Journal of Cellular Biochemistry Supplement 34:73–79 (2000)

Quality Control for HPLC Assay and Surrogate End Point Biomarkers From the Fenretinide (4-HPR) Breast Cancer Prevention Trial

Franca Formelli*

Istituto Nazionale Tumori, Via Venezian 1, 20133 Milan, Italy

Abstract The Fenretinide (4-HPR) Breast Cancer Study is a randomized multicenter clinical trial designed to evaluate the effectiveness of the synthetic retinoid 4-HPR, at a dose of 200 mg per os every day for 5 years, in reducing the incidence of contralateral breast cancer in patients previously operated on for T1-T2 N-MO breast cancer. During the trial, blood samples were collected at baseline and on a yearly basis from most of the patients. Evaluation of drug and retinol concentrations by HPLC assay has been performed for all the samples to obtain 4-HPR pharmacokinetic information as well as information on the effect of 4-HPR in lowering retinol plasma levels. The most important criteria for validation and quality control of the HPLC assay are summarized in order to provide a guide and practical recommendations for analytical procedures to be performed during prevention trials. Studies have been performed on subsets of patients participating in the trial in order to identify circulating biomarkers predictive of breast cancer. Evidence has been obtained on a lowering effect of 4-HPR on biologically active IGF-I only in premenopausal women. This was due to a decrease of IGF-I, associated with a trend to an increase in IGF-I binding protein 3 (IGFBP-3). An interim analysis of the ongoing trial indicates that 4-HPR reduces the incidence of contralateral breast cancer only in premenopausal women. Analyses of total and unbound IGF-I are being performed on plasma samples collected at baseline and during intervention from women ≤ 50 years old. The relationship between the incidence of a second breast cancer and the changes in IGF-I plasma levels will be assessed in order to validate IGF-I as a surrogate end point of contralateral breast cancer. The preliminary results of other studies on the effects of 4-HPR on tissue plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1), and urokinase plasminogen activator (uk-PA) and on the relevance of circulating p53 antibodies with relapse will be also presented. J. Cell. Biochem. Suppl. 34:73–79, 2000.

Key words: 4-HPR; breast cancer; circulating surrogate endpoint biomarkers; prevention

The Fenretinide (4-HPR) Breast Cancer Study is a randomized multicenter clinical trial originally designed and conducted by the investigators of the Istituto Nazionale Tumori of Milan (INT-Milan) [De Palo et al., 1997]. The study is sponsored by the National Cancer Institute of Bethesda and by the Italian National Research Council. The trial was designed to evaluate the effectiveness of the synthetic retinoid 4-HPR, at a dose of 200 mg per os every day for 5 years, in reducing the incidence of contralateral breast cancer in a population of patients previously operated on for breast cancer. Between 1987 and 1993, the INT-Milan and nine other collaborating centers enrolled 2,972 women between the ages of 30 and 70 years who had been previously operated on for T1-T2 N-M0 breast cancer. During the trial, blood samples were collected at baseline and on a yearly basis from most of the patients. Evaluation of drug and retinol concentrations in all the samples has been performed with the aim of assessing whether such concentrations are related to 4-HPR toxicity and/or activity and in order to perform a control of patient reliability in relation to compliance [Mariani et al., 1997]. From the analysis of these samples, information has been obtained on the pharmacokinetics of 4-HPR [Formelli et al., 1993], on its effect in lowering retinol plasma levels [Formelli et al., 1989, 1993], and on the association between

Contract grant sponsor: NIH/NCI; Contract grant numbers: CA 38567 and CA 72286.

*Correspondence to: Franca Formelli, Istituto Nazionale Tumori, Via Venezian 1, 20133 Milan, Italy. E-mail: mailto: formelli@istitutotumori.mi.it

Received 2 September 1998; Accepted 25 March 1999

retinol level reduction and visual side effects [De Censi et al., 1994; Mariani et al., 1996]. Evidence has also been obtained on a lowering effect of 4-HPR on biologically active IGF-I [Torrisi et al., 1993, 1998]. Additional analyses are being performed on the collected samples with the aim of validating IGF-I as a surrogate end point biomarker of breast cancer and in order to identify circulating biomarkers predictive of breast cancer relapse. The most important criteria for validation and quality control of the HPLC assay, performed to detect drug and retinol concentrations, are summarized in order to provide a guide and practical recommendations for analytical procedures to be performed during prevention trials. The ongoing studies on SEB are also summarized.

QUALITY MANAGEMENT OF THE HPLC ASSAY

Bioanalytical support for chemoprevention studies usually involves a large number of samples. Collection of the samples may last for several years, and more than one center may be involved in sample collection and/or assay. In the 4-HPR breast cancer prevention trial, blood samples have been collected from 1987 to present. During these 11 years, approximately 10,000 samples were collected. Samples have been collected at baseline and on a yearly basis only from patients followed at the INT-Milan, which account for 61% of the participants in the trial. At the time of planning the study, we decided not to draw blood from patients followed at peripheral centers because it was considered preferable to rely upon uniform standards of sampling, preparation, and storage and to avoid possible thawing during transportation. The phase I study, which was run before the phase III trial, gave us the chance to set up all the procedures for quality management of the assay to be routinely performed during the trial [Formelli et al., 1989]. The main aims of quality management were to ensure that: a) the samples were properly collected and preserved, b) the analysis was carried out using a validated technique, and c) the results were properly recorded and reported.

Sample Collection

In regard to sampling, the effect of anticoagulants on the assay to be performed was first checked. Afterwards, instructions for blood collection, storage and transport within the INT-Milan were clearly described and provided to all the operators involved in order to achieve sampling homogeneity. Plasma samples to be stored and assayed were identified in the simplest way: that is with progressive numbers of collection. All the information related to the samples was clearly reported in a form designed for an interactive data entry of the analytical results with clinical data. The form to be completed by clinicians included: subject identification code number, date and time of last dose, date and time of blood sampling (for calculation of interval from drug intake), reason for sampling, co-administered drugs, and a space for possible notes. Each plasma sample was divided in subsamples in the event that other assays were required. The amount to be analyzed by HPLC was kept frozen at –20°C until analysis, which was performed within 3 weeks. The stability of the samples at this temperature had been determined previously. The remaining subsamples were frozen at –80°C. Due to the large number of samples collected and kept in this kind of study, it is fundamental that samples be stored in freezers used only for this purpose and which are continuously under control. A freezer should also be available for emergency use.

Validation Procedures

The levels of 4-HPR in plasma were measured by an HPLC assay taken from the literature. The procedure was, however, validated before its routine use. It is a tedious process, but the quality of the data usually depends on the quality of the validation process. The validation procedure included first the validation of the analytical method, that is the process of proving that the method is acceptable for its intended purpose, and second, the validation of analyte stability. In general, validation of the method includes studies on specificity/selectivity, linearity, accuracy, precision, detection, and quantitation limits. Specificity and selectivity are the abilities to measure and separate, respectively, the analyte from other products. Specificity evaluation was performed by analyzing different sources of the sample matrix (i.e., six plasma samples) before and after the addition of three different drug concentrations. Linearity is the concentration range in which the analyte response is linearly proportional to concentrations. The analysis was performed with five concentration levels in the range of the

expected concentrations of the analyte. Accuracy is the closeness of the measured value to the true value of the sample. It was checked with spiked samples with three different concentrations (intra-assay accuracy) and evaluated in three separate runs (interassay accuracy). Precision is the amount of scatter in the results obtained from multiple analyses of a homogenous sample. It is particularly important for assays to be performed over long periods of time, as may be the case for analytical procedures of chemoprevention studies. Quality control (QC) samples were prepared at the beginning of the study by spiking three different analyte concentrations into pools of plasma. The pools were then divided into aliquots to be stored under the same conditions as the clinical samples to be analyzed. Their stability has been demonstrated over 11 years. For each assay, two replications of three QC samples (six samples) were included and analyzed. They were used to accept or reject the run. The detection limit was established by assessing the lowest analyte concentration which produced a response detectable above the noise level of the system (three times the noise level was considered acceptable). The quantitation limit, i.e., the lowest analyte level that can be precisely measured, was thus assessed.

Validation of the stability of the biological samples included: short-term stability of the analyte at 4°C in the biological matrix (i.e., in plasma) and in the final extract, freeze and thaw stability and long-term stability at –20°C and at –80°C.

At the end of the validation process, all the standard procedures were clearly written so as to be easily understood and performed. During the 11 years of blood sample collection and analysis, three different technicians have performed the assay, one following the other. Despite turnover of personnel, no problems have arisen in the assay. This is due to the skill of the personnel and the validation of the entire procedure, from blood sampling to plasma assay, performed before starting the routine analysis. Quality of the analytical procedure also depends on the instruments used. We have contracts with qualified specialists for maintenance controls of all major equipment. This saved us time and in the long run also money.

The final issue of quality management of any analytical assay is to ensure that the results of the assay are properly recorded and reported. The results of each HPLC assay were reported in the form which accompanied each blood sample, previous control of each chromatogram by the person responsible for the pharmacologic studies. EDP (electronic data processing) operators, introduced the data in the data base of the trial and printed the summary form for each patient. The person responsible for pharmacologic data verified that data were entered correctly.

CIRCULATING SURROGATE END POINT BIOMARKERS

When the 4-HPR breast cancer prevention study was started, a decision was made to investigate, in subsets of patients participating in the trial, the effects of 4-HPR on circulating biological markers bearing some relationship to breast cancer. There was also a decision to save samples of plasma collected from most of the patients at baseline and during treatment in order to measure the identified biomarkers in the samples. The relationship between the changes of these markers and the incidence of contralateral breast will be assessed in order to validate the markers as surrogate end points.

Insulin-Like Growth Factor-I

Insulin-like growth factor I (IGF-I) is a potent mitogen that can affect the proliferation of breast epithelial cells [Yee et al., 1989]. There is now substantial evidence that the IGF family, including IGF-I and IGF-II, their receptors and their binding proteins play a crucial role in the growth of several human malignancies including breast cancer [Werner et al., 1996]. Just recently, in a prospective study, a strong association was found between circulating IGF-I concentrations and the relative risk of breast cancer in premenopausal but not in postmenopausal women [Hankinson et al., 1998].

Pilot studies by Andrea Decensi on a subset of patients participating in the trial demonstrated that 4-HPR lowers circulating IGF-I levels and that the decrease is particularly pronounced in premenopausal patients [Torrisi et al., 1993]. They also found that 4-HPR treatment was associated with a trend to an increase in IGFBP-3 as compared with controls [Torrisi et al., 1998], suggesting that the bioavailability of IGF-I is further decreased in premenopausal women.

In a pilot case-control study performed in a small group within the trial [Formelli et al.,

1996], IGF-I plasma levels were measured at baseline and at tumor relapse in patients aged ≤50 and in patients aged >50 years treated with 4-HPR. IGF-I plasma levels were compared with those of patients who had not relapsed and who were matched pairwise for age, length of 4-HPR treatment, and interval from primary tumor removal. In patients aged >50 years, IGF-I average levels were lower than in patients ≤50 years, and they did not change during treatment in patients who relapsed or in those who did not. In patients ≤50 years, mean levels before and after treatment were unchanged in those who relapsed, whereas in patients who did not relapse, there was a slight (12%) but significant reduction in mean IGF-I levels.

The effect of the association of 4-HPR at high doses (500, 600, 700 mg/day) with tamoxifen (TAM; 20 mg/day) on IGF-I plasma levels was also evaluated. The determinations were performed on blood drawn from seven metastatic breast cancer patients participating in a phase I trial [Cobleigh et al., 1993]. The samples had been drawn and sent to us for 4-HPR analysis. In the patients aged >50 years, baseline IGF-I levels were higher than in patients of the same age operated on for stage I breast cancer and participating in the 4-HPR breast cancer prevention trial. After 9 months of treatment (range 4–10 months), baseline levels were significantly reduced in all patients with a mean reduction of 60%. The contribution of 4-HPR and TAM as single agents to the marked reduction is not known.

The results of these pilot studies and the recently published finding of a positive association between IGF-I plasma levels and breast cancer in premenopausal women [Hankinson et al., 1998] strongly suggest a role of IGF-I as a surrogate end point biomarker for breast cancer in premenopausal women. The hypothesis is being verified in a study (supported by a U.S. NIH/NCI grant), whose aim is to validate IGF-I as a surrogate end point within the phase III trial. An interim analysis of the phase III trial at a median of 64 months indicated that 4-HPR reduces breast cancer in premenopausal women. The potential association between the incidence of breast cancer and the change in plasma concentrations of total and unbound IGF-I will be evaluated in women aged ≤50 years. The sample under study will include a subgroup of 586 women aged ≤50 years (360 on 4-HPR intervention and 226 untreated controls) whose plasma aliquots are available at baseline and during the intervention period. An exploratory analysis of the intervention effect on contralateral tumors by the Cox model provided no statistical support for a bias in the selection of the 586 subjects on study. From these subjects we randomly selected for an exploratory analysis a subgroup of 60 cases (30 4-HPR-treated and 30 controls: total number of aliquots 307). The aims of the analysis are: a) to confirm the 4-HPR-induced decline of plasma IGF-I; b) to assess the effects of 4-HPR on other components of the IGF system, namely IGF-II and IGFBP-1–2-3; c) to study the time profile of the modulation of the whole IGF system by 4-HPR. Afterwards, IGF-I and any other component of the IGF system proven to be modulated by 4-HPR will be measured in all the plasma samples.

Retinol

The potential relationship between vitamin A (i.e., retinol) and cancer has been extensively investigated in humans. A number of prospective studies have shown a significant negative correlation between serum retinol concentrations and risk of cancer, in particular that of the lung and stomach [Wald et al., 1980]. In breast cancer, few studies have been reported. In a prospective study, no relationship was found between retinol and subsequent development of breast cancer [Russell et al., 1988]. In another study, the retinol plasma levels of patients with T1-T2N-MO breast cancer were no different from those of control subjects [Marubini et al., 1988]. Retinol levels have also been measured as a potential predictive index of tumor recurrence. Studies in postoperative breast cancer patients [Basu and Sasmal, 1988] and in premenopausal women with node-positive breast carcinoma receiving adjuvant chemotherapy [Mehta et al., 1987] have shown that patients who displayed low retinol serum levels after completion of treatment were more likely to relapse. We investigated whether the retinol plasma levels of the patients participating in the trial, i.e., of patients previously operated on for T1-T2N-MO breast cancer, were different from those of healthy subjects and might be predictive markers of breast cancer relapse. Retinol plasma levels were evaluated at baseline in 1,574 patients followed at the INT-Milan. Patients randomized to the 4-HPR group

and to the control group did not differ for baseline retinol levels (that is, the two groups were well balanced for the parameter) and their retinol levels did not differ from those of healthy subjects. A significant positive correlation between age and retinol levels was observed. Taking into account only patients who have been followed for 7 years (i.e., 5 years of treatment plus 2 years of follow-up), there was no significant difference between the baseline retinol plasma levels of patients who relapsed and of those who did not. Similar results were found for retinol levels during and at the end of 4-HPR treatment or follow-up. The results indicated that, in these kind of patient's, retinol plasma levels were not predictive of breast cancer relapse.

Plasminogen Activator

Plasminogen activator (PA) is a serine protease which exists in two forms known as tissue-type plasminogen activator (t-PA) and urokinase plasminogen activator (uk-PA). Retinoids stimulate the production of t-PA by endothelial cells [Kooistra et al., 1991]. Elevated t-PA levels were found in vivo in rat plasma and tissues after supplementation with retinol or retinoic acid [Kooistra et al., 1991]. The two types of PA seem to be associated with prognosis in breast cancer. The uk-PA as well as its main inhibitor, plasminogen activator inhibitor (PAI-1), have been shown to have a prognostic value in breast cancer, i.e., high tumor levels are associated with a poor prognosis [Duffy et al., 1990]. Contrary to experience with uk-PA and PAI-1, t-PA levels were not indicators of a poor prognosis. Breast cancer patients with tumors with a high t-PA content had a better prognosis than those with low t-PA [Duffy et al., 1988]. In any case, the mean plasma levels of t-PA have been found to be higher in breast cancer patients than in healthy donors [Grondahl-Hansen et al., 1990; Mannucci et al., 1990], with a positive correlation between the mean t-PA plasma concentration and extent of the disease. In a subset of patients participating in the trial, we investigated the effects of 4-HPR on circulating PA levels. The study was conducted in collaboration with Dr. T. Kooistra (Gaubius Laboratory, Leiden, The Netherlands). t-PA, uk-PA, and PAI-1 levels were measured in plasma collected at baseline and after 1, 6, and 12 months in 20 4-HPR-treated and 18 control patients. The same measurements were also performed in plasma collected from patients who relapsed (21 4-HPR-treated and 26 controls) and from patients who did not relapse (19 4-HPR-treated and 38 controls) and who were pair-matched for age and treatment or follow-up. Patients were asked to abstain from caffeine-containing beverages and from physical activities for 12 h before blood collection. Average, t-PA, uk-PA, and PAI-1 plasma levels of 4-HPR-treated patients were no different from those of controls, and no differences were found between relapsed and not relapsed patients. Analysis of the results is ongoing.

Plasma p53-Antibodies

The p53 tumor-suppressor gene is a negative regulator of cell proliferation. The protein encoded by normal p53 has a life-span too brief to permit its detection in cells. The protein encoded by mutant p53 has longer life-span which leads to higher levels detectable by immunohistochemical methods. Expression of mutant p53 is the most common genetic defect found in human cancers. In breast cancer, mutations of p53 have been identified in 15–50% of invasive duct carcinomas [Elledge and Alfred, 1994], and its expression has been shown to be an unfavorable prognostic factor [Silvestrini et al., 1993; Allred et al., 1993]. Patients with various types of cancer may have p53 antibodies in their sera. The frequency of p53 antibodies in breast cancer patients may range from 9 to 25.6% [Crawford et al., 1982; Green et al., 1994]. In some studies, the presence of p53 antibodies in breast cancer patients was correlated with poor prognostic factors [Schlichtholz et al., 1992] and with worse overall survival [Peyrat et al., 1995]. In another study, there were no differences in the recurrence-free interval between p53 antibody positive and negative patients [Regidor et al., 1995]. In collaboration with Dr. G.E. Trivers (Laboratory of Human Carcinogenesis, Division of Cancer Etiology, NCI, Bethesda, MD), Dr. V.M.G. De Benedetti and Dr. S. Pilotti (INT-Milan), we are investigating, in a subset of patients participating in the trial, the levels of p53 antibodies in plasma and the presence of elevated p53 protein in the tumor. The aims of the study are: a) to evaluate the prevalence and the time course of p53 antibodies under 4-HPR treatment or follow-up, b) to investigate the association between serum p53 antibodies and elevated p53 protein in the tumor, and c) to assess the clinical relevance of p53 antibodies

for breast cancer relapse. For this purpose, 35 patients (26 4-HPR-treated and 9 controls) who relapsed during the trial and with three plasma samples collected consecutively were selected. Thirty-one patients with no evident disease (25 4-HPR-treated and 6 controls) were also selected (total, 66 patients). They were matched with the cases for age, length of treatment or follow-up, and intervals of plasma sampling from random, and length of plasma storing. The criteria for selection were that: a) the blood sample, drawn at random, had been drawn shortly (≤1 months) after primary tumor removal, b) a blood sample was drawn at relapse, and c) a blood sample was drawn between the two previous samples. Plasma was analyzed in triplicate assays (enzyme-linked immunoassay, immunoprecipitation, and immunoblot). The samples were considered p53 antibody-positive when at least two tests were positive. Tumor tissues were examined by immunohistochemistry for abnormal p53 protein accumulation. Tumor DNAs of patients who were positive either for tumor p53 protein or for plasma p53 antibodies are being screened in exons 5–8 by PCR and direct sequence analysis. Evaluation of the results is ongoing.

ACKNOWLEDGMENTS

Supported, in part, by the Associazione Italiana per la Ricerca sul Cancro

REFERENCES

Allred DC, Clark GM, Elledge R, Fuqua SA, Brown RW, Chamness GC, Osborne CK, McGuire WL. 1993. Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. J Natl Cancer Inst 85:200–206.

Basu TK, Sasmal P. 1988. Plasma vitamin A, retinol-binding protein, and prealbumin in postoperative breast cancer patients. Int J Vit Nutr Res 58:281–283.

Cobleigh MA, Dowlatshahi K, Deutsch TA, Mehta RG, Moon RC, Minn F, Benson AB, Rademaker AW, Ashenhurst JB, Wade JL. 1993. Phase I/II trial of tamoxifen with or without fenretinide, an analog of vitamin A, in women with metastatic breast cancer. J Clin Oncol 11: 474–477.

Crawford LV, Pim DC, Bulbrook RD. 1982. Detection of antibodies against the cellular protein p53 in sera from patients with breast cancer. Int J Cancer 15:403–408.

Decensi A, Torrisi R, Polizzi A, Gesi R, Brezzo V, Rolando M, Rondanina G, Orengo MA, Formelli F, Costa A. 1994. Effect of the synthetic retinoid fenretinide on dark adaptation and the ocular surface. J Natl Cancer Inst 86:105–110.

De Palo G, Camerini T, Marubini E, Costa A, Formelli F, Del Vecchio M, Mariani L, Miceli R, Mascotti G, Magni A, Campa T, Di Mauro MG, Attili A, Maltoni C, Del Turco MR, Decensi A, D'Aiuto G, Veronesi U. 1997. Chemoprevention trial of contralateral breast cancer with fenretinide. Rationale, design, methodology, organization, data management, statistics and accrual. Tumori 83:884–894.

Duffy MJ, O'Grady P, Devancy D, O'Siorain L, Fennelly JJ, Lijnen HR. 1988. Tissue-type plasminogen activator, a new prognostic marker in breast cancer. Cancer Res 48:1348–1349.

Duffy MJ, Reilly D, O'Sullivan C, O'Hoggins N, Fennelly JJ, Andreasen P. 1990. Urokinase-plasminogen activator, a new and independent prognostic marker in breast cancer. Cancer Res 50:6827–6829.

Elledge RM, Allred DC. 1994. The p53 tumor suppressor gene in breast cancer. Breast Cancer Res Treat 32:39–47.

Formelli F, Carsana R, Costa A, Buranelli F, Campa T, Dossena G, Magni A, Pizzichetta M. 1989. Plasma retinol level reduction by the synthetic retinoid fenretinide: A one year follow-up study of breast cancer patients. Cancer Res 48:6149–6152.

Formelli F, Clerici M, Campa T, Di Mauro MG, Magni A, Mascotti G, Moglia D, De Palo G, Costa A, Veronesi U. 1993. Five-year administration of fenretinide: Pharmacokinetic and effects on plasma retinol concentrations. J Clin Oncol 11:2036–2042.

Formelli F, Cleris L, Cavadini E, Camerini T, Di Mauro MG, Campa T, Magni A, Costa A, De Palo G, Veronesi U. 1996. Analysis of fenretinide (4HPR) effects on plasma IGF-1 levels in relation with age and chemopreventive efficacy in breast cancer patients. Proc Am Assn Cancer Res 37:185.

Green JA, Mudenda B, Jenkins J, Leinster SJ, Tarunina M, Green B, Robertson L. 1994. Serum p53 auto-antibodies: Incidence in familial breast cancer. Eur J Cancer 30:580–584.

Grondahl-Hansen J, Bach F, Munkholm-Larsen P. 1990. Tissue-type plasminogen activator in plasma from breast cancer patients determined by enzyme-linked immunosorbent assay. Br J Cancer 61:412–414.

Hankinson SE, Willett WC, Colditz GA, Hunter DJ, Michaud DS, Deroo B, Rosner B, Speizer FE, Pollak M. 1998. Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. Lancet 351:1393–1396.

Kooistra T, Opdenberg JP, Toet K, Hendriks HF, van den Hoogen RM, Emeis JJ. 1991. Stimulation of tissue-type plasminogen activator synthesis by retinoids in cultured human endothelial cells and rat tissues in vivo. Thromb Hemostasis 65:565–572.

Mannucci PM, Cugno M, Bottasso B, Marongiu F, Maniezzo M, Vaglini M, Cascinelli N. 1990. Changes in fibrinolysis in patients with localized tumors. Eur J Cancer 26:83–87.

Mariani L, Formelli F, De Palo G, Manzari A, Camerini T, Campa T, Di Mauro MG, Crippa A, Delle Grottaglie M, Del Vecchio M, Marubini E, Costa A, Veronesi U. 1996. Chemoprevention of breast cancer with fenretinide (4HPR): Study of long-term visual and ophthalmologic tolerability. Tumori 82:444–449.

Mariani L, Miceli R, Formelli F, Marubini E. 1997. Direct and indirect assessment of treatment compliance: an empirical comparison of the two methods based on data from a randomized clinical trial. J Epidemiol Biostat 2:219–224.

Marubini E, Decarli A, Costa A, Mazzoleni C, Andreoli C, Barbieri A, Capitelli E, Carlucci M, Cavallo F, Monferroni N. 1988. The relationship of dietary intake and serum levels of retinol and beta-carotene with breast cancer. Cancer 61:173–180.

Mehta RR, Hart G, Beattie CW, Das Gupta TK. 1987. Significance of plasma retinol binding protein levels in recurrence of breast tumors in women. Oncology 44:350–355.

Peyrat JP, Bonneterre J, Lubin R, Vanlemmens L, Fournier J, Soussi T. 1995. Prognostic significance of circulating p53 antibodies in patients undergoing surgery for locoregional breast cancer. Lancet 345:621–622.

Regidor PA, Regidor M, Callies R, Schindler AE. 1996. 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 Gyn Oncol 3:192–199.

Schlichtholz B, Legros Y, Gillet D, Marty M, Lane D, Calvo F, Soussi T. 1992. The immune response to p53 in breast cancer patients is directed against immunodominant epitopes unrelated to the mutational hot spot. Cancer Res 52:6380–6384.

Silvestrini R, Benini E, Daidone MG, Veneroni S, Boracchi P, Cappelletti V, Di Fronzo G, Veronesi U. 1993. p53 as an independent prognostic marker in lymph node-negative breast cancer patients. J Natl Cancer Inst 85:965–970.

Torrisi R, Parodi S, Fontana V, Pensa F, Casella C, Barreca A, De Palo G, Costa A, Decensi A. 1998. Effect of fenretinide on plasma IGF-1 and IGFBP-3 in early breast cancer patients. Int J Cancer.

Torrisi R, Pensa F, Orengo MA, Catsafados E, Ponzani P, Boccardo F, Costa A, Decensi A. 1993. The synthetic retinoid fenretinide lowers plasma insulin-like growth factor I levels in breast cancer patients. Cancer Res 53:4769–4771.

Wald NJ, Boreham J, Bailey A. 1980. Low serum vitamin A and subsequent risk of cancer. Preliminary results of a prospective study. Lancet 11:813.

Werner H., Leroith D. 1996. The role of insulin-like growth factor system in human cancer. Adv Cancer Res 68:184–223.

Yee D, Paik S, Lebovic GS, Marcus RR, Favoni RE, Cullen KJ, Lippman ME, Rosen N. 1989. Analysis of insulin-like growth factor I gene expression in malignancy: Evidence for a paracrine role in human breast cancer. Mol Endocrinol 3:509–517.