Cell Prof. 1998, **31,** 113–126

REVIEW

Prognosis and prediction of response in breast cancer: the current role of the main biological markers

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(*Received* 20 *May* 1998; *accepted* 19 *June* 1998)

Abstract. In the medical literature there are frequently conflicting reports on the utility of biological tumour markers available in the clinical management of breast cancer. In this review we analyse current information on the relationships between the most widely investigated breast cancer biological markers including oestrogen and progesterone receptors, p53, Bcl-2, c-*erb*B-2, cyclin expression, proliferative activity, DNA ploidy and the urokinase plasminogen activation system, as well as their relevance to prognosis and response to clinical treatment. By biological prognostic indicator, we mean a marker that correlates with survival and disease-free survival; the term predictor marker indicates a marker that is capable of predicting tumour sensitivity or resistance to various therapies. Similarly to other authors' experiences, our analysis suggests that oestrogen receptors are weak prognostic indicators and good predictors of response to endocrine therapy. Furthermore, there are consistent data suggesting that proliferation indices are good indicators of prognosis, and that they are directly related to response to chemotherapy and closely related to response to hormonotherapy. On the contrary, there is no evidence or conflicting data for all of the other biological markers. These should be considered in the context of randomized trials in order to precisely define their prognostic and predictive roles. p53 and c-*erb*B-2 seem to be the most promising factors, but their use in routine practice still needs validation.

In the clinical management of breast cancer, the identification of prognostic indicators and predictors of response to therapy may have an important impact on treatment decision making and improve clinical outcome. However, although some clinicians have introduced a number of laboratory-derived factors into their clinical decision-making process for entry criteria, co-operative group trials clearly reflect a more cautious approach that is generally based on classic tumour node metastasis (TNM) information and steroid hormone receptor status in order to define eligibility and stratification subsets (Ravdin 1997). Various biological variables have been investigated, but there is no general consensus as to their acceptability for therapeutic management. For this reason, a decisional model for the clinical validation of biological variables was proposed by Silvestrini (Fig. 1), and a tumour marker utility grading

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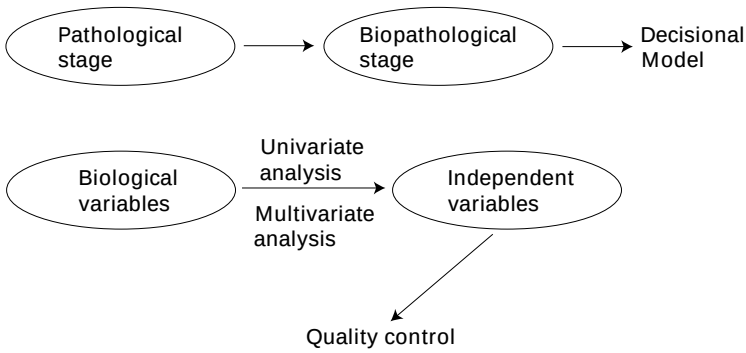

Figure 1. Decisional model for the clinical validation of biological markers. With the permission of Rosella Silvestrini, *Le Scienze* 1994.

scale (TMUGS) was recently designed as a working document for organizing the results of tumour marker studies (Hayes 1997). Bearing these recommendations in mind, we have reviewed the value of tumour markers reflecting various biological features of breast cancer that are currently being investigated in a clinical setting. This review cites published data relating to *p53* tumour suppressor gene, Bcl-2 apoptosis inhibitor, c-*erb*B-2 oncogene, cyclin cell-cycle regulatory proteins, DNA ploidy, urokinase plasminogen activation system and proliferation markers such as ^{3}H-thymidine labelling index (TLI), S-phase fraction determined by flow cytometry (FCM-S), and the Ki-67/MIB index. In particular, we have analysed these variables as both indicators of prognosis and predictors of clinical response to hormone and/or chemotherapy.

OESTROGEN AND PROGESTERONE RECEPTORS

Multivariate analyses in studies involving node-negative breast cancer patients have shown that oestrogen receptors (ER) and progesterone receptors (PgR) are not strong predictors of prognosis (Fisher *et al.* 1988, Arriagada *et al.* 1992, Silvestrini *et al.* 1995). However, on the basis of data deriving from meta-analysis, the American Society of Clinical Oncology suggests that ER can be used to identify both pre and postmenopausal women most likely to benefit from endocrine therapy (American Society of Clinical Oncology 1996).

A large number of reports have described improved disease-free or overall survival following adjuvant tamoxifen treatment in postmenopausal women with ER-positive tumours (Early Breast Cancer Trialists' Collaborative Group 1988, 1992, Hilf *et al.* 1980, Fisher *et al.* 1983, Raemaekers *et al.* 1987). Various trials have also shown that pre-menopausal patients with ER-positive tumours achieve a better clinical outcome after hormonal therapy, although the advantage is less than that observed in the postmenopausal group (Early Breast Cancer Trialists' Collaborative Group 1988, Stewart 1992). However, two separate studies have indicated that postmenopausal women receiving adjuvant tamoxifen treatment survive longer, even if their oestrogen receptor status is negative or unknown (Early Breast Cancer Trialists' Collaborative Group 1988, 1992, Breast Cancer Trials Committee 1987, Stewart 1992). Many gaps still exist in understanding the mechanisms underlying oestrogen and progesterone inhibition of breast cancer cell growth (King 1993). One of the major unresolved issues concerns the reason as to why nearly 50% of ER-positive tumours fail to respond to hormonal treatment, and why 10% of ER-negative patients are responsive to treatment with

anti-oestrogens (Hoskins & Weber 1994). Various hypotheses have been proposed in an attempt to explain these discrepancies, such as presence of negative oestrogen receptor subpopulations which escape hormonal therapy in positive receptor tumours, loss of receptors during hormonotherapy, molecular modification of receptors, alterations in postreceptorial events or alterations in drug metabolism (Murphy 1995).

A prospective study on patients with metastatic ER-positive breast cancer reported an ≈50% response rate to front-line endocrine therapy, but PgR can refine this estimate by splitting ER positive patients into corresponding subsets with 40% (ER+, PgR−) and 60% (ER+, PgR+) response rates (Ravdin *et al.* 1992). On the contrary, patients who are both ER and PgR negative show a less than 5% response rate to endocrine therapy (Ravdin 1997).

The relationship between ER or PgR levels and response to cytotoxic chemotherapy has been less intensively analysed in retrospective studies (Raemaekers *et al.* 1987, Hilf *et al.* 1980). In a recent adjuvant trial of CMF chemotherapy vs. ovarian ablation, pre-menopausal women with ER negative tumours showed a better clinical outcome after chemotherapy, whereas those with ER positive tumours responded better to ovarian ablation (Scottish Cancer Trials Breast Group & ICRF Breast Unit, Guy's London 1993). There are many retrospective studies showing the value of ER and PgR as predictors in an adjuvant setting. In particular, steroid receptor positivity seems to be an indicator of response to hormone therapy in mainly post menopausal women, whereas the lack of steroid receptors is only a weak predictor of response.

p53

Alterations in the *p53* gene are the most common genetic changes found in human malignancies, including breast cancer. The function of p53 nuclear phosphoprotein is not fully understood, but there is evidence to suggest that it plays the part of a tumour suppressor gene by controlling transcription, cell cycle and apoptosis. In particular, *p53* molecular mutations lead to an increase in *p53* gene protein expression that is detectable immunohistochemically. Large retrospective studies on breast cancer patients with (N+) or without (N−) lymph node involvement have shown that *p53* overexpression is often (Thor *et al.* 1992, Borg *et al.* 1995, Silvestrini *et al.* 1996a, Fresno *et al.* 1997), but not always (Lipponen *et al.* 1993, Schimmelpenning *et al.* 1994), an independent marker of poor prognosis. Moreover, there is also recent evidence from a study on node-negative breast cancer patients with a minimum follow-up of 10 years, that *p53* expression has no prognostic value whatsoever (Bianchi *et al.* 1997).

There are two retrospective studies concerning p53 and response to radiotherapy. Jansson *et al.* found a better clinical outcome in irradiated node-negative breast cancer patients with *p53* mutations, and suggested that irradiation can induce cell death regardless of the presence of *p53* alterations (Jansson *et al.* 1995). This finding was confirmed by Silvestrini *et al.* who found a benefit from radiation therapy in preventing local breast cancer relapse in node-negative patients with high levels of *p53* expression (Silvestrini *et al.* 1997a).

Experimental data have shown that cells containing the wild-type *p53* gene undergo death by apoptosis after treatment with anticancer drugs (5-fluorouracil, etoposide and adriamycin) and ionizing radiation, whereas those lacking wild-type *p53* show resistance. These observations suggest that *p53* mutation may be one of the factors responsible for drug resistance in cancer patients (Lowe *et al.* 1993). However, the results from *in vivo* studies on the relationship between *p53* status and chemosensitivity to clinical treatment are still contradictory.

Formenti *et al.* reported a correlation between high *p53* expression and low responsiveness to neoadjuvant 5-fluorouracil chemotherapy plus radiotherapy (Formenti *et al.* 1996). However, another neoadjuvant trial using mitoxantrone, methotrexate and tamoxifen did not highlight any relationship (Makris *et al.* 1997). In an adjuvant setting, Stal *et al.* suggest that pre-menopausal breast cancer patients with lymph node metastases benefit more from CMF chemotherapy than postoperative radiotherapy if the primary tumour has abnormal p53 accumulation (Stal *et al.* 1995). This could mean that p53-dependent apoptosis is not the only, and perhaps not the major mechanism of response to CMF. It has also been shown that specific *p53* gene mutations correlate with primary resistance to doxorubicin and early relapse (Aas *et al.* 1996).

A recent pilot study showed that postmenopausal women with node-positive ER+ breast cancers treated with radical or conservative surgery plus radiotherapy, followed by adjuvant tamoxifen, had a significantly lower relapse-free and distant metastases-free survival rate after 5 years of follow-up if their tumours overexpressed p53 (Silvestrini *et al.* 1996b). Other authors did not find any relationship between p53 expression and response to tamoxifen therapy in ER-positive metastatic breast carcinoma (Elledge *et al.* 1997), or to tamoxifen, zoladex, zoladex plus tamoxifen or megestrol acetate in patients with locally advanced or metastatic breast cancer (Archer *et al.* 1995).

Bcl-2

The Bcl-2 oncogene is a member of a family of genes involved in the regulation of cell death as it inhibits apoptosis, and has been found to be frequently overexpressed in breast cancer.

In two retrospective studies, univariate analysis revealed that Bcl-2 expression is associated with favourable clinicopathological parameters, and that it is a favourable prognostic indicator in node-negative and node-positive breast cancers (Sivestrini *et al.* 1994, Joensuu, Pylkanen & Toikkanen 1994).

Moreover, experimental *in vitro* studies have shown that the expression of Bcl-2 leads to cell death resistance following treatment with a variety of cytotoxic agents (Walton *et al.* 1993). This evidence is confirmed by the results of a study in which oestrogens were found to be capable of inducing resistance to anticancer agents in ER+ breast cancer cell lines through a mechanism regulating Bcl-2 expression (Teixeira, Reed & Pratt 1995). On the contrary, a clinical study by Gasparini *et al.* reported that women with node-positive cancer and high Bcl-2 levels had a more favourable outcome when treated with adjuvant chemotherapy (CMF) and/or hormone therapy (tamoxifen) than those with node-positive and Bcl-2 negative tumours (Gasparini *et al.* 1995). These results suggest that Bcl-2 protein expression and number of metastatic lymph nodes are independent predictors of clinical response to chemotherapy and hormonotherapy. Silvestrini *et al.* reported a significantly lower relapse-free and distant metastasis-free survival at 5 years in tamoxifen-treated node-positive patients with weak or absent *Bcl-2* expression than in those with tumours whose level of Bcl-2 expression was high. However, in this study, Bcl-2 did not provide any further prognostic information to that obtained from tumour size, axillary node involvement, steroid receptors, or ^{3}H-thymidine labelling index (Silvestrini *et al.* 1996b). On the contrary, Van Slooten *et al.* reported that the outcome of premenopausal node-negative breast cancer patients with a moderate to high expression of Bcl-2 treated with one cycle of pre-operative CMF does not seem to be improve (Van Slooten *et al.* 1996). More recently, Silvestrini *et al.* reported a greater benefit from radiation therapy in preventing local breast cancer relapse in

node-negative patients with tumours that express little or no Bcl-2 protein than in those with Bcl-2 positive tumours (Silvestrini *et al.* 1997a).

C-*erb*B-2

HER-2/*neu*, the protein produced by the c-*erb*B-2 oncogene, is a transmembrane tyrosine kinase which is a receptor for a family of peptide ligands thought to stimulate cell growth. In breast cancer, retrospective studies have shown that overexpression of this gene is associated with a poorer prognosis in patients with positive axillary nodes (Toikkanen *et al.* 1992), but its prognostic role as an independent marker in node-negative patients appears to be controversial (Lovekin *et al.* 1991, Kallioniemi *et al.* 1991, Bianchi *et al.* 1993, Press *et al.* 1994).

There is growing evidence that c-*erb*B-2 expression is an important predictor of resistance to tamoxifen in node-negative (Carlomagno *et al.* 1996) and metastatic patients (Leitzel *et al.* 1995). A possible explanation for this finding comes from experimental data showing cross-regulation between c-*erb*B-2 and oestrogen receptors (Lupu & Lippman 1993).

Conflicting data have been reported concerning the correlation between c-*erb*B-2 expression and adjuvant chemotherapy. In a randomized Cancer and Leukaemia Group B study comparing three different doses of adjuvant fluorouracil, doxorubicin and cyclophosphamide (FAC), it was found that, unlike DNA ploidy, S-phase fraction and p53 accumulation, the overexpression of c-*erb*B-2 was associated with a better disease-free survival (DFS) and overall survival (OS) in patients receiving higher-dose chemotherapy. This suggests that response to FAC is dose-related only for patients with c-*erb*B-2 overexpressing tumours (Muss *et al.* 1994). On the other hand, two large randomized adjuvant trials (Gusterson *et al.* 1992, Allred *et al.* 1992) found that c-*erb*B-2 overexpression was associated with resistance to CMF-based regimens. The Ludwig Breast Cancer Study Group trial compared one cycle of peri-operative CMF (short therapy) with six cycles of postoperative CMFp (cyclophosphamide, methotrexate, fluorouracil and prednisone) with or without one cycle of pre-operative CMF (long therapy) in node-positive patients, and found that the c-*erb*B-2-negative patients receiving the long therapy had a significantly better DFS and OS. There was no difference between the two treatment arms in c-*erb*B-2-positive patients (Gusterson *et al.* 1992). Similarly, in a study of high-risk node-negative patients (tumour size > 3 cm and ER negative), Allred *et al.* reported better survival after CMFp treatment only in c-*erb*B-2-negative patients, whereas there was no difference between treated and untreated c-*erb*B-2-positive patients (Allred *et al.* 1992).

CYCLINS

The deregulation of cell cycle control is one of the most evident alterations in tumour growth. The transition from one phase of the cell cycle to the next (G1 to S, S to G2, G2 to mitosis) is normally regulated by members of a family of proteins known as cyclins, whose concentration rises and falls during the cell cycle. An aberration in cyclin D1 protein may cause the loss of the G1-S phase control check point and thus induce abnormal cell proliferative activity which contributes to genomic instability during breast tumour development (Zhou *et al.* 1996). Immunohistochemical studies have found that the percentage of tumours overexpressing this protein ranges from 30% to 73% (Gillet *et al.* 1994, Michalides *et al.* 1996). Neither the relation between cyclin D1 expression and other biological characteristics, nor its prognostic significance, have been adequately investigated and defined (McIntosh *et al.* 1995, Gillet *et al.* 1996).

Cyclin E is a protein related to the G1/S phase transition and may consequently be a potentially deregulated molecule in tumours. It has recently been observed that it is overexpressed in breast cancer, and significantly correlates with an increased risk of death and relapse (Nielsen *et al.* 1996). Cyclin D1 overexpression has been associated with a better clinical outcome in node positive breast cancer patients treated with adjuvant hormonal treatment than in those treated with chemotherapy (Pelosio *et al.* 1996).

PROLIFERATIVE ACTIVITY MARKERS

In breast cancer, tumour proliferative activity expressed as ^{3}H-thymidine labelling index (TLI), flow cytometric S-phase fraction (FCM-S) and Ki-67/MIB index, is a potential prognostic factor. Highly proliferative tumours are generally associated with shorter disease-free and overall survival. Besides its prognostic relevance, the proliferative rate of tumour cells can also provide information on the response to clinical treatment, as has emerged from the analysis of retrospective and prospective clinical studies.

^{3}H-Thymidine labelling index

One direct way of measuring cell proliferation rate is to evaluate the incorporation of tritiated thymidine in DNA. Autoradiography is then used to assess the percentage of tumour nuclei that are in active nucleic acid synthesis.

A recent study on a series of 3800 node negative breast cancer patients identified TLI as an important prognostic factor by revealing that rapid cell proliferation is associated with a high risk of locoregional relapse and the development of distant metastases regardless of tumour size and oestrogen receptor status (Silvestrini *et al.* 1997b).

In terms of predictability of clinical response in early and advanced disease, it has been shown that the benefit of hormonotherapy is greater in patients with a low TLI, a result that has been observed in postmenopausal node-positive patients treated with hormonal adjuvant therapy (Silvestrini *et al.* 1993a) as well as in postmenopausal ER+ patients with advanced disease (Paradiso *et al.* 1990).

As far as cytotoxic treatment is concerned, retrospective analyses have shown that TLI in locally advanced breast cancer patients is not related to response to primary chemotherapy with doxorubicine and vincristine, and the proliferative index thus emerges as an indicator of long-term clinical outcome (Silvestrini *et al.* 1987).

A higher objective response rate has been observed in highly proliferating locally advanced breast cancer treated with chemotherapy including antimetabolites (FAC or CMF) (Gardin *et al.* 1994), and in metastatic breast carcinomas treated with FAC±vincristine (Sulkes, Livingston & Murphy 1979).

In an adjuvant setting, rapid cell proliferation is associated with a high risk of relapse and/or death in patients with node-positive resectable tumours treated with CMF±adriamycin (Silvestrini *et al.* 1990).

Furthermore, Gardin *et al.* reported that patients who still had high TLI tumours after neoadjuvant FAC-chemotherapy (FAC) were more likely to benefit from subsequent adjuvant chemotherapy (FAC and CMF) (Gardin *et al.* 1994).

A recent prospective study on metastatic breast cancer patients treated with FEC (5-fluorouracil, epirubicin and cyclophosphamide) or CMF as front-line therapy showed that the objective clinical response rate was significantly higher in patients with rapidly proliferating than in those with slowly proliferating primary tumours, but this was only true when chemotherapy was not preceded by hormonal treatment. This finding suggests that TLI

represents a consistent indicator of response to chemotherapy over time, and that hormonotherapy may alter the proliferative activity of tumours and, consequently, their chemosensitivity (Amadori *et al.* 1997).

FCM-S-phase fraction

A widely used means of assessing tumour proliferation is the evaluation of S-phase cell fraction from flow cytometric DNA histograms. In a recent retrospective study, it was found that the S-phase fraction is directly related to tumour size, and inversely related to steroid hormone receptor status (Dettmar *et al.* 1997). However, although this variable is an indicator of DFS and OS, the power of the positive correlation with prognosis has still to be confirmed because of retrospective nature of analyses (Bosari *et al.* 1992, Merkel *et al.* 1993, Balslev *et al.* 1994, Witzig *et al.* 1994, Pfisterer *et al.* 1995, Dettmar *et al.* 1997, Bergers *et al.* 1997).

The predictive value of FCM-S in cytotoxic treatment has also been investigated. In the case of anthracycline-neoadjuvant therapies and front-line FEC chemotherapy, tumours with a high FCM-S respond better than those with a low FCM-S (O'Reilly *et al.* 1992, Remvikos *et al.* 1993, Hietanen *et al.* 1995). However, in two prospective randomized trials of adjuvant therapy with cyclophosphamide, fluorouracil and prednisone (CFP) ± tamoxifen, univariate analysis showed that relapse-free survival (RFS) and overall survival (OS) were better in patients with a low FCM-S (Witzig *et al.* 1993), thus demonstrating that a high FCM-S is not a long-term predictor of response to chemotherapy.

Ferno *et al.* described the correlation between FCM-S and response to adjuvant endocrine therapy, reporting a higher benefit from tamoxifen for patients with PgR positive and high S-phase tumours than for those with PgR-positive and low FCM-S tumours (Ferno *et al.* 1995).

Ki-67/MIB index

Ki-67 and MIB1 monoclonal antibodies react with different epitopes of the nuclear Ki-67 antigen (Weidner *et al.* 1994). This marker is expressed during all cell cycle phases apart from the quiescent phase (G0), and is regarded as a marker of active cell proliferation. MIB1 is a murine monoclonal antibody prepared against epitopes of recombinant Ki-67 antigen. Unlike conventional Ki-67 antibodies, it recognizes the antigen in formalin-fixed, paraffin-embedded tissue sections (Cuevas *et al.* 1993).

In two recent studies, a high cell kinetic activity evaluated by means of the Ki-67 monoclonal antibody was found to be an effective indicator of poor prognosis in node-negative breast cancer patients (Pierga *et al.* 1996, Railo *et al.* 1997). Furthermore, Ki-67 staining has been reported to be an independent prognostic indicator when compared with tumour size, nodal status and steroid receptor expression (Bouzubar 1989), although a recent study found that Ki-67, evaluated by means of MIB1 monoclonal antibody, loses its significant prognostic value after multivariate analysis (Dettmar *et al.* 1997).

The relationship between the Ki-67 index and response to chemotherapy is difficult to define because it has only been studied in a small series of patients. In a prospective study of neoadjuvant chemoendocrine therapy with mitoxantrone, methotrexate (± mitomycin C) and tamoxifen, tumours with high Ki-67 levels responded better to chemotherapy, whereas slowly proliferating tumours responded better to endocrine treatment (Makris *et al.* 1997). In advanced breast carcinoma patients, response rate to first line chemotherapy (including CMF, FEC, cisplatin and etoposide, vinorelbine, and carboplatin with 5-fluorouracil and folinic acid) was also significantly higher in those with rapidly proliferating tumours, than in

those with a low Ki-67 index; however, this difference did not lead to a statistically significant improvement in overall survival (Bonetti *et al.* 1996).

DNA ploidy

Flow cytometric evaluation of DNA ploidy provides information concerning the presence or absence of abnormal DNA content as well as the extent of alteration. The frequency of aneuploid breast tumours ranges from 56% to 75% in different studies. There are numerous reports addressing the potential usefulness of DNA flow cytometry-based information in defining prognostically distinct populations of node-negative patients (Early Breast Cancer Trialists' Collaborative Group 1992). These studies do not strongly support the importance of ploidy as a prognostic variable as only a few authors have found it to be an independent indicator of survival (Clark *et al.* 1989, Silvestrini *et al.* 1993b, Duffy *et al.* 1996).

Contradictory results have been reported concerning the correlation between ploidy and clinical treatment response. When ploidy was analysed as a response predictor, no significant differences in overall survival or response rates were observed in metastatic breast cancer patients receiving FEC chemotherapy (Hietanen *et al.* 1979), patients given neoadjuvant chemo-endocrine therapy with mitoxantrone, methotrexate ($\pm$mitomycin C) and tamoxifen (Makris *et al.* 1997), or node-positive patients receiving adjuvant chemo-endocrine therapy (CFP $\pm$ tamoxifen) (Witzig *et al.* 1993). Furthermore, a pilot study conducted by O'Reilly failed to detect a significantly higher response rate of aneuploid with respect to diploid tumours to pre-operative anthracycline-based chemotherapy (O'Reilly *et al.* 1992).

Urokinase plasminogen activation system

Cancer cell invasion is accomplished by means of the concerted action of a number of extracellular proteolytic enzyme systems, one of which is the urokinase plasminogen activation system. This complex consists of the urokinase plasminogen activator (uPA), its receptor (u-PAr), its main type 1 (PAI-1) and type 2 (PAI-2) inhibitors, all of which would appear to be of prognostic value in breast cancer.

In particular, high uPA levels have been found to be predictors of tumour aggressiveness (Duffy *et al.* 1996) and poor prognosis from multivariate analysis of a subgroup of patients not treated with adjuvant therapy (Ferno *et al.* 1996). Univariate analysis has likewise shown that high levels of u-PAr are significantly associated with a shorter overall survival, although no statistically significant differences have been found in terms of relapse-free survival (Grondahl-Hansen *et al.* 1995). In postmenopausal patients, Grondahl-Hansen *et al.* more recently observed that a high PAI-1 level is an independent prognostic variable predicting short overall survival, and that high uPA levels predict short recurrence-free survival (Grondahl-Hansen *et al.* 1997). PAI-2 levels were not found to be significantly associated with prognosis in a population of more than one thousand breast cancer patients; however, selected patients with high uPA and PAI-2 levels showed prolonged relapse-free survival, metastasis-free survival, and overall survival (Foekens *et al.* 1995a). This finding was confirmed in another study, which showed that high PAI-2 levels correlate with a good prognosis in patients with node-positive or ER-negative tumours, as well as in those with high uPA levels (Duggan *et al.* 1997).

One pilot study described possible associations between uPA or PAI-1 expression and the efficacy of tamoxifen treatment; the authors reported that the level of uPA would seem to be capable of predicting the response of metastatic disease (Foekens *et al.* 1995*b*). A recent paper (Kosaka & Sumiyoshi 1995) has suggested that 5′-deoxy-5-fluorouridine modifies the degree of malignancy and metastatic activity of breast cancer by inducing fluctuations in the

plasminogen activator system. Another study has shown that PAI-1 is not a predictive marker of response to FAC or FEC neoadjuvant chemotherapy, and that its levels are not altered by these regimens (Pierga *et al.* 1997).

CONCLUSION

Breast cancer biological markers can be divided into prognostic factors and predictors of response to locoregional or systemic chemo and/or hormonotherapy. The acceptance or rejection of a tumour marker for clinical purposes depends on whether it can be considered a reliable means upon which to base therapeutic decisions. The clinical guidelines adopted by the American Society of Clinical Oncology for the validation of tumour markers in breast cancer is based on a modification of the scale developed by the Canadian Task Force on Periodic Health Examination, which identifies different levels of evidence for the clinical use of biological variables (Table 1).

According to such criteria and on the basis of available information, we tried to classify the most widely investigated markers at different levels for clinical utilization (Table 2).

Table 1. Levels of evidence for the clinical utilization of biological variables

LEVEL I	derived from meta-analysis or high-power controlled studies in which the primary objective of the trial design was to test the utility of the marker.
LEVEL II	derived from prospective clinical trials designed to test a therapeutic hypothesis in which tumour marker evaluation was a secondary, but prospectively described objective.
LEVEL III	derived from retrospective studies that are characterized by their large size and/or the inclusion of multivariate analyses.
LEVEL IV	considered less reliable than level III evidence, because the study was smaller or no multivariate analysis was provided.
LEVEL V	derived from studies that were small, retrospective, and not designed to correlate the marker with clinical outcome.

From the Canadian Task Force on Periodic Health Examination, with modifications (ASCO, *J. Clin. Oncol.* 1996).

Table 2. Prognostic value and predictivity of response to clinical treatment of biological markers

	Level of evidence	Prognosis	Predictivity of response	
			Chemotherapy	Hormonotherapy
Hormonal receptors	I/II	+	*+	+++
L.I.	II/II	+++	++	++
p53	II/III	++	±	±
C-*erb*B-2	II/III	+	++	++
S-phase fraction	II/III	±	±	±
DNA ploidy	II/III	±	±	±
Bcl-2	III/IV	+	+	++
Ki-67/MIB1	III/IV	++	±	±
Urokinase plasminogen system activator	III/IV	++	±	+
Cyclins	>III	±	±	+

*Studies with level IV evidence; – absence of indication; ± lack of a general consensus; + weak indicator; ++ good indicator; +++ strong indicator.

Oestrogen and progesterone receptor levels fall within categories I and II. ER and PgR values should be examined in both pre and postmenopausal patients, and this information can be used to identify those who are most likely to benefit from adjuvant hormone therapy. However, there is insufficient data to support the use of oestrogen and progesterone receptors to predict the favourable impact of adjuvant cytotoxic chemotherapy because to date only level IV evidence studies have directly addressed this question. Evidence at levels II and III suggests that TLI has a predictive power which is suitable for the clinical management of breast cancer, and *p53* expression is considered to be a prognostic indicator in this disease. Although there are several lines of evidence suggesting that c-*erb*B-2 may be a predictor of treatment response, the need for a more aggressive approach with anthracycline-based chemotherapy in node positive c-erbB-2 positive patients is not supported by adequate data for its use outside research protocols. Likewise, there is still not sufficient evidence to recommend the use of FCM-S determinations in assigning patients to different prognostic groups in the standard setting. DNA flow cytometry-derived ploidy is also not advisable. Moreover, these flow cytometry parameters should not be used to select the type of adjuvant therapy to be given or, only in the case of DNA ploidy, to select which treatment options should be used in metastatic disease.

It must be pointed out that studies of other biological variables (including *Bcl-2* expression, Ki-67/MIB index, the plasminogen activator system and cyclins) have an evidence level of less than III, since the information has been derived from retrospective analyses on small size series of patients, and different cut-off points have been used for the definition of high and low risk patients. Methodological standardization and large confirmatory studies with sufficient statistical power are needed for these last markers in order to verify their real clinical value.

ACKNOWLEDGEMENTS

The authors wish to thank Professor Rosella Silvestrini for the careful revision of this manuscript. This study was supported by the Istituto Oncologico Romagnolo grant no. 97.288.1.

REFERENCES

Aas T, Breeson AL, Geisler S. (1996) Specific p53 mutations are associated with de novo resistance to doxorubicin in breast cancer patients. *Nat. Med.* **2**, 811.

Allred DC, Clark GM, Tandon AK *et al.* (1992) *Her*-2/neu in node-negative breast cancer: prognostic significance of overexpression influenced by the presence of in situ carcinoma. *J. Clin. Oncol.* **10**, 599.

Amadori D, Volpi A, Maltoni R *et al.* (1997) Cell proliferation as a predictor of response to chemotherapy in metastatic breast cancer: A prospective study. *Breast Cancer Res. Treat.* **43**, 7.

American Society of Clinical Oncology. (1996) Clinical practice guidelines for the use of tumor markers in breast and colorectal cancer. *J. Clin. Oncol.* **14**, 2843.

Archer SG, Eliopolous A, Spandidos D *et al.* (1995) Expression of *ras*, p53 and c-*erb*B-2 in advanced breast cancer and response to first line hormonal therapy. *Br. J. Cancer* **72**, 1256.

Arriagada R, Rutqvist LE, Skoog L *et al.* (1992) Prognostic factors and natural history in lymph node-negative breast cancer patients. *Breast Cancer Res. Treat.* **21**, 101.

Balslev I, Christensen IJ, Rasmussen BB *et al.* (1994) Flow cytometric DNA ploidy defines patients with poor prognosis in node-negative breast cancer. *Int. J. Cancer* **56** (1), 16.

Bergers E, Baak JPA, Van Diest PJ *et al.* (1997) Prognostic implications of different cell cycle analysis models of flow cytometric DNA histograms of 1,301 breast cancer patients: results from the multicenter morphometric mammary carcinoma project (MMMCP). *Int. J. Cancer* **74**, 260.

BIANCHI S, CALZOLARI A, VEZZOSI V *et al.* (1997). Lack of prognostic value of p53 protein expression in node-negative breast cancer. *Tumori* **83**, 699.

BIANCHI S, PAGLIERANI M, ZAMPI G *et al.* (1993) Prognostic significance of *c-erb*B-2 expression in node negative breast cancer. *Br. J. Cancer* **67**, 625.

BONETTI A, ZANINLLI M, RODELLA S *et al.* (1996) Tumor proliferative activity and response to first-line chemotherapy in advanced breast carcinoma. *Breast Cancer Res. Treat.* **38**, 289.

BORG A, LENNERSTRAND J, STENMARK-ASKMALM M *et al.* (1995) Prognostic significance of p53 overexpression in primary breast cancer; A novel luminometric imunoassay applicable on steroid receptor cytosols. *Br. J. Cancer* **71**, 1013.

BOSARI S, LEE AK, TAHAN SR *et al.* (1992) DNA flow cytometric analysis and prognosis of axillary lymph node-negative breast carcinoma. *Cancer* **70**, 1943.

BOUZUBAR N, WALKER KJ, GRIFFITHS K *et al.* (1989) Ki-67 immunostaining in primary breast cancer: pathological and clinical associations. *Br. J. Cancer* **59**, 943.

BREAST CANCER TRIALS COMMITTEE. (1987) Adjuvant tamoxifen in the management of operable breast cancer: The Scottish Trial. *Lancet* **2**, 171.

CARLOMAGNO C, PERRONE F, GALLO C *et al.* (1996) *C-erb*B2 overexpression decreases the benefit of adjuvant tamoxifen in early-stage breast cancer without axillary lymph node metastases. *J. Clin. Oncol.* **14**, 270.

CLARK GM, DRESSER LG, OWENS MA *et al.* (1989) Prediction of relapse or survival in patients with node-negative breast cancer by DNA flow cytometry. *N. Engl. J. Med.* **320**, 627.

CUEVAS E, JONES DB, WRIGHT DH *et al.* (1993) Immunohistochemical detection of tumor growth fraction (Ki-67 antigen) in formalin-fixed and routinely processed tissues. *J. Pathol.* **169**, 477.

DETTMAR P, HARBECK N, THOMSSEN C *et al.* (1997) Prognostic impact of proliferation-associated factors MIB1 (Ki-67) and S-phase in node-negative breast cancer. *Br. J. Cancer* **75**, 1525.

DUFFY MJ, DUGGAN C, MAGUIRE T *et al.* (1996) Urokinase plasminogen activator as a predictor of aggressive disease in breast cancer. *Enzyme Protein* **49**, 85.

DUGGAN C, KENNEDY S, KRAMER MD *et al.* (1997) Plasminogen activator inhibitor type 2 in breast cancer. *Br. J. Cancer* **76**, 622.

EARLY BREAST CANCER TRIALISTS' COLLABORATIVE GROUP. (1988) Effects of adjuvant tamoxifen and of cytotoxic therapy on mortality in early breast cancer. *N. Engl. J. Med.* **319**, 1681.

EARLY BREAST CANCER TRIALISTS' COLLABORATIVE GROUP. (1992) Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. *Lancet* **339**, 71.

ELLEDGE RM, GREEN S, HOWES L *et al.* (1997) Bcl-2, p53, and response to tamoxifen in estrogen receptor-positive metastatic breast cancer. *J. Clin. Oncol.* **15**, 1916.

FERNO M, BALDETORP B, BENDAHL PO *et al.* (1995) Recurrence-free survival in breast cancer improved by adjuvant tamoxifen especially for progesterone receptor positive tumors with a high proliferation. *Breast Cancer Res. Treat.* **36**, 23.

FERNO M, BENDAHL PO, BORG A *et al.* (1996) Urokinase plasminogen activator, a strong independent prognostic factor in breast cancer, analysed in steroid receptor cytosols with a luminometric immunoassay. *Eur. J. Cancer* **32A**, 793.

FISHER B, REDMOND C, BROWN A *et al.* (1983) Influence of tumor estrogen and progesterone receptor levels on the response to tamoxifen and chemotherapy in primary breast cancer. *J. Clin. Oncol.* **4**, 227.

FISHER B, REDMOND C, FISHER ER *et al.* (1988) Relative worth of estrogen or progesterone receptor and pathologic characteristics of differentiation as indicators of prognosis in node-negative breast cancer patients: Findings from National Surgical Adjuvant Breast and Bowel Project protocol. B-06. *J. Clin. Oncol.* **6**, 1076.

FOEKENS JA, BUESSECKER F, PETERS HA *et al.* (1995a) Plasminogen activator inhibitor-2: prognostic relevance in 1012 patients with primary breast cancer. *Cancer Res.* **55**, 1423.

FOEKENS JA, LOOK MP, PETERS HA *et al.* (1995b) Urokinase-type plasminogen activator and its inhibitor PAI-1. predictors of poor response to tamoxifen therapy in recurrent breast cancer. *J. Natl. Cancer Inst.* **87**, 751.

FORMENTI SC, LENZ J, DUNNINGTON G *et al.* (1996) Initial p53 status as potential predictor for pathological response to the combination of 5FU and radiotherapy in locally advanced breast cancer. *Proc. Ann. Meet. Am. Soc. Clin. Oncol.* **15**, A174.

FRESNO R, MOLINA R, PEREZ MJ *et al.* (1997) P53 expression of independent predictive value in lymph node-negative breast carcinoma. *Eur. J. Cancer* **33**, 1268.

GARDIN G, ALAMA A, ROSSO R *et al.* (1994) Relationship of variation in tumor cell kinetics induced by primary chemotherapy to tumor regression and prognosis in locally advanced breast cancer. *Breast Cancer Res. Treat.* **32**, 311.

GASPARINI G, BARBARESCHI M, DOGLIONI C *et al.* (1995) Expression of Bcl-2 protein predicts efficacy of adjuvant treatments in operable node-positive breast cancer. *Clin. Cancer Res.* **1**, 189.

GILLET C, FANTL V, SMITH R *et al.* (1994) Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. *Cancer Res.* **54**, 1812.

GILLET C, SMITH P, GREGORY W *et al.* (1996) Cyclin D1 and prognosis in breast cancer. *Int. J. Cancer* **69**, 92.

GRONDAHL-HANSEN J, HILSENBECK SJ, CHRISTENSEN IJ *et al.* (1997) Prognostic significance of PAI-1 and uPA in cytosolic extracts obtained from node-positive breast cancer patients. *Breast Cancer Res. Treat.* **43**, 153.

GRONDAHL-HANSEN J, PETERS HA, VAN PUTTEN WL *et al.* (1995) Prognostic significance of the receptor for urokinase plasminogen activator in breast cancer. *Clin. Cancer Res.* **1**, 1079.

GUSTERSON BA, GELBER RD, GOLHIRSCH A *et al.* (1992) Prognostic importance of c-*erb*B-2 expression in breast cancer. *J. Clin. Oncol.* **10**, 1049.

HAYES DF. (1997) When is a tumor marker ready for prime time? The tumor marker utility grading scale. In: Perry MC, ed. *Thirty-third Annual Meeting. American Society of Clinical Oncology.* Alexandria VA: Educational Book, 206.

HIETANEN P, BLOMQVIST C, VASENIUS V-M, NISKANEN E, FRANSSILA K, NORDLING S. (1995) Do DNA ploidy and S-phase fraction in primary tumor predict the response to chemotherapy in metastatic breast cancer? *Br. J. Cancer* **71**, 1029.

HILF R, FELDSTEIN ML, GIBSON SL *et al.* (1980) The relative importance of estrogen receptor analysis as a prognostic factor for recurrence or response to chemotherapy in women with breast cancer. *Cancer* **45**, 1993.

HOSKINS K, WEBER BL. (1994) The biology of breast cancer. *Curr. Opin. Oncol.* **6**, 554.

JANSSON T, INGANAS M, SJOGREN S *et al.* (1995) P53 status predicts survival in breast cancer patients treated with or without postoperative radiotherapy: a novel hypothesis based on clinical findings. *J. Clin. Oncol.* **13**, 2745.

JOENSUU H, PYLKKANEN L, TOIKKANEN S. (1994) Bcl-2 protein expression and long-term survival in breast cancer. *Am. J. Pathol.* **145**, 1191.

KALLIONIEMI OP, HOLLI K, VISAKORPI T *et al.* (1991) Association of *c-erb*B-2 protein overexpression with high rate of cell proliferation, increased risk of visceral metastasis and poor long-term survival in breast cancer. *Int. J. Cancer* **49**, 650.

KING RJ. (1993) Estrogen and progestin effects in human breast carcinogenesis. *Breast Cancer Res. Treat.* **27**, 3.

KOSAKA A, SUMIYOSHI K. (1995) Fluctuations of plasminogen activator system in patients with breast cancer (1st report)—especially the effects of preoperative administration of 5′-deoxy-5-fluorouridine (5′-dfur) and pyrimidine nucleoside phosphorylase (pynpase) activity. *Can. J. Infectious Dis.* **6C**, 226.

LEITZEL K, TERAMOTO Y, KONRAD K *et al.* (1995) Elevated serum *c-erb*B-2 antigen levels and decreased response to hormone therapy of breast cancer. *J. Clin. Oncol.* **13**, 1129.

LIPPONEN P, JI H, AALTOMAA S, SYRJANEN S, SYRJANEN K. (1993) P53 protein expression in breast cancer as related to histopathological characteristics and prognosis *Int. J. Cancer* **55** (1), 51.

LOVEKIN C, ELLIS IO, LOCKER A *et al.* (1991) c-*erb*B-2 oncoprotein expression in primary and advanced breast cancer. *Br. J. Cancer* **63**, 439.

LOWE SW, RULEY HE, JACKS T *et al.* (1993) P53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* **74**, 957.

LUPU R, LIPPMAN ME. (1993) The role of erb-B2 signal transduction pathways in human breast cancer. *Breast Cancer Res. Treat.* **27**, 83.

MAKRIS A, POWLES TJ, DOWSETT M *et al.* (1997) Prediction of response to neoadjuvant chemoendocrine therapy in primary breast carcinomas. *Clin. Cancer Res.* **3**, 593.

MCINTOSH GG, THOMAS MD, ANDERSON JJ, ANGUS B, HORNE CH. (1995) Determination of the prognostic value of cyclin D1 overexpression in breast cancer. *Oncogene* **11**, 885.

MERKEL DE, WINCHESTER DJ, GOLDSCHIMD RA *et al.* (1993) DNA flow cytometry and pathologic grading prognostic guides in axillary lymph node negative breast cancer. *Cancer* **72**, 1926.

MICHALIDES R, HAGEMAN P, VAN TINTEREN H *et al.* (1996) A clinicopathological study on overexpression of cyclin D1 and of p53 in a series of 248 patients with operable breast cancer. *Br. J. Cancer* **73**, 728.

MURPHY LC. (1995) Mechanisms associated with the progression of breast cancer from hormone dependence to independence. *The Pezcoller Foundation J.* **6**, 6.

MUSS HB, THOR AD, BERRY DA *et al.* (1994) C-erbB-2 expression and response to adjuvant therapy in women with node-positive early breast cancer. *N. Engl. J. Med.* **330**, 1260.

NIELSEN NH, ARNERLOV C, EMDIN SO *et al.* (1996) Cyclin E overexpression, a negative prognostic factor in breast cancer with strong correlation to oestrogen receptor status. *Br. J. Cancer* **74**, 874.

O'REILLY SM, CAMPLEJOHN RS, RUBENS RD *et al.* (1992) DNA flow cytometry and response to preoperative chemotherapy from primary breast cancer. *Eur. J. Cancer* **28**, 681.

PARADISO A, TOMMASI S, MANGIA A *et al.* (1990) Tumor proliferative activity, progesterone receptor status, estrogen receptor level and clinical outcome of estrogen receptor-positive advanced breast cancer. *Cancer Res.* **50**, 2958.

PELOSIO P, BARBARESCHI M, BONOLDI E *et al.* (1996) Clinical significance of cyclin D1 expression in patients with node-positive breast carcinoma treated with adjuvant therapy. *Ann. Oncol.* **7**, 695.

PFISTERER J, KOMMOSS F, SAUERBREI W *et al.* (1995) DNA flow cytometry in node-positive breast cancer Prognostic value and correlation with morphologic and clinical factors. *Anal. Quant. Cytol. Histol.* **17**, 406.

PIERGA JY, LAINE″-BIDRON C, BEUZEBOC P *et al.* (1997) Plasminogen activator inhibitor-1 (PAI-1) is not related to response to neoadjuvant chemotherapy in breast cancer. *Br. J. Cancer* **76**, 537.

PIERGA JY, LEROYER A, VIEHL P *et al.* (1996) Long term prognostic value of growth fraction determination by Ki-67 immunostaining in primary operable breast cancer. *Breast Cancer Res. Treat.* **37**, 57.

PRESS MF, PIKE MC, CHAZIN VR *et al.* (1994) Her-2/neu expression in node-negative breast cancer: direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease. *Cancer Res.* **53**, 4960.

RAEMAEKERS JMM, BEEX LVAM, PIETERS GFFM *et al.* (1987) Progesterone receptor activity and relapse-free survival in patients with primary breast cancer. The role of adjuvant chemotherapy. *Br. Cancer Res. Treat.* **9**, 191.

RAILO M, LUNDIN J, HAGLUND CAJ *et al.* (1997) Ki-67, P53 ER-receptors, ploidy and s-phase as prognostic factors in t1 node negative breast cancer. *Acta Oncol.* **36**, 369.

RAVDIN PM. (1997) Prognostic factors in breast cancer. In: Perry MC, ed. *Thirty-third Annual Meeting. American Society of Clinical Oncology.* Alexandria VA: Educational Book, 217.

RAVDIN PM, GREEN S, DORR TM *et al.* (1992) Prognostic significance of progesterone receptor levels in estrogen receptor-positive patients with metastatic breast cancer treated with tamoxifen: Results of a prospective Southwest Oncology Group study. *J. Clin. Oncol.* **10**, 1284.

REMVIKOS Y, JOUVE M, BEUZEBOC P *et al.* (1993) Cell cycle modifications of breast cancer during neoadjuvant chemotherapy: a flow cytometry study on fine needle aspirates. *Eur. J. Cancer* **29A**, 1943.

SCHIMMELPENNING H, ERIKSSON ET, ZETTEMBERG A, AVER GU. (1994) Association of immunohistochemical p53 tumor suppressor gene protein overexpression with prognosis in highly proliferative human mammary adenocarcinomas. *World J. Surg.* **18**, 827.

SCOTTISH CANCER TRIALS BREAST GROUP ICRF BREAST UNIT, GUY'S LONDON. (1993) Adjuvant ovarian ablation vs. CMF chemotherapy in premenopausal women with pathological stage II breast carcinoma: the Scottish Trial. *Lancet* **341**, 1293.

SILVESTRINI R, BENINI E, VENERONI S *et al.* (1996b) P53 and Bcl-2 expression correlates with clinical outcome in a series of node-positive breast cancer patients. *J. Clin. Oncol.* **14** (5), 1604.

SILVESTRINI R, DAIDONE MG, BENINI E *et al.* (1996a) Validation of p53 accumulation as a predictor of distant metastasis at 10 years of follow-up in 1400 node-negative breast cancers. *Clin. Cancer Res.* **2**, 2007.

SILVESTRINI R, DAIDONE MG, DEL BINO G *et al.* (1993b) Prognostic significance of proliferative activity and ploidy in node-negative breast cancer. *Ann. Oncol.* **4**, 213.

SILVESTRINI R, DAIDONE MG, LUISI A *et al.* (1995) Biologic and clinicopathologic factors as indicators of specific relapse types in node-negative breast cancer. *J. Clin. Oncol.* **13**, 697.

SILVESTRINI R, DAIDONE MG, LUISI A *et al.* (1997b) Cell proliferation in 3800 node-negative breast cancers: consistency over time of biological and clinical information provided by ^{3}H-thymidine labelling index. *Int. J. Cancer* **74**, 122.

SILVESTRINI R, DAIDONE MG, MASTORE M *et al.* (1993a) Cell kinetics as a predictive factor in node-positive breast cancer treated with adjuvant hormone therapy. *J. Clin. Onc.* **11**, 1150.

SILVESTRINI R, DAIDONE MG, VALAGUSSA P *et al.* (1987) Cell kinetics as a prognostic marker in locally advanced breast cancer. *Cancer Treat. Rep.* **71**, 375.

SILVESTRINI R, DAIDONE MG, VALAGUSSA P *et al.* (1990) ^{3}H-thymidine-labeling index as a prognostic indicator in node-positive breast cancer. *J. Clin. Oncol.* **8**, 1321.

SILVESTRINI R, VENERONI S, BENINI E *et al.* (1997a) Expression of p53, glutathione s-transferase-π and bcl-2, proteins and benefit from adjuvant radiotherapy in breast cancer. *J. Natl. Cancer Inst.* **89**, 639.

SILVESTRINI R, VENERONI S, DAIDONE MG *et al.* (1994) The Bcl-2 protein: a prognostic indicator strongly related to p53 protein in lymph node-negative breast cancer patient. *J. Natl. Cancer Inst.* **86**, 499.

STAL O, ASMALM MS, WINGREN S *et al.* (1995) P53 expression and the result of adjuvant therapy of breast cancer. *Acta Oncologica* **34**, 767.

STEWART HJ. (1992) The Scottish trial of adjuvant tamoxifen in node-negative breast cancer. *Monograf. J. Natl. Cancer Inst. Mono* **11**, 117.

SULKES A, LIVINGSTON RB, MURPHY WK. (1979) Tritiated thymidine labeling index and response in human breast cancer. *J. Natl. Cancer Inst.* **62**, 513.

TEIXEIRA C, REED JC, PRATT MAC. (1995) Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. *Cancer Res.* **55**, 3902.

THOR AD, MOORE IIDH, EDGERTON SM *et al.* (1992) Accumulation of p53 tumor suppressor gene protein: an independent marker of prognosis in breast cancer. *J. Natl. Cancer Inst.* **84**, 845.

TOIKKANEN S, HELIN H, ISOLA J *et al.* (1992) Prognostic significance of HER-2 oncoprotein expression in breast cancer: a 30-year follow up. *J. Clin. Oncol.* **10**, 1044.

VAN SLOOTEN HJ, CLAHSEN PC, VAN DIERENDOCK JH *et al.* (1996) Expression of Bcl-2 in node-negative breast cancer is associated with various prognostic factors, but does not predict response to one course of perioperative chemotherapy. *Br. J. Cancer* **74**, 78.

WALTON MI, WHYSONG D, O'CONNOR PM *et al.* (1993) Constitutive expression of human bcl-2 modulates nitrogen mustard, camptothecin induced apoptosis. *Cancer Res.* **53**, 1853.

WEIDNER N, MOORE DH, VARTANIAN R *et al.* (1994) Correlation of Ki-67 antigen expression with mitotic figure index and tumor grade in breast carcinomas using the novel 'paraffin' reactive MIB1 antibody. *Hum. Pathol.* **25**, 337.

WITZIG TE, INGLE JN, CHA SS *et al.* (1994) DNA ploidy and the percentage of cell in S-phase as a prognostic factors for women with lymph node negative breast cancer. *Cancer* **74**, 1952.

WITZIG TE, JAMES NI, SCHAID DJ *et al.* (1993) DNA ploidy and percent s-phase as prognostic factors in node-positive breast cancer: results from patients enrolled in two prospective randomized trials. *J. Clin. Oncol.* **11**, 351.

ZHOU P, JIANG W, WEGHORST CM *et al.* (1996) Overexpression of cyclin D1 enhances gene amplification. *Cancer Res.* **56**, 36.