, 452-459 (2003)
176. Nieto Y, S. Nawaz, R.B. Jones, E.J. Shpall, P.J. Cagnoni, P.A. McSweeney, A. Baron, C. Razook, S. Matthes & S.I. Barman: Prognostic model for relapse after high-dose chemotherapy with autologous stem-cell transplantation for stage IV oligometastatic breast cancer. *J Clin Oncol* 20, 707-718 (2002)
177. Miserez A.R, I. Gunes, J. Muller-Brand, E. Walther R Fridrich & H. Macke: Clinical value of a mucin-like carcinoma-associated antigen in monitoring breast cancer patients in comparison with CA 15-3. *Eur J Cancer* 27, 126-131 (1991)
178. Gunes I, A.R. Miserez, J. Muller-Brand & H. Maecke: The diagnostic value of mucinous carcinoma-associated antigen (MCA) tests in breast carcinomas. *Int J Biol Markers* 7, 222-224 (1992)
179. Tobias, R. Rothwell, J. Wagner, A. Green & Y.S. Liu: Development and evaluation of a radioimmunoassay for the detection of monoclonal antibody defined breast tumor associated antigen 115D8/DF3. *Clin Chem* 31, 986 (1985)
180. Jezersek B, J. Cervek, Z. Rudolf & S. Novakovic: Clinical evaluation of potential usefulness of CEA, CA 15-3, and MCA in follow-up of breast cancer patients. *Cancer Lett* 110, 137-144 (1996)
181. Nicolini A, M. Ferdeghini, C. Colombini & A. Carpi: Evaluation of serum CA549, CA M26 and CA M29 levels in the post-operative follow-up of breast cancer patients. *J Nucl Med Allied Sci* 34, 309-313 (1990)
182. Nicolini A, M. Ferdeghini & C. Colizzi: Evaluation of CEA, TPA, CA 15-3, CA 549 and TPS in the monitoring of metastatic breast cancer. *J Nucl Biol Med* 37, 126-133 (1993)
183. Cooper E.H, M.A. Forbes, A.K. Hancock, J.J. Price & D. Parker: An evaluation of mucin-like carcinoma associated antigen (MCA) in breast cancer. *Br J Cancer* 59, 797-800 (1989)
184. Luthgens M & G. Schlegel: Tissue polypeptide antigen and carcinoembryonic antigen in the management of tumor patients treated with cytotoxic chemotherapy. *Tumor Diagnostic & Therapie* 3, 120-126 (1982)
185. Dixon A.R, M.R. Price, C.W. Hand, P.E. Sibley, C. Selby, R.W. Blamey: Epithelial mucin core antigen (EMCA) in assessing therapeutic response in advanced breast cancer-a comparison with CA15.3. *Br J Cancer* 68, 947-949 (1993)
186. Carney W.P, R. Neumann, A. Lipton, K. Leitzel, S. Ali & C.P. Price: Potential clinical utility of serum HER-2/neu oncoprotein concentrations in patients with breast cancer. *Clin Chem* 49, 1579-1598 (2003)
187. Mehta R.R, J.H. McDermott, T.J. Hieken, K.C. Marler, M.K. Patel, L.D. Wild & T.K. Das Gupta: Plasma c-erbB-2 levels in breast cancer patients: prognostic significance in predicting response to chemotherapy. *J Clin Oncol* 16, 2409-2416 (1998)
188. Saghatchian M, S. Guepratte, K. Hacene, R. Neumann, J.L. Loiras, & M.F. Pichon: Serum HER-2 extracellular domain: relationship with clinicobiological presentation and prognostic value before and after primary treatment in 701 breast cancer patients. *Int J Biol Markers* 19, 14-22 (2004)
189. Fehm T, W. Jager, S. Kraemer, C. Sohn, G. Solomayer-Meyberg, E.F. Solomayer, R. Kurek, D. Wallwiener & G. Gebauer: Changes of serum HER2 status during clinical course of metastatic breast cancer patients. *Anticancer Res* 24, 4205-4210 (2004)
190. Pichon M.F, K. Hacene, S. Guepratte & R. Neumann: Serum HER-2 extracellular domain (ECD) before the first metastasis in 128 breast cancer patients. *Clin Lab* 50, 163-170 (2004)
191. Jensen B.V, J.S. Johansen & P.A. Price: High levels of serum HER-2/neu and YKL-40 independently reflect aggressiveness of metastatic breast cancer. *Clin Cancer Res* 9, 4423-4434 (2003)
192. Luftner D, P. Henschke, B. Flath, C. Akrivakis, S. Schnabel, B. Prinz, R. Geppert, K.D. Wernecke & K. Possinger: Serum HER-2/neu as a prediction and monitoring parameter in a phase II study with weekly paclitaxel in metastatic breast cancer. *Anticancer Res* 24, 895-906 (2004)
193. Kostler W.J, B. Schwab, C.F. Singer, R. Neumann, E. Rucklinger, T. Brodowicz, S. Tomek, M. Niedermayr, M. Hejna, G.G. Steger, M. Krainer, C. Witschke & C.C. Zielinski: Monitoring of serum Her-2/neu predicts response and progression-free survival to trastuzumab-based treatment in patients with metastatic breast cancer. *Clin Cancer Res* 10, 1618-1624 (2004)
194. Lipton A, S.M. Ali, K. Leitzel, L. Demers, V. Chinchilli, L. Engle, H.A. Harvey, C. Brady, C.M. Nalin, M. Dugan, W. Carney & J. Allard: Elevated serum Her-2/neu level predicts decreased response to hormone therapy in metastatic breast cancer. *J Clin Oncol* 20, 1467-1472 (2002)
195. Luftner D, P. Henschke, A. Kafka, I. Anagnostopoulos, K. Wiechen, R. Geppert, H. Stein, K.D. Wernecke, R. Kreienberg, K. Possinger: Discordant results obtained for different methods of HER-2/neu testing in breast cancer--a question of standardization, automation and timing. *Int J Biol Markers* 19, 1-13 (2004)
196. Luftner D, C. Luke & K. Possinger: Serum HER-

2/neu in the management of breast cancer patients. *Clin Biochem* 36, 233-40 (2003)

197. Vojtesek B, J. Kovarik, H. Dolezalova, R. Nenutil, P. Havlis, R.R. Brentani & D.P. Lane: Absence of p53 autoantibodies in a significant proportion of breast cancer patients. *Br J Cancer* 71, 1253-1256 (1995)

198. Regidor P.A, M. Regidor, R. Callies & A.E. Schindler: Detection of p53 auto-antibodies in the sera of breast cancer patients with a new recurrence using an ELISA assay. Does a correlation with the recurrence free interval exist? *Eur J Gynaecol Oncol* 17, 192-199 (1996)

199. Sangrajrang S, W. Arpornwirat, A. Cheirsilpa, P. Thisuphakorn, A. Kalalak, A. Sornprom & T. Soussi: Serum p53 antibodies in correlation to other biological parameters of breast cancer. *Cancer Detect Prev* 27, 182-186 (2003)

200. Angelopoulou K, H. Yu, B. Bharaj, M. Gai & E.P. Diamandis: p53 gene mutation, tumor p53 protein overexpression, and serum p53 autoantibody generation in patients with breast cancer. *Clin Biochem* 33, 53-62 (2000)

201. Metcalfe S, T.K. Wheeler, S. Picken, S. Negus & A. Jo Milner: P53 autoantibodies in 1006 patients followed up for breast cancer. *Breast Cancer Res* 2, 438-443 (2000)

202. Regele S, F.D. Vogl, T. Kohler, R. Kreienberg & I.B. Runnebaum: p53 autoantibodies can be indicative of the development of breast cancer relapse. *Anticancer Res* 23, 761-764 (2003)

203. Hayes D.F, R.C. Bast, C.E. Desch, H. Jr Fritsche, N.E. Kemeny, J.M. Jessup, G.Y. Locker, J.S. Macdonald, R.G. Mennel, L. Norton, P. Ravdin, S. Taube & R.J. Winn: Tumor marker utility grading system: a framework to evaluate clinical utility of tumor markers. *J Natl Cancer Inst* 16, 1456-1466 (1996)

204. Lambrechts A.C, L.J. van't Veer & S. Rodenhuis: The detection of minimal numbers of contaminating epithelial tumor cells in blood or bone marrow: use, limitations and future of RNA-based methods. *Ann Oncol* 9, 1269-1276 (1998)

205. Pantel K, R.J. Cote & O. Fodstad: Detection and clinical importance of micrometastatic disease. *J Natl Cancer Inst* 91, 1113-1124 (1999)

206. Fields K.K, G.J. Elfenbein, W.L. Trudeau, J.B. Perkins, W.E. Janssen & L.C. Moscinski: 1996. Clinical significance of bone marrow metastases as detected using the polymerase chain reaction in patients with breast cancer undergoing high-dose chemotherapy and autologous bone marrow transplantation. *J Clin Oncol* 14, 1868-1876 (1996)

207. Schoenfeld A, K.H. Kruger, J. Gomm, H.D. Sinnett,

J.C. Gazet, N. Sacks, H.G. Bender, Y. Luqmani & R.C. Coombes: The detection of micrometastases in the peripheral blood and bone marrow of patients with breast cancer using immunohistochemistry and reverse transcriptase polymerase chain reaction for keratin 19. *Eur J Cancer* 33, 854-861 (1997)

208. Slade M.J, B.M. Smith, H.D. Sinnett, N.C. Cross & R.C. Coombes: Quantitative polymerase chain reaction for the detection of micrometastases in patients with breast cancer. *J Clin Oncol* 17, 870-879 (1999)

209. Cristofanilli M, G.T. Budd, M.J. Ellis, A. Stopeck, J. Matera, M.C. Miller, J.M. Reuben, G.V. Doyle, W.J. Allard, L.W. Terstappen & D.F. Hayes: Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med* 351, 781-791 (2004)

210. Gaforio J.J, M.J. Serrano, P. Sanchez-Rovira, A. Sirvent, M. Delgado-Rodriguez, M. Campos, N. de la Torre, I. Algarra, R. Duenas & A. Lozano: Detection of breast cancer cells in the peripheral blood is positively correlated with estrogen-receptor status and predicts for poor prognosis. *Int J Cancer* 107, 984-90 (2003)

211. Stathopoulou A, I. Vlachonikolis, D. Mavroudis, M. Perraki, Ch. Kouroussis, S. Apostolaki, N. Malamos, S. Kakolyris, A. Kotsakis, N. Xenidis, D. Reppas & V. Georgoulas: Molecular detection of cytokeratin-19-positive cells in the peripheral blood of patients with operable breast cancer: evaluation of their prognostic significance. *J Clin Oncol* 20, 3404-3412 (2002)

212. Terstappen L.W, C. Rao, S. Gross & A.J. Weiss: Peripheral blood tumor cell load reflects the clinical activity of the disease in patients with carcinoma of the breast. *Int J Oncol* 17, 573-578 (2000)

213. Brankin B, T.C. Skaar, M. Brotzman, B. Trock & R. Clarke: Autoantibodies to the nuclear phosphoprotein nucleophosmin in breast cancer patients. *Cancer Epidemiol Biomarkers Prev* 7, 1109-1115 (1998)

214. Skaar T.C, S.C. Prasad, S. Sharareh, M.E. Lippman, N. Brunner & R. Clarke: Two-dimensional gel electrophoresis analyses identify nucleophosmin as an estrogen regulated protein associated with acquired estrogen-independence in human breast cancer cells. *J Steroid Biochem Mol Biol* 67, 391-402 (1998)

215. Mills P.J, B. Parker, V. Jones, K.A. Adler, C.J. Perez, S. Johnson, M. Cohen-Zion, M. Marler, G.R. Sadler, J.E. Dimsdale & S. Ancoli-Israel: The effects of standard anthracycline-based chemotherapy on soluble ICAM-1 and vascular endothelial growth factor levels in breast cancer. *Clin Cancer Res* 10, 4998-5003 (2004)

216. Mancuso P, A. Calleri, C. Cassi, A. Gobbi, M. Capillo, G. Pruneri, G. Martinelli & F. Bertolini: Circulating endothelial cells as a novel marker of angiogenesis. *Adv Exp Med Biol* 522, 83-97 (2003)

217. Byrne G.J, A. Ghellal, J. Iddon, A.D. Blann, V. Venizelos, S. Kumar, A. Howell & N.J. Bundred: Serum soluble vascular cell adhesion molecule-1: role as a surrogate marker of angiogenesis. *J Natl Cancer Inst* 92, 1329-1336 (2000)
218. O'Hanlon D.M, H. Fitzsimons, J. Lynch, S. Tormey, C. Malone & H.F. Given: Soluble adhesion molecules (E-selectin, ICAM-1 and VCAM-1) in breast carcinoma. *Eur J Cancer* 38, 2252-2257 (2002)
219. Banks R.E, A.J. Gearing, I.K. Hemingway, D.R. Norfolk, T.J. Perren & P.J. Selby: Circulating intercellular adhesion molecule-1 (ICAM-1), E-selectin and vascular cell adhesion molecule-1 (VCAM-1) in human malignancies. *Br J Cancer* 68, 122-124 (1993)
220. Sheen-Chen S.M, H.L. Eng, C.C. Huang & W.J. Chen: Serum levels of soluble E-selectin in women with breast cancer. *Br J Surg* 91, 1578-1581 (2004)
221. Eichbaum M.H, T.M. de Rossi, S. Kaul & G. Bastert: Serum levels of soluble E-selectin are associated with the clinical course of metastatic disease in patients with liver metastases from breast cancer. *Oncol Res* 14, 603-610 (2004)
222. Vogel C.L, M.A. Cobleigh, D. Tripathy, J.C. Gutheil, L.N. Harris, L. Fehrenbacher, D.J. Slamon, M. Murphy, W.F. Novotny, M. Burchmore, S. Shak, S.J. Stewart & M. Press: Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J Clin Oncol* 20, 719-726 (2002)
223. Pegram M.D, G.E. Konecny, C. O'Callaghan, M. Beryt, R. Pietras & D.J. Slamon: Rational combinations of trastuzumab with chemotherapeutic drugs used in the treatment of breast cancer. *J Natl Cancer Inst* 96, 739-749 (2004)
224. Albain K, R. Elledge & W.J. Gradishar: Open-label, phase II, multicenter trial of ZD1839 ("Iressa") in patients with advanced breast cancer. *Breast Cancer Res Treat* 76, S33 (2002)
225. Winer E, M. Cobleigh & M. Dickler: Phase II multicenter study to evaluate the efficacy and safety of tarcevaTM (erlotinib, OSI-774) in women with previously treated locally advanced or metastatic breast cancer. *Breast Cancer Res Treat* 76, S115 (2002)
226. Maung K: Inhibition of ErbB1 and ErbB2 by lapatinib ditosylate, a dual kinase inhibitor; promising activity in pretreated advanced breast cancer. *Clin Breast Cancer* 4, 398-400 (2004)
227. Agus D.B, M. Gordon & C. Taylor: Clinical activity in a phase I trial of HER-2 targeted rhuMab2C4 (pertuzumab) in patients with advanced solid malignancies (AST) *Proc Annu Meet Am Soc Clin Oncol* 22, (2003)
228. Cobleigh M.A, V.K. Langmuir, G.W. Sledge, K.D. Miller, L. Haney, W.F. Novotny, J.D. Reimann & A. Vassel: A phase I/II dose-escalation trial of bevacizumab in previously treated metastatic breast cancer. *Semin Oncol* 30, 117-124 (2003)
229. Miller K.D, L.I. Chap, F.A. Holmes, M.A. Cobleigh, P.K. Marcom, L. Fehrenbacher, M. Dickler, B.A. Overmoyer, J.D. Reimann, A.P. Sing, V. Langmuir & H.S. Rugo: Randomized phase III trial of capecitabine compared with bevacizumab plus capecitabine in patients with previously treated metastatic breast cancer. *J Clin Oncol* 1, 792-799 (2005)

Abbreviations: AF1, AF2, AF-3: activation function-1, 2, 3 , ASCO: American Society of Clinical Oncology , BrdUrd: bromodeoxyuridine , CA15.3: carbohydrate antigen 15.3, CA549: cancer antigen 549, cdk: cyclin-dependent kinase , CE: constant elevation , CEA: carcinoembryonic antigen, CK-19: cytokeratin 19 , CT: computed tomography , CTCs: circulating tumor cells , DBA: DNA binding activity , DCIS: ductal carcinoma in situ , DFS: disease-free survival, DR5: death receptor 5, ECD HER2/neu: extracellular domain of epidermal growth factor receptor, EGFR: epidermal growth factor receptor, EIA: enzyme immunoassay, ELISA: enzyme-linked immunoassay, ER: estrogen receptor, ERE: estrogen response element , FISH: fluorescent in situ hybridization , GF: growth factor, HMFGM: human milk fat globule membranes , hPR-B, hPR-A: human progesterone receptors A, B, ICA: immunocytochemistry assay , ICAM-1: intracellular adhesion molecule 1 , IEV: isolated elevated value , IGF-BP3: insulin growth factor-binding protein 3, IGF-I and IGF-II: insulin growth factors I and II, IHC: immunohistochemistry , LBD: ligand binding domain, LI: labeling index , MABs: monoclonal antibodies , MAPK: mitogen-activated protein kinase, MCA: mucin-like carcinoma associated antigen , MRI: magnetic resonance imaging, N+: node positive , N-: node negative, NB: northern blot , NK cell: natural killer cell, NPM: nucleophosmin, OS: overall survival , PCR: polymerase chain reaction , PI: progressive increase , PI3K: phosphatidylinositol-3-kinase, PR: progesterone receptor, RIA: immunoradiometric assay, RT-PCR: reverse transcription polymerase chain reaction , SB: Southern blotting, SERMs: selective estrogen receptor modulators, SPF: S-phase fraction, TAA: tumor associated antigen, TAM: tamoxifen, TGF-alpha: transforming growth factor alpha, TK: thymidine kinase, TMUGS: tumor marker utility grading system, TPA: tissue polypeptide antigen, VCAM-1: vascular cell adhesion molecule 1 , VEGF: vascular endothelial growth factor, VPF: vascular permeability factor , WB: Western blotting

Key Words: Her2, Neu, Ki67, ER, PR, Estrogen receptor, Progesterone receptor, CEA, carcinoembryonic antigen, MAA, Mucin-like carcinoma associated antigen, Tissue polypeptide antigen, TPA, Vascular endothelial growth factor, VEGF, Selectin, Intracellular adhesion molecule 1, (ICAM-1, vascular cell adhesion

Biomarkers of Breast cancer

molecule Breast, Cancer, Neoplasia, Tumor,
Biomolecular Markers, Review

Send correspondence to: A Nicolini MD, Department
of Internal Medicine, University of Pisa, Via Roma 67,
56126 Pisa, Italy, Tel: 050 992141, Fax: 050- 553414,
E-mail: a.nicolini@int.med.unipi.it

<http://www.bioscience.org/current/vol11.htm>