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Tissue Glycolytic Enzymes in Primary Breast Cancer Patients Receiving Adjuvant Chemotherapy

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Abstract—*Primary breast cancers from 85 patients undergoing post-surgical adjuvant chemotherapy were analyzed for five glycolytic enzymes: lactate dehydrogenase (LDH); phosphohexose isomerase (PHI); glucose-6-phosphate dehydrogenase (G-6PD); pyruvate-kinase (PK); and 6-phospho-gluconate dehydrogenase (6-PGD). The purpose of this study was to determine whether biochemical parameters could offer a prognostic index to determine outcome of therapy. The patients were followed up to a maximum of 54 months; during this period 30 of them developed recurrent or metastatic disease. The enzyme activities were expressed by the three following reference parameters:* units/g *proteins,* units/g *tissue weight and* units/mg *DNA. Two methods of analysis were compared: firstly, univariate analysis using life tables; and secondly, multivariate analysis using the Cox's model, where enzyme levels were tested for each mode of expression in addition to node status, histological features, receptor and menopausal status. Life table analyses appear limited when subsets of patients were studied because the sample size tends to become too small to warrant firm conclusions. Using the Cox's model, a prognostic index 1 was proposed, including the number of involved nodes and the product of logarithms of G-6PD and 6-PGD expressed as* units/mg *DNA. Compared to the number of involved nodes, this index gives a slightly better discrimination of the patients at 2 yr after mastectomy.*

INTRODUCTION

ACCURATE estimation of prognosis in breast cancer patients is of particular importance when considering the necessity for adjuvant therapy after initial surgery. If prognosis could be determined more precisely than at present, unnecessary treatment could be avoided and appropriate therapy could be reserved for high-risk patients. It is well established that tumor size, grade and axillary nodal status are important prognostic indicators [1–5]. A number of trials provided data on the relation between the length of disease-free interval and several other factors, particularly hormone receptors [5–7]. Attempts have also been made to use tissue glycolytic enzyme levels as a prognostic factor [8] or as an index of response to chemotherapy [9, 10].

In this study five glycolytic enzymes were measured in 85 primary breast cancer patients presenting a high risk of recurrence (evaluated by the usual clinical and pathological criteria) and undergoing postsurgical chemotherapy: lactate dehydrogenase (LDH); phosphohexose isomerase (PHI); glucose-6-phosphate dehydrogenase (G-6PD); pyruvate kinase (PK); and 6-phosphogluconate dehydrogenase (6-PGD). The enzyme values were expressed by the three following reference parameters: units/g proteins, units/g tissue and units/mg DNA. In order to assess their actual value in clinical practice, two methods of analysis were performed: univariate, using life tables, and multivariate, using the Cox's model.

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Abbreviations: ER, estrogen receptor; PR, progesterone receptor; DTT, dithiothreitol; EDTA, ethylenediamine tetracetate; DNA, deoxyribonucleic acid.

MATERIALS AND METHODS

Patients

Between January 1980 and April 1982, 85 women with early breast cancer (age range 32–67 yr; average age 50 yr) underwent modified radical or segmental mastectomies. The stage of the disease was classified according to the TNM system [11]. The diagnosis of carcinoma was confirmed by histological examination in each case. All patients were staged pathologically by examination of the axillary lymph nodes found in the mastectomy specimen. The malignancy grade of infiltrating carcinomas was determined according to Bloom and Richardson [12].

The following criteria were used in selecting the population for this study: grading II according to Bloom and Richardson, with more than three axillary nodes invaded; grading III irrespective of

the number of axillary nodes invaded.

Because of their nodal invasion or their unfavorable histological grading, the 85 patients underwent adjuvant chemotherapy. For 55 patients this comprised 12 cycles of a combination of CMF (cyclophosphamide, methotrexate, and 5-fluorouracil) and FAC (5-fluorouracil, adriamycin and cyclophosphamide); and for 30 patients, 12 cycles of FAC. No difference between these two groups was observed with regard to TNM, nodal, menopausal or receptor status. The treatment started 3–4 weeks after surgery.

None of the patients had demonstrable metastases at the beginning of the study. They were followed up at Centre René Huguenin (St-Cloud, France) for up to 54 months. Physical examinations were performed and routine chest X-rays were made at 3-month intervals over a period of 2 yr, then subsequently once a year. Liver scintigraphy, bone scans and mammograms were taken at yearly intervals. The length of disease-free intervals was defined as the time from mastectomy until the first detection of recurrent or metastatic disease, or to the end of the investigation (30 June 1984). Thirty out of the 85 patients had a failure during the follow-up period. The median follow-up time was 811 days.

Sample preparation

Breast tumours, trimmed of excess fat and non-tumorous tissue, were frozen immediately after surgery and stored in liquid nitrogen until use. A portion of each tumor was examined for pathology. Tissue was powdered in a Thermovac autopulverizer and homogenized in Tris–HCl buffer 0.01 M, pH 7.4, containing DTT 0.5 mM and EDTA 1.5 mM. The high-speed supernatant (cytosol) was prepared by ultracentrifugation at 105,000 ***g*** for 60 min at 4°C. It was then used for estradiol and progesterone receptor assays, for enzyme studies and for the estimation of proteins. The pellet was used for DNA determinations.

Receptor assay

The assay of estrogen and progesterone receptors was carried out using the method described by the European Organization for Research and Treatment for Cancer (EORTC) [13]. The detection limit was set at 10 fmol/mg cytosol proteins.

Assay for glycolytic enzymes

The enzyme activities were measured at 37°C according to Shonk and Boxer [14], with a Beckman spectrophotometer model 34. Under these conditions, the enzyme values were expressed as μmol NADPH produced/min or as μmol NADH oxidized (or produced)/min. The DTT necessary for receptor assays is not used in the Shonk and Boxer method; it does not alter the enzyme activity measurements (results not shown).

The protein content of the cytosol was determined by the method of Bradford [15] using human serum albumin for the calibration curve. The pellet was used for DNA estimation according to Gross-Bellard *et al.* [16] using human lymphocytes as a standard.

All the chemicals, substrates and enzymes were purchased from Boehringer Mannheim.

Expression of the results

The results were analyzed as units of enzyme activity in three different ways: units/g total protein; units/g tissue; and units/mg DNA.

Statistical analysis

For patients having an enzyme activity either above or below the median value calculated for the entire group, the life tables [17] were calculated for each enzyme and each mode of expression. Tests of differences between curves were made with the log-rank test [18]. Firstly, all patients were classified in a single population. They were then classified according to their nodal, histological, menopausal and hormone receptor status. The multivariate analysis is based upon the Cox regression model [19] and allows identification, in a stepwise manner, of the most significant prognostic factors related to the length of disease-free interval. From multiple factors the method selects the combination of variables that, when taken together, give the best fit to the data. The order of selection is determined by using the maximum log-likelihood value as a measure of the importance of variables not yet in the regression equation. The statistical significance of adding variables at each step was evaluated by a chi-square test of change in maximized log-likelihood. Data as enzyme activities and estrogen and progesterone binding levels underwent a logarithmic transformation before the multivariate analysis was carried out.

RESULTS

Clinical characteristics of the patients

A summary of various clinical and histological parameters of the patients in each stratification is presented in Table 1.

Relation of tumor glycolytic enzymes to disease-free survival following chemotherapy

Life-table analyses. The median values of each enzyme and each reference parameter are presented in Table 2.

All patients. In the total population, no significant difference in recurrence pattern was attributable to

Table 1. *Characteristics of the patient population receiving chemotherapy*

Characteristics	No. of patients	failures
Age (yr)		
≤ 50	44 (52)*	17 (57)
> 50	41 (48)	13 (43)
Tumour size		
T1	7 (8)	4 (13)
T2	52 (61)	17 (57)
T3+T4	25 (29)	9 (30)
Unknown	1 (1)	0 (0)
Histologic grade		
II	28 (33)	9 (30)
III	57 (67)	21 (70)
No. of involved nodes		4 (13)
≤ 3	26 (31)	24 (80)
> 3	57 (67)	2 (7)
Unknown	2 (2)	
Menopausal status		
Pre-menopause	49 (58)	16 (53)
Post-menopause	36 (42)	14 (47)
Estrogen receptor status		
Negative	40 (47)	14 (47)
Positive†	45 (33)	16 (53)
Progesterone receptor status		
Negative	55 (65)	21 (70)
Positive†	30 (35)	9 (30)
Treatment		
FAC	30 (35)	13 (43)
FAC+CMF	55 (65)	17 (57)
Total	85	30

* Figures in parentheses are the percentages of patients.
† Specimens considered receptor-positive if they contained more than 10 fmol/mg protein.

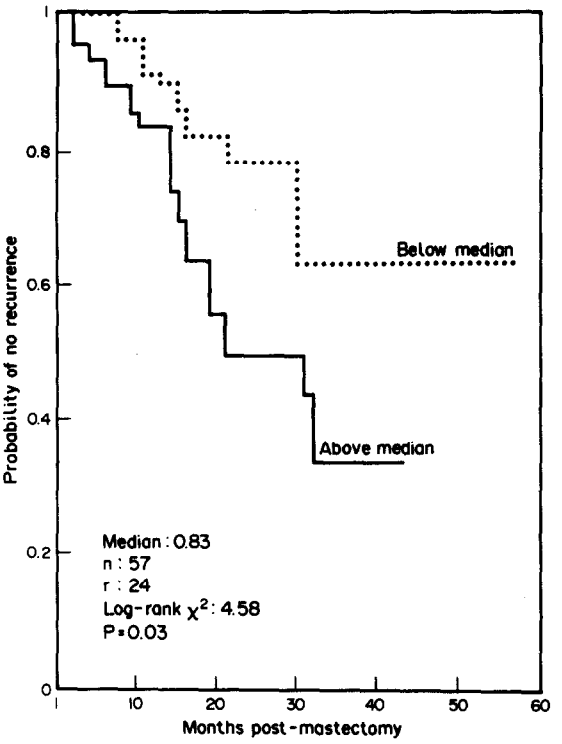

Fig. 1. *Patients with more than three invaded lymph nodes: life tables for* 6-*PGD content of carcinomas expressed as* u/mg *DNA (N: total No. of patients; R: No. of recurrences).*

enzyme status, irrespective of the mode of expression.

The state of involvement and the histological grading are now well-established prognostic factors in breast cancer. It is therefore important to determine whether glycolytic enzyme measurements could provide further information in patient subgroups defined by these criteria. The patients in this study were also classified on the basis of their menopausal status and hormone receptors content.

Patients separate according to the number of axillary invaded lymph nodes (1–3 *vs* > 3). In the group of patients having up to three invaded lymph nodes, no clear difference was observed when considering recurrence in relation to the five enzyme levels expressed by the three modes of expression.

In the group of patients with more than three invaded lymph nodes (Table 3), no significant difference was observed for LDH and PHI. High values of G6PD expressed/g tissue, of PK expressed/g tissue and mg DNA, and of 6-PGD expressed/g protein, g tissue and mg DNA (Fig. 1) were significantly associated with higher recurrence risk.

Patients classified according to their histological grading. No significant difference for the five enzyme

Table 2. *Activities of glycolytic enzymes in the total population*

	P.K.	G-6PD	6-PGD	PHI	LDH
Units/g proteins	308* (62–563)†	71 (17–190)	53 (20–210)	2148 (864–4447)	1711 (465–4038)
Units/g tissue	4.37 (0.79–8.07)	1.06 (0.18–2.61)	0.72 (0.19–2.80)	30.4 (8.68–44.4)	22.8 (8.9–59.4)
Units/mg DNA	5.32 (0.66–9.63)	0.82 (0.17–2.41)	0.83 (0.15–1.90)	20.9 (7.02–68.41)	18.6 (4.03–57.40)

No difference in the medians was observed in patient subgroups defined by their nodal, histological and receptor status.
* Median.
† Range.

Table 3. Summary of the results of life table analyses

Enzymes		LDH	PHI	G-6PD	PK	6-PGD
Patients with > 3 lymph nodes invaded	u/g total proteins	0.55	0.58	0.32	0.10	0.03
	u/g tissue	0.21	0.55	0.04	0.009	0.004
	u/mg DNA	0.57	0.96	0.43	0.002	0.03
Patients with positive PR	u/g total protein	N.S.*	N.S.	0.04	N.S.	N.S.
	u/g tissue	N.S.	N.S.	0.04	0.003	N.S.
	u/mg DNA	0.01	N.S.	N.S.	N.S.	N.S.

Life tables were calculated for patients having an enzyme activity either above or below the median value calculated for the entire group. *P* values were calculated by the log-rank test. Patients having low values of LDH and high values of G-6PD, PK and 6-PGD have a greater tendency to recurrence.
* N.S. = not significant.

values were observed in histological gradings II and III.

Patients classified according to their menopausal status. Analyses relating patient disease-free survival following chemotherapy to glycolytic enzymes failed to demonstrate that there was any difference in outcome between pre- and post-menopausal subgroups.

Patients classified according to the estrogen and progesterone receptor status. No significant difference was observed when the patients were grouped according to the ER status. In the patients whose tumors contained PR, high values of PK expressed/g tissue ($P = 0.003$), high values of G-6PD expressed/g proteins ($P = 0.04$) or g tissue ($P = 0.04$) were significantly associated with higher recurrence risk. Low values of LDH expressed per mg DNA were associated with higher recurrence risk ($P = 0.01$) (Fig. 2).

Multivariate analysis. In another approach to evaluate the relationship of enzyme levels to the length of disease-free interval, enzymes factors were tested in Cox's regression model in addition to grade of Bloom, tumor size, histologic number of involved nodes, menopausal status, and estrogen and progesterone binding levels. Treatment (FAC vs FAC + CMF) was also included as a potential variable in Cox's regression model. All the possible ratios and products of one enzyme activity to another were also retained as candidate factors in Cox's model.

Three multivariate analyses were performed, each of them being related to the enzyme level expression. Table 4 presents the results obtained for each mode of expression. In all models the histologic number of involved nodes is the most significant prognostic factor. This factor is the single important factor delineated by the multivariate analysis when the enzyme levels were expressed either as g total protein or as g tissue, although for

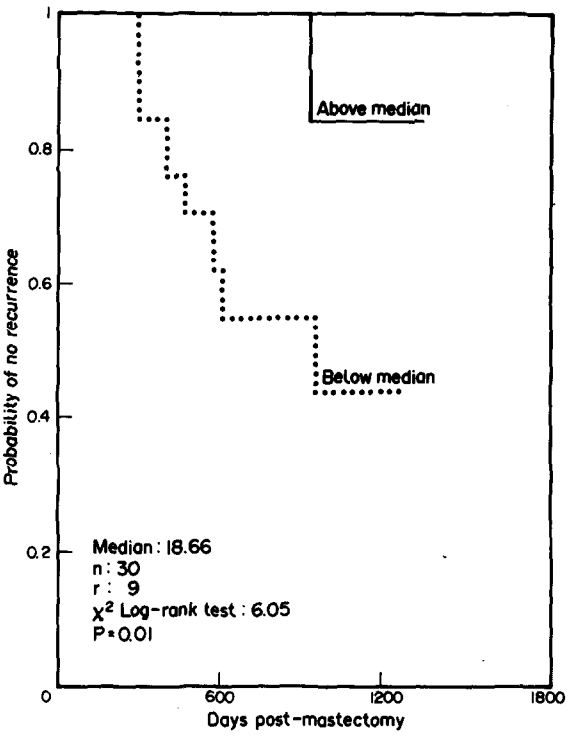

Fig. 2. Patients whose tumours contained PR: life tables for LDH content of carcinomas as u/mg *DNA (N: total no. of patients, R: no. of recurrences).*

that latter node the logorithm of G-6PD was of borderline significance ($P = 0.06$). The Cox's model that offered the greater maximization was one that contained the histologic number of involved nodes and the product of logarithms of G-6PD and 6-PGD expressed in mg DNA. The statistical significance of the entering patient factors and other details concerning the fit of the model, when enzyme level is expressed in mg DNA, are given in Table 5. Additional variables which did not result in a significant change in maximized log-likelihood ($P > 0.05$) were not included. The model that performed best was then

Table 4. Multivariate analysis: summary of results obtained according to each mode of enzyme expression

Enzyme levels expression	No. of patients	Selected factors	1st eliminated factor
g/total protein	79*	nodes (0.019)†	PK‡ (0.10)
g/tissue	79	nodes (0.019)	G-6PD (0.06)
		nodes (0.008)	
mg/DNA	75	G-6PD × 6-PGD (0.02)	PHI (0.21)

* Not every patient had complete information on all candidate variables.
† *P* value of adding variables at each step in the model.
‡ Enzyme levels underwent a logarithmic transformation.

Table 5. Prognostic factors related to disease-free interval when enzyme activities are expressed in u/mg *DNA*

Step	Factors	Regression coefficient	Maximized log-likelihood	Chi-square	*P* value
0	None	—	−94.312	—	—
1	No. of involved nodes	0.129 (0.04)*	−90.850	6.925	0.0083
2	G-6PD × 6-PGD	−3.801 (1.85)	−88.239	5.222	0.0223

* Figures in parentheses are the standard deviations.

taken as the full working model. Knowledge of the two most significant prognostic variables and their associated regression coefficients allows one to obtain a prognostic index for each patient. The index I for each patient is then:

$$I = \ln\left(\frac{\lambda(t; z)}{\lambda_0(t)}\right) = 0.129 \times (\text{nodes}) - 3.801 \times (\log(\text{G-6PD}) \times \log(\text{6-PGD})),$$

where $\lambda(t;z)$ is the hazard rate for being disease-free at time t and $\lambda_0(t)$ is the underlying hazard computed at the average values of the variables z in the model.

The larger the value of I, the worse the prognosis for the patients. If G-6PD value is less than 1 u/mg DNA and the 6/PGD value is greater than or equal to 1 u/mg of DNA, and, conversely, the larger the value of number of involved nodes, the worse the prognosis for the patient. In all other cases, the larger the value of the product G-6PD × 6-PGD, the better the prognosis for the patient.

The I was computed for each patient, and the patients then arranged in order of decreasing values of I. We have compared the performance of the index I with that of the number of involved nodes (the most significant single factor) alone. The patient group consisted of 25 with nodes ≤ 3 and 50 with nodes >3. Figure 3 shows the disease-free curves for these groups (curves 3 and 4) together with those for subgroups containing the same numbers of patients but selected according to their I value (i.e. the 25 with the lowest values and the 50 with the highest values). The I gives a slightly better discrimination at about 2 yr after mastectomy. The difference between the two risk groups is significant, with $P = 0.02$.

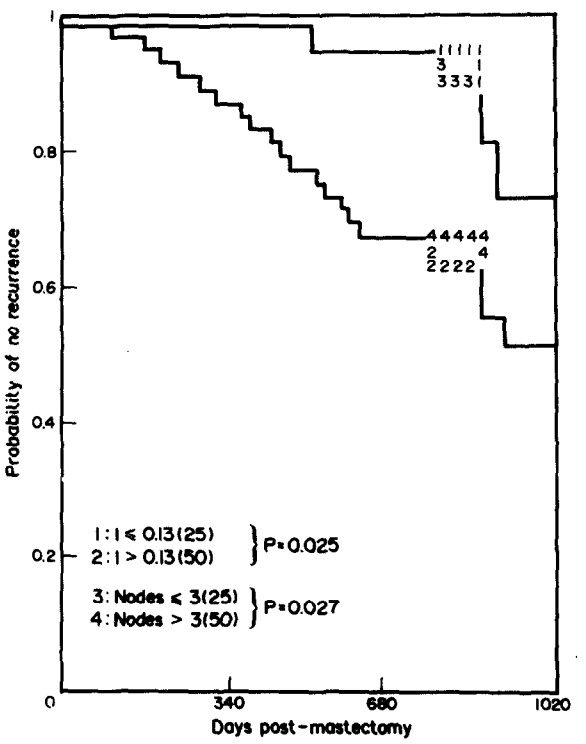

Fig. 3. Evaluation of the index compared with the number of involved nodes, using disease-free curves.

DISCUSSION

Previous studies have shown that the activities of a number of glycolytic enzymes were altered in patients with breast cancer and some correlations were found between enzyme activities, prognosis and response to chemotherapy [8–10, 20].

We have attempted here to determine whether the tumor levels of glycolytic enzymes could identify patients most likely to benefit from adjuvant therapy. If the enzyme parameters could offer such a distinction, the patients would avoid being exposed to the side-effects of unnecessary cytotoxic treatment. Our patient population presented primary breast cancer and was homogeneous regarding histopathological features and treatments. Surgical treatment, pathological control, clinical follow-up, and biochemical assays including hormone receptors and enzymes were all performed at the same institute. In order to investigate whether our enzyme parameters could predict the patient outcome following chemotherapy, we compared univariate (life table analyses) and multivariate methods (Cox's model).

The results of the first study, after 54 months of life table analyses, support in part the conclusions drawn by previous reports.

In a study of 54 primary breast cancer patients, Savlov *et al.* [9] found that low levels of glycolytic enzymes, including LDH, PHI, G-6PD and PK, occurred in patients failing to benefit from adjuvant chemotherapy; however, the statistical analysis of the data (not including life tables) was probably not sufficient to make firm conclusions regarding the relation of glycolytic enzymes to patient outcome. In our study low enzyme values were observed for LDH expressed per mg DNA, but the *P* values (log-rank test) were significant only in patients whose tumours contained PR.

Savlov's working hypothesis was based on *in vitro* studies [21] demonstrating that reduced activities of glycolytic enzymes were shown to be correlated with less rapid tumour growth. It was then postulated that slower growing lesions with low enzyme levels might be less affected by cytotoxic agents than faster growing lesions. However, the concept observed in *in vitro* studies [21] is not necessarily transferable to the *in vivo* clinical situation. Moreover, lower levels of glycolytic enzymes are found in normal tissue and benign breast lesions than in carcinomas [22–24]; the low levels of enzymes observed in benign lesions probably being indicative of their slower growth.

Like Savlov *et al.* [9], we did not observe PHI, G-6PD and PK low levels of in patients failing to benefit from chemotherapy. We observed high values of PK associated with higher recurrence risk in patients with more than three invaded lymph nodes and in patients with positive PR. Several explanations for these discrepancies need to be considered: firstly, the duration of treatment, the drug and dosages used, and the criteria for determining response to chemotherapy all vary considerably between studies; and secondly, the statistical analyses of the results are quite different.

We found significant results for 6-PGD in the patiens with more than three invaded lymph nodes. These results confirm those obtained by Deshpande *et al.* [8] in a population of primary breast cancer patients. 6-PGD associated with alpha-GPDH was found to be useful as an aid to prognosis, high levels of 6-PGD being associated with higher risk of recurrence (post-surgical treatment was not taken into account in Deshpande's study). However, in our population the univariate factor analysis appears to be insufficient to warrant firm conclusions regarding the usefulness of these enzymes as an aid to evaluate outcome of patients following chemotherapy. The use of the median to separate population is probably not adequate and wastes a lot of information.

Using the Cox's regression model, a prognosis index was proposed using the number of involved nodes and levels of G-6PD and 6-PGD. The validity of this model has now to be treated in a true prospective manner.

In conclusion, the multivariate model as performed in this study is a demonstration of one method of dealing with the interrelationships existing between prognostic variables in breast cancer and illustrates the limitations of the univariate factor analysis.

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REFERENCES

1. Blamey RW, Davis CJ, Elston CW, Johnston J, Haybittle JL, Maynard PV. Prognostic factors in breast cancer, the formation of prognosis index. *Clin Oncol* 1979, **5**, 227–236.
2. Nealon TF, Nkongho A, Grossi C, Gilloley J. Pathologic identification of poor prognosis stage I (TINOMO) cancer of the breast. *Ann Surg* 1979, **190**, 129–132.
3. Fisher B, Redmond C, Fisher ER, and participating N.S.A.B.P. investigators. The contribution of recent N.S.A.B.P. clinical trials of primary breast cancer therapy to an understanding of tumor biology. An overview of findings. *Cancer* 1982, **46**, 1009–1025.

4. Haybittle JL, Blamey RW, Elston CW *et al.* A prognosis index in primary breast cancer. *Br J Cancer* 1982, **45**, 361–366.
5. Clark GM, Mc Guire WL. Prognostic factors in primary breast cancer. *Breast Cancer Res Treat*, 1983, **3** (Suppl.), 69–72.
6. Pichon MF, Pallud C, Brunet M, Milgrom E. A relationship of presence of progesterone receptors to prognosis in early breast cancer. *Cancer Res* 1980, **40**, 3357–3360.
7. Saez S, Cheix F, Asselain B. Prognostic value of estrogen and progesterone receptors in primary breast cancer. *Breast Cancer Res Treat* 1983, **3**, 345–354.
8. Deshpande N, Mitchell I, Millis R. Tumour enzymes and prognosis in human breast cancer. *Eur J Cancer*, 1981, **17**, 443–448.
9. Savlov ED, Hilf R, Gibson BS, Feldstein M. Low tissue enzyme activity seen in breast cancers of patients who fail adjuvant chemotherapy. *Cancer* 1981, **47**, 2214–2217.
10. Hilf R, Feldstein ML, Gibson SL, Savlov E. A logistic model based on enzyme activities for the prediction of response of breast cancer patients to chemotherapy. *Cancer* 1982, **50**, 1734–1738.
11. *TNM Classification of Malignant Tumors*. Geneva, Union Internationale Contre le Cancer, 47–53, 1979.
12. Bloom HJG, Richardson WW. Histological grading and prognosis in breast cancer. A study of 1,409 cases of which 359 have been followed for 15 years. *Br J Cancer* 1957, **11**, 35–77.
13. EORTC Breast Cooperative Group. *Revision of the Standards for the Assessment of Hormone Receptors in Human Breast Cancer: Report of the Second EORTC*. Netherlands, Cancer Institute, Workshop, 1979.
14. Shonk CE, Boxer GE. Enzyme patterns in human tissues. I. Methods for the determination of glycolytic enzymes. *Cancer Res* 1964, **24**, 709–721.
15. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 1976, **72**, 248–254.
16. Gross-Bellard M, Oudet P, Chambon P. Isolation of high molecular weight DNA from mammalian cells. *Eur J Biochem* 1973, **36**, 32–36.
17. Kaplan EL, Meier P. Non parametric estimation from incomplete observation. *J Am Statis Assoc* 1958, **53**, 457–481.
18. Peto R, Pike MC, Armitage P *et al.* Design and analysis of randomized clinical trials requiring prolonged observation of each patient. II. Analysis and examples. *Br J Cancer* 1977, **35**, 1–39.
19. Cox DR. Regression models and life tables. *J R Statist Soc (Series B)* 1972, **34**, 187–220.
20. Deshpande N, Mitchell I, Millis R. Enzymes, lymph node involvement and possible prognosis in human breast cancer. *Eur J Cancer* 1978, **14**, 183–187.
21. Weber G. Molecular correlation concept. In: Bush H, ed. *The Molecular Biology of Cancer*. New York, Academic Press, 1974, 488–521.
22. Hilf R, Wittliff JL, Rector WD, Savlov ED, Hall TC, Orlando RA. Studies on certain cytoplasmic enzymes and specific estrogen receptors in human breast cancer and in nonmalignant diseases of the breast. *Cancer Res* 1973, **33**, 2054–2062.
23. Deshpande N, Mitchell I, Millis R. Enzyme studies in human breast tumours. *Eur J Cancer*, 1977, **13**, 1261–1267.
24. Balinsky D, Platz CE, Lewis JW. Isozyme patterns of normal, benign and malignant breast tissues. *Cancer Res* 1983, **43**, 5895–5901.