

**PREOPERATIVE SYSTEMIC TREATMENT OF LARGE OPERABLE
PRIMARY BREAST CARCINOMA: RELATIONSHIP BETWEEN
TUMOUR CHARACTERISTICS AND RESPONSE TO THERAPY.**

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The writing and composition of this thesis has been my own effort and the work described has been carried out by myself except where otherwise indicated in the acknowledgements.

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ABSTRACT

By administering systemic therapy before definitive local surgery in patients with large operable breast cancers (T2, T3, N0, N1, M0) an in vivo human tumour model system has been devised which allows direct objective assessment of response to systemic therapy in the individual patient. Response was assessed following 12 weeks of systemic therapy by linear regression analysis of changes in tumour volume measured at weekly intervals. Definitive locoregional surgery (mastectomy n = 82, wide local excision n = 6) was performed on completion of systemic therapy (3-6 months). Response to both endocrine (n = 61) and cytotoxic therapies (n = 47) have been studied. Twenty-seven patients received both forms of treatment.

A biopsy of the tumour (fine-needle aspirate and open wedge biopsy) was performed prior to initiating systemic therapy. Using the human tumour model system it has been possible to evaluate the predictive value for response of several pretreatment tumour indices. These have included tumour ER concentration assayed by either dextran-coated charcoal method or immunocytochemical staining (ERICA), the activity of enzymes involved in local steroidogenesis (i.e. tumour aromatase activity and peritumour fat 17 β hydroxysteroid dehydrogenase activity), tumour grade and tumour ploidy. By obtaining a biopsy of tumour after systemic therapy it has also been possible to determine the effects of systemic therapy on these potential indices of response.

Response was observed in 24 (39%) of the 61 patients who received endocrine therapy and 34 (72%) of the 47 patients who received cytotoxic therapy. Of the predictive indices examined within this study only ER concentration and possibly tumour aromatase activity have been shown to be of any value in relation to response to endocrine therapy and aromatase inhibitors respectively. The other parameters studied have been uniformly disappointing in their prophetic value.

When systemic therapy is given as the preferred first line therapy, response to therapy can be assessed on an individual basis and hence allow determination of appropriate systemic therapy. By using this principle a selective protocol for adjuvant systemic therapy has been devised. Endocrine therapy should be reserved for patients with tumours of ER concentration of ≥ 20 fmol/mg cytosol protein. Patients with ER-poor/negative tumours or those which fail to respond to endocrine therapy should receive cytotoxic therapy. Using this protocol the overall survival rate at 4 years was

77% (+/- 5%) with 83 (+/-4%) remaining free from local relapse. This treatment policy outlined within this paper is now being tested against orthodox management by controlled randomised trial.

AIMS OF STUDY

It has now been established from statistical analyses of large controlled randomised trials that the long term survival of patients with operable breast cancer can be improved by systemic endocrine or cytotoxic therapy (Early Breast Cancer Trialists' Collaborative Group 1988; 1992a; 1992b). To date it has been more usual to give adjuvant systemic therapy after definitive surgery. Once the primary tumour has been removed however there is no direct index of the efficacy of systemic therapy and as yet these trials have not defined which therapy is most suitable for an individual patient. For this reason it was decided to reverse the conventional treatment sequence for patients with large operable breast cancers, a group of patient with a poor prognosis, and give systemic therapy before definitive locoregional surgery with the following objectives;

1. to devise an in vivo human tumour model system to allow direct objective assessment of response to systemic therapy in the individual patient.
2. to test the feasibility and efficacy of this novel approach in allowing selection of appropriate systemic therapy.
3. to use the in vivo model system to evaluate potential predictive indices of tumour response.
4. to determine the effect of systemic therapy on those indices of response

INTRODUCTION

CHAPTER 1

BREAST CANCER AS A SYSTEMIC DISEASE

For the last 35 years breast carcinoma has been one of the leading causes of cancer death among women of the western world (Cancer statistics, 1988). In 1986 breast cancer accounted for the deaths of 15,250 women in the United Kingdom alone, with 1 in 12 women developing breast cancer. Despite improvements in the diagnosis and treatment of breast cancer epidemiological data show that mortality has not reduced (Cancer statistics, 1988). It would therefore be complacent to continue our current therapeutic practice without critical review.

The Natural History of Untreated Breast Cancer

So few patients with breast cancer remain without some form of therapy that adequate information on the natural history of breast cancer is difficult to obtain and has relied on retrospective analyses (Greenwood 1926, Daland 1927, Nathanson & Welch 1936, Phillips 1959, Bloom Richardson & Harries 1962). The 3 year survival figures within these studies have varied considerably from 19 - 44%. More agreement was noted after the third year with a 5 year survival of approximately 20% and 5% of patients remaining alive at 10 years. The median survival of untreated patients from onset of symptoms was approximately 2 years and has varied little over the preceding one and a half centuries (Bloom 1967).

Breast Cancer as a Systemic Disease

Halsted's (Halsted 1898) initial use of radical mastectomy as the preferred treatment for breast cancer emphasised the management of the local tumour. Halsted initially applied radical mastectomy to all patients with breast cancer, but in 1943 Haagensen defined disease characteristics which if treated by radical mastectomy alone were associated with high local recurrence and poor survival rates (Haagensen 1943). Such 'grave signs' included solid fixation of the tumour to the chest wall, oedema or ulceration of the skin and massive involvement or fixation of the axillary lymph nodes and were associated with 5 year survival rates of less than 40% with 40% of patients developing local recurrence despite radical mastectomy. Even lower overall survival (0-5% at 5 years) and higher local relapse rates (50-60% at 5 years) were observed

with more advanced local disease, including the presence of two or more 'grave signs', satellite skin nodules, inflammatory carcinoma, involvement of supraclavicular or parasternal lymph nodes or oedema of the arm. The presence of any one of these signs is now considered evidence of inoperability.

For patients whose tumours do not exhibit any of Haagenson's 'grave signs', surgery remains the conventional form of primary management. Mastectomy with axillary clearance (Turner et al. 1981; Maddox et al. 1983), and more recently breast-conserving surgery (Fisher et al. 1985; 1989; Veronessi, Zucali & Del Vecchio 1985; Veronessi 1987), in association with radiotherapy, has been found to give control of the primary tumour and the associated axillary lymph node metastasis in the majority of cases when the primary tumour is less than 2 or 4 cm respectively (Veronessi et al. 1981; Veronessi, Zucali & Del Vecchio 1985; Veronessi 1987; , Fisher et al. 1985,1989).

The precise effect of definitive local surgery on survival however has been difficult to interpret since no case-control studies exist. Direct comparison with historical control series is difficult since these contain a high proportion of patients with larger tumour or more advanced disease. This has led to conflicting opinions with evidence for both improved short and long term survival rate in favour of surgical management (Bloom, Richardson & Harries 1962) and no overall survival advantage (McKinnon 1954; Park & Lees 1951). Duncan and Kerr (1976) have suggested that the survival benefit associated with adequate locoregional surgery was limited to those patients whose tumours measured 3 cm or less in diameter at initial presentation.

The latest national survival figures for England and Wales have demonstrated that approximately 60% of women diagnosed with breast cancer in 1981 were alive 5 years later (Cancer Statistics 1988). Other series report 5 year survival rates for technically operable breast cancer, treated by local means alone, of between 51 and 74% (Delarue, Anderson & Starr 1969; Meyer, Smith & Potter 1978), with 43% of patients surviving to 10 years (Meyer, Smith & Potter 1978).

Long term survival data for patients with so called "operable" breast cancer has indicated however that despite adequate locoregional therapy women still show an excess mortality from breast cancer 15 years, (Mueller & Jeffries 1975; Bond 1968; Myers 1973; Ariel 1979; Haagenson & Bodian 1984) 20 years (Adair et al. 1974; Mueller, Ames & Anderson 1978) and even 30 years (Brinkley & Haybittle 1975,1984;

Hibberd, Horwood & Wells 1983) post-initial treatment. One study of patients aged 40 years or less at diagnosis has demonstrated that for this selected group of patients the excess mortality from breast cancer persisted for up to 40 years (Rutqvist & Wallgren 1985). Although statistical 'cure' (annual death rate from all causes is similar to that of a normal population group of similar sex and age distribution, Eason & Russel 1968) for patients with "operable" breast cancer has yet to be demonstrated, personal 'cure' (freedom from clinically detectable disease during the remainder of a patient's life-span) does occur. The proportion effecting personal cure has been reported as around 20-30% (Meuller & Jeffries 1975; Muller, Ames & Anderson 1978; Brinkley & Haybittle 1984; Rutqvist & Wallgren 1985). These data imply that in the majority of patients presenting with palpable breast cancer, tumour cells have already disseminated into the systemic circulation.

Breast cancer constitutes a very heterogeneous collection of disease entities with survival patterns which vary greatly between individuals. Since no direct method exists by which the exact timing of metastasis can be studied within individual patients, the precise factors which determine tumour cells dissemination and successful establishment of metastasis, are not yet fully understood. It is generally accepted that the development of a metastasis is the culmination of a whole series of complex events. The enormous disparity between the relatively large number of cells released from the primary lesions (Butler & Gullino 1975; Liotta, Kleinermann & Saidel 1974) and the relatively small number of overt metastases resulting from this release indicates that metastasis is a very inefficient process. The disparity could be accounted for by the fact that only a small subpopulation of aggressive cancer cell clones are capable of surviving 'ordeal by metastasis' or it may be that the trauma inherent in the various steps of metastasis, including cell separation, dissemination and arrest in lymphatic or vascular channels, adherence and tissue infiltration and eventual establishment with vascularisation may result in a random survival of cancer cells (Weiss 1977; 1979).

Factors Which Relate to Probability and Timing of Metastatic Dissemination

Tumour Size and Metastatic Potential

A direct relationship can be demonstrated between the size of the tumour at initial presentation and prognosis (Fisher, Slack & Biass 1969; Duncan & Kerr 1976; Ariel 1979; Adair et al. 1974; Foster & Costanza 1984; Daly, Clark & McLoure 1974; Fracchia et al. 1985; Pascval et al. 1983, Harveit, Thoresen & Maehle 1984;

Ketterhagen, Quackenbush & Haushalter 1984; Coulson et al., 1984; Peterson et al. 1982; Carter et al. 1978; Blamey et al. 1979; Rosen et al. 1981; Wallgren, Silfversward & Eklund 1976; Atkinson, Brown & Montague 1986; Carter, Allen & Henson 1989). A progressively worsening prognosis and incidence of local relapse has been shown as tumour size at initial presentation increases from 1 cm to 4 cm in diameter. Thereafter no further decline in prognosis was observed (Duncan & Kerr 1976). The expected survival for tumours greater than 4 cm at initial presentation and treated by mastectomy is shown in Table 1. The equivalent incidence of local recurrence following radical mastectomy for tumour of ≥ 4 cm is between 24-31% at 5 years (Danforth & Lippman 1988).

The probability of distant metastasis therefore appears to be a function of tumour volume. A detailed study of the relationship between the size of the primary tumour at initial treatment and the incidence of distant metastasis was performed on 2648 breast cancer patients treated at the Institut Gustave Roussy between 1954 and 1972 (Koscielny, Tubiana & Le, 1984). A remarkable linear relationship was demonstrated between tumour volume (log) at the time of detection and metastatic probability to either regional lymph nodes or distant sites (Table 2) and by the time a tumour reached a diameter of 3.5cm, 50% had established systemic metastases. A similar relationship between metastatic potential and tumour volume has been shown by Atkinson (Atkinson, Brown & Montague 1986).

By retrograde extrapolation of the growth rate of metastasis it has been suggested by other workers, that metastatic spread occurs even earlier in the life cycle of breast tumours (Igot & Le Gal 1968). Bauer and Le Gal (Bauer & Le Gal 1973), estimated that axillary metastases were established in 346 out of 348 primary tumours when the primary tumour was less than 1cm in diameter. Such calculations have however been criticised since they presume that tumour growth is exponential in type and the growth rate of a primary tumour and its metastasis are equal. The doubling time of a metastasis has been demonstrated to be shorter than its primary tumour (Charbit, Malaise & Tubiana 1971; Steel 1977). Tumours consisting of only 10 cells have however been shown to be large enough to successfully establish metastatic growth (Bond 1968).

Table 1

Review of overall survival of patients with operable breast cancers of diameter 4 cm or greater treated by mastectomy with or without radiotherapy.

	Patient group	Rx	Survival	
			3yrs	5yrs
Duncan and Kerr 1976	>4cm	Mx/XRT	75%	65%
Seidman & Mushinski 1987 (1940-1943, n=1458)	>4cm	Mx	-	55%
Seidman & Mushinski 1987 (1940-1956, n=1978)	>5cm	Mx	-	50%
Fentiman et al. 1984 (n=42)	>5cm	Mx	-	60%
Ariel 1979 (n=266)	≥4cm	Mx		44-63%
Veronesi et al. 1981	>5cm	Mx		N- 60-91% N+ 23-50%
Carter, Allen & Henson 1989	>5cm	Mx	75%	60%

Rx = treatment given; Mx = mastectomy; XRT = radiotherapy; N- = axillary nodes free from metastases; N+ axillary nodes contained metastases by histological examination.

Axillary Lymph Node Involvement, Histological Grade and Metastatic Potential

Histological examination of axillary node status is the single most reliable factor in predicting the timing of recurrence and death (Carter, Allen & Henson 1989). The

absolute number of nodes involved, the level and size of lymph node involvement are all associated with prognosis (Fisher et al. 1969; Daly, Clark & McLoure 1974; Fracchia et al. 1985; Pascual et al. 1983, Harveit, Thoresen & Maehle 1984; Ketterhagen, Quackenbush & Haushalter 1984; Coulson et al., 1984; Peterson et al. 1982; Carter et al. 1978; Rosen et al. 1981; Wallgren, Silfversward & Eklund 1976; Atkinson, Brown & Montague 1986; Carter, Allen & Henson 1989). Prognosis however is not related to either the fraction of lymph nodes involved or the total number of lymph nodes identified.

The presence of axillary lymph node metastasis is a marker of, but not synonymous with systemic dissemination. It correctly predicts for recurrence in only 75% of all patients during the first 10 years post-mastectomy. The probability of systemic metastasis has been shown to strongly correlate with number of involved axillary lymph nodes (Koscielny, Tubiana & Le 1984; Tubiana & Koscielny 1986; Atkinson Brown & Montague 1986) and histological grade of the primary tumour (Koscielny, Tubiana & Le 1984; Tubiana & Koscielny 1986). Tumours with a large number of involved axillary lymph nodes have a high probability of systemic metastases. Well differentiated tumours (histological grade 1) demonstrate a particularly low potential for dissemination while no significant difference could be demonstrated between moderately or poorly differentiated tumours (grade 2 and 3; Tubiana & Koscielny 1986). These effects were related to but could be demonstrated independent of tumour size (Table 2 and 3). These data confirm the prognostic significance of axillary node status and histological grading and stress their influence on the probability of dissemination.

Table 2

Volume (V₅₀) for which 50% of the tumour have metastasised in relation to histological grade and number of axillary lymph nodes involved (Tubiana & Koscielny 1986).

Group	Number of patients	V50 (ml)	Corresponding Diameter (cm)	Variation Interval(ml)
Overall	2648	23.6	3.56	19.3-28.8
Histological Grade				
known	1596	41.0	4.27	30.5-54.8
1	298	584	10.4	191 - 1765
2	766	29.5	3.83	19.5-44.7
3	532	23.0	3.53	14.6-35.0
Number of involved axillary lymph nodes				
known	1722	32.8	3.97	24.5-43.8
0	560	290	11.0	217-2180
1 to 3	657	30.3	3.87	19.0-48.4
>3	505	7.2	2.4	4.0-13.1

Table 3.

Theoretical diameter for which 50% of the tumours have initiated distant metastases, according to histological grade and the number of invaded axillary lymph nodes at equivalent diameters (Tubiana & Koscielny 1986).

	Number of Invaded Axillary Lymph Nodes			
	0	1 to 3	4 to 10	>10
Histological Grade 1	9 cm	4.8 cm	3.1 cm	2.5 cm
Histological Grade 2 or 3	3.7 cm	2.6 cm	2 cm	1.8 cm

Other Prognostic Variables

Data has suggested that the metastatic potential of a tumour evolves as the tumour grows, and that nodal status simply reflects the ability of a tumour to spread. The evolution of metastatic potential is not the same for all tumours and a newer generation of prognostic markers have been based on measurement of tumour aggressiveness, of which growth rate is an integral part. The introduction of the concept of tumour doubling time enabled a quantitative estimation of tumour growth rate. A higher incidence of positive axillary lymph nodes has been recorded in patients with tumours showing rapid as compared to slow growth rates (Geshon-Cohen, Berger & Klickstein, 1963; Heuser, Spratt & Polk, 1979). Tumour doubling time has also been correlated to long term survival after surgical treatment (Kusama et al., 1972), relapse-free interval and duration of survival after relapse (Malaise et al., 1974). However the direct measurement of doubling time necessitates sequential clinical and mammographic examinations over several months and this type of assessment in an untreated patient is not ethically acceptable for routine use. More indirect methods have therefore been employed by which to measure tumour kinetics.

An inverse relationship has been documented between the indices of proliferation and the degree of tumour differentiation or tumour grade (Steel 1977; Meyer & Connor 1977). The clinical relevance of histological grade as a prognostic index was initially reported by Bloom (Bloom 1950a 1950b) and since then there have been numerous studies which confirm that patients with well differentiated tumours have a considerable better survival when compared to those with poorly differentiated tumours (Elston et al., 1982; Fisher, Gregorio & Fisher 1975; Haybittle et al., 1982; Freedman et al. 1979). The use of histological grading has however been criticised because of inconsistency and lack of reproducibility in assessment (Cutler et al. 1966; Gresham 1976; Gilchrist et al. 1985). More objective automated systems for the evaluation of proliferative activity have been developed. The use of radiolabelled tritiated iodine labelled precursors of DNA synthesis have allowed the measurement of the fraction of cells synthesising DNA (Tubiana & Malaise 1976; Chavaudra, Richard & Malaise 1979). The thymidine labelling index, as determined by this method is directly correlated to prognosis such that patient with a high labelling index have a very high incidence of relapse and death (Tubiana & Koscielny 1987; Meyer et al. 1983; Silvestrini, Diadone & Gasparini 1985; Silvestrini et al., 1989). It would appear that this cannot be explained simply by differences in the growth rates of metastases but

appears to be due at least partly to differences in the metastasising potential of the neoplastic cells (Tubiana & Koscielny 1988).

Although effective thymidine labelling techniques requires autoradiography, are time consuming and inconvenient. Furthermore the proportion of cells present within the S-phase can be directly computed from quantitative analysis of a flow cytometric DNA histogram and although a practical problem exists for aneuploid tumours. The % S-phase calculated from flow cytometric histograms has tended to be slightly higher than the corresponding thymidine labelling index, and this excess has been attributed largely to debris in the histogram (Meyer et al., 1987). Although a high % S phase has been associated with poor prognostic features, that is a poor histological grade (Hery et al., 1987; Olszewski et al., 1981; Kallioniemi et al., 1987), the absence of ER or PR (Kallioniemi et al., 1987; Dressler et al., 1988) and positive lymph node status (Dressler et al., 1988; Kallioniemi et al., 1987; Silvestrini et al., 1985) it has been shown to be of independent prognostic significance for both overall survival and disease-free survival in patients with (Kintenburg et al., 1986; Kallioniemi et al., 1987) or without axillary node metastases (Hery et al., 1987; Silvestrini et al., 1985; 1986; 1989; Clark et al., 1989). Some authors have suggested that the prognostic impact of % S phase in relation to disease recurrence decreases with time (Stal et al., 1989). Recently, more specific methods for measuring tumour cell kinetics have been described using antigens incorporated or present within cycling cells and detected by simple immunocytochemical techniques. Such antibodies include bromodeoxyuridine (Gratzner 1982; Haag et al. 1987; Khochbin et al. 1988; Lacombe et al. 1988; Wilson et al. 1988) and Ki 67 (Gerdes et al. 1984,1986; Lelle et al. 1987; Barnaud et al. 1987; Charpin et al. 1988; Walker & Campjohn 1988; Sasaki et al. 1988). In general the bromodeoxyuridine labelling index relates to that of the Ki-67 labelling index (mean ratio 0.58, Sasaki et al. 1988). The clinical relevance of the kinetic parameters remains to be demonstrated.

Measurement of cellular DNA content by flow cytometry is a simple and rapid technique which provides information about tumour ploidy and proliferative activity. The mean labelling index of aneuploid tumour is significantly higher than that of diploid tumour (Dean et al., 1984; Coulson et al., 1984; Dressler et al., 1986) and breast tumours with diploid DNA content tend to be of low histologic grade and oestrogen rich, whereas those with a high ploidy are more anaplastic and poorer in ER proteins (McDivitt et al., 1986; Olszewski et al., 1981; McGuire et al., 1986, Daver et al., 1983). The value of tumour DNA content as an independent prognostic variable is a

little more controversial. DNA aneuploidy has been shown to be significantly associated with a shorter recurrence free survival by some (Cornelisse et al., 1987; Fallenius 1986; Hedley, Rugg & Gelber 1987; Hedley et al., 1984; Kallioniemi et al., 1987) but not all studies (Stal et al., 1989; Hedley, Rugg & Gelber 1987; Klintenberg et al., 1986), while in yet others the predictive effect was confined only to node-positive postmenopausal women (Cornelisse et al., 1987; Fallenius 1986). Kallioniemi and colleagues have demonstrated an independent association with overall survival (Kallioniemi et al., 1987). Tetraploid tumours would appear to show a better prognosis than other hyperdiploid tumours (Kintenberg et al., 1986; Fallenius 1986; Kallioniemi et al., 1987; Baildam et al., 1987b; Stal et al., 1989).

Hormone Receptors

The maintenance of the ER and the presence of progesterone receptors (PR) are more common associated with well differentiated tumours (Fisher, Sass & Fisher 1987; Kamby et al. 1988; Singh et al. 1988). The expression of ER has been inversely correlated with high proliferation rates, whether measured by ploidy, flow cytometry or thymidine labelling index (Stal et al., 1989; Kute et al., 1985; Tubiana et al., 1989). Some studies have however disputed the correlation of ER with ploidy (Fossa et al., 1984; Taylor et al., 1983) and as yet no correlation between Ki-67 staining and ER expression has been demonstrated (Bouzubar et al., 1989).

The value of steroid receptors as prognostic indicators remains controversial. Several authors have concluded that patients with ER-positive tumours have a longer overall and disease-free survival (Spyratos et al. 1989; Sutton et al. 1987; Hawkins et al. 1987; Chevalier et al. 1988; Shek & Godolphin 1989; Raemakers et al. 1985; Knight et al. 1977; Parl et al. 1984; Godolphin, Elwood & Spinelli 1981; Mason et al. 1983). Only six studies however have assess the effects of hormone receptors on prognosis by multivariant analysis (Spyratos et al. 1989; Sutton et al. 1987; Stewart et al. 1983; McGuire et al. 1986; Caldarola et al. 1986; Todd et al. 1987). Other studies with a longer follow-up suggest that the beneficial effect of positive ER status on survival (Spyratos et al. 1989; Todd et al. 1987) had no independent value or gradually diminished with time (Howat et al. 1983). In node negative patients, measurement of steroid receptors has also mostly failed to identify those patients who relapsed (Williams et al. 1987; Maki & Hoehn 1989). In addition relatively few studies compare both ER and PR, and at present controversy exists as to which hormonal receptor actually conveys the favourable prognosis (Sutton et al. 1987; Mason et al.

1983; Howat et al. 1983; Stewart et al. 1983; McGuire et al. 1986; Caldarola et al. 1986).

Oncogenes

More recent investigations have called attention to the possible prognostic relevance of oncogenes. In breast cancer, a variety of specific alterations in DNA or gene expression (messenger RNA or protein) have been demonstrated in breast cancer (MacKay et al., 1990) some of which have already been associated with either early disease recurrence and death e.g. *c-myc* (Varley et al., 1987) and *erb B-2* (Slamon et al., 1984; 1987; Wright et al., 1989) or very aggressive growth i.e. *Int2* on 11q13p (Zhou, Casey & Cline, 1988). High levels of Harvey-ras, a gene which encodes for proteins of approximately 21,000 daltons (p21) are thought to be involved in signal transduction and have been found in the majority of breast tumours (Horan-Hand et al. 1984). Although activation of Harvey-ras may be rare in breast cancer (Rochlitz, Scott & Dodson 1989), it is possible to demonstrate a concentration-dependent effect of p21 on tumorigenicity (Redmond et al. 1988) and elevated levels of Harvey-ras mRNA in malignant compared to normal breast tissue (Spandidos & Agnatis 1987; Slamon et al., 1989). In the experimental setting transfection of v-Harvey-ras into MCF-7 cells changes their hormone sensitivity such that they become oestrogen independent (Kasid, Knabbe & Lippman, 1987) suggesting that Harvey-ras may be involved in hormone independence rather than initiation of breast cancer (Agnatis et al., 1986; Lundy et al., 1986). At present there is little evidence that p21 expression significantly affects the metastasing potential of breast cancer cells. A significant association has however been demonstrated between increasing levels of p21 (or its related mRNA) and the presence of lymph node metastasis (Watson et al. 1990; Clair, Miller & Cho- Chung 1987, Querzoli et al. 1988).

Reduced expression of a gene named *nm23* and its messenger RNA have been observed in highly metastatic cells in experimental animal systems (Steeg et al. 1988; Liotta, Steeg & Steller-Stevenson 1991). In human breast cancer the loss of *nm23* messenger RNA has been strongly associated with metastatic involvement of regional lymph nodes (Bevilacqua et al. 1989) and poor survival (Hennessy et al. 1991). The exact effect of the protein encoded for by the *nm23* gene is still uncertain but the amino acid sequence is similar to nucleoside diphosphate kinase (Rosengard et al. 1989; Munoz-Darado, Inouge & Inouge 1990a, 1990b).

Enhanced expression of growth factors and growth factor receptors is also a possible prognostic indicator for human tumours. Epidermal Growth factor (EGF) is a 53-aminoacid polypeptide (Carpenter & Cohen 1979) originally characterised from rodent salivary glands (Cohen 1962) and now thought to be more widely expressed in human tissues. Epidermal growth factor (EGF) is a potent mitogen for many tissues (Carpenter 1981; Carpenter & Cohen 1976) including mammary carcinoma cells in vitro (Osborne et al. 1980; Imai et al. 1982, Davidson et al. 1987). Cellular events are induced by EGF via its cell membrane receptor (EGFR, Carpenter et al. 1983). These receptors have a molecular weight of approximately 170 kD, are glycoprotein in nature (Cohen et al. 1982) and are possibly a product of c-erb B proto-oncogene (Downward et al. 1984; Lin et al. 1984). TGF- α is a growth factor produced by human breast cancer cells under the control of oestrogen (Bates et al. 1988). It contains a protein sequence homologous with that of EGF and although not recognised by all anti-EGF antibodies, competes with EGF for binding to its receptor to produce its effect (Todaro et al. 1980; Carpenter et al. 1983). Suppression of growth of MCF-7 cell lines can be produced by antibodies directed against TGF- α or EGF-receptors (Bates et al. 1988). The EGF-receptor may therefore have a pivotal role in control of tumour cell growth at an autocrine paracrine or level.

In human breast cancer tissue a 35-50% excess EGFR expression has been detected in primary tumours and several cultured cells by means of biochemical binding (Fitzpatrick et al. 1984a, 1984b; Sainsbury et al. 1985; Fabio et al. 1986; Perez et al. 1984, Macias et al. 1986). A significantly higher proportion of cycling cells has been found in EGFR-positive compared to negative tumours and an inverse relationship between EGFR and ER (Toi et al. 1991; Sainsbury et al. 1985; Fitzpatrick et al. 1984; Nicholson et al. 1988, 1989) and PR (Delarue et al. 1988) can be demonstrated. Expression has also been correlated with other markers of poor prognosis such as a high Bloom and Richardson grade (Sainsbury et al. 1985b). In clinical studies EGFR expression has been associated with a poor prognosis (Sainsbury et al. 1987; Toi et al. 1991; Spyrtos et al. 1990) and it has been suggested that the overexpression of EGFR may be important in determining a biologically high malignant potential but this remains to be defined.

In summary, breast cancer differs from most other epithelial tumours in that it can run a very variable clinical course which may extend over thirty years. Although there has been an increasing understanding of the factors which can be used to predict prognosis following the diagnosis of breast cancer, the exact factors which determine metastatic

potential are complex and as yet not fully understood. It is likely however that breast cancer cells disseminate early in the life history of the tumour and in the majority of patients who present with clinically palpable lumps, breast cancer is already a systemic disease.

CHAPTER 2

SYSTEMIC THERAPY FOR BREAST CANCER

The aim of all primary cancer therapy is "cure". This requires the total eradication of all cancer cells. If a tumour can be detected before it disseminates, then appropriate surgical removal with or without additional radiotherapy may suffice. In the majority of patients presenting with palpable breast cancer however, the tumour has already spread outwith the breast and locoregional lymph nodes. In such patient "cure" can only be achieved by the additional use of effective tumouricidal systemic therapy. Many systemic approaches to the management of breast cancer have been developed since the introduction of endocrine therapy nearly a century ago and there is now substantial clinical data demonstrating antitumour activity of both endocrine and cytotoxic therapies. Initially systemic therapy was reserved for those patients with recurrent or locally advanced disease. Evidence from both "in vitro" and "in vivo" studies have suggested that the ability of chemotherapeutic drugs to produce cytoeradication is inversely related to tumour burden (Goldin et al. 1956). This has formed the theoretical basis for adjuvant chemotherapy, where the systemic therapy is given before the clinical signs of metastasis become apparent. The precise effect of adjuvant systemic therapy on overall survival has not been easily reached. Many trials, when taken in isolation, contained only small number of patients (Stewart 1989) and so a major overview of adjuvant systemic therapy was recently undertaken (Early Breast Cancer Trialists' Collaborative Group 1988). This involved central collation of data from the major randomised trials worldwide and was based on summation of the deaths observed in the treatment group minus those in the control group (observed minus expected deaths) within those individual studies which have addressed related questions. Significance testing can then be performed on the variance of this summation. By using this method the favourable trend in each study reinforces the trends of others to provide a total which is less subject to random error than that of individual trials. This technique has facilitated the study of major subgroups of patients and their treatment schedules, with confirmation of conclusions tentatively deduced from individual trials. Many questions however still remain to be answered.

THE ROLE OF OESTROGENS AND ANTIOESTROGENS IN BREAST CANCER

The essential role of ovarian function in normal glandular development of the breast was originally described by Sir Percival Pott in 1775 (Pott 1775). Although Schinzinger, a German surgeon, was first to postulate that oophorectomy might lead to regression of breast cancer (Schinzinger 1889), it was Beatson who confirmed the relationship in clinical practice (Beatson 1896). In 1923 the ovarian hormone oestrogen (Allan & Doisy 1923), was identified and it was not long before the stimulatory effect of oestrogen on the growth of mammary cancer in mice was demonstrated (Murray 1928; Lacassagne 1932). The proliferative effect was later verified for human breast cancer by Pearson who demonstrated an increase in urinary calcium excretion following the administration of oestrogens to patients with bone metastases (Pearson et al. 1955). Epidemiological studies have shown that oophorectomy, especially early in life, reduces the risk of breast cancer suggesting a primary promotional effect of ovarian oestrogen in the development of breast cancer (Feinleib 1968). Although the mechanisms by which oestrogens favour breast cancer development are largely unknown the direct stimulatory effect of oestradiol on at least a proportion of breast cancers has now been well established. On average one third of tumours are known to regress when subjected to hormonal manipulation. The following section reviews the relevance of local oestrogen production in breast cancer and therapeutic forms of oestrogen deprivation.

Tissue Oestradiol and its Synthesis

Glandular and Extraglandular Production

In premenopausal women the principal source of plasma oestradiol is the ovary. Lesser amounts of oestradiol arises from extraglandular aromatisation of androstenedione and to a lesser extent testosterone (Siiteri & MacDonald 1973). In the ovary the enzyme aromatase, catalyses the conversion of androstenedione to oestrone and testosterone to oestradiol. The amount of oestradiol produced depends on two factors, the amount of substrate present and the magnitude of enzyme activity. Luteinising hormone (LH) provides the primary control for the amount of substrate present, whereas follicle stimulating hormone (FSH) regulates the activity of the aromatase enzyme (Channing & Segal 1981).

After the menopause, the production of both androstenedione and oestradiol by the ovary is substantially reduced and the oestrogens are primarily derived from extraglandular aromatisation of adrenal precursors (Siiteri & MacDonald 1973; Judd et al. 1974). Androgens such as androstenedione, dehydroepiandrosterone and its sulphate are then the major precursor and are secreted by the adrenal gland. Their production varies with stress and positively correlates with the degree of obesity (Kirschner 1979; Meldrum et al. 1981). Peripheral tissues such as fat (Perel, Wilkin & Killinger 1980), skin (Schweikert, Milewich & Wilson 1976), liver and muscle (Longcope 1972) the breast and its tumours (Miller & Forrest 1976; Geier et al. 1975; Perel, Wilkin & Killinger 1980), all have the potential to synthesise and metabolise oestrogens. Peripheral conversion of circulating androstenedione to oestrone increases not only with obesity but also age (Grodin, Siiteri & MacDonald 1973; MacDonald et al. 1978). Oestrone is then converted to oestradiol by the enzyme 17- β -hydroxysteroid dehydrogenase. The activity of this enzyme does not appear to be regulated by the degree of obesity or by age (Santen et al. 1986).

Approximately 40% of plasma oestrone and oestradiol are converted to oestrone sulphate, a compound with a slow metabolic turnover and consequently relatively high plasma levels (Longcope 1972). Approximately 15% of the oestrone sulphate is then recycled back to oestrone. This storage pool pathway may provide an important source of precursor substrate for local oestradiol production in breast cancer. Most of the circulating oestrogens are protein bound, however it is the non-protein bound or 'free' hormone which is biologically active. It has been suggested that high levels of free oestradiol may be associated with increased risk of breast cancer but this remains controversial (Fishman et al. 1978; Dao 1979; Van Landeghem et al. 1981; Moore et al. 1982; Reed et al. 1983; Bruning, Bonfrer & Hart 1985). Biological effects are more likely however to be a function of the levels of endogenous steroids within the target tissues.

Oestrogen Concentration in Breast Tissues

Marked differences in the concentrations and/or patterns of major oestrogens between the plasma and breast tissues have been observed, particularly in postmenopausal women (Van Landeghem et al. 1985; Vermeulen 1976). In postmenopausal women, endogenous levels of oestrogen were significantly higher in breast tissue when compared to plasma, mean tissue : plasma ratios of about 3 for oestrone and 20 for oestradiol having been demonstrated (Vermeulen 1976). In addition oestradiol levels in normal or malignant breast tissue were found not to change significantly with

advancing age, in contrast to the large decrease in plasma concentration which occurs after the menopause (Millington et al. 1974; Van Landeghem et al. 1985; Vermeulen 1976). Malignant breast tissue was found to contain higher concentrations of oestradiol when compared to benign breast tissue, irrespective of the ER status of the tumour (Fishman et al. 1977). The mean oestradiol level did tend to be higher in ER-positive compared to ER-negative tumours, but a significant quantitative correlation between ER status and oestradiol levels has not been demonstrated (Fishman et al. 1977; Edery et al. 1981; Van Landeghem et al. 1985; Thijsen & Blankenstein 1989). In contrast no relationship between oestrone levels and ER status of breast tumours has been demonstrated (Fishman et al. 1977). The levels of endogenous oestrogen in breast fat also exceed plasma concentrations, although the difference was observed mainly for oestrone (Feher et al. 1982).

The differential in oestrogen concentrations between plasma and breast tissue may either be a function of selective uptake from the circulation against a concentration gradient or active synthesis and metabolism of oestrogens within the breast. A positive arterio-venous gradient, suggesting active uptake from the plasma to mammary acinar tissue has been demonstrated for several androgens including androstenedione and testosterone (Deslypere 1984; Van Landeghem et al. 1981; Vermeulen & Deslypere 1989). Some (Duvivier et al. 1981) but not all workers (Vermeulen & Deslypere 1989) have demonstrated a positive arteriovenous gradient for oestradiol.

Local Oestrogen Synthesis

The degree to which active synthesis occurs in individual tumours is highly variable and the percentage of oestrogen accounted for by local biosynthesis may vary between 0 to 78% of the total oestrogen content (James et al. 1989). Oestrone can be produced either from aromatisation of androstenedione, or hydrolysis of the oestrone sulphate. The resulting oestrone is then converted to the more active oestrogen, oestradiol, by the enzyme 17 β -hydroxysteroid dehydrogenase (James & Reed 1980). No consistent relationship however has been demonstrated between in vitro activity of any of the above enzymes and the levels of endogenous oestrogens within the breast (Bonney et al. 1983; Vermeulen 1986; Hawkins, Thijsen & Miller 1987) or tumour tissue (Miller & O'Neill 1989).

Aromatase. This enzyme catalyses the conversion of C19 androgens to C18 oestrogens with the concomitant aromatisation of the steroid A ring. Aromatase activity can be detected in normal glandular tissue (Miller 1986; Vermeulen et al. 1986), breast

fat (Perel, Wilkin & Killinger 1980; O'Neill, Elton & Miller 1988) and at least 60-70% of breast tumours (Miller 1986). The activity within cancer tissue has been found to be equivalent to, or higher than that in the surrounding breast tissue (Abul-Hajj, Iverson & Kiang 1979; Perel, Wilkin & Killinger 1980; Vermeulen & Deslypere 1989). The level of aromatisation in the breast fat of patients with breast cancer is significantly higher when compared to that of patients with benign disease (O'Neill & Miller 1987) and within an individual breast appears to be higher in the quadrant of breast in which the cancer was located (O'Neill, Elton & Miller 1988). Although the latter observation may be a spurious finding related to contamination with tumour cells which are known to have higher aromatase activity (Abul-Hajj, Iverson & Kiang 1979; Perel, Wilkin & Killinger 1980). Studies on the expression of the aromatase enzyme in human adipose stromal cells have indicated that its activity may be regulated by a number of factors including cAMP, phorbol esters, and the growth factors, EGF, TGF- α , TGF- β and TNF (Simpson et al. 1989). Several of these factors are produced by breast cancer cells in response to oestrogens (Lippman et al. 1976; Knabbe et al. 1987). The possibility of paracrine and autocrine feedback system exists within the breast. Growth of a tumour may be regulated by oestrogen produced locally in the adipose cells of the surrounding tissues, which in turn is regulated by growth factors produced by the tumour as a result of oestrogenic action. Unlike oestradiol production in the ovary, gonadotrophins do not appear to be the principal regulation system for aromatase activity in adipose tissue.

Sulphatase/sulphokinase axis. These two enzymes are concerned with the interconversion of unconjugated and sulphated oestrogens. The enzymes are relatively ubiquitous and can be found in both benign and malignant tumours, as well as most types of breast tissues (Hawkins, Thomson & Killen 1985; Dao & Libby 1972; Adams et al. 1979). In disrupted cell preparations it can be shown that sulphatase activity exceed that of sulphokinase. In whole cell preparations under physiological conditions sulphokinase activity may be more important giving a positive gradient towards free oestrogens (Santner, Feil & Santen 1984; Vermeulen & Deslypere 1989).

17 β -hydroxysteroid dehydrogenase. The interconversion of oestrone and oestradiol is under control of this enzyme and activity is higher in carcinomatous than normal glandular tissue (Bonney et al. 1983; Vermeulen et al. 1986). Unlike aromatase activity no association has been demonstrated between the level of 17 β hydroxysteroid dehydrogenase activity in breast fat and the presence or location of the tumour within the breast (Miller & O'Neill 1989). High 17 β hydroxysteroid dehydrogenase activity

in breast fat has however been associated with indices of advanced tumour stage, and in particular the presence of axillary node metastasis (Miller & O'Neill 1989) and larger tumour size (Beranek et al. 1985).

The position of equilibrium between oestrone and oestradiol is unclear. In vitro studies support oxidation to oestrone (Bonney et al. 1983, Vermeulen et al. 1986), whereas in vivo perfusion studies have favoured reduction to oestradiol (McNeill et al. 1986a). Both oestrone and oestradiol directly stimulate reductive activity but have no effect on the oxidative reaction (James et al. 1989) while homogenates from breast cancers cells (McNeill et al. 1986; James et al. 1989) also influence the activity of the enzyme in the direction of the reduction. This suggests a degree of autocrine or paracrine control in the in vivo situation which favours oestradiol production.

Therapeutic Inhibition of Oestrogen Synthesis

Surgical (Beatson 1896; Pearson et al. 1954) and later radiotherapeutic induction of ovarian ablation (De Cormelles 1922) was the first form of hormone manipulation available for systemic control of breast cancer. With the recognition of the important role of the adrenal glands in maintaining breast cancer growth, total bilateral adrenalectomy (Huggins & Scott 1945) was introduced but was never a viable proposition until the discovery and widespread availability of synthetic glucocorticoids. Hypophysectomy was introduced in 1952 by Luft (Luft, Olivecrona & Sjorgen 1952) and the procedure later refined to a transnasal implantation of radioactive material by Forrest (Forrest & Peebles-Brown 1955). These ablative procedures however are not without significant morbidity, and even mortality in a population of patients who may already be systemically unwell from their tumour and the response rate is only around 30%. They have now mainly been succeeded by new and potent pharmacological agents.

Ovarian ablation

Following the initial enthusiasm for oophorectomy in the management of patients with advanced breast cancer, it fell into temporary disfavour only to be resurrected by Pearson in the 1950's (Pearson et al. 1955). Reasons for its demise included introduction of photon irradiation to ablate ovarian function (De Cormelles 1922) and development of pharmacological methods of influencing the endocrine environment

such as testosterone (Loeser 1938; Ulrich 1939), the synthetic oestrogen diethylstilbestrol (Haddow, Watkinson & Paterson 1944), progestogens (Taylor & Morris 1951) and corticosteroids (Pearson et al. 1954). In addition these pharmacological methods were effective in control of advanced breast malignancy in post-menopausal women. Although now mainly replaced by the LHRH analogues, oophorectomy still has an occasional and effective role in the management of advanced breast cancer.

In the adjuvant situation, both surgery (Ravdin et al. 1970; Lewison 1978; Bryant & Weir 1981) and radiotherapy (Paterson & Russel 1959; Cole 1975; Meakin et al. 1983; Meakin 1986; Nissen-Meyer 1968) have been used to secure premature menopause with good effect. With the exception of the Ravdin study (Ravdin et al. 1970) these trials have all shown a statistically significant improvement in both relapse-free and overall survival in favour of ovarian ablation for premenopausal patients with evidence of axillary lymph node metastasis. The order of magnitude of improved survival at 10 years in crude survival rates was around 11%. The addition of 7.5mg of prednisolone per day to ovarian radiation in the Meakin study was found to further improve survival (17% at 10 years) over that achieved by ovarian irradiation alone (Meakin et al. 1983).

The beneficial effect of ovarian ablation on survival was not evident until after 3 years in the Meakin study (Meakin et al. 1983) and 5 years in the Bryant study (Bryant & Weir 1981) and it may be that the limited follow-up period in the Ravdin study accounts for its failure to demonstrate a positive effect.

The Nissen Meyer group (Nissen-Meyer 1968) have been unique in demonstrating a positive effect in postmenopausal women.

A meta-analysis of the results of adjuvant oophorectomy has recently been published (Early Breast Cancer Trialist's Collaborative Group 1992a). Data was available for 10 of the 12 trials of ablation began before 1985 (with a total of 3000 women randomised) Within this analysis age has been used as a surrogate for menopause, and the results analysed separately for women aged over 50 (most of whom were presumably postmenopausal) and under 50 (most of whom were presumably premenopausal) when randomised. For recurrence-free survival among all women aged under 50, the predominant effect was seen within the first 10 years. A small but insignificant difference was still apparent after 10 years. By year 15, the overall difference in

recurrence-free survival was 10.6 %, SD 2.7, log rank 2p = 0.00007, proportional reduction in annual risk 26% SD6).

For survival, the effect of treatment on the average annual death rate was similar up to and beyond year 10. By year 15 the absolute difference in overall survival for the treatment arm was 10.2% SD 2.7 (logrank test 2p = 0.0004, proportional reduction in annual risk 25% SD7).

The size of the absolute benefit was dependent on axillary node status. Among node positive women aged < 50, the improvement in recurrence-free survival and overall survival at 15 years are highly significant at 10.5% SD 3.4 and 13% SD 3.6 respectively. For node-negative women, the number of positive events were smaller and when each trial was analysed separately no benefit was demonstrated. When all the trials were analysed by meta-analysis however a small but significant effect can be seen with treatment for both recurrence-free survival and overall survival.

Among the 1326 women aged over 50 no significant effect either on recurrence-free survival or overall survival was demonstrated. The addition of cytotoxic chemotherapy to ovarian ablation appeared to reduce the beneficial effect from ovarian ablation. This interaction however was not statistically significant, and even in the presence of cytotoxic chemotherapy, ovarian ablation still reduced both recurrence and mortality to an extent which just reached significance.

No significant effect of ovarian ablation was demonstrated on causes of death other than breast cancer.

Tamoxifen

With the advent of tamoxifen, a competitive inhibitor for oestrogen at its target receptor (Harper and Walpole 1967), a simpler and less invasive method became available to reduce the biological effectiveness of oestrogen in vivo. The first indication of the value of tamoxifen came from its use in the palliation of advanced breast cancer. After Cole, Jones and Todd published their results in 1971, many studies were quickly undertaken to determine the likely overall response rate and confirm the low toxicity. A recent comprehensive review of these studies (Litherland & Jackson 1988) has shown that one-third of 5353 patients with advanced breast cancer obtained a complete or partial response and that the likelihood of response increased with age. In over 30 studies objective evidence of response was demonstrable after six to eight weeks and

lasted on average twenty-four months. About 12% of patients with ER-negative tumours can be expected to benefit from tamoxifen therapy in the advanced disease situation, the response rate increases to near 50% when ER levels are above 5 fmol/mg protein.

At least 57 randomised trials testing the efficacy of adjuvant tamoxifen have now been undertaken (Stewart 1989). The collaborated results from 28 of the most major trials, involving 16,513 women, were initially reported in 1988 (Early Breast Cancer Trialists' Collaborative Group 1988) and have recently been updated to include 42 randomised trials involving 30,000 women (Early Breast Cancer Trialists' Collaborative Group 1992a). Overall the administration of adjuvant tamoxifen is associated with a highly significant reduction in recurrence-free and overall survival. The main benefit to recurrence-free survival would appear to take place during the first 5 years, such that at the end of the fifth year an 8.3% difference in overall relapse-free survival can be demonstrated (proportional reduction in annual risk 25% SD 2). This gain is not significantly increased or decreased during the next 5 years. After year 10 there are as yet few data. When overall survival was considered however, the mortality difference between the tamoxifen and control groups grew steadily throughout years 1 to 10, such that at year 5, the absolute difference in survival is 3.6%. In the next 5 years a highly significant additional mortality reduction can be demonstrated such that by 10 years the overall difference in survival is 6.2% (SD 0.9). The proportional reduction in annual risk was similar during the first 5 and subsequent 5 years at 17% (SD 2).

The most extensively tested tamoxifen regimens, involved doses of at least 20mg per day given for a minimum of 2 years. There is no evidence that a dose of more than 20mg per day provided additional benefit but within the overview a highly significant benefit could be demonstrated with respect to relapse and survival with more prolonged courses of tamoxifen (Early Breast Cancer Trialists' Collaborative Group 1992). Within individual randomised trials currently addressing the effect of duration of tamoxifen therapy survival results are at present immature. At present results are only available from one randomised trial. This has demonstrated a significant benefit from the addition of a third year of tamoxifen therapy over no further therapy beyond two years, in patients over the age of 50 with involved axillary nodes (Fisher et al. 1987).

Although the absolute benefits at 10 years are larger for women who are axillary node-positive than those who are axillary node negative (recurrence-free survival absolute improvement in node-positive 8.8% SD 1.1, versus 5.1% SD 1.4 for node-negative women, overall survival 8.2% SD 1.1 for node-positive women versus 3.5% SD 1.4 for node-negative women) the proportional reductions in annual risk was independent of axillary node status (Early Breast Cancer Trialists' Collaborative Group 1992a). This has also been demonstrated in individual trials (Scottish Cancer Trials Office 1987, Ribeiro & Swindell 1988, Nolvadex Adjuvant Trial Organisation 1988, Bianco et al. 1988, CRC Adjuvant Breast Trial Working Party 1988).

Tamoxifen in general is well tolerated with few major side effect (Litherland & Jackson 1988; Fisher et al. 1989). An increased incidence of endometrial cancers in patients receiving long term tamoxifen has however been demonstrated and was more pronounced in those treated for over 2 years (Fournander et al. 1989). This has not been supported in other studies (Scottish Cancer Trial Office - personal communication; Fisher et al. 1989; Early Breast Cancer Trialists' Collaborative Group 1992a) and may be a function of the high dose (40mg per day) of tamoxifen given within that particular trial (Fournander et al. 1989). Within the recent overview, tamoxifen was associated with a significant reduction in non-breast-cancer deaths (12% SD 6, Early Breast Cancer Trialists' Collaborative Group 1992a). The reduction in deaths was highest amongst vascular causes at 25% SD 13 ($p = 0.06$). In addition a highly significant reduction in contralateral breast cancers was demonstrated with tamoxifen (odds reduction 39% SD 9), with a nonsignificant trend towards a greater effect for long-term treatment (Early Breast Cancer Trialists' Collaborative Group 1992a). The morbidity as well as the benefits of longterm tamoxifen therapy obviously require to be under continuous review.

The clinical efficacy of adjuvant tamoxifen in relation to irradiation induced menopause has been tested in 1005 premenopausal patients with operable breast cancer (Ribeiro & Swindell 1988). At 10 years the analysis shows no significant difference in overall or disease free survival between either treatment group. For premenopausal node negative patients however there was a trend in favour of the tamoxifen treated patients with a 93% ten year survival versus 82% for the irradiation menopause group ($p=0.09$). In this study however irradiation menopause, a permanent method of oestrogen deprivation was compared to only one year of tamoxifen therapy. Since there is evidence to suggest that more prolonged use of tamoxifen may be more effective (Early Breast Cancer Trialist's Collaborative Group 1988, 1992a; Fisher et al. 1987), the

results may therefore be unfairly biased in favour of the group receiving an irradiation menopause.

Aromatase Inhibitors

Aminoglutethimide was the first clinically useful drug available which utilised the strategy of inhibition of oestrogen production. Initially developed as an anticonvulsant, it was shown to produce adrenal insufficiency and later introduced as a method of providing "medical adrenalectomy" in breast cancer patients (Cash et al. 1967).

Although at high doses of aminoglutethimide, inhibition of the the adrenal desmolase enzyme system could be demonstrated, the drug was still efficacious at lower doses which produced little effect on adrenal steroids. It was thus suggested that aminoglutethimide might inhibit the production of oestrone from androstenedione (Samojlik, Santen & Wells 1977) and in 1978 Santen and his coworkers confirmed that aminoglutethimide caused a 95-98% inhibition of aromatase activity in post-menopausal patients with breast cancer (Santen et al. 1978). This led to the introduction of the term 'aromatase inhibition' as a mechanism of endocrine treatment for breast cancer. The question of optimum dosage for aminoglutethimide remains open. Results from the daily use of 250mg of aminoglutethimide alone seems to be inferior to those using the conventional 1000mg/day plus hydrocortisone (Stuart-Harris et al. 1985; Murray & Pitt 1985). The addition of hydrocortisone to low dose aminoglutethimide may give rise to further suppression of oestrogen synthesis, by reduction in the availability of androstenedione as a substrate in a setting of incomplete aromatase inhibition (Dowsett et al. 1985; Harris et al. 1986). At higher dosages of aminoglutethimide, the addition of glucocorticoids does not seem to improve response (Ceci et al. 1989). Furthermore a randomised trial comparing aminoglutethimide 500mg/day to 1000 mg/day revealed no significant difference in response rate (Boneterre et al. 1985). In summary the optimal aminoglutethimide regime is not yet clearly established, until further data from randomised trials are available a dose of 250-500mg aminoglutethimide daily with 40 mg hydrocortisone appears to be sufficient. Treatment trials using this agent in postmenopausal patients with breast cancer revealed an efficacy equal to that achieved by surgical adrenalectomy (Newsome et al. 1977; Santen & Henderson 1982; Brodie & Santen 1986) and tamoxifen (Harvey et al. 1982; Lipton et al. 1982; Smith et al. 1981) but at the expense of substantial side effects including skin rash, ataxia, drowsiness and lethargy; in addition when used at high doses glucocorticoid supplementation was required. For these reasons further inhibitors have been developed in an attempt to enhance aromatase inhibitory potency while improving specificity and reducing side-effects.

4- Hydroxyandrostenedione

4-hydroxyandrostenedione (4OHA) is a structural analogue of androstenedione and is a specific, selective suicide or non-reversible inhibitor for the aromatase enzyme. In vitro studies have shown it to have a sixty-fold greater activity when compared to aminoglutethimide as an inhibitor of placental aromatase (Brodie, Schwarzel & Brodie, 1976; Brodie et al. 1977). Unfortunately 4OHA is rapidly inactivated by hepatic glucuronidation, and this has limited its efficacy when administered by the oral route (Goss et al. 1986; Dowsett et al 1989). The drug is therefore usually given parenterally, by deep intra-muscular injection into the buttock. Efficacy of a daily oral dose of 500mg per day has been reported in postmenopausal patients with advanced breast cancer (Cunningham et al. 1987; Coombes et al. 1987).

Endocrinological Effects

The predominant endocrinological effect of 4OHA administration is a reduction in plasma oestradiol concentration (Goss et al. 1986; Dowsett et al. 1987). A paradoxical failure to lower plasma oestrone, as measured by radioimmunoassay, was recorded (Goss et al. 1986) but when measured by gas chromatography-mass spectrophotometry, oestrone levels were found to be suppressed by a mean value of 60% (Dowsett et al. 1989). The failure of initial results to show a fall in oestrone is thought to be related to interference of 4OHA and its metabolites in the radioimmunoassay technique.

Following a single intramuscular injection of 4OHA (500mg), serum oestradiol levels have been found to fall to a nadir, by 4-7 days, of approximately one third pretreatment concentrations, the effect being maintained for longer than 14 days in the majority of patients. A similar degree of oestradiol suppression was seen following a single injection of 125mg 4OHA but recovery of serum oestradiol levels occurs more rapidly, rising at the end of the second week to 54 +/- 6.4% of the pretreatment values (Coombes, Stein & Dowsett 1989). The optimum clinical dose is not fully established but the response rates in 55 patients were equivalent irrespective of whether the patients had received 250mg (i.m.) at 14 day intervals, 500mg (i.m.) at weekly intervals, or 500mg orally daily (Coombes, Stein & Dowsett 1989).

Effects on Local Metabolism

Little information is available concerning the direct effect of aromatase inhibitors on local oestrogen biosynthesis within the breast tissue. James and colleagues (1989)

have reported the effect of 4-hydroxyandrostenedione in the tumours of 6 postmenopausal women and suggested that peripheral production was substantially inhibited, with a marked reduction in aromatase activity in 4 of the 5 patients. The levels of DNA polymerase α , an indicator of mitotic activity also showed a marked reduction, suggesting a reduction in the growth rate of the tumour (James et al. 1989). Conversion of androstenedione to oestrone was below the limits of detection in 6 postmenopausal patients following 4OHA therapy.

Clinical studies

In postmenopausal patients with advanced disease one third will demonstrate regression to 4OHA and this relates to the ER status of the tumour, in that of the 43 patients with ER-negative tumours, only 3 demonstrated clinical regression (Coombes, Stein & Dowsett 1989). The incidence of side effects (29%) experienced by patients treated by 4OHA is low when compared to aminoglutethimide (40-80%, Smith et al. 1981, Coombes et al. 1986). Side effects relate mainly to the local administration site with the development of sterile abscesses and painful lumps (8%). Systemic reactions are usually mild and transient and included flushing, perioral oedema, lethargy, drowsiness, myalgia and one skin rash. Two cases of anaphylactoid reaction have been noted (Coombes, Stein & Dowsett 1989). In experimental systems it is also known to have weak androgenic effects (Brodie & Santen 1986) but this has not been a marked problem in vivo.

Recently, a non-steroidal compound, CGS 16949A has been identified as a potent specific inhibitor of aromatase activity without intrinsic androgenic or oestrogenic properties (Steele et al. 1987; Schieweck, Bhatnager & Matter 1988). This drug is effective when administered orally (Santen 1989). Its value in the clinical situation is currently under test (Santen 1989; Coombes, Stein & Dowsett 1989).

Gonadotrophin-Releasing Hormone Superagonist Analogues

Analogues of the gonadotrophin-releasing hormone (GnRH) have been developed which, even when given intermittently, appear to mimic the effects of a constant infusion of GnRH and produce a paradoxical inhibition of gonadotrophins (Santen, Manni & Harvey 1986). Under normal physiological conditions GnRH is secreted in pulses at approximately two hourly intervals. The prolonged half-life of clearance after subcutaneous injection (Clayton et al. 1985) and more importantly, the longer duration of binding of the superagonist analogues to the GnRH receptor, produce a state of constant GnRH receptor occupancy. This desensitises the pituitary to further GnRH

stimulation and following an initial transient rise causes paradoxical inhibition of LH and FSH secretion. This in effect produces a reversible medical oophorectomy (Maynard & Nicholson 1979; Nicholson et al. 1984). Several analogues are now available for clinical practice including Zoladex (ICI 118630; D-ser(Bu[†])⁶, Azgly¹⁰, LH-RH; goserelin), Buserelin (D-Ser (Bu[†])⁶, LH-RH proethylamide⁹), Leuprolide (D-Leu⁶, LH-RH proethylamide⁹) and Decapeptyl (D-Trp⁶, LH-RH). These appear to vary little in their efficacy (Klijn & De Jong 1987; Nicholson et al. 1984,1987; Harvey 1988; Hoffken et al. 1986).

Endocrinological Effects

The pattern of gonadotrophins released during treatment with the Gn-RH agonists appear largely independent of whether treatment is initiated during follicular or luteal phase of the menstrual cycle (Nicholson & Walker 1989). Following initial exposure to the drug a short burst of elevated gonadotrophin levels are observed which is translated into a transient rise of serum oestradiol and progesterone concentration. On continued treatment plasma gonadotrophin levels fall to low levels within 3 days for follicle-stimulating hormone and within 3 weeks for lutenising hormone (Klijn 1984; Klijn & De Jong 1982,1987; Klijn et al. 1984; Harvey, Lipton & Max 1984; Manni et al. 1986; Nicholson et al 1984, 1987; Walker et al. 1986). Serum oestradiol and progesterone concentrations fall to the castrate or postmenopausal range within 3-4 weeks (Nicholson et al. 1987). The rate of decline in serum sex steroids is not as rapid as that following surgical oophorectomy, where postmenopausal levels are reached within 7 days (Vermeulen 1976, Beksac et al. 1983). Plasma gonadotrophin levels have been kept low for several years on maintenance treatment with no ovulatory peaks being observed (Klijn & Foekens 1986). A depot preparations of the LHRH agonist Zoladex is now available and provides sustained release of the drug over a 4 week period (ICI Zoladex).

A reduction in the plasma levels of oestrone, androstenedione, and testosterone to postmenopausal levels also occurs with Gn-RH agonist therapy (Nicholson et al. 1987; Harvey 1988). Long term administration of Zoladex in contrast to Buserelin or Leuprolide also causes a reduction in the circulating levels of prolactin (Nicholson & Walker 1989).

Clinical Studies

To date clinical trials of GnRH analogues in relation to breast cancer have been mainly restricted to patients with advanced disease. An overall response rate of around 40%

has been reported in premenopausal women (Nicholson & Walker 1989; Klijn & De Jong 1987; Nicholson et al. 1987; Harvey et al. 1984; Hoffken et al. 1986).

Regression has been restricted mainly to women with ER-positive tumours in whom the response rate is around 53% (Harvey 1988, Nicholson & Walker 1989). The observed response is equivalent in extent and duration to that observed following oophorectomy (Nicholson et al. 1987b). The side-effects to GnRH agonist therapy are minimal and associated mainly with a hypo-oestrogenic state. They include cessation of menstruation (usually by two months), hot flushes and occasional nausea (Williams et al. 1986, Harvey, Lipton & Max 1987; Klijn & De Jong 1987).

Tumour remissions have also been recorded in 10 % of a total of 118 postmenopausal patients with ER-negative tumours (Nicholson & Walker 1989). The mechanism of induction of regression in these women, is unclear. Possible explanations include a reduction of the androgen output of the ovary which is still under gonadotrophin control in the postmenopausal women (Dowsett et al. 1988), or a direct inherent antitumour action of the GnRH analogues themselves (Miller et al 1987). GnRH binding sites have been identified in human breast tumours (Eidne, Flanagan, Miller 1985), while a direct inhibitory action of Buserelin has been demonstrated on some (Miller et al. 1985, 1987; Blankenstein, Henkelman & Klijn 1985) but not all (Wilding, Chen & Gelmann 1987) clones of MCF-7 cell lines.

Clinical studies are currently underway comparing GnRH analogues to either surgical and X-ray induced menopause in pre- and perimenopausal women (ICI study 2301, Harvey 1988). In addition the efficacy of GnRH analogues is being examined in combination with other endocrine therapies, in particular tamoxifen (Walker et al. 1989).

Other Endocrine Therapy

Other endocrine treatments have received relatively little attention in the adjuvant setting. A prospective comparative trial of androgens did not show any benefit from this treatment (Karydas et al. 1987), but early results from a trial of aminoglutethimide and hydrocortisone show that this treatment can delay first recurrence (Coombes et al. 1986).

CYTOTOXIC THERAPY

It is now twenty-eight years since Greenspan and colleagues first suggested the combination of multiple drugs to manage advanced breast cancer (Greenspan et al. 1963). Since that observation, innumerable studies have appeared in the literature demonstrating that a variety of drug combinations can all be expected to result in fairly high initial response rates in patients with metastatic breast cancer (Carbone et al. 1977; Bonadonna & Valagussa 1983). While there is considerable variability in overall response rates between one study and another several conclusions can be drawn. First, virtually no combination chemotherapy studies have reported response rates in excess of approximately 65-70% in the management of metastatic disease. The median duration of response is usually less than one year (Carbone & Tormey 1977; Jones, Durie & Salmon 1975) and few studies demonstrate a clinical complete response rate of over 25%. While it is true that complete responders have an increased response duration as compared to partial responders, there are very few patients who remain in durable complete remission for prolonged periods of time (Legha et al. 1979; Decker et al. 1979). These overall results appear to be independent of specific agents chosen.

The first trials of adjuvant cytotoxic therapy studied the value of perioperative chemotherapy and were based on the theory that malignant cells were disseminated during surgery and their subsequent implantation could be inhibited by chemotherapy given around the time of challenge. Two randomised trials of perioperative chemotherapy in operable breast cancer were started in the 1960s (Fisher et al. 1975; Nissen-Meyer et al. 1978). The National Surgical Adjuvant Breast and Bowel Project demonstrated that a postoperative course of thiotepa significantly increased survival in premenopausal patients with four or more metastatic nodes (Fisher et al. 1986). In the Scandinavian Adjuvant Chemotherapy Study Group a short perioperative of cyclophosphamide 5mg/kg for 6 days, given after closure of the surgical wound (Nissen-Meyer et al. 1978) was found to produce a survival advantage after 5 years which became significant after 12 years for both relapse-free and crude survival. The effect was found to be independent of nodal or menopausal status (Nissen-Meyer et al. 1986). More recently performed studies have further confirmed the advantage of a short perioperative course of chemotherapy. In the Ludwig study a single perioperative cycle of cyclophosphamide, methotrexate, flurouracil and leucovorin was shown to produce a significant improvement in disease-free survival at 4 years; the advantage was observed for both pre and post menopausal women with the magnitude of effect being largest among patients with no or low oestrogen- receptor content in the primary

tumour (Ludwig Breast Cancer Study Group 1989). The CRC Adjuvant Trial also reports a survival advantage for patients receiving a 6 day course of immediate postoperative cyclophosphamide versus no systemic therapy (CRC Adjuvant Breast Trial Working Party 1988).

With the recognition from long term survival data that breast cancer is already a systemic disease by the time of initial clinical presentation, it was thought that prolonged postoperative chemotherapy may be more effective in preventing recurrent disease. The cardinal trials which pioneered this principle were from the National Surgical Adjuvant Breast Project in the United States (Fisher et al. 1986) and the Istituto Nazionale Tumori in Milan (Bonadonna et al. 1985; Bonadonna & Valagussa 1987). Numerous trials are now available and the collaborative results from 40 trials involving a total of 13442 women have been reviewed (Early Breast Cancer Trialists' Collaborative Group 1988) and now updated (Early Breast Cancer Trialists' Collaborative Group 1992b). Thirty-one of these trials compared adjuvant chemotherapy with no chemotherapy, the remainder involved a combination of cytotoxic drugs with other systemic agents, particularly tamoxifen. Clear benefits can be seen in recurrence-free survival and overall survival for patients receiving adjuvant polychemotherapy when compared to those who did not receive chemotherapy. The main effect on recurrence-free survival is seen during the first 5 years, after which no additional gains can be demonstrated. The absolute difference at 5 years is 9.2% SD 1.2. while the average proportional reduction in annual risk is 28% SD3. The beneficial effect on survival however continues at a steady rate (reduction in annual hazard of 16% SD 3) up to 10 years, such that the absolute mortality difference at 5 years (3.2% SD 3.3) has doubled by 10 years (6.3% SD 1.4 2p , 0.00001). An additional benefit can be demonstrated past 10 years but as yet there exists too little extra information beyond year 10 for this additional benefit to be statistically reliable.

Improving The Efficacy of Chemotherapy

In the absence of new and more effective chemotherapeutic agents for breast cancer, efforts are currently being made to explore the pharmacodynamics and scheduling of the presently available chemotherapeutic agents.

Dose and Duration of Treatment

Undoubtedly dose intensity is important and when this is as high as possible

commensurate with acceptable side effects, better therapeutic results can be expected (Hrynuik, Levine & Levin 1986). Firm prospective data confirming this has recently become available in metastatic disease (Carmo-Periera et al. 1987, Engelsman et al. 1987) but the optimal dose for adjuvant therapy still remains undefined. Prolonged (6 to 24 months) cytotoxic therapy have been found to offer no further benefit over less prolonged (3-6 months) regimes (Early Breast Cancer Trialists' Collaborative Group 1988; 1992b). A nonsignificant difference of 10(+/-8)% was in fact found in favour of the less prolonged therapy (Early Breast Cancer Trialists' Collaborative Group 1988).

Efficacy of Combination Chemotherapy

Second generation chemotherapy trials have evolved to compare combination with single agents regimes and regimes of different durations. These have shown that polychemotherapy is more effective than single agent chemotherapy (Early Breast Cancer Trialists' Collaborative Group 1988; 1992b). The most extensively studied cytotoxic regimes consisted of CMF or CMF. The regimes have varied considerably in their dose and intensity of treatment. No conclusive statement can therefore be made about the relative efficacy of other regimes and in particular those containing adriamycin, a drug which is known from advanced breast cancer (Hoogstraten et al 1976; Livingston & Carter 1970) to be one of the most active single agents available for the control of human breast cancer.

Theoretical and Experimental Evidence for Early Administration of Systemic Therapy

There is increasing evidence from several biological systems which suggests that the timing of adjuvant systemic therapy may be crucial; systemic therapy being more likely to achieve cure if it is administered early in the life history of a cancer. The following section will review the theoretical, experimental and clinical evidence relating the potential importance of the timing of systemic therapy.

Chemosensitivity and Tumour Burden

An inverse relationship has been demonstrated between tumour burden and the ability to obtain cyto-eradication (Goldin et al. 1956; Skipper 1960; Skipper, Schabel & Wilcox 1964). Drug therapy fails because the tumour cells are resistant. Conceptually two forms of resistance exist, temporary resistance, due to either pharmacological sanctuaries or altered cell kinetics, and permanent resistance with the emergence of

resistant mutant lines. Since both are mass related, the greater the tumour mass or number of cell divisions the tumour has gone through the more likely a tumour is to be resistant to anticancer drugs.

Pharmacological Sanctuaries

As a tumour increases in size areas will outgrow their blood supply which are the source of systemically derived drugs and so can provide sanctuary site in which otherwise sensitive tumour cells can survive (Devita 1983). Resistance can be also be imparted by physiological barriers such as the blood-brain barrier (Devita 1983; West et al. 1980).

Alteration in Tumour Kinetics

The overall growth pattern of the malignant tumour is best characterised by Gompertzian function, although unpredictable phases of growth early in the life history of a primary tumour and its micrometastases are suspected (Skipper 1960). With expansion of overall tumour cell burden, the proportion of cells actively proliferating will decrease. Since many anti-cancer drugs are specific for cycling cells, tumours which have a larger cell burden would theoretically be less susceptible to anticancer drugs.

Noncurative cytoreduction, whether by means of surgery, chemotherapy, or radiotherapy has been shown in experimental animal models to lead to an increase in the labelling index of the metastatic deposits (Tyzzer 1973; Gorelik, Segal & Feldman 1978; Simpson-Herren, Sanford & Holmquist 1976; DeWys 1972; Gunde, Fisher & Saffer 1979; Fisher, Gunde & Saffer 1983). Although the data is not as conclusive as for animal models, similar kinetic changes have been shown in human malignancies (El Rifi et al. 1965; Lange et al. 1980). This kinetic perturbation through acceleration of metastatic growth is potentially harmful. A shift in the proliferation phase from the flat back to the steep slope of the Gompertzian curve (Ragaz et al. 1985; DeVita 1983, Lloyd 1975) may explain this phenomenon but the effect can be reproduced in the tumours of other animals by transfer of serum (Fisher, personal communication). The burst of proliferative activity may be mediated by, as yet undefined growth factors (DeWys 1972) and can be blocked by prior treatment of the donor animal with chemotherapy (Schabel et al. 1979, Corbett et al. 1981).

Phenotypic Resistance

Phenotypically resistant mutants arise spontaneously, early in the natural history of tumours even before exposure to anticancer drugs. The likelihood of a resistant line developing appears closely related to the number of previous cell divisions (Ling 1978). It has been suggested by mathematical modelling (Goldie and Coldman 1979) and confirmed in biological systems (Skipper 1960) that within 5 doublings, or less than a 2-log increase in the microscopic burden, the risk of drug-resistant mutant cells may increase from 5% to 95%. The doubling time of microscopic tumours may be shorter than their overt counter parts (Shackney, McCormack & Cuchural 1978) providing further theoretical argument for systemic treatment to being given as early as possible.

Acquired resistance

The ability to resist environmental chemical stresses represents a major evolutionary driving force and a wide variety of defence mechanisms have evolved to protect cells against such cytotoxic insults. Potential mechanisms including changes in membrane permeability (Kartner et al. 1983), DNA repair (Karran & Lindahl 1986), gene amplification (Ozols & Cowan 1986) and drug detoxification (Hayes & Wolf 1988). Spontaneous genetic mutations occur in all living cells at a rate of between one in 10^{5-8} new cells. The probability that a spontaneous drug mutant would emerge which was resistant to two separate drugs is much lower and this has formed the theoretical basis for combination chemotherapy. Clinical trials have confirmed improved response rates with combination chemotherapy for the treatment of breast cancer by systemic chemotherapy in both the adjuvant (Early Breast Cancer Trialist's Collaborative Group 1988) and the advanced disease situation (Carter 1972).

Clinical Studies on the Relationship Between Efficacy and Timing of Chemotherapy

In theory, timing of the first cycle of chemotherapy may be critical. By observing the alteration in labelling index associated with noncurative cytoreduction in mouse-mammary tumours, Fisher and his colleagues were able to investigate the relationship between interval from primary tumour removal to administration of a single dose of cyclophosphamide and the effect on growth of residual tumour cells and animal

survival (Fisher, Gunde & Saffer 1983). The greatest effect was noted when the cyclophosphamide was given prior to operation when it completely prevented the increase in labelling index resulting from tumour removal, more effectively suppressed the growth of residual tumour and prolonged survival to a greater extent than noted under any other circumstance. Cyclophosphamide given on the day of tumour removal was more effective than when given 3 days later and least effective if given 7 days following primary tumour excision.

The literature on the clinical importance of minor delays in starting adjuvant chemotherapy in operable breast tumours of human patients shows no uniform consensus. Only two randomised trials compare the efficacy of perioperative to conventionally timed chemotherapy. In the Scandinavian Adjuvant Chemotherapy Study a short perioperative course of cyclophosphamide (5mg/kg for 5 days) started immediately after mastectomy was compared to a 12 month schedule of cyclophosphamide, methotrexate and 5-fluorouracil in 1026 patients. A significant advantage was noted for relapse-free survival in favour of perioperative chemotherapy ($p=0.002$, Nissen-Meyer et al. 1986). This finding was not however confirmed by the Ludwig Breast Cancer Study Group (Ludwig Breast Cancer Study Group 1989). In this trial the chemotherapy regime used included cyclophosphamide, methotrexate, fluorouracil and leucovorin. One thousand and twenty-patients with operable node-positive breast cancer were randomised to one of three treatment schedules. The first group received a single perioperative course of chemotherapy within 36 hours of mastectomy, the second received six cycles of conventionally timed chemotherapy starting 25-32 days after operation and the third received both the perioperative cycle and the conventionally timed regimen. Tamoxifen was added to the conventionally timed chemotherapy in postmenopausal women. With a median follow-up period of 42 months, the estimated 4 year disease-free survival was 40% for the group of women who received a single perioperative cycle, 62% for the longer conventionally timed regimen and 60% for the combined program ($p<0.0001$). Overall survival also favoured longer treatment ($p=0.011$). The authors concluded from this study that a single perioperative cycle of adjuvant combination chemotherapy was less effective than prolonged therapy in patients with operable breast cancer with involved axillary nodes. Furthermore starting the prolonged chemotherapy in the perioperative period added little to beginning treatment 4 weeks after mastectomy (Ludwig Breast Cancer Study Group 1989).

Several groups have retrospectively evaluated the relationship between delay in initiation of chemotherapy after surgery and disease-free survival (Senn, Jungi & Amgwerd 1981; Buzdar et al. 1982; Glucksberg et al. 1982). Because these studies were not primarily set up to answer this specific question their evidence is less substantial. In the OSAKO trial (Senn, Jungi & Amgweid 1981) patients with newly diagnosed cancer of the breast were treated with LMFP (leukeran, methotrexate, flurouracil, prednisone) chemotherapy regime with or without BCG. A review of the first 117 patients has shown that the median delay between diagnosis and the first day of chemotherapy was 32 days in the relapsing and 23 days in nonrelapsing patients ($p=0.05$; Ragaz 1986). Other workers (Jones et al. 1984; Brooks et al. 1983) have also shown that long as opposed to short delays adversely affect outcome. The South West Oncology Group (Glucksberg et al. 1982) and the M.D. Anderson Hospital (Buzdar et al. 1982) however have failed to demonstrate any correlation. Buzdar and his colleagues (Buzdar et al. 1982) subdivided their study of 460 patient with stage II and III breast cancer into 4 subgroups according to the length of delay in initiation of chemotherapy following regional therapy. These groups included patients who had received their chemotherapy <10weeks, 10-13 weeks, 14-17 weeks and ≥ 18 weeks post surgery. Their respective overall four year disease-free survival was 64%, 68%, 60%, and 63%. The Southwest Oncology Group (Glucksberg et al. 1982) in their randomised trial comparing one year of adjuvant combination chemotherapy with continuous cyclophosphamide, methotrexate, flurouracil, vincristine and prednisone (CMFVP) to two years of intermittent L-PAM in women with operable breast cancer with positive axillary nodes also analysed the relationship between interval from mastectomy to onset of chemotherapy (between 1-6 weeks) and rate of recurrence. No correlation was demonstrated. Animal experiments suggest that it is the first 7 days which are of greatest importance in relation to the effectiveness of perioperative chemotherapy (Fisher, Gunduz & Saffer 1983) and it must be noted that in both the study reported by Buzdar (Buzdar et al. 1982) and the Southwest Oncology Group (Glucksberg et al. 1982) the importance of giving chemotherapy within the first postoperative week has not been separately analysed .

Thus despite the convincing theoretical and experimental data indicating superiority of early administration of systemic therapy the value of giving chemotherapy early in the postoperative period as opposed to 4-6 weeks latter has not yet been sufficiently tested in human malignancy and requires further definition. The results of further prospective ongoing randomised trials will be vital (Ragaz 1986).

Preoperative Chemotherapy

Several workers have demonstrated both increased survival and improved locoregional control in mice-mammary carcinoma when chemotherapy was administered before, rather than after surgical removal of the primary lesion. (Corbett et al. 1981; Fisher, Gunduz & Saffer 1983; Schabel et al. 1979). Combination chemotherapy prior to local therapy was first introduced for human breast cancer by the Milan group (DeLena et al. 1978, 1981).

Investigation of preoperative chemotherapy regimes in breast cancer have until recently been uncontrolled individual studies concentrating on patients with locally advanced disease; a group of patients known to have a poor overall survival and early relapse when treated by surgery or radiotherapy alone. Improved 5 year overall (57% to 73%) and distant-free (50% to 87.5%) survival was demonstrated for patients with operable stage III breast cancer (T3, N0-2) receiving 6 cycles of combination chemotherapy therapy as opposed to radiotherapy after modified radical mastectomy (Grohn et al. 1984). Combination chemotherapy prior to local therapy for human breast cancer was introduced by the Milan group (DeLena et al. 1981). In 110 patients a 70% objective response rate (complete response rate of 15%) was achieved with 4 cycles of adriamycin and vincristine chemotherapy prior to local therapy. This produced an overall 3 year survival of 53% compared to 41% for a historical control group receiving radiation alone. In subsequent studies comparing surgery versus radiation after primary chemotherapy, equivalent findings were achieved with respect to local control, patterns of recurrence, and overall survival (DeLena et al. 1981). A similar multimodality treatment strategy has been used in 174 patients with locally advanced noninflammatory stage III breast cancer at the M D Anderson Hospital (Hortobagyi et al. 1988). All patients received as their initial therapy 3 cycles of cyclophosphamide, adriamycin and flurouracil. Local treatment in the form of mastectomy, axillary dissection radiotherapy or both was then carried out after which chemotherapy was continued using the induction regime until a total cumulative dose of adriamycin of 450 mg/m² was reached. Thereafter chemotherapy was continued using cyclophosphamide, methotrexate and flurouracil for a further 20 cycles. Following induction chemotherapy, objective remission was achieved in 87.4% (complete remission 16.7%) of patients and following local therapy all but 6 of the 174 patients were rendered disease-free. With a median follow-up period of 59 months, the 5 year disease-free survival was 84% for patient with stage IIIA disease and 33% for patients

with stage IIIB disease. Five year overall survival rates were 84% and 44% respectively; 26 patients (15.3%) had locoregional recurrence. The quality of response to induction chemotherapy correlated prominently with prognosis.

At the department of Medical Oncology of the Salpetriere Hospital and the Department of Radiotherapy of the Necker Hospital in France, 98 patients with locally advanced breast cancer (stage IIIA-IIIB) have received intensive induction chemotherapy, locoregional radiotherapy and maintenance chemotherapy with or without hormonotherapy (Jacquillat et al. 1988). The chemotherapy used consisted of vincristine, thiotepa, methotrexate, flurouracil, adriamycin for both induction and maintenance chemotherapy. In addition tamoxifen was given to all postmenopausal women; premenopausal women received this drug by random allocation. Mastectomy was not an integral part of this study. Tumour regression with reduction in tumour size in excess of 50% was observed in 91% of patients after chemotherapy and complete clinical remission in 100% of patients after irradiation. The rate of local relapse was 13% at 3 years. The 3-year disease-free survival was 62% with an overall survival of 77%. Again they demonstrated that initial tumour regression of >75% was the main predictive factor for disease-free survival (Jacquillat et al. 1988).

The National Cancer Institute, since 1977, has given women with locally advanced breast cancer chemotherapy to the point of maximum objective clinical response in an attempt to produce considerable tumour regression to enhance the ability to perform surgical resection or deliver a tumouricidal dose of radiation (Swain et al. 1987). Concomitant endocrine and chemotherapy were used in such a way so that tamoxifen and premarin were administered sequentially before the cell cycle specific agents in the hope of synchronising DNA synthesis in the tumour cells and thus make them more susceptible to cytotoxic chemotherapy (Weichselbaum et al. 1978; Aitken & Lippman 1982). The chemotherapy regime used included cyclophosphamide, adriamycin, methotrexate, flurouracil, and leucovorin and initial doses of chemotherapy were escalated to targeted myelosuppression. Patients were treated to maximum clinical response before preceding to any local therapy. Patients achieving a complete response with a negative biopsy received radiation only while patients with residual disease received debulking surgery prior to irradiation. All patients received at least 6 months of additional chemotherapy following local therapy. Seventy-six patients with locally advanced breast cancer have now received this form of management, 31 with stage IIIA, 41 with stage IIIB and 4 with stage IV disease. The objective response rate to induction chemotherapy was 93% with 49% complete response, 44% partial response

and 7% no change. With a median follow-up period of 26.4 months (range 0-66months), 24 (32%) patients have relapsed with a 14% incidence of locoregional recurrence. The median time to progression was 35.9 months for stage IIIA and 34.2 months for stage IIIB. The median survival was 35.3 months for stage IIIB and indeterminate for stage IIIA. The high response rate within this series reported by Swain (Swain et al. 1987) is due in part to an increased proportion of patients achieving complete response and relates to the increased number of cycles of chemotherapy given before surgery.

After the success of preoperative therapy in advanced disease, this approach was extended to operable tumours, with the aim of downstaging them to facilitate a more conservative surgical approach. Such a study was started in Milan in 1989 and the results of 165 patients, with operable tumours of diameter greater than 3 cm have recently been reported (Bonadonna et al. 1990). Five different chemotherapeutic regimens were tried with similar effect. In 127 (81%) of patients a reduction in tumour size to diameter of less than 3cms was achieved allowing a breast-saving procedure. Mansi and colleagues (Mansi et al. 1989) have also reported results from primary medical therapy in operable breast cancer. Of the 15 patients treated with primary chemotherapy, 60% achieved a response and of the 42 patients 47% achieved regression as defined by UICC criteria. Subsequent mastectomy was only performed in 18% of patients, 30% had radiotherapy and or conservative surgery and the rest are still on medical therapy. With a median follow-up of 19 months (range 6- 42 months) only two patients have had a local recurrence and none have developed uncontrolled local disease. Eight have developed distant recurrence and 4 (7%) have died of metastatic disease. In both of these studies therefore, although downstaging of local disease allowing more conservative surgery has been achieved in a high proportion of patients, the followup is too short to be sure of the ultimate impact of primary chemotherapy, used in this way, on local control. In addition given the design of the study, the impact of primary chemotherapy on relapse-free and overall survival is not likely to be conclusive.

Interest has also been shown in the initial use of tamoxifen in operable breast cancer of elderly (>70 years) patients (Gazet et al. 1988; Ashby et al. 1988; Robertson et al. 1988; Gaskell et al. 1989; Horobin et al. 1991). Only two however are prospective randomised trials comparing primary tamoxifen to surgery. Preliminary data (median follow-up 3 years) from a study of 116 elderly patients with breast cancer that has shown no difference in either time to progression or survival and suggested that

tamoxifen was as effective as surgery (Gazet et al. 1988). In contrast Robertson and colleagues in a study comparing wedge mastectomy to primary tamoxifen therapy have demonstrated that with a median follow-up time of 2 years that a high proportion of the group treated by tamoxifen alone required surgery for local control (Robertson et al 1988). This lead them to the conclusion that optimal treatment for elderly patients should include both surgery and tamoxifen. The use of tumour characteristics to predict both an increased likelihood of initial response, by determination of ER status by immunocytochemical assay (Gaskell et al. 1989), or persistence of response as shown only in those patients who achieve complete clinical response (Horobin et al. 1991) may help select a subgroup of patients with operable breast tumours in whom primary tamoxifen is the treatment of choice.

In conclusion, studies of preoperative chemotherapy (Table 4) in advanced disease have suggested improved survival rates as compared to corresponding figures from stage matched control populations. Direct evaluation of preoperative chemotherapy can however only come from randomised control studies. There are at present two ongoing controlled randomised trials testing the efficacy of pre- versus post- operative chemotherapy in operable breast cancer. In the NSABP study four cycles of preoperative adriamycin and cyclophosphamide given at 3 weekly intervals followed by surgical therapy is being compared to surgery followed by the same chemotherapeutic regime. This study is still in its infancy and as yet no results as yet available. In 1983 a randomised trial of preoperative therapy was started by Ragaz in British Columbia (Ragaz 1986). In this study premenopausal women with operable breast cancer are randomised following a tissue diagnosis of breast cancer (cytological or histological) to receive either a preoperative course of CMF as soon as the staging investigations are ordered or to proceed onto initial definitive locoregional surgery. After surgery the high risk patients (axillary lymph node involvement or histological evidence of vascular or lymphangitic spread in the primary tumour) from both groups receive eight (preoperative arm) or nine (no preoperative CMF) courses of CMF at 3 weekly intervals. The median interval between diagnosis and the first cycle of chemotherapy was 6 and 24 days respectively. The disease free interval at 2 years followup (n=68) has shown no statistically significant difference between the randomised arms.



Table 4

Overview of the studies of induction chemotherapy in locally advanced breast cancer.

	No. of patient	Response rate %(CR)	3 Year			5 Year		
			OS	DFS	LR	OS	DFS	LR
DeLena et al. 1981	110	70%(15)	53%	-	-	-	-	-
Hortobagyi et al. 1988	174	87%(17)						15%
stage IIIA			-	-	-	84%	84%	-
stage IIIB			-	-	-	33%	44%	-
Swain et al. 1987	76	93%(49)			14%			
stage IIIA	31		-	50%	-	-	-	-
stage IIIB	41		50%	45%	-	-	-	-
stage IV	4		-	-	-	-	-	-
Jacquillat et al. 1988	98	91%(23)	77%	62%	13%	-	-	-
Keiling et al. 1985 (inflammatory)	41	-	-	-	-	63%	54%	-
Thomas et al. 1989 (inflammatory)	61	61%	-	-	-	-	27%	58%

OS = overall survival; DFS = disease-free survival; LR = local relapse rate; CR = complete remission from chemotherapy

CHAPTER 3

SELECTING APPROPRIATE SYSTEMIC THERAPY

The goal of adjuvant systemic therapy for breast cancer is to effect a significant prolongation of survival, while maintaining an acceptable quality of life. From adjuvant studies it is apparent that not all patients benefit equally from systemic therapy and given the high morbidity associated with cytotoxic therapy (Glass et al. 1981) a non selective policy is not ideal. One of the most difficult aspects of assessing the success of any therapeutic approach is in knowing how successful that approach has been. In no area is the data more confusing than in the assessment of factors which predict for response to systemic therapy, since not only is the ground confused by differing therapeutic approaches but breast cancer itself is a heterogeneous collection of disease patterns with very variable courses. This section reviews current understanding of factors which will predict for response to systemic therapy in both the advanced and adjuvant setting.

PREDICTING SENSITIVITY TO ENDOCRINE THERAPY

A critical step forward in our understanding of the mechanism of oestrogen action on mammary tissue came with the discovery of high-affinity binding sites for oestrogen, originally in rat mammary tumours (King, Cowan & Inman 1965) and subsequently in normal (Desphande et al. 1967) and eventually malignant breast (Jensen et al. 1971) tissue. Oestrogen receptors (ER) can be detected in 60-80% of human breast cancers, and their concentration is related to age, menstrual status and the degree of differentiation of the tumour (McGuire et al. 1975). The highest receptor values are found in more differentiated, highly cellular tumours (Elston et al. 1980) or those from postmenopausal patients (Hawkins, Roberts & Forrest 1980).

Structure and Measurement of the Oestrogen Receptor

The ER has been characterised by sucrose-gradient centrifugation as an 8S protein (Gorski et al. 1968; Jensen et al. 1968). The unoccupied receptor sites are loosely bound within the nucleus. Oestradiol diffuses to the nucleus, where receptor binding produces a configurational change causing tight binding to genomic DNA and transcription of messenger RNA (King & Greene 1984).

Ligand-saturation assays were developed by which unoccupied sites of oestrogen receptor (ER) could readily be quantified (Hawkins et al. 1975; 1981). These ligand binding assays have however been associated with certain methodological difficulties (Raam et al 1981; Leclercq et al. 1975; Thorpe 1987). The development of an antibody to the oestrogen receptor (Greene, Fitch & Jensen 1980; Greene & Jensen 1982) has allowed newer and more reliable radioimmune, immunohistochemical and immunocytochemical assays to be developed. These assays offer several advantages over the conventional steroid-binding biochemical assay since they are rapid, do not require complex laboratory materials and can be performed on small tissue samples or fine-needle aspirates (Flowers et al. 1986; Hawkins et al. 1987; McLelland et al. 1987; Weintraub et al. 1987). They also provide valuable information concerning the heterogeneity of ER expression within a tumour population.

Several studies have examined the relative sensitivities and specificities of the biochemical and immunochemical assay methods. Although in general these have demonstrated a good correlation, several differences have been noted (King et al. 1985; McCarty et al. 1985; McLelland et al. 1985, 1986; Hawkins et al. 1987). Recent data has shown that the immunocytochemical ER status also correlates with mitotic activity of the tumour, patient age and prognosis. (Walker et al. 1988).

Oestrogen Receptor and Response to Endocrine Therapy

The concept that assay of oestrogen receptors may be of value in the clinical management of breast cancer originates from the work of Folca and colleagues (Folca, Glascock & Irvine 1961) who related uptake of synthetic oestrogens by tumour tissue to patient response to simultaneous oophorectomy and adrenalectomy. Experiments in rat mammary tumours indicated that tumours which contain appreciable concentrations of ER regressed after endocrine ablative therapy whereas tumours without ER failed to respond (McGuire, Pearson & Segaloff 1975). That ER concentration predicts for response to endocrine therapy in human advanced breast cancer has been confirmed in many studies (McGuire, Pearson & Segaloff 1975; Brooks et al. 1980, Jensen, 1975, Dao & Nemoto, 1980, Oriana et al. 1986) and the results from over 1800 patients reviewed by Hawkins (Hawkins, Roberts & Forrest 1980). The chances of tumour regression in response to endocrine therapy in ER-negative tumours are small (4 - 14%), while tumours with detectable ER have a response rate of between 49-58%. In the absence of ER data, response to endocrine therapy can be expected in approximately one third of patients.

A good correlation between response to tamoxifen and the proportion of tumour cells which stain with ER antibody has also been demonstrated in patients with advanced disease (Coombes et al. 1987) and elderly patients treated by primary endocrine therapy (Gaskell et al. 1989).

The value of ER status in predicting benefit from adjuvant endocrine treatment however remains controversial. While several studies have demonstrated that only patients with ER-positive tumours treated by adjuvant tamoxifen have a statistically significant survival advantage (Hubay et al. 1980; Palshof et al. 1985, Rose et al., 1985, Fisher et al., 1986, Cummings et al. 1986; Rutquist et al., 1987, Meakin, 1985, Marshall et al., 1987, Bianco et al., 1988 (disease-free survival only), the NATO trial (Nolvadex Adjuvant Trial Organisation, 1988) has demonstrated a benefit independent of ER status. An intermediate view has been suggested by the Scottish (Scottish Cancer Trials Office 1987) and NSABP trials (Fisher et al. 1986) in which all patients who received tamoxifen benefitted, but the level of benefit was greatest in those patients with tumours of ER concentration ≥ 100 fmol/mg cytosol protein and >9 fmol/mg cytosol protein respectively (Table 5).

When the results of the individual studies are combined in an overview a significant benefit can be demonstrated in recurrence-free survival and overall survival with adjuvant tamoxifen in both patients with ER-positive (≥ 10 fmol/mg cytosol protein) and ER-negative (<10 fmol/mg cytosol protein) tumours (Early Breast Cancer Trialists' Collaborative Group 1992). When recurrence-free survival was analysed, the level of benefit achieved in ER-positive tumours was significantly higher than for ER-negative tumours (ER-negative 13% SD 4 versus ER-positive 32% SD3). The difference in mortality benefit between ER-positive and ER-negative however was not significant. These data suggest that adjuvant tamoxifen produced a beneficial effect irrespective of the ER status of the primary tumour but the effect on long-term outcome was largest when the primary tumour was ER-positive.

Improving the Predictive Value of Oestrogen Receptor

The presence of ER in a breast tumour is thus an indicator but not synonymous with hormonal sensitivity. Various theories have been proposed to explain why the correlation between the presence of ER and response to endocrine manipulation is not more exact. These include tumour heterogeneity of ER concentration both at a cellular (McClelland et al. 1986; Charpin et al. 1988) and architectural level (Hawkins et al. 1977; Senbanjo, Miller & Hawkins 1986), lability of the ER protein, sensitivity of the

assay (Thorpe 1987), difficulty with reliable assessment of tumour response in advanced disease (Hawkins 1985) and possible cytotoxic effects of endocrine drugs which are not dependent on the ER (Sutherland, Watts & Ruenttz 1986; Etienne et al. 1989). The receptor remains however the primary site of interaction between the cell and oestrogen. Biochemical detection of the ER however does not provide information about the function of the receptor and it is possible that some failures of ER-positive tumours to respond to endocrine therapy are due to a nonfunctioning receptor complex (Jensen et al. 1982).

Table 5

Summary of the relationship between ER status of the primary tumour and survival benefit from adjuvant tamoxifen.

Trial	Number with ER	% of total trial intake	ER subgroup benefitting
NATO (Nolvadex Adjuvant Trial Organisation 1988)	513	45	all levels Nolvadex
Scottish (Scottish Breast Cancer Trials Committee 1987)	742	57	all levels- >99fmol best
NSABP B09 (Fisher et al. 1986)	1079	82	all levels- ER or PR >9fmol best
Copenhagen (Pashlof et al. 1985)	225	59	>9fmol, >60fmol best
ECOG (Cummings et al. 1986)	144	85	>9fmol only
GUN (Bianco et al. 1988)	308	71	ER or PR >9fmol
Danish (Rose et al. 1985)	291	18	>99 fmol only

In sensitive breast cancer cells oestrogen is known not only to stimulate cell proliferation and reduce cell cycle time (Weichselbaum et al. 1978; Lykkesfeldt et al. 1984), but also induce at a transcriptional level, the synthesis of specific proteins. Some of these proteins are thought to coordinate the trophic effect of oestrogens at an autocrine or paracrine level (Lippman & Dickson 1989). Detection of these oestrogen-induced proteins, or their messenger RNA, now possible with monoclonal antibodies and probes, would give at least partial information on the integrity of ER-mediated pathways and may improve the accuracy for predicting response to endocrine therapy.

The progesterone receptor was the first oestrogen-induced protein to be studied in breast cancer (Sherman, Corval & O'Malley 1970; Milgrom et al. 1972, Horwitz & McGuire 1977; Horwitz, Koseki & McGuire 1978). Other proteins include a pS2 protein (Masiakowski et al. 1982, Brown et al. 1984), a 52kDa protein precursor of cathepsin D (Westley et al. 1984; Rochefort et al. 1986), a 160 kDa protein (Westley et al. 1984), insulin like growth factors IGF-I and II, transforming growth factors TGF- α , TGF- β , fibroblast growth factors, and platelet derived growth factors (Lippman & Dickson 1989).

The Progesterone Receptor

In 1972 Milgrom and colleagues first demonstrated that oestradiol induces progesterone receptors (PR) in the rat uterus (Milgrom et al. 1972). Several workers have now confirmed this observation for human breast cancer cells (Horwitz & McGuire 1977; 1978; Horwitz, Koseki & McGuire 1978; Rao & Meyer 1977). PR can be detected in approximately two thirds of ER-positive tumours, but is exceptional in ER-negative tumours (8%, McGuire & Horwitz 1977, Hawkins et al. 1987). The incidence of PR-positivity also tends to rise as the ER concentration increases (Hawkins et al. 1987).

The presence of PR has been shown to improve the prediction of objective response to endocrine manipulation in advanced disease by up to 70 - 80% (Hawkins, Roberts & Forrest 1980; Siebert & Lippman 1982; Lippman 1988). Interestingly, about 30% of ER-positive but PR-negative tumours respond to endocrine therapy. It has been suggested that this may be due to problems in detecting the PR. In postmenopausal women levels of circulating oestradiol are low and this may account for the failure to detect PR in some elderly women. In premenopausal women levels of circulating progesterone during the luteal phase are high and this may mask the receptor sites (Saez, Martin & Chouvet 1978). These dilemmas may be solved with the recent

development of antibodies to the PR which bind irrespective of the occupancy of the receptor (Pertschuk et al. 1988).

The relationship between progesterone receptor (PR) status and efficacy of adjuvant tamoxifen is shown in Table 5. Analysis of disease-free survival in the Gun study (Bianco et al. 1988). showed a near significant interaction between tamoxifen-treatment effect and a PR concentration of ≥ 10 fmol/mg cytosol protein ($p = 0.11$) which was significant interrelated to ER status ($p = 0.04$). In the NSABP B09 study (Fisher et al. 1986) the progesterone receptor content was found to be a stronger predictor of the effectiveness of PFT (L-phenylalanine mustard, 5-fluorouracil, tamoxifen) therapy than ER status.

PS2 and Response to Endocrine Therapy

The transcription of pS2 mRNA is stimulated by oestrogen (Masiakowski et al. 1982, Brown et al. 1984) and inhibited by tamoxifen (Westley et al. 1984) in MCF-7 cell lines. The mature protein is secreted probably as a 58 amino acid peptide, although its exact function remains unknown (Nunez et al. 1987). A DNA probe is available for the mRNA (Masiakowski et al. 1982) and a specific monoclonal antibody for the protein (Nunez et al. 1987).

Not all breast cancers have positive staining for pS2. Ninety-six percent of ER-negative tumours neither express PR or pS2 (Rio et al. 1987). Around 67% of ER-positive tumours express the pS2 protein and although expression appears independent of PR status, an increased likelihood of expression of pS2 in ER-PR-positive tumours has been noted (Rio et al. 1987). No data is as yet available on its value as a predictive index for hormonal sensitivity.

Epidermal Growth Factor Receptor and Response to Endocrine Therapy

The relationship between EGF-receptor expression and response of recurrent breast cancer to tamoxifen has recently been reported in 72 patients (Nicholson et al. 1989). Tumours which contained ≥ 10 fmol/mg cytosol protein were significantly less (8.5%) likely to respond to tamoxifen than those tumours which were EGF-receptor negative (30%, $p < 0.05$). The effect was independent and additive to the ER status, such that EGF-receptor-positive, ER-negative were least likely to respond (11%), EGF-receptor-negative, ER-positive tumours were most likely to respond (44%) and if both receptors were either positive or negative the response rate was intermediate at around 30%.

Correlation Between Local Metabolism and Endocrine Responsiveness

Little direct data is available concerning the relationship of local oestradiol production and hormone sensitivity but several studies have related the pattern of endogenous steroidogenesis to the established marker of hormone sensitivity, namely ER-status (McGuire, Pearson, Segalof 1975).

Sulphokinase Activity

High sulphating activity is invariably associated with ER-positive tumours, whereas ER-negative tumours usually have low activity (Adams et al. 1979). A direct and absolute relationship between the presence of steroid sulphurylation and response to endocrine therapy has been demonstrated in one major study of response to adrenalectomy and patients with advanced breast cancer (Dao & Libby 1972), but this has not been confirmed by other workers (Leung et al. 1973).

Aromatase Activity

Aromatase activity can be demonstrated in both ER-positive and ER-negative tumours. While some studies have failed to demonstrate a significant association between these two parameters (Varela & Dao 1978, Li & Adams 1981, Tilson-Mallet et al. 1983), others have demonstrated a positive association. Vermeulen & Deslypere (Vermeulen & Deslypere 1989) have suggested a higher level of aromatase activity exists in ER-positive tumours while Miller & O'Neill have demonstrated a higher incidence of significant activity in ER-positive tumours but a similar level of activity in both ER-positive and ER-negative tumours (Miller & O'Neill 1987).

Correlation between response to aminoglutethimide therapy and aromatase activity has been investigated in 23 postmenopausal patients with ER-positive tumours by Miller (Miller & O'Neill 1989). Of the 5 patients in whose tumours aromatase activity was undetectable, there were no responders; response was observed in 11 of the 18 patients whose tumours exhibit significant aromatase activity. It was concluded that while the detection of aromatase activity in a tumour may not be an absolute marker for response to aminoglutethimide, it would seem that the drug, which is known to produce at least some of its effect through aromatase inhibition, is more likely to produce response in patients whose tumours contain significant aromatase activity. A similar relationship between aromatase activity and response to oophorectomy in premenopausal women or tamoxifen in postmenopausal women has not been demonstrated (Miller & O'Neill

1989). Tumour aromatase activity may therefore be a marker of hormonal sensitivity specific to the aromatase inhibitors.

17B hydroxysteroid Dehydrogenase Activity

17B hydroxysteroid dehydrogenase activity is significantly higher in ER-positive when compared to ER-negative tumours (Vermeulen & Deslypere 1989). In ER-positive tumours the enzyme activity can be induced by progestins and the increase has been suggested to be a further putative marker for hormonal dependency (Fournier et al 1985).

Rate of Cell Proliferation and Endocrine Sensitivity

Histological Grade

Several methods have been developed for the histological grading of breast carcinomas (Elston 1988). The best known is that described by Bloom and Richardson (Bloom 1950a, 1950b, Bloom & Richardson 1957), which relies on the subjective assessment of the number of mitosis present, the level of nuclear pleomorphism and the degree of tubular formation to classify tumours into three categories; well-differentiated grade 1, moderately-differentiated grade 2 and poorly-differentiated grade 3. This method was later refined by Elston (Elston 1984, 1988) Black (Black, Opler & Speer 1955; Black, Barklay & Hankey 1975) developed an alternative grading system which was derived from the work of Bloom, but assessed tubular structures and nuclear appearances separately, with the conclusion that tubular differentiation did not contribute to prognosis but nuclear appearances did. Fisher (Fisher, Gregorio & Fisher 1975; Fisher 1977) have advocated a method which combines both systems. All three methods have been shown to correlate with prognosis (Bloom & Field 1971; Black, Barklay & Hankey 1975; Fisher, Redmond & Fisher 1980). The distribution of cases within this grading system which can be expected for any given series of patients is given in Table 6.

Table 6

Comparison of the relative percentages of cases in each histological grade from different studies.

Study	Histological grade %		
	I	II	III
Bloom & Richardson (1957)	26	45	29
Wolff (1966)	33	33	34
Tough et al. (1969)	11	51	38
Champion et al. (1972)	23	52	25
Fisher et al. (1980)	3	30	67
Fisher et al. (1984)	11	23	66
Elston (1984)	17	37	46
Masters et al. (1987)	4	58	38

An inverse relationship has been demonstrated between the degree of differentiation and frequency of response to endocrine therapy (Millis et al. 1981, Masters, Millis & Ruben 1986) and survival (Williams et al. 1986) in advanced disease. Response of advanced breast cancer to endocrine therapy is known to correlate with such features as a long postoperative disease-free interval, the presence of steroid receptor activity and elastosis (Masters et al. 1979) within the tumour. These features are all interrelated and it was not possible to determine, on the small sample study whether the positive association with grade was independent of the other factors. Williams and colleagues (Williams et al. 1986), following a study of survival in patients with metastatic breast carcinoma treated by endocrine therapy, suggested that the addition of low grade to high ER status could define a subpopulation which had an improved survival when compared to simply a population with high ER status.

Information relating the importance of histological tumour grade to benefit from adjuvant tamoxifen is scanty but is available in a subsection of 546 cases from the NATO trial (Singh et al. 1988). The greatest benefit from adjuvant tamoxifen was observed in those patients with better differentiated tumours (Grade I and II). No survival advantage was noted in Grade III tumours which were twice as likely to be ER-negative than ER-positive. In this trial benefit from adjuvant tamoxifen was independent of ER status (Nolvadex Adjuvant Trial Organisation 1988) and therefore the relationship between grade to benefit may be real but requires further definition.

Similarly the NSABP study (Fisher et al. 1986) has shown that a good nuclear grade was predictive of a better disease-free and overall survival in patients receiving chemoendocrine therapy (L-pam, 5 FU and tamoxifen). The effect was independent of but additive to the predictive value of hormone receptor status.

DNA Analysis and Labelling Indices

Changes in cell cycle distribution following hormone treatment may be elicited in model systems for example, MCF-7 breast cancer cell lines which when given oestrogen show a dose dependent increase in the rate of proliferation, with recruitment of nondividing cells from the resting G₀/G₁ phase into the actively dividing population and also shortening of the cell cycle time due to a decrease in the G₁ phase of the cell cycle (Weichselbaum et al. 1978). Administration of tamoxifen, a competitive inhibitor of oestrogen for its receptor, causes a dose dependent inhibition of these effects, with reduction of the cells within the S-phase and accumulation of cells in the growth arrest phase of G₀/G₁ (Green et al. 1981; Sutherland et al. 1983; Bruno et al. 1988, Lykkesfeldt et al. 1984) and G₂/M phase (Lykkesfeldt et al. 1984, Bruno et al. 1988). In vitro the effect of tamoxifen can be detected within 36 hours of challenge and if it were possible to detect such changes in cell cycle kinetics in vivo it would prove a useful early predictor of response (Brunner et al. 1984). As yet this has not been possible (Engleholm et al. 1987) and may relate to the heterogeneity within the tumour.

However information from DNA analysis may relate to likelihood of response. A higher incidence of complete response to endocrine therapy has been shown in tumours which are both ER-positive and have a low proliferation rate (Meyer & Lee 1980; Paradiso et al. 1988). Although the time taken to reach maximal response was shorter with tumours which exhibit a faster proliferation rate, these tumours were more likely to escape hormonal control (Paradiso et al. 1988). Tetraploid tumours when treated by adjuvant endocrine therapy have shown improved survival in relation to other aneuploid tumours and they may represent a distinct entity of endocrine responsive tumours (Baildam et al. 1987a 1987b; Stuart-Harris et al. 1985). It is unclear whether this represents an independent effect or is a function of the better prognosis or higher ER-positivity of tetraploid tumours (Baildam et al. 1987b).

Efficacy of Adjuvant Tamoxifen in Relation to Patient Characteristics Age, Menopausal Status and Axillary Node Status

The relationship between age and effect from adjuvant tamoxifen has also been reviewed by recent meta-analysis. The beneficial effect of tamoxifen on both recurrence and mortality is highly significant in women over the age of 50 at randomisation (Early Breast Cancer Trialist's Collaborative Group 1992a). Among women under the age of 50, tamoxifen produced a highly significant delay in recurrence (12% SD 4), but the size of the benefit was significantly less than for women over the age of 50 (Early Breast Cancer Trialist's Collaborative Group 1992a). For survival the general pattern resembles that for recurrence-free survival, but with smaller risk reductions and hence less statistical significance to the pattern. The effect of tamoxifen on overall survival in women under the age of 50 was favourable but did not reach statistical significance. The trend towards less effect in younger age groups only just reaching conventional significance ($2p = 0.04$). Improved survival has however been demonstrated in premenopausal women within several individual trials by subgroup analysis (Pashlof et al. 1985; Scottish Breast Cancer Trials Committee 1987; Nolvadex Adjuvant Trial Organisation 1988; Bianco et al. 1988).

PREDICTING SENSITIVITY TO CYTOTOXIC THERAPY

Sensitivity to anticancer drugs may either be tested directly by in vitro panel testing or indirectly by relating specific histological and biochemical parameters to response in vivo. The following section summarises the data which is presently available in relation to cytotoxic drugs.

Efficacy of Cytotoxic Therapy and Relationship to Patient Characteristics

Axillary Node Status

The proportional reduction in annual risk for both recurrence-free and overall survival was similar in extent irrespective of the axillary node status. The absolute benefits at 5 and 10 years however appear greater in node-positive disease and was highly significant (Early Breast Cancer Trialists' Collaborative Group 1988, 1992b). The meta-analysis at 10 years has now confirmed that even in node-negative disease the survival benefit produced is significant. In relation to individual randomised studies two large series containing over 500 patient, with a followup period in excess of 3 years have been published on the effect of polychemotherapeutic regimes in patients with high risk (ER-negative or ER-positive diameter >3cms) node negative disease (Fisher et al. 1989, Mansour et al. 1989). Each have shown a statistically significant prolongation of disease-free survival in favour of women who received cytotoxic therapy. As yet no significant difference in survival has been observed but the trials are still immature in relation to overall survival.

Patient Age and Menopausal Status

Clear survival benefits from cytotoxic therapy were evident in the overview for women less than 50 years of age (Early Breast Cancer Trialists' Collaborative Group 1988, 1992b). The effect of polychemotherapy on mortality in women ≥ 50 years old was also significant but appear to be only half as large as the benefit achieved in younger women (Early Breast Cancer Trialists' Collaborative Group 1992b). A significant reduction in recurrence rates has been demonstrated with adjuvant cytotoxic therapy irrespective of age (Early Breast Cancer Trialists' Collaborative Group 1988, 1992b). Age is related to menopausal status but the relationship was so close that it was not possible to disentangle the effects of these two factors on the response to polychemotherapy.

Oestrogen and Progesterone Status

Controversy exists regarding the relationship between ER content and response to chemotherapy in patients with advanced breast cancer. Some workers have reported a negative correlation between ER presence and response to chemotherapy (Jonat & Maass 1978; Hilf et al. 1980), while others have noted a positive correlation (Kiang et al. 1978; Young, Ehrlich & Einhorn 1980; Rosenbaum et al. 1980, Samal et al. 1980; Mortimer et al. 1981) and yet others have been unable to demonstrate any relationship at all (Rubens et al. 1980; Jonat et al. 1980; Bonadonna et al. 1980; Stewart et al. 1983).

The relationship between hormone receptor status and the value gained from adjuvant chemotherapy is also illdefined. Padmanabhan (Padmanabhan, Howell & Rubens 1986) in his review of the literature on adjuvant chemotherapy noted that the benefit gained from adjuvant chemotherapy was equivalent to that obtained by oophorectomy, with the predominant effect seen in premenopausal women, especially when permanent amenorrhoea was achieved. Adjuvant chemotherapy induces permanent cessation of menstruation in 70% of premenopausal patients by 6 months of treatment. This is accompanied by a concomitant decrease in circulating oestrogens and a rise in gonadotrophins (Rubens 1988). Moreover in Rubens study (Rubens 1988) the effect of chemotherapy was confined to patients with progesterone receptor positive tumours. These observations lead them to postulate that the major effect of adjuvant chemotherapy was mediated by ovarian suppression, with only a small contribution from direct cytotoxicity. Debate on this philosophy continues. Randomised trials are currently ongoing comparing the effects of oophorectomy directly with that of a CMF regime in node-positive premenopausal women (Scottish Breast Cancer Trials Committee - Trial A personal communication). Unfortunately hormonal studies are not an integral part of this study and so the study may not help to resolve this precise problem.

Two recent studies of chemotherapy however, in oestrogen receptor negative tumours (Fisher 1988, Bonadonna et al 1987), have shown a significant improvement in disease-free survival in the treatment arm suggesting some effect independent of the presence of the ER and therefore not hormonally mediated. Other studies have relied on retrospective analyses of subpopulations not stratified for ER at the time of randomisation. In a randomised trial of a single perioperative cycle of combination chemotherapy with no adjuvant therapy, involving 1275 patients with no axillary node metastasis, not only was an improvement in disease-free survival noted in pre- and postmenopausal women but the magnitude of effect was largest among patients

with no or low oestrogen-receptor content of their primary tumour (Ludwig Breast Cancer Study Group 1989).

In Vitro Test of Drug Sensitivity - The Clonogenic Assay

The "human tumour stem-cell assay" has been proposed as an in vitro method by which the activity of anticancer drugs could be tested against tumour cells from the patient and is analogous to sensitivity testing for antibiotics in patients with bacterial infections. Tumours contain a small proportion of stem cells which are capable of unlimited self-renewal and are responsible for tumour growth and metastasis (Mendelsohn & Dethlefsen 1968; Steel 1977). Inherent drug sensitivity of these tumour stem cells is a prerequisite for treatment success and a major determinant of clinical response.

Stem cells when grown in enriched semisolid agar will produce cell colonies or clones (Hamburger & Salmon 1977; Courteney et al. 1978; Salmon et al. 1978) against which a panel of anticancer drugs can be tested. Using this clonogenic assay it has been shown that human tumours of a single histological type exhibit a pattern of response in vitro which is similar to their known clinical behaviour (Von Hoff et al. 1981). For any given histological type however, tumours display heterogeneity in sensitivity patterns both in vivo and in vitro. Direct comparison of in vitro sensitivity and clinical response has shown that when the in vitro assay predicts for resistance it is correct in 84-98% of cases, whereas the prediction of drug sensitivity is correct in 40-70% of cases (Salmon et al. 1980; Von Hoff et al. 1981a; Mann et al. 1982; Meyskens et al. 1981; Hug et al. 1986). Both clinical response rates (Von Hoff et al. 1981b) and survival (Alberts et al. 1982; Durie, Young & Salmon 1983) have been shown to be improved by selecting drugs by in vitro panel testing but the majority of these trials have been retrospective and uncontrolled and to date there is no study which has unequivocally demonstrated that the assay has benefited the patient.

There are also several technical problems associated with the clonogenic assay which would make its routine application impracticable. Sufficient in vitro growth for drug testing can be obtained from less than 50% of tumour biopsies (Selby, Buick & Tannock 1983; Von Hoff 1983a; 1983b) and Selby (Selby, Buick & Tannock 1983) has calculated that in only 5% of the total patients tested would an improvement be achieved in drug selection.

In summary although this method of testing may have potential for improving the selection of appropriate chemotherapeutic agents improved methods are clearly still needed before a definitive trial can be undertaken.

Multidrug Resistance

Multidrug resistance describes the phenomenon of simultaneous resistance to several structurally and functionally unrelated cytotoxic drugs. A specific form of multidrug resistance has been identified from tumour cell lines, in which there was overexpression, due to gene amplification (Gros, Croop & Housman 1986; Chen-Jie et al. 1986), of a high molecular weight, membrane bound glycoprotein named P-glycoprotein (Gerlach et al. 1986; Pastan & Gottesman 1987). Resistant cells contain decrease levels of intracellular cytotoxic drug and it is now thought that the P-glycoprotein is an energy-dependent membrane transport protein which extrudes toxic drugs from the cell. This effect is directly inhibited by calcium channel blockers which may represent a potential method of increasing the sensitivity of tumour cells to cytotoxic drugs (Tsuruo et al. 1981, 1982).

Monoclonal antibodies are now available against the P-glycoprotein and a cDNA probe against its gene. These have allowed the relevance of P-glycoprotein to be studied in a small number of human tumours in vivo. P-glycoprotein has been identified in around 10-20% of tumours tested and although increased amounts have been demonstrated with increasing resistance to therapy (Kartner & Ling 1989) no study has yet demonstrated a clear role for the multidrug resistant phenotype in clinical drug resistance.

Topoisomerases and Atypical Multidrug Resistance

Cells lines have been developed which display resistance to different classes of antineoplastic agents in the absence of any apparent P-glycoprotein gene expression (Marsh & Center 1987; Danks, Yalowich & Beck 1987). In these cells the drug influx and steady-state intracellular drug concentrations were similar to the parent drug-sensitive cells. This new pattern of cross-resistance has been termed "atypical multidrug resistance" and although the precise mechanism is not known, there is strong evidence implicating altered topoisomerase activity in the resistant cells (Moscow & Cowan 1988).

Topoisomerases are enzymes which catalyse changes in the secondary and tertiary structure of DNA and are necessary for DNA replication. Topoisomerase II is thought

to be the enzyme target of drugs which act as DNA-intercalating agents (Glisson et al. 1986). They may represent the final common pathway of cytotoxicity for several different classes of antineoplastic agents. No studies have yet determined whether altered topoisomerase activity plays a role in clinical drug resistance.

Glutathione S-Transferase Enzymes

There is evidence that overexpression of glutathione and glutathione dependent enzymes are important mechanisms of acquired drug resistance in both normal and tumour cells (Hirono 1960; Calcutt & Connors 1963; Arrick & Nathan 1984; Fabrer 1984; Wolf et al. 1987; Hayes & Wolf 1988; Lewis et al. 1988, 1989). Although the direct involvement of these enzymes in the detoxification of anticancer drugs has not been clearly established, glutathione S-transferase mediated conjugation of melphalan has been reported (Dulik, Feneselau & Hilton 1986). It is likely that the enzyme will also be able to inactivate other structurally related nitrogen mustards. Certainly cell lines resistant to nitrogen mustards, such as chlorambucil and cyclophosphamide (Wang & Tew 1985; McGowan & Fox 1986; Robson et al. 1987; Buller, Clapper & Tew 1987), cisplatin (Green et al. 1984) and adriamycin (Green et al. 1984) have been shown to have elevated levels of GST. Such elevated levels have been shown to be associated with an amplification of alpha class glutathione S-transferase genes (Lewis et al. 1989). The role of glutathione S-transferase in clinical drug resistance has yet to be determined.

Glutathione Peroxidase

Glutathione peroxidase catalyses the reduction of potentially toxic peroxides to alcohols at the expense of oxidising GSH to its disulphide form. Adriamycin-resistant MCF-7 cells have shown a 13-fold increase in glutathione peroxidase activity relative to the parent cell line suggesting a possible causal relationship (Sinha et al. 1987).

Rate of Cell Proliferation and Chemosensitivity

Histological Grade

Proliferating cells are known to be more sensitive to the lethal activities of most cytotoxic drugs than cells which are not proliferating (Valeriote & van Putten 1975). One would therefore expect that rapidly growing tumours respond more readily to therapy whereas tumours with a low rate of proliferation very often show no reaction to

therapy. Clinical studies in human solid tumours (Silvestrini et al 1985) and leukemias (Hart et al. 1977) have demonstrated that a direct relationship exists between the DNA labeling index and clinical therapeutic success such that well-differentiated tumours, with the possible exception of lymphomas and chronic lymphocytic leukemias, are relatively resistant to chemotherapy, while poorly-differentiated tumours are more likely to respond (Whitehouse 1984). A study of the relationship between response to chemotherapy and tumour grade, elastosis, and ploidy, in 125 patients with advanced breast disease has however failed to confirm any significant relationship, although there was a trend for patients with tumours which were diploid or had a high elastosis content to have a higher response rate (Masters et al. 1987).

Nuclear grade was shown to have an independent influence on both disease-free and overall survival in breast cancer patients receiving adjuvant L-pam, 5 fluorouracil with or without tamoxifen (Fisher et al. 1986). Those having tumours with either receptors ≥ 10 fmol or a good nuclear grade had a better relative 5 year survival. The magnitude of difference was similar for all three discriminants but the effect was independent for each and the predictive value improved if more than one marker was used, particularly in those patients who received tamoxifen.

DNA Analysis and Labelling Indices

It has been observed that in the treatment of leukemia, patients with tumours which had a high thymidine labelling index (TLI), were more likely to achieve complete response (Zittoun et al. 1975). A significant reduction in the TLI of tumours 48 hours post-therapy has been shown to predict for clinical response to cytotoxic therapy in some tumours (Murphy et al. 1975; , Zittoun et al. 1975; Thirlwell et al. 1976). A relationship between labelling index and clinical response in a small number of patients to combination chemotherapy has been demonstrated (Sulkes, Livingston & Murphy 1979). None of the patients with a labelling index of less than 9% regressed with therapy, in contrast to 11 of 16 patients with a labelling index greater than 9%. There has been no evidence to support the value of tumour ploidy or % S-phase as useful indices of chemoresponsiveness in breast cancer in vivo (Masters et al. 1987).

Thymidine Kinase Activity

Thymidine kinase is the essential enzyme involved in the salvage pathway of DNA synthesis. Levels within malignant tissue are significantly elevated (Kit 1976) and correlate directly with the proliferation rate (Kit 1976, Bronzert et al. 1981). A rise in

thymidine kinase activity can also be demonstrated in ER-positive cells when growth is stimulated by oestrogens (Bronzert et al. 1981).

In a study of 45 patients with breast cancer treated by combination chemotherapy, a significant difference was demonstrated in the response rates of tumours containing greater or less than 80 pmol/mg/min of thymidine kinase activity (86 vs 13%, Zhang, Kennedy & Kiang 1984). The effect was independent of the ER-status of the tumour. Within this study, thymidine kinase activity was not useful in determining response to endocrine therapy but the numbers were small (n=12) and a higher median concentration was found in those with regression.

CHAPTER 4

ASSESSMENT OF TUMOUR RESPONSE

The principal aim of new therapies is to improve survival. For the individual patient, to use survival as an end point is too complex since it takes years of followup, may be confused by multiple therapies and the biological heterogeneity of breast cancer itself. For this reason a more objective method of assessment of tumour response has been sought and if the primary tumour is left in situ during systemic therapy, it can be used as an assessable parameter of the disease.

The natural course of a solid tumour is to grow. The rate of growth is variable in breast cancer and doubling times may vary between a week and a year, with a median of around 60 days (Steel 1977). It is however rare for breast tumours to undergo spontaneous remission (Wyand 1925; Greenwood 1926; Daland 1927; Everson & Cole 1966) and it is therefore likely that any sustained reduction in tumour size is due to therapy. Provided an accurate method of determining tumour size can be achieved, response to therapy could be followed. The problem comes with accurate measurement of tumour size.

Direct measurement of changes in clinical tumour size

Direct measurement of tumour mass with a ruler or engineers calipers, has been a basic method of testing the effect of anti-tumour agents in animals. Tumour size has been shown to correlated with weight and volume in these models (Scholler, Phillips & Bittner 1955). Using simulated tumour nodules it has been shown that if response is assessed from measurement of a single diameter, a significant false positive response rate exists (Moertel & Hanley 1976) such that if a reduction of 25% in diameter was used, the placebo response rate was 19% if the same observer had measured the tumour and as high as 25% if the second measurement had been made by a different observer. When a 50% reduction in diameter was used to define response, the false response rate due to measurement error was reduced to 7.8%. On the basis of these findings in 1977 guide lines were introduced by the UICC for the evaluation of response to treatment in advanced disease (Hayward et al. 1977; Ward et al. 1977). In the case with a clinically measurable lesion, a 50% or greater reduction in diameter was required in order to specify regression although the exact period of time was not defined. Progression was defined as a greater than 25% increase in tumour diameter with tumours of intermediate

response being categorised as no change. A 50 % reduction in diameter however represents over an 80% reduction in tumour volume and if the measurement error could be reduced a less stringent index of tumour response may be defined.

Several pertinent points were made by Brindley and his colleagues in their review of tumour measurements in patients with inoperable breast cancer (Brindley, Markoff & Schneiderman 1959). They demonstrated that two observers, measuring the same lesion may differ by a considerable amount in the absolute measurement, but if each remained consistent in their method, the noted changes in lesion size were very similar. Measurements taken at frequent intervals also allowed discrimination of transient changes in tumour size from a real therapeutic effect. Only those lesions which were discrete and easy to measure gave reproducible results and since an observer may be influenced by knowledge of their previous values, measurements should be made without referring to previous results. Other workers (Warr, McKinney & Tannock 1984; 1985) have confirmed that the false positive rate could be reduced if two or more successive measurements showed a consistent change, or if the pretreatment diameter of the lesion was greater than 3.2 cm.

By utilising the theory of multiple measurements by the same observer over a period of treatment, Thomlinson (Thomlinson 1982; 1987) has devised an alternative method of assessing response based on the statistical regression analysis of the observed changes in tumour volume. Over 62,000 measurements were made in 239 tumours. The average number of measurement points in each regression line was 7 and the correlation coefficient of the exponential regression lines were extremely high. This represent a more precise method of assessing regression in the individual patient while also allowing appraisal of the rates of regression.

Imaging

A further objective measurement of tumour size can be gained from imaging the tumour and the most commonly used technique in breast disease is mammography. The relationship between clinical and mammographic response to systemic therapy has been reviewed in 49 patients with primary breast cancer (Cocconi et al. 1984) with some disagreement being noted in quantifying the magnitude of response. Physical examination overestimated the overall response rate in relationship to mammography in 22.9% of cases, while in 8.6% of cases the reverse was true. Although physical examination may be less objective than mammography, a mammographic abnormality does not allow differentiation between malignant or reactive processes within the

imaged lesion. It was concluded that both techniques should be used together since the most favourable response was the best predictor of complete pathological response. Like clinical examination, mammography only gives a two dimensional view of the tumour. The developing technique of breast ultrasound may be a potential method of obtaining estimation of the third elusive dimension, depth (Nishimura et al. 1988)

Pathological Assessment of Response

Pathological assessment of a change in tumour size is difficult since in order to assess initial tumour size it would require complete removal of the tumour. Complete histological disappearance of a tumour is however a definite end point and it has been suggested that this is a better predictor for survival than complete clinical response (Feldman et al. 1986).

Survival

It is common practice in cancer literature to compare the survival experience of patients who respond with those who do not respond to therapy and assume that the difference is due to the efficacy of therapy (Anderson, Cain & Gelber 1983; Anderson & Davis 1986). This type of direct analysis is invalid since survival may be associated with response because of some basic underlying factor which merely identifies those more prognostically favourable patients. To test the efficacy of new therapies properly controlled randomised trials are required.

METHODS

CHAPTER 5

STUDY PROTOCOL

Patient Population

Patients were considered for entry into the study if they presented with an invasive adenocarcinoma of the breast, 4 cms or greater in clinical diameter (T2, T3, N0, N1, M0). Patients with evidence of tumour fixation to underlying muscle or chest wall, skin involvement, breast lymphoedema, or detectable metastasis on routine clinical and radiological investigations were excluded from the study as were patients with tumours of classical lobular carcinoma, because of the inherent difficulty in clinical and mammographic measurement of these tumours. Due to the undetermined effect on the psychological state of patients undergoing this form of management, patients with a history of psychological morbidity were not included. Patients required to be medically fit for and have no history of previous chemotherapy. All patients were Karnofsky grade 0.

During the 4 year period between April 1985 and April 1989, 136 patients presented to the Breast Unit of Longmore Hospital with a primary breast carcinoma of clinical diameter greater than 4cm; 88 have been included in this study. Sixteen patients failed to fulfil the selection requirements due either to psychological (n = 7) or medical (n = 3) infirmity, or the wedge biopsy revealed lobular carcinoma (n = 3) or carcinoma in situ (n = 3). In 5 patients, a large amount of tissue had already been removed to establish diagnosis so leaving insufficient tissue for reliable measurement; in a further 13 patients the tumour was either multifocal, partly cystic, bilateral or just difficult to measure reliably. Seven patient were excluded because they lived greater than 50 miles from the hospital. Only 7 patients refused preoperative therapy, preferring immediate mastectomy.

The mean age of the study population was 53.1 years (range 33 - 69 years). Thirty-eight patients were premenopausal (less than one year since their last menstrual period), 50 were postmenopausal. Determination of menopausal status in patients who had undergone hysterectomy was based on serum gonadotrophin levels; patients were defined as postmenopausal if their follicle- stimulating hormone was greater than 30u/l.

Procedure

Initial Assessment

At initial presentation, tumour size was assessed both clinically and mammographically. The diagnosis of malignancy was made from cytological examination of a fine-needle aspirate. Staging assessment was performed in all patients and involved a thorough clinical examination supplemented by haematological (erythrocyte sedimentation rate, full blood count) biochemical (urea and electrolytes, liver function tests, serum calcium, phosphate and albumin) and radiological (chest X-ray and radioisotope bone scan) investigation. Any patient with abnormal liver function tests had a liver ultrasound to exclude the presence of overt metastasis. The philosophy of the study was explained to all suitable patients both verbally and by written document and informed consent obtained.

Pretreatment tumour material for histological and biochemical evaluation, was obtained by incisional wedge biopsy performed under general anaesthesia. Approximately 0.6cm³ of tumour was removed and a biopsy of 2 grams of peritumour fat also obtained; all specimens were transported to the laboratory on ice. In order to standardise the technique this procedure has been performed by one person (EA) in the last 50 patients and to aid post-therapeutic localisation of the tumour area, the tumour bed was marked by metal ligaclips. Early in the study an involved axillary node was excised in preference to biopsy of the primary tumour in 15 patients. Although not initially performed, formal axillary node sampling has been routine in the last 29 patients.

Systemic therapy

Endocrine therapy

Of the 88 patients included within this study 61 received primary endocrine therapy (Table 12) and 47 cytotoxic therapy. Twenty patients received both endocrine and cytotoxic therapy. Systemic therapy was commenced within 10 days of the wedge biopsy.

Initially a small pilot study was conducted in which all patients (n = 36) received endocrine therapy; a variety of endocrine agents being assessed. Ovarian function was ablated in premenopausal women initially by surgical bilateral oophorectomy (n = 5) and subsequently by means of the lutenising-hormone releasing-hormone analogue

goserelin (Zoladex ICI 118630, subcutaneous implantation 3.6mg depot preparation at 28 day intervals following 4ml lignocaine local anaesthetic, n = 7). Postmenopausal women received either tamoxifen (20mg per day, n = 11) or the aromatase inhibitor aminoglutethimide (500mg plus 40mg hydrocortisone acetate, n = 10). Three postmenopausal patients received goserelin as their primary therapy. Following the demonstration that no patient with an ER concentration of <20 femtomols/mg cytosol protein showed significant regression to endocrine therapy (Table), a more formal selective policy was instituted on 1st April 1987. Endocrine therapy was reserved only for those patients with ER-moderate/rich tumours (≥ 20 fmol/mg cytosol protein), patient with ER-poor tumours (ER<20fmol/mg cytosol protein) or those patients with tumours unresponsive to endocrine therapy received cytotoxic therapy. In this formalised protocol premenopausal patients received goserelin (n = 9) and postmenopausal patients received the selective peripheral aromatase inhibitor, 4-hydroxyandrostenedione (250mg intramuscular injection to alternate buttocks at 14 day intervals; Ciba-Geigy CGP 32349, n = 16).

Cytotoxic Therapy

Forty-seven patients received cytotoxic therapy. The regime used was 4 cycles of CHOP (cyclophosphamide 1g/m², adriamycin 50mg/m², vincristine 1.4mg/m², maximum 2g, all by iv bolus and oral prednisolone 40mg per day for 5 days) administered at 21 day intervals. If cytopenia (white blood count <3,000 ml⁻³ or platelet count of < 100,000 ml⁻³) was present on day 21, therapy was delayed until the cytopenia had resolved. A dose adjusted course was then given. Twenty-seven patients, all with tumours of ER concentration of < 20 fmols/mg cytosol protein were given chemotherapy as their primary treatment. The remaining 20 patients received chemotherapy following failed endocrine therapy; 10 had ER-negative/poor and 10 had ER-moderate/rich tumours (Table).

Assessment of Response to Therapy

Clinical Assessment

Patients were reviewed at weekly intervals by one clinician and serial mean clinical diameters obtained. Although formal assessment of tumour response was calculated following completion of 12 weeks systemic therapy, detection of any interim signs of local progression (n = 16), i.e. de novo breast lymphoedema or increasing size of tumour, ensured that endocrine therapy was immediately stopped and the patient either

transferred to cytotoxic therapy or proceeded immediate to mastectomy (n = 2). Clinical response to therapy was assessed either as clinical progression (de novo presentation of breast lymphoedema), residual palpable tumour or complete clinical regression when there was no residual tumour palpable.

Statistical Assessment of Response

Statistical evaluation of response was by linear regression analysis (Apple Mac, Statview) of the changes in tumour volume between treatment weeks 4 to 12 (earlier measurements were not considered in order to allow the reaction caused by tumour biopsy to subside, Figure 1, 3, 5 and 7).

Mean tumour volume was calculated by assuming that the tumour was a sphere ($\frac{4}{3}\pi r^3$) and mean clinical tumour diameter was calculated by taking the mean of measurements performed in 8 differing axes taken at 22.5° angles using engineers calipers. Care was taken to apply a consistent amount of pressure. All measurements within the same patient were performed when possible by the same investigator and without direct knowledge of previous measurements.

Linear regression analysis is a standard statistical test (Rees 1985) which allows determination of the relationship between two variables (in this case time and log of tumour volume) drawn on a scattergram. It is based on the linear regression equation

$$y = a + bx$$

where y is the variable on the y-axis, x is the variable on the x-axis, b the slope (gradient or regression coefficient) of the line and a the intercept. By application of this equation to a scattergram of two variables, a 'best fit' or regression line can be drawn. This is a line for which the sums of the squares of distances from the points to the line in the y direction are minimised. Application of regression equations allow not only estimation of y given x within the allocated range but also by application of hypothesis testing to the slope of the regression line one can calculate whether it is significantly greater than 0 (5% level). The designated formula is

$$t = \frac{b}{\frac{S^r}{\sqrt{\sum x^2 - \frac{(\sum x)^2}{n}}}}$$

Where b = slope of the line, S^r is the residual standard deviation (residual is the distance from the points to the line in the y direction) and n is the number of points. The significance with which the line deviates from zero is calculated by comparing the

observed value of t to tables of the Student t distribution with $n-2$ degrees of freedom. There are numerous statistical packages for calculation of linear regression of which the AppleMac Statview is only one.

When the scatter diagram shows a nonlinear pattern it may be possible to produce a linear pattern by transforming one of the variables (Rees1985). If a standard proportional reduction in tumour volume was to occur for every given period of time a nonlinear scattergram would be obtained for this reason the volume data has been log transformed (\log_{10}) when calculating regression.

Response was graded as i) significant regression (reduction in tumour size where the probability that the regression line deviated from the horizontal was greater than 95%)
ii) progression (significant increase in tumour diameter or signs of local advancement)
iii) no change (regression slope intermediate to significant regression and progression).

Mammographic Assessment

Alterations in tumour size were also assessed radiologically by a single mammogram, performed at 4 weekly intervals, in the view known to give the best perspective of the tumour.

Pathological Assessment

Pathological assessment of tumour response was obtained by histological examination of both the incisional wedge biopsy and the definitively excised specimen. In the latter the macroscopic size of any residual tumour was measured by ruler. If no macroscopic tumour was visible the specimen was graded according to whether or not there was any evidence of microscopic tumour (complete or non-complete pathological response). Axillary lymph nodes were also examined for the presence of metastatic tumour.

Hormonal Assays

Hormonal assays were performed in patients who received either goserelin or 4-hydroxyandrostenedione. Twenty mls of blood (lithium heparin tube) was spun at 4000 rpm. (4°C for 10 mins) and the resulting plasma pipetted off and stored at -20°C until analysis. In patients who were treated with goserelin, samples were obtained weekly for the first month and fortnightly thereafter. The samples were assayed for plasma FSH, LH, oestradiol and progesterone. In patients undergoing treatment with 4-hydroxyandrostenedione plasma samples were obtained before initiating therapy and

at 14 day intervals just prior to the next depot injection. Plasma oestrone levels were assayed by gas chromatography-mass spectrometry performed by Dr Mitch Dowsett and his staff at the Department of Academic Biochemistry of the Royal Marsden Hospital, London (Dowsett et al. 1989).

Side-effects

Side-effects to therapy were assessed from the weekly clinical interview. The morbidity associated with cytotoxic therapy was reported using the WHO toxicity grading system (Miller et al. 1981). Blood was taken when clinically indicated and on day 21 for patients receiving cytotoxic therapy and day 28 of the cycle in patients receiving goserelin or 4-hydroxyandrostenedione, to check haematological and biochemical indices.

Survival

All patients were followed with respect to disease-free survival, distant disease-free survival and overall survival. Recurrences of tumour in the chest wall, ipsilateral breast or axillary nodes were classified as local recurrences. Tumour reappearance in any other area were considered as distant failure. Overall survival was defined as survival with or without recurrence of disease. The followup period has been expressed from entry into the study, dating from the time of initiating systemic therapy to the date of analysis.

Survival has been analysed according to Kaplan Meier life analysis (Kaplan & Meier, 1958) and statistical differences between parameters calculated by the Generalised Wilcoxon Test.

Definitive Locoregional Surgery

On completion of systemic therapy (3-6 months) mastectomy with extensive skin removal (3cm clear of tumour) and axillary node clearance (level 3) was performed in 82 patients, of whom 55 have had simultaneous reconstruction by latissimus dorsi myocutaneous flaps. In 6 patients, with complete clinical response, wide local excision of the previous tumour site was preferred: this was followed by radical radiotherapy in 5 cases.

Postoperative Systemic Therapy

Patients who had shown significant regression to preoperative endocrine therapy were continued on endocrine therapy following definitive locoregional surgery.

Premenopausal patients proceeded to oophorectomy, postmenopausal patients received tamoxifen at a daily dose of 20mg. Further cytotoxic therapy was not given after definitive local surgery.

CHAPTER 6

MEASUREMENT OF POTENTIAL PREDICTIVE INDICES

Biochemical Method of Oestrogen Receptor Estimation

In both pre- and post-treatment specimens, a section was cut from the face of the tissue portion used for receptor analysis, fixed in formol-saline and stained with haematoxylin and eosin to permit histopathological confirmation of the presence of tumour.

Oestrogen receptor activity was determined by saturation analysis (Hawkins et al. 1975, 1981). In brief, tumour was homogenised in tris-monothioglycerol-glycerol buffer and centrifuged at low speed; aliquots of tumour cytosol were incubated at 4°C overnight with ³H oestradiol +/- 8 concentrations of nonradioactive oestradiol (0.031-62.3 nMolar). After separation of the bound fraction by adsorption with dextran-coated charcoal, and scintillation counting, the concentration of receptor sites and dissociation constant of binding were calculated by Scatchard analysis (Scatchard 1949) using a programmed BBC microcomputer. Protein concentration was determined in a separate aliquot of each tumour cytosol by the method of Bradford (Bradford 1976); using serum albumin as a standard, with 5 quality controls. Receptor concentration was finally expressed as femtomoles of binding sites/mg extract protein.

Correlation of Changes in ER with Changes in Histology

To examine the correlation between any changes in ER concentration and histopathology, 12 paired (pre- and post-treatment) tumours samples were independently examined by Dr. T.J. Anderson to exclude major differences in morphology of the pre- and post-treatment paired specimens.

Statistical analysis

The relationship between pre- and post-treatment specimen ER concentrations was examined using the paired "t" test after logarithmic transformation of the data.

Determination of Oestrogen Receptor Status by Cytochemical Assay

Smears for cytochemical assay were prepared from the fine needle aspirate for diagnostic cytopathology, and any material left within the syringe and aspiration needle was flushed through and into Roswell Park Memorial Institute (RPMI) tissue culture medium (0.4ml). Distilled water was added to lyse erythrocytes, further medium was added to restore osmolarity and the remaining cells were harvested by centrifugation (5

mins at 230g) and resuspended in RPMI (>300ul). Three 100µl aliquots were then cytospun (4 min at 1000rpm) onto slides precoated with polylysine, air-dried for 2 minutes, fixed and later stained with the immunocytochemical assay system (ERICA, Abbott laboratories). Control slides were processed with each batch of aspirates. Slides were independently assessed by two observers and following correction for nonspecific staining, scored for both the proportion of epithelial cells staining, expressed as a percentage, and the average staining intensity. The staining intensity was graded visually on an arbitrary scale of 0 (no staining)- 3 (strong staining). To determine whether correlation with either the biochemically determined ER concentration or response could be improved by integrating these scores, their product the "staining index" (% epithelial cells x average staining intensity x 0.01) was also calculated.

Statistics

The relationship between two variables has been examined using the Spearman's Rank Correlation Coefficient.

Measurement of 17B-hydroxysteroid Dehydrogenase Activity

Determination of 17B-hydroxysteroid dehydrogenase activity was based on in vitro assay of the conversion of radioactive labelled androstenedione to testosterone and has been expressed in pmols testosterone formed/mg protein/hour of incubation.

Harvesting

Breast adipose tissue was obtained from the vicinity of the primary tumour either at the time of incisional biopsy or at the time of mastectomy. (If complete tumour regression had occurred, tissue was taken from the area surrounding the previous tumour biopsy site). All biopsy tissue was rapidly processed and kept on ice until stored or fixed. Adipose tissue was dissected free from breast parenchyma and fibrous tissue by teasing with a metal forceps and stored in liquid nitrogen until assayed.

Extraction and Incubation

Two grams of fat were homogenised in ice cold buffer (2ml, 0.1M phosphate buffer, pH 7.4) then centrifuged (800g, 5 mins at 4°C). The soluble fraction below the lipid layer was extracted and further centrifuged (100,000g 60 mins). The particulate fraction was resuspended in 600µl of ice cold phosphate buffer and a 100µl aliquot was retained for protein estimation. The remaining 500µl was incubated for 3 hours at 37°C in phosphate buffer (0.1M; pH 7.4) containing in a total volume of 1.1mls, an NADPH

generating system (NAD, ATP, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase), $1\text{B } ^3\text{H}$ 4-androstenedione (10^6 dpm) and cold androstenedione (final concentration 100nM). Blank incubations were also set up using 500 μl of bovine serum albumin (2.0mg/ml) in place of the particulate fraction. The incubation was terminated by the addition of ice cold chloroform. Testosterone fractions were purified from the chloroform layer and aromatase activity derived from processing of aqueous fractions.

Purification of Metabolites

Testosterone fractions were purified by thin layer chromatography and characterised by chemical derivative formation. "Cold" carrier steroids androstenedione (500 μg) and testosterone (500 μg) were added at this stage so that losses during purification and characterisation could be determined.

Chloroform extracts were evaporated to dryness and the residue reconstituted in 2 mls of ethanol. Two aliquots (40 μl) were removed, dried down, reconstituted in Scintol 7 (10ml) and the radioactivity measured in a liquid scintillation counter to check distribution of radioactivity between organic and aqueous fractions. The metabolites in the remaining solution were separated by fractionation with thin layer chromatography (chloroform: acetone 92.5:7.5 on silica gel HF 254/360 for 1hour). Testosterone and androstenedione fractions were identified by exposure to UV light. Both fractions were eluted but only the testosterone fraction was purified further by sequential acetylation and hydrolysis. Testosterone was acetylated by the addition of pyridine (3 drops) and acetic anhydride (6 drops) and incubation for 1hour at 60°C. The incubation was halted by the addition of methanol. The mixture was evaporated to dryness, reconstituted in ethanol and the derivative fractionated by thin layer chromatography (cyclohexane: ethyl acetate 70:30). The area corresponding to the testosterone acetate was identified under UV light, scraped off the plate and eluted with ethanol. Aliquots were taken for estimation of radioactivity and concentration of testosterone acetate determined by UV spectrophotometry. The testosterone acetate was then hydrolysed by incubation overnight with 0.25mls of 2% K_2CO_3 at 37°C. Steroids were extracted with ethyl acetate (5ml x 2). The bulked ethyl acetate extracts were evaporated to dryness, reconstituted in ethanol and run on thin layer silica gel coated chromatography plates in a solution of chloroform and acetone (92.5:7.5). The hydrolysed testosterone acetate was identified under UV light and eluted before being reconstituted in ethanol. The resulting testosterone was reacylated to obtain the third derivative. For each of

the three derivatives, specimen samples were removed to determine the amount of cold and radioactive testosterone, and sample specimens were obtained to assess background radioactivity and steroid contamination. Radioactivity was read in a liquid scintillation counter and the amount of cold testosterone assessed by U.V. absorption at 240 nm against a known standard solution of testosterone. Specific activities were then calculated for the derivatives from each incubation and results only accepted when the specific activities for all three derivatives were within a 10% error. Activity was described as pmol testosterone formed/mg protein/hour incubation.

Formulae Used in Calculation of 17B-Hydroxysteroid Dehydrogenase Activity

Estimation of radioactive testosterone in disintegrations per minute

$$= \frac{\text{counts per minute} \times 100 \times \text{dilution correction}}{\% \text{ efficiency of solvent}}$$

Estimation of cold testosterone in nmol

$$= \frac{(\text{reading} \times - \text{blank}) \times 694^* \times \text{dilution correction}}{\text{reading of standard}}$$

* amount of testosterone in 200µl of 1mg/ml solution (standard)

Specific activity of derivative

$$= \frac{\text{radioactivity (dpm)}}{\text{amount of cold testosterone (nmol)}}$$

% conversion/3hour incubation

$$= \frac{\text{mean specific activity of derivatives} \times 1736^* \times 100}{\text{total amount of radioactivity}}$$

* amount of cold testosterone added in 500µl of 1mg/ml.

The results have been expressed as pmol testosterone formed/mg protein/hour incubation.

Statistical Analysis

The relationship between peritumour 17 β -hydroxysteroid dehydrogenase activity and ER concentration of the primary tumour was analysed using the Spearman Rank Test. Correlation of with menopausal status and clinical response to systemic therapy have been assessed from nonparametric analysis using the Mann-Whitney U Test. The changes in 17 β -hydroxysteroid dehydrogenase activity following systemic therapy have been analysed using the Wilcoxon-signed rank test of log transformed data.

Measurement of Tumour Aromatase Activity

Tumour for assay was obtained from either the pretreatment biopsy or the mastectomy specimen which had been rapidly transported to the laboratory on ice. A portion of the specimen was taken to histologically confirm the presence of malignancy. 500mgs of tumour was finely sliced and incubated for 2 hours at 37°C in Krebs-Ringer phosphate buffer pH 7.4 containing, in a total volume of 7.5ml, an NADPH generating system and 7 α -³H testosterone (22.5 uCi, 8.9 Ci/mmol). The reaction was stopped by the addition of methanol and the incubations stored at -10°C until processed.

Determination of protein content was by an adaption of the method of Bradford (Bradford 1976).

Before extraction, 500 μ g of non-radioactive oestradiol was added to allow monitoring of procedural losses. The mixture was then homogenised, centrifuged (2500 rpm for 2 mins) and the resulting supernatant decanted off. The residue was rehomogenised with 2 x 20ml. acetone, centrifuged for a further 2min at 2500 rpm and the acetone supernatant combined with the aqueous methanol supernatant and evaporated to dryness. The residue was dissolved in ethyl acetate (20ml) and partitioned with distilled water (2 x 20ml). The ethyl acetate fraction was evaporated to dryness dissolved in a few drops of alcohol and spotted onto thin-layer silica gel coated chromatography plates (TLC). The TLC plates were run in a continuous elution system with chloroform:acetone (98:2) for 3 hours. The area corresponding to standard oestradiol was scraped off and eluted with alcohol, evaporated to dryness and the precipitate redissolved in 4ml ether:pet ether 40-60 (1:1). NaOH (1ml N x 3) was used to extract the steroid and the organic layer washed with 1ml of water. The NaOH and aqueous extract were combined and neutralised with 0.56 g NaHCO₃. The oestrogen fraction was extracted using ethyl acetate (2 x 2 ml), dried down and spotted on to TLC plates and run for 1 hour in benzene: ethylacetate (4:1). The oestradiol was eluted with alcohol, evaporated to dryness and then acetylated by incubation for an hour

at 60°C with acetic anhydride (6 drops) and pyridine (3drops). Methanol was then added and the mixture evaporate to dryness, redissolved in a few drops of ethanol and rerun on TCL plates in cyclohexane: ethyl acetate (7:3) for 1 hour. The fraction corresponding to oestradiol diacetate was eluted with ethanol, evaporated to dryness and then hydrolysed by incubating overnight (37°C) in 1ml of methanol and 0.25ml 2% K₂CO₃. Distilled water (3ml) was added and the steroid extracted with ethyl acetate (2 x 5ml). The purified oestradiol fraction was then rerun on TCL in benzene:ethyl acetate (4:1) for 1 hour and eluted. This represented the first derivative and aliquots were taken for counting of radioactivity and estimation of cold oestradiol by UV spectrophotometry (282nm) using a known oestradiol concentration as a standard. The specific radioactivity of the derivative was expressed as dpm³H/nmol oestradiol. The remaining oestradiol was reacylated, run on TCL plates in cyclohexane:ethyl acetate (7:3) for an hour, the oestradiol diacetate eluted and specific radioactivity measured. The third derivative was obtained by hydrolysis as previously described and the free oestradiol run in benzene:acetate (4:1) for an hour. The free oestradiol was eluted and the specific radioactivity measured. If this was not within 10% of that of the diacetate then the derivative formation and characterisation was continued until it was. A fourth derivative was obtained by methylation of the free oestradiol. Distilled water (10ml) were added to the dried steroid, followed by boric acid (0.18g), NaOH (0.8ml, 20%) and dimethylsulphate (0.2ml). The steroid was dissolved by shaking in a water bath (37°C) and then incubated for 30 mins. A further 0.4mls of NaOH (20%) and 0.2mls dimethyl sulphate were then added, the solution shaken and reincubated for 30 mins (37°C). The incubation was culminated by the addition of NaOH (2ml, 20%) and hydrogen peroxide (0.5ml, 30%). The methylated oestradiol was extracted with 25ml pet. ether, redissolved in a spot of alcohol and run on TCL in cyclohexane:ethyl acetone (1:1), eluted and its specific radioactivity determined. This required to be within a 10% error of the specific activity of the third derivative. When the specific activities were in agreement, the % conversion was determined using the following formula

$$\% \text{ conversion} = \frac{\text{specific radioactivity} \times 1838 \times 100}{\text{dpm added initially}}$$

Aromatase activity was calculated from the % conversion based on the measurement of radioactivity in the purified oestradiol fraction and has been expressed as pmol E₂ produced/g/hr. Using these methods, conversions in excess of 0.02% (0.5 pmol

E²/g/hr) were measurable and values below this figure have been considered as negative.

Statistics

The relationship between the presence of aromatase and oestrogen receptor activity has been analysed using the Fishers exact test. The changes in tumour aromatase activity following systemic therapy have been analysed using the Wilcoxon-signed rank test.

Determination of Protein Concentration

This was adapted from the method of Bradford (Bradford 1976). Coomassie Blue Reagent (5ml) was added to 0.1ml blank, standards (10, 20, 30, 40, 50, 60, 70, 80 and 100mg bovine serum albumin/ 100mls 0.9% NaCl), quality controls (2 dilutions) and the samples. Following thorough mixing the solutions were left for 10mins at room temperature. The absorbance of each sample was read at 595nm in a spectrophotometer against a reagent blank. A plot of absorbance (595nm) is an almost linear curve. The protein concentration in each cytosol was established by reading from the standard curve, correcting for the dilution employed. All samples were measured in duplicate and the mean estimated.

Determination of Histological Grade

Tissue for grading was fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin wax and 6µ sections stained with haematoxylin and eosin. Grading was carried out on invasive cancer only using the modification to the criteria of Bloom and Richardson (Elston 1988). In order to check the degree of consistency all slides were graded by the same reader on two separate occasions without knowledge of the previous results. In cases of discrepancy the tumours were graded for a third time and a definitive grade assigned. Histological grading was obtained by analysis of the following three features, each of which was given a score of 1-3.

Tubule formation

Care was taken to differentiate between clefts due to tumour tissue shrinkage and tubule formation.

Score 1 - great majority of tumour composed of tubules with clearly visible lumina

2 - moderate amount of tubule formation but with areas of solid tumour growth

3 - little or no tubule formation, the cells mainly growing in sheets or cords.

Nuclear pleomorphism

The variability of both size and shape of tumour nuclei was assessed and scored.

Score 1 - little variation in size and shape of nuclei

2 - moderate variation without extremes of cell size or shape

3 - marked variation present with large bizarre nuclei and multiple nucleoli.

Mitotic Rate

Using a magnification of x 300 the number of mitoses per 10 fields were counted.

Score 1 - <10 mitoses per 10 fields

2 - 10 -19 mitoses per 10 fields

3 - ≥ 20 mitoses per 10 fields

To obtain the overall tumour grade the scores for each category were added, giving a possible score of between 3 and 9 points. Grade was then allocated on the following basis;

Grade 1 - well differentiated - 3-5 points

Grade 2 - moderately differentiated - 6-7 points

Grade 3 - poorly differentiated - 8-9 points.

Statistics

The relationship between histological grade, ER status, patient menopausal status, axillary node status and response to systemic therapy has been analysed using χ^2 test.

DNA Analysis

Fresh tumour was transported to the laboratory on ice and processed immediately. A portion of tumour was submitted for histological analysis and FNA biopsies performed on the remaining tumour. Using a 1cm³ syringe with a 23 guage needle the tumour was pierced, negative pressure applied and a gentle pumping action used to free cells. After releasing the negative pressure, the needle was withdrawn and any material held within the needle tip expelled into and flushed through with 200µl of citrate buffer. The specimen was rapidly frozen and stored at -40°C until analysis (Vindelov et al. 1983b).

The method used for DNA staining is an adaption of that used by Vindelov (Vindelov, Christensen, Nissen 1983; Vindelov et al. 1983a). Thawed cell suspension (100µl) was mixed with an internal standard (Vindelov, Christensen, Nissen 1983) of chicken red blood cells (15µl) and incubated for 10 minutes at room temperature with 0.003% solution of trypsin (450µl). A solution of trypsin inhibitor (375µl, 0.05%w/w) and RNAase (0.01%w/w) was then added and a further 10 minutes incubation carried out. The cells were then stained with a solution of propidium iodide (250µl, 416µl/ml) and spermine tetrahydrochloride (1.16mg/ml). Samples were kept on ice and run in the flow cytometer 30 to 120 minutes after addition of the propidium iodide solution. In order to break-up any particulate matter the cell suspension was vigorously passed through a 23 guage needle prior to insertion into the flow cytometer. Cellular DNA content was measured using an EPICS C flow cytometer (Coulter Electronics Ltd, Hialeah, Florida). A 5 watt argon laser was used to create a 250mW beam of 488nm light. Twenty thousand cellular events were acquired on a single parameter red fluorescence histogram. The chicken red blood cell internal control helped identify the Go/G1 diploid population. Analysis of the DNA histograms was using the para 2 software package (Coulter Electronics).

Samples for flow cytometric analysis of paraffin-embedded tumour blocks were prepared using the method of Hedley and colleagues (Hedley, Freidlander & Taylor, 1983). The presence of representative tumour tissue was confirmed on a 4µm haematoxylin and eosin section. Adjacent 30µm sections were cut, dewaxed in two changes of xylene (each for 10 min), rehydrated through decreasing concentrations of ethanol (100, 95, 70, 50%, each for 10 min) and washed twice in distilled water. Nuclei were released by cytoplasmic digestion, using 0.5% pepsin in 0.9% NaCl (pH 1.5) for 30min at 37°C. Release of nuclei was enhanced by intermittent vortex mixing. Samples were resuspended in 1µg of 4',6-diamidino-2-phenylindoldihydrochloride

(DAPI) per millilitre of RPMI tissue culture medium. DAPI is a fluorescent dye that binds to DNA. The quantity of DAPI bound is directly proportional to DNA content. Stained nuclear suspensions were filtered through 35µm nylon mesh and analysed on a the EPICS C flow cytometer, using ultra-violet excitation and blue fluorescent emission (408nm long pass filter). A total of 20,000 nuclei were analysed in each sample.

The diploid population was given a DNA index of 1.0. DNA content was defined as aneuploid, if there were 2 discrete Go/G1 peaks, the aneuploid peak containing at least 10% of the 20,000 cell events and there was an associated G2M peak. The aneuploid populations were subclassified as follows, DNA index (DI)<1.0 = hypodiploid, 1.0<DI< 1.9 = hyperdiploid, 1.9<DI<2.1 = tetraploid, DI>2.1 = hypertetraploid. By definition a tetraploid population had at least 20% of cellular events and a corresponding G2M peak. Tumours with more than one aneuploid peak were designated as multiploid. Histograms were categorised as “acellular” when there was no distinct Go/G1 peak or “unassessable” when either the coefficient of variation for the peak was >5 or the peaks could not be satisfactorily resolved. No attempt was made to determine % S-phase in aneuploid tumours.

The qualitative reproducibility of flow cytometric DNA analysis, using fine-needle aspiration (FNA) as a method of biopsy was measured by obtaining 4 separate FNA biopsies from each of 20 tumours.

Statistics

To express the variation in the measurement DI and % total cell count in aneuploid cell lines due to measurement error, the pooled variance and standard deviation has been used. All percentages are absolute figures i.e. % total cell counts.

The relationship between ploidy status and response to systemic therapy has been analysed by means of the Fishers exact test.

RESULTS AND DISCUSSION

CHAPTER 7

ASSESSMENT OF RESPONSE TO TREATMENT

Reproducibility of Clinical Mean Tumour Diameter

In order to assess the reliability of clinical mean tumour diameter, multiple measurements were performed in forty patient on 4 separate occasions before therapy had been instituted or a surgical biopsy performed. At least 4 hours were allowed to elapse between measurements. Results for both mean clinical tumour diameter and resulting volumes have been analysed in relation to standard deviation and an indication of variance is given by the pooled standard deviation (Table 7 and Table 8). For mean tumour diameter the pooled standard deviation was 0.0072 (SD range 0 - 0.183). For tumour volume the pooled standard deviation was 9.87 (SD range 0 - 17.1).

Statistical Assessment of Response

The reduction in tumour volumes achieved with systemic therapy in association with the gradients of the regression lines and corresponding statistical analysis as derived from linear regression analysis are shown in Table 9 for all 88 patients. Examples of regression lines are given in Figures 1, 3, 5, 7. The mean reduction in tumour value of those tumours categorised as having undergone significant regression was 72% (range 14 - 100%).

Table 7

Reliability of clinical measurements of mean tumour diameter by comparison of measurement taken at 4 different occasions from each of 40 tumours. Mean tumour diameter was calculated by computing the mean of 8 diameters taken at 22.5° angles with engineers calipers.

Clinical tumour diameter(cm)				Mean	Standard deviation	CV
1	2	3	4			
3.9	4.1	4.1	3.9	4.0	0.115	2.89
5.2	5.3	5.1	5.2	5.2	0.082	1.57
6.5	6.3	6.5	6.4	6.42	0.096	1.49
3.9	4.1	4.0	4.0	4.0	0.082	2.04
4.9	4.9	5.0	5.1	4.97	0.096	1.92
4.3	4.3	4.2	4.4	4.3	0.082	1.89
5.1	5.0	5.0	5.0	5.02	0.05	0.99
5.9	5.7	6.0	5.8	5.85	0.129	2.21
5.0	5.0	5.1	5.0	5.02	0.05	0.99
4.0	4.2	4.2	4.0	4.1	0.115	2.82
6.7	6.5	6.7	6.6	6.62	0.096	1.44
4.0	4.0	4.0	4.0	4.0	0	0.00
6.6	6.5	6.7	6.6	6.6	0.082	1.24
4.0	4.1	4.0	4.0	4.02	0.05	1.24
4.7	4.7	4.8	4.8	4.75	0.058	1.21
4.6	4.6	4.5	4.5	4.55	0.058	1.27
4.7	4.8	4.6	4.8	4.75	0.096	2.03
9.7	10.0	10.0	9.8	9.87	0.15	1.52
5.8	5.5	5.5	5.7	5.62	0.15	2.67
3.8	4.1	4.0	3.9	3.95	0.129	3.27
5.6	5.6	5.8	5.9	5.72	0.15	2.62
4.6	4.6	4.5	4.7	4.6	0.082	1.77
4.3	4.4	4.4	4.4	4.37	0.05	1.14
6.2	6.3	6.3	6.2	6.25	0.058	0.92
4.8	4.9	5.0	5.0	4.92	0.096	1.94
5.2	5.2	5.2	5.1	5.17	0.05	0.96
4.7	4.7	4.8	4.8	4.75	0.058	1.21
4.9	4.7	4.8	4.8	4.77	0.096	2.00

5.1	5.0	5.0	5.1	5.05	0.058	1.14
4.5	4.5	4.4	4.5	4.47	0.05	1.12
5.1	5.0	5.2	5.1	5.1	0.08	1.60
5.7	5.8	5.7	5.8	5.75	0.058	1.00
4.8	4.7	4.8	4.6	4.75	0.096	2.03
4.3	4.0	4.2	4.3	4.2	0.141	3.37
4.1	4.2	4.0	4.1	4.1	0.083	1.99
4.8	4.6	4.5	4.9	4.7	0.183	3.88
4.8	4.7	4.8	4.7	4.75	0.058	1.21
4.2	4	4.1	3.9	4.05	0.129	3.19
5.6	5.4	5.6	5.7	5.57	0.126	2.26
4.4	4.4	4.4	4.5	4.42	0.05	1.14
Mean				5.03	0.16	2.10

“Pooled” standard deviation = 0.0072

Table 8

Reliability of clinical measurements of volume as calculated from mean tumour diameter by comparison of measurement taken on 4 different occasions for each of 40 tumours. Mean tumour diameter was calculated by computing the mean of 8 diameters taken at 22.5° angles with engineers calipers. Volume was calculated from $4/3\pi r^3$.

Clinical tumour volume(cm ³)				Mean	Standard deviation	CV
1	2	3	4			
31.0	36.1	36.1	31.0	33.6	2.94	8.78
73.6	77.9	69.4	73.6	73.6	3.47	4.71
143.7	130.9	143.7	137.2	138.9	6.14	4.42
31.0	36.1	33.5	33.5	33.5	2.08	6.21
61.6	61.6	65.4	69.4	64.5	3.72	5.78
41.6	41.6	38.8	44.6	41.6	2.37	5.69
69.4	65.4	65.4	65.4	66.4	2.00	3.01
107.4	96.9	113.0	102.1	104.9	6.94	6.62
65.4	65.4	69.4	65.4	66.4	2.00	3.01
33.5	38.8	38.8	33.5	36.1	3.06	8.46
157.4	143.7	157.4	150.5	152.2	6.56	4.30
33.5	33.5	33.5	33.5	33.5	0	0
150.5	143.7	157.4	150.5	150.5	5.60	3.70
33.5	36.1	33.5	33.5	34.1	1.30	3.80
54.3	54.3	57.9	57.9	56.1	2.08	3.71
50.9	50.9	47.7	47.7	49.3	1.85	3.75
54.3	57.9	50.9	57.9	55.2	3.36	6.08
358.2	392.5	392.5	369.4	378.2	17.1	4.50
102.1	87.1	87.1	96.9	93.3	7.46	8.00
28.7	36.1	33.5	31.0	32.3	3.19	9.86
91.9	91.9	102.1	107.4	98.3	7.73	7.86
50.9	50.9	47.7	54.3	50.9	2.69	5.29
41.6	44.6	44.6	44.6	43.8	1.5	3.42
124.8	130.9	130.9	124.8	127.8	3.52	2.75
57.9	61.6	65.4	65.4	62.6	3.59	5.74
73.6	73.6	73.6	69.4	72.6	2.1	2.89
54.3	54.3	57.9	57.9	56.1	2.08	3.71

61.6	54.3	54.3	57.9	57.0	3.49	6.12
69.4	65.4	65.4	69.4	67.4	2.3	3.43
47.7	47.7	44.6	47.7	46.9	1.55	3.3
69.4	65.4	73.6	69.4	69.4	3.35	4.82
96.9	102.1	96.9	102.1	99.5	3.0	3.01
57.9	54.3	57.9	50.9	55.2	3.36	6.08
41.6	33.5	38.8	41.6	38.9	3.82	9.82
36.1	38.8	33.5	36.1	36.1	2.16	5.99
57.9	50.9	47.7	61.6	54.5	6.36	11.66
57.9	54.3	57.9	54.3	56.1	2.10	3.70
38.8	33.5	36.1	31.0	34.9	3.40	9.60
91.9	82.4	91.9	96.9	90.8	6.06	6.68
44.6	44.6	44.6	47.7	45.3	1.55	3.42
Mean				73.9	3.72	5.05

“Pooled” standard deviation = 9.76

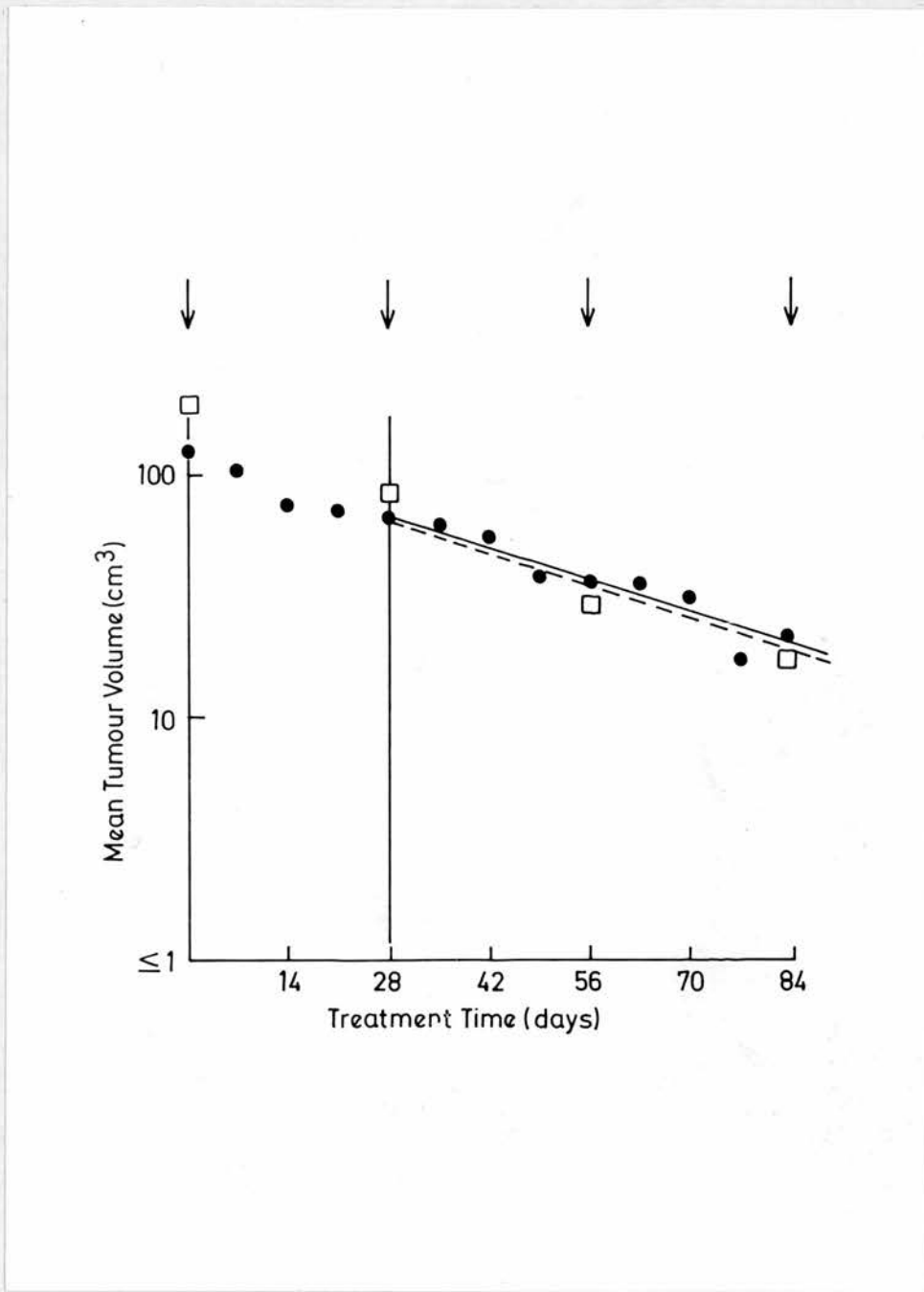


Figure 1
 Response of a premenopausal woman with a large operable breast cancer of ER concentration 46 fmol/mg cytosol protein following subcutaneous LHRH analogue goserelin (3.6mg) at 28 day intervals shown by arrows. Each closed point represents tumour volume as calculated from the mean clinical tumour diameter, while each open point is the volume calculated from the mean mammographic diameter. The calculated regression line had a correlation coefficient of -0.96 and a slope of $-9 \times 10^{-3} \text{ cm}^3(\log)/\text{day}$. This indicates statistically significant regression ($p < 0.003$, Student's t-test).

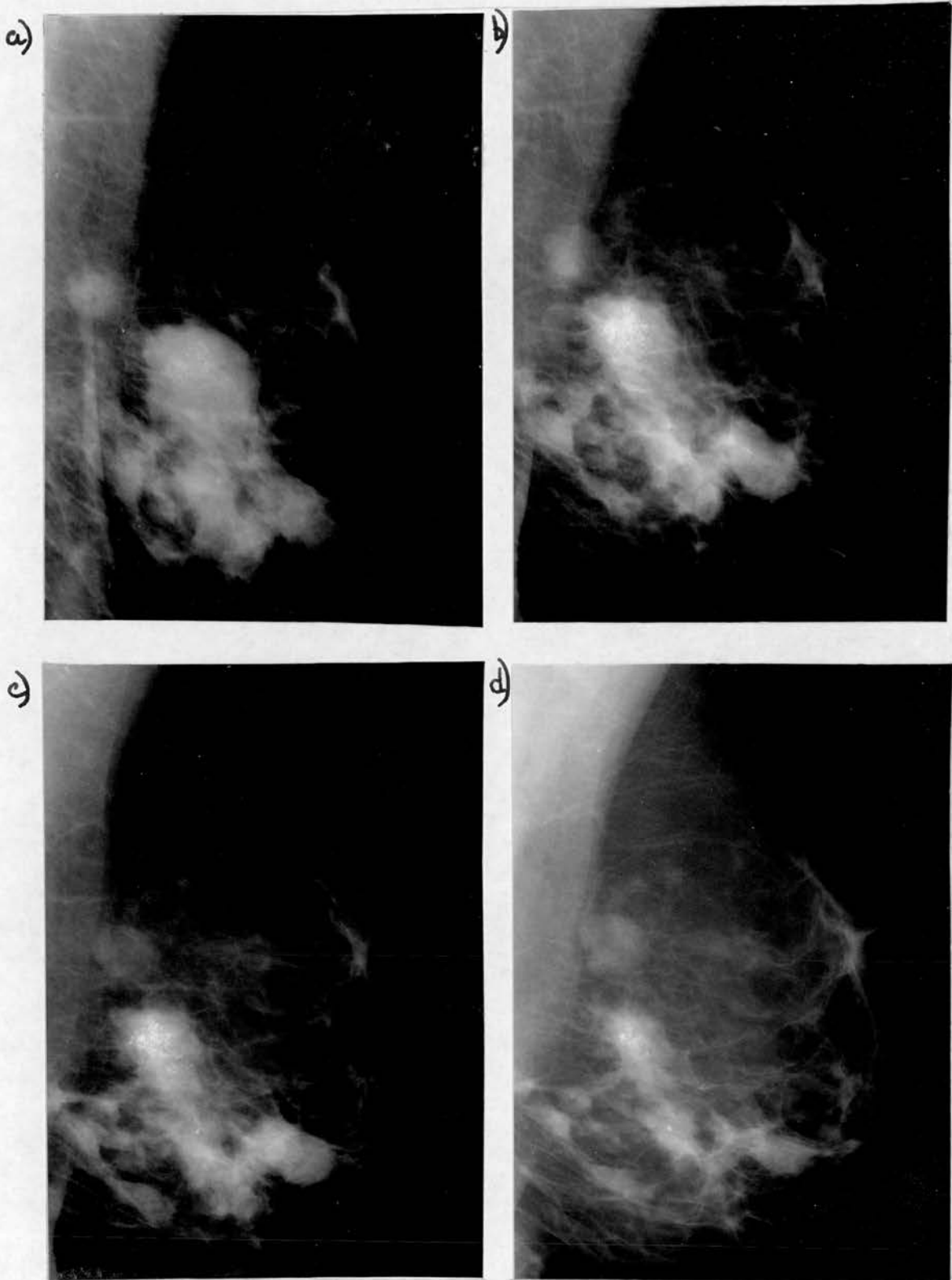


Figure 2

Response of a premenopausal woman with a large operable breast cancer of ER concentration 46 fmol/mg cytosol protein following subcutaneous LHRH analogue goserelin (3.6mg) at 28 day intervals as shown by serial changes in mammographic tumour size at weeks a) 0, b) 4, c) 8, and d) 12. A significant reduction in tumour volume was observed (Figure 1).

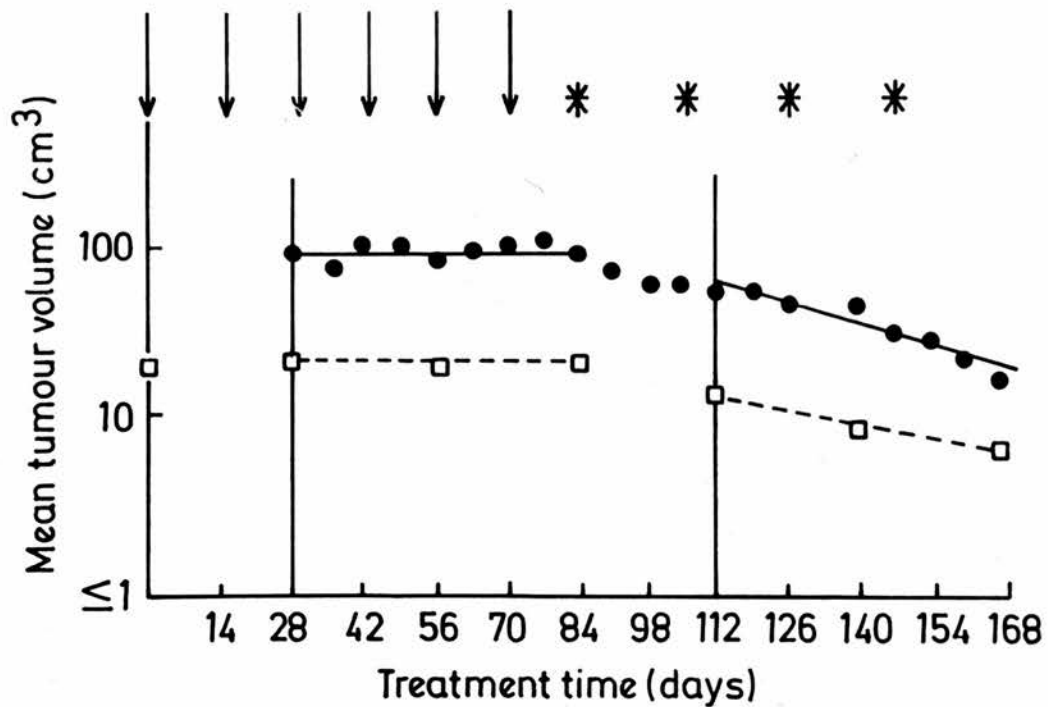


Figure 3

Response of a postmenopausal woman with a large operable breast cancer of ER concentration 81 fmol/mg cytosol protein following intramuscular 4-hydroxyandrostenedione (250mg) at 14 day intervals as shown by arrows. Each closed point represents tumour volume as calculated from the mean clinical tumour diameter, while each open point is the volume calculated from the mean mammographic diameter. The calculated regression line had a correlation coefficient of 0.24 and a slope of $1 \times 10^{-3} \text{ cm}^3(\log)/\text{day}$. This indicates non significant regression ($p = 0.53$ Student's t-test). The patient then went on to receive 4 cycles of the cytotoxic regime CHOP as shown by the stars. The calculated regression line had a correlation coefficient of -0.92 and a slope of $-6 \times 10^{-3} \text{ cm}^3(\log)/\text{day}$. This indicates statistically significant regression ($p < 0.004$, Student's t-test).

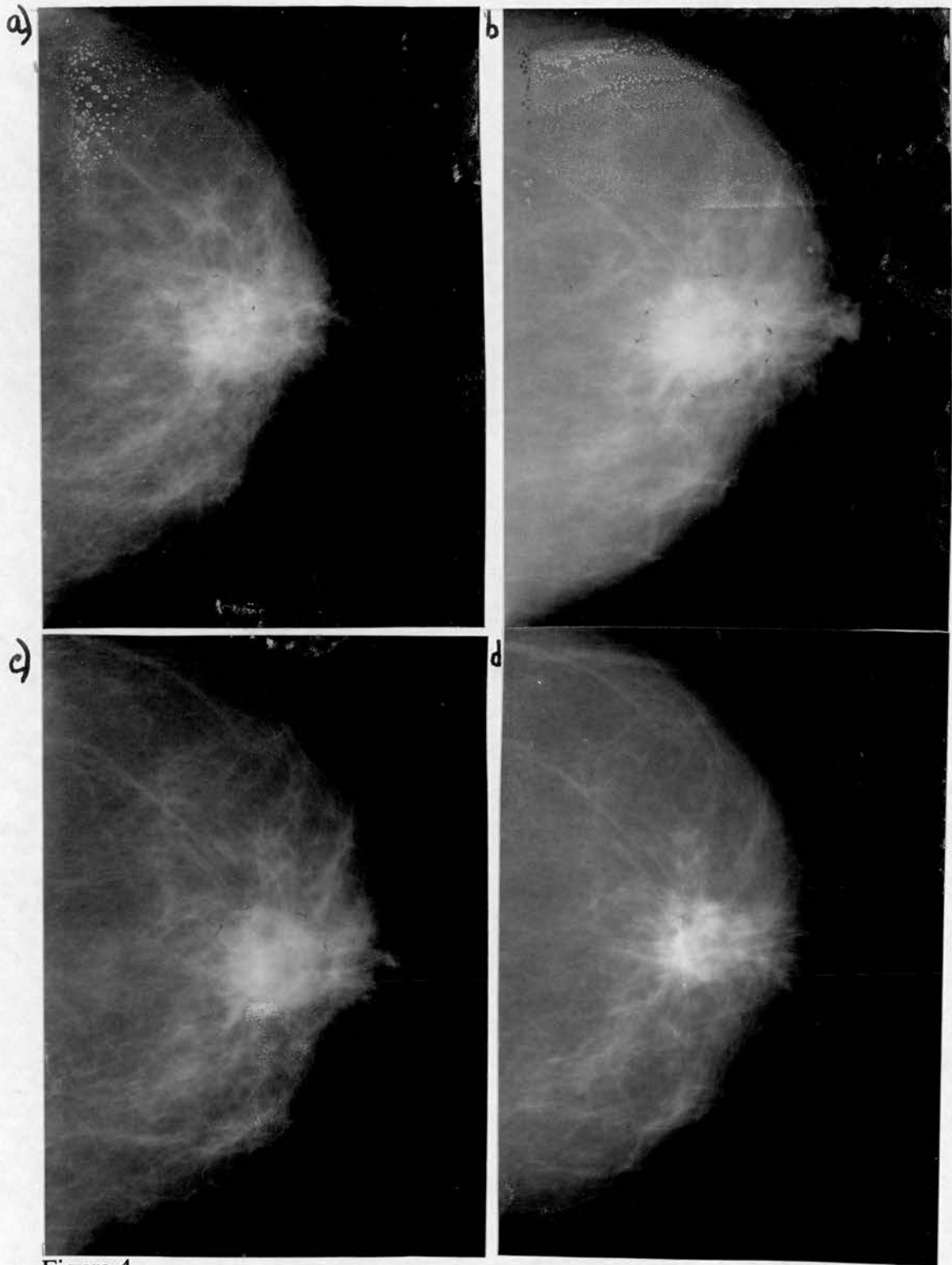


Figure 4

Response of a postmenopausal woman with a large operable breast cancer of ER concentration 81 fmol/mg cytosol protein following intramuscular 4-hydroxyandrostenedione (250mg) at 14 day intervals for 84 days followed by 4 cycles of the cytotoxic regime CHOP at 3 weekly intervals, as shown by serial changes in mammographic size at weeks a) 0, b) 12, c) 16, and d) 24. No change in tumour size was observed between treatment weeks 0 and 12. There was a significant reduction in tumour size following chemotherapy (Figure 3).

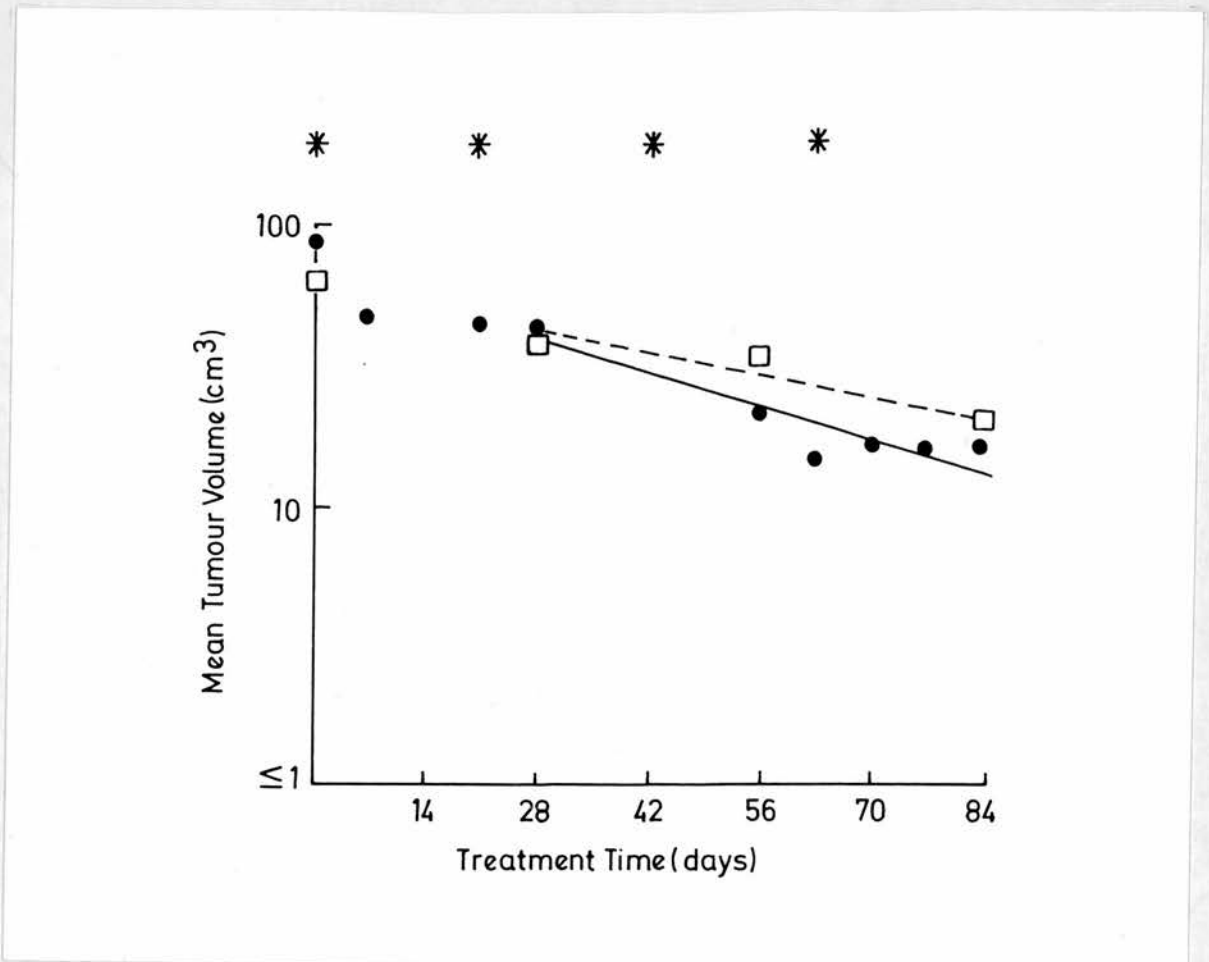


Figure 5. Response of a postmenopausal woman with a large operable breast cancer of ER concentration 19 fmol/mg cytosol protein following 4 cycles of the cytotoxic regime CHOP (shown by star). Each closed point represents tumour volume as calculated from the mean clinical tumour diameter, while each open point is the volume calculated from the mean mammographic diameter. The calculated regression line had a correlation coefficient of -0.80 and a slope of $-2.4 \times 10^{-2} \text{ cm}^3(\log)/\text{day}$. This indicates statistically significant regression ($p < 0.003$, Student's t-test).

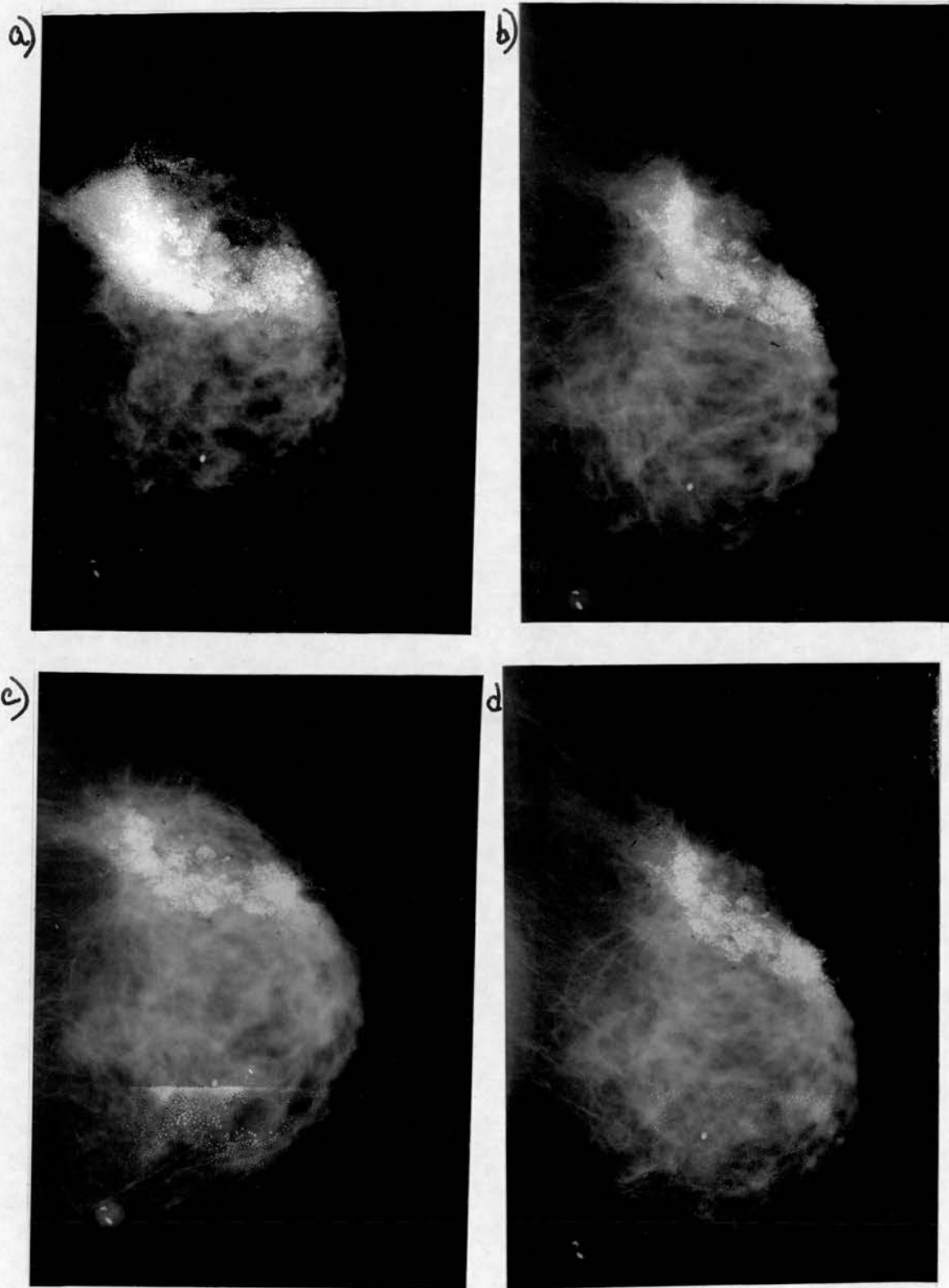


Figure 6.
Response of a postmenopausal woman with a large operable breast cancer of ER concentration 19 fmol/mg cytosol protein (Figure 5) following 4 cycles of the cytotoxic regime CHOP as shown by serial changes in tumour size at a) 0, b) 4, c) 8 and d) 12 weeks. This patient achieved significant regression, note the gradual disappearance of both opacification and microcalcification.

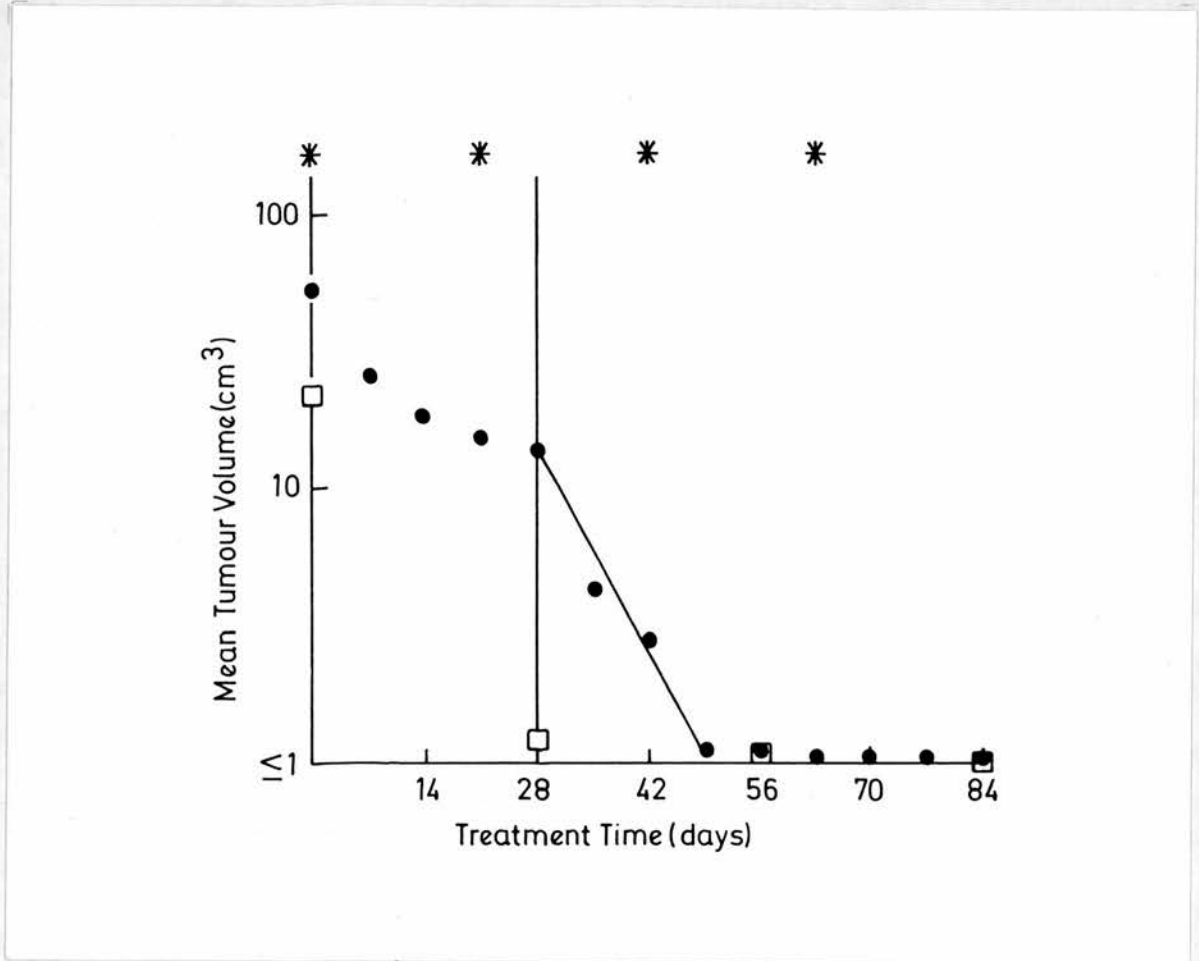


Figure 7

Response of a premenopausal woman with a large operable breast cancer of ER concentration 8 fmol/mg cytosol protein following 4 cycles of the cytotoxic regime CHOP (shown by star). Each closed point represents tumour volume as calculated from the mean clinical tumour diameter, while each open point is the volume calculated from the mean mammographic diameter. The calculated regression line had a correlation coefficient of -0.70 and a slope of $-4 \times 10^{-2} \text{ cm}^3(\log)/\text{day}$. This indicates statistically significant regression ($p < 0.04$, Student's t-test). The patient achieved a clinically and histologically complete regression.

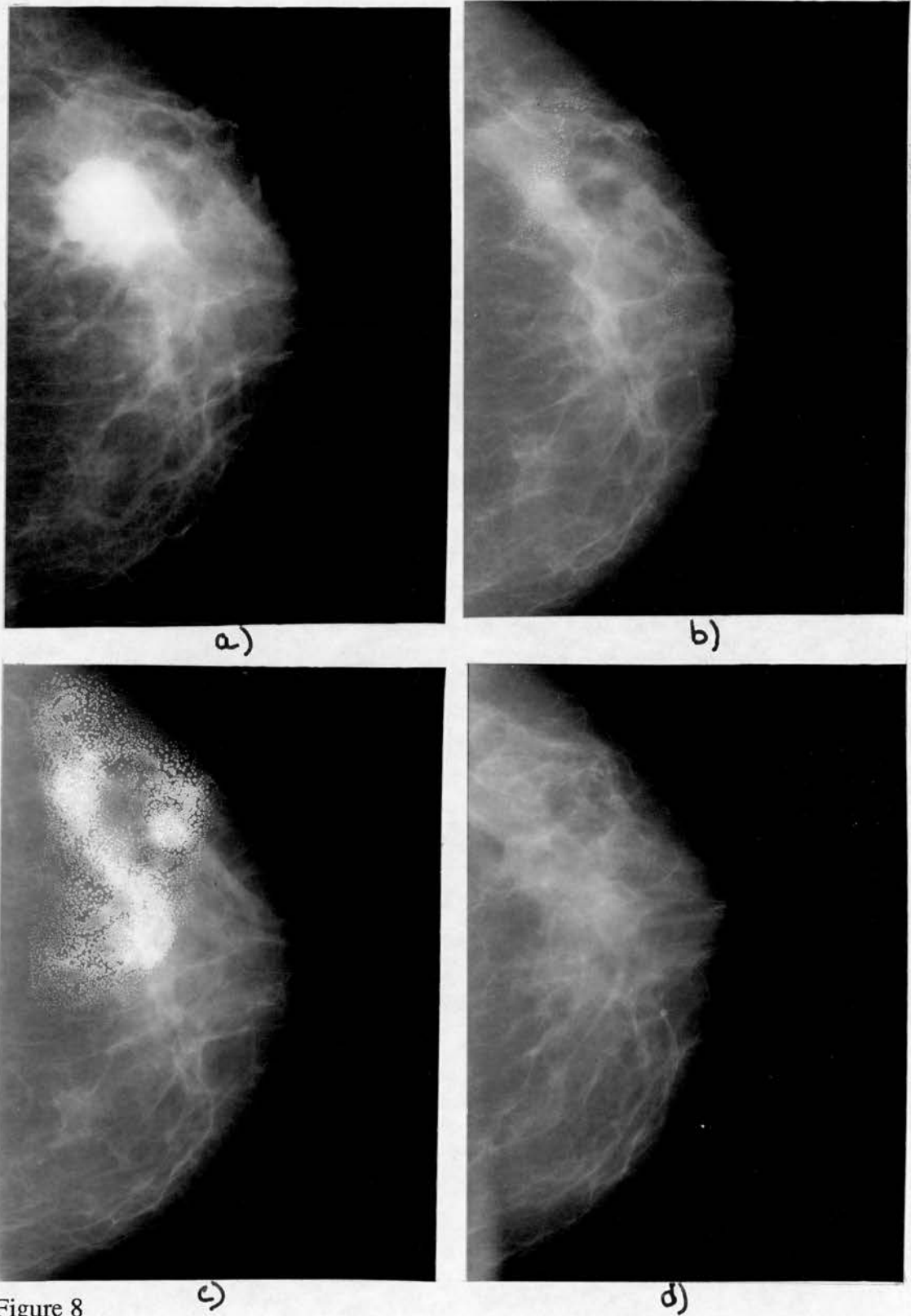


Figure 8
Response of a premenopausal woman with a large operable breast cancer of ER concentration 8 fmol/mg cytosol protein following 4 cycles of the cytotoxic regime CHOP as shown by serial changes in mammographic tumour size at a) 0, b) 4, c) 8 and d) 12 weeks. This patient achieved a clinically and histologically complete regression (Figure 7).

Table 9

Individual patient details of tumour volumes, regression analysis and clinical response in relation to systemic therapy. Tumour volume was calculated from $\frac{4}{3}\pi r^3$ mean clinical volume.

Tumour volume (cm ³)				gradient	t ^a	p	Clinical response
Day 0	28	84	168				
Oophorectomy							
33.5	36.1	28.7	-	-0.002	2.48	0.04	R
91.9	57.9	26.5	-	-0.005	5.83	0.01	R
102	54.3	57.8	-	+0.00005	1.07	0.33	NC
Oophorectomy + CHOP							
113.1	77.9	107.4	-	+0.005	2.85	0.07	NC
-	-	107.4	107.4	-0.0002	0.13	0.9	NC
69.4	54.3	50.9*	-	+0.002	0.89	0.4	P
-	-	50.9*	14.1	-0.06	3.1	0.01	R
Zoladex							
41.6	24.4	4.2	-	-0.017	3.73	0.02	R
96.9	38.8	8.2	-	-0.12	6.53	0.0006	R
28.7	15.6	4.8	-	-0.009	2.68	0.04	R
239	172	4.2	-	-0.029	9.3	0.0007	R
36.1	22.4	12.8	-	-0.003	1.57	0.17	NC
41.6	24.4	22.4	-	+0.001	0.91	0.4	NC
50.9	44.6	47.7	-	+0.001	1.49	0.19	P
Zoladex + Oophorectomy							
91.9	50.9	22.4	-	-0.006	7.8	0.0001	R
57.9	36.1	1.77	-	-0.02	4.75	0.003	R
157	65.4	22.4	-	-0.009	5.4	0.003	R
Zoladex + CHOP							
33.5	31.0	22.4	-	-0.002	2.26	0.06	NC
-	-	22.4	0	-0.034	6.13	0.002	CR

Tumour volume (cm ³)				gradient	t ² a	p	Clinical response
Day 0	28	84	168				
92	113	143*	-	+0.007	1.08	0.47	P
-	-	143*	120	+0.002	2.19	0.09	NC
77.9	47.7	73.6	-	+0.002	1.87	0.1	NC
-	-	73.6	54.3	0.0009	0.938	0.39	NC
44.6	24.4	22.4	-	-0.0006	0.40	0.77	NC
-	-	73.6	69.4	-0.0002	0.314	0.77	NC
50.9	73.6*	-	-	+0.006	-	-	P
-	73.6*		57.8	+0.003	1.27	0.24	NC
143	131	187*	-	+0.002	1.77	0.14	P
-	-	187*	0	-0.06	6.25	0.001	CR
151	195	204*	-	+0.001	0.16	0.88	P
-	-	204*	65.4	-0.009	3.45	0.04	R
57.9	69.4	87.1*	-	+0.14	0.15	0.67	P
-	-	87.1*	0	-0.028	5.46	0.002	CR
41.6	44.6	61.6*	-	+0.006	1.69	0.189	P
-	-	61.6*	8.1	-0.01	5.13	0.004	R

Aminoglutethimide + Hydrocortisone

54.3	41.6	20.6	-	-0.005	3.95	0.007	R
77.9	82.4	38.8	-	-0.003	3.11	0.01	R

Day 0	Tumour volume (cm ³)			gradient	t ² a	p	Clinical response
	28	84	168				
26.5	24.4	8.2	-	-0.007	2.7	0.03	R
54.3	50.9	24.4	-	-0.006	7.95	0.001	R
57.9	57.9	57.9	-	+0.0002	0.221	0.83	NC
87.1	73.6	73.6	-	-0.0004	0.394	0.72	NC
87.1	38.8	28.7	-	-0.002	1.47	0.2	NC

Aminoglutethimide + Hydrocortisone + CHOP

47.7	38.8	54.3	-	+0.003	2.21	0.09	NC
-	-	54.3	4.2	-0.015	8.88	0.003	R
96.9	54.3	57.9*	-	+0.00004	0.04	0.97	P
-	-	57.9*	24.4	-0.005	2.3	0.06	NC
50.9	44.6	57.9	-	+0.004	2.21	0.11	P
-	-	57.9	0	-0.023	5.17	0.007	CR

4-Hydroxyandrostenedione

38.8	24.4	8.2	-	-0.01	4.4	0.004	R
91.9	65.4	44.6	-	-0.004	3.14	0.02	R
41.6	44.6	18.8	-	-0.009	8.58	0.0001	R
57.9	18.8	7.2	-	-0.006	2.48	0.04	R
38.8	33.5	7.2	-	-0.011	7.25	0.0003	R
31	24.4	14.1	-	-0.004	7.9	0.0002	R

Day 0	Tumour volume (cm ³)			gradient	t ^a	p	Clinical response
	28	84	168				
33.5	20.6	8.2	-	-0.005	3.14	0.02	R
61.6	33.5	15.6	-	-0.007	1.92	0.09	NC
28.7	17.1	17.1	-	+0.0005	0.50	0.631	NC
41.6	36.1	26.5	-	-0.004	2.1	0.08	NC
33.5	18.8	12.8	-	-0.002	1.89	0.11	NC
77.9	47.7	54.3	-	+0.0002	0.334	0.75	NC
54.3	33.5	22.4	-	-0.002	3.89	0.008	P [^]

4-hydroxyandrostenedione + CHOP

113	96.9	96.9	-	+0.001	0.66	0.53	NC
-	-	96.9	22.4	-0.006	5.2	0.003	R
65.4	87.1*	-	-	+0.007	-	-	P
-	87.1*		61.6	-0.001	0.468	0.66	NC
73.6	69.4	69.4	-	+0.001	0.82	0.439	P
-	-	69.4	65.4	-0.002	0.88	0.42	NC

Tamoxifen

96.9	36.1	0	-	-0.03	4.04	0.0001	R
96.9	41.6	10.3	-	-0.012	4.15	0.004	R
44.6	38.8	22.4	-	-0.003	6.2	0.0002	R

Tumour volume (cm ³)				gradient	t ^a	p	Clinical response
Day 0	28	84	168				
38.8	22.4	14.1	-	-0.003	3.62	0.02	R
44.6	44.6	38.8	-	-0.001	0.244	0.82	NC
31	41.6	33.5	-	-0.0003	2.17	0.7	NC
118.8	77.9	54.3	-	-0.004	1.8	0.06	NC
150	157	477.7	-	+0.007	6.76	0.02	P
Tamoxifen + CHOP							
50.9	18.8	14.1	-	-0.002	1.05	0.40	NC
-	-	14.1	8.2	-0.002	1.47	0.1	NC
41.6	38.8	41.6*	-	+0.0004	0.293	0.79	P
-	-	41.6*	4.2	-0.011	4.58	0.006	R
65.4	77.9	64.5*	-	-0.006	3.4	0.18	P
-	-	64.5*	0	-0.022	6.07	0.004	CR
CHOP							
47.7	0	0	-	-0.12	~	~	CR
33.5	14.1	0	-	0.024	4.0	0.003	CR
54.3	14.1	0	-	-0.04	2.0	0.04	CR
131	3.1	0	-	-0.038	6.6	0.0001	CR

Day 0	Tumour volume (cm ³)			gradient	"t" ^a	p	Clinical response
	28	84	168				
44.6	1.44	0	-	-0.033	6.2	0.0003	CR
523	0	0	-	0.065	~	~	CR
33.5	2.57	0	-	-0.032	6.3	0.0002	CR
143.7	50.9	0	-	-0.021	2.93	0.02	CR
82.4	41.6	14.1	-	-0.008	7.96	0.001	R
69.4	65.4	41.6	-	-0.004	4.37	0.007	R
125	102.1	50.9	-	-0.005	6.87	0.001	R
41.6	6.4	1.77	-	-0.015	4.9	0.006	R
65.4	33.5	24.4	-	-0.004	5.2	0.001	R
157	61.6	17.1	-	-0.009	3.9	0.002	R
44.6	22.4	1.8	-	-0.016	3.1	0.01	R
54.3	28.7	7.2	-	-0.015	5.7	0.0008	R
57.9	31	18.8	-	-0.006	3.8	0.004	R
102.1	50.9	14.1	-	-0.012	6.1	0.0003	R
65.4	6.4	0.52	-	-0.013	5.7	0.0008	R
57.9	17.1	0.5	-	-0.022	5.2	0.001	R
36.1	8.2	0.5	-	-0.028	2.0	0.04	R

Day 0	Tumour volume (cm ³)			gradient	"t" ^a	p	Clinical response
	28	84	168				
57.9	17.1	1.2	-	-0.018	5.2	0.001	R
57.9	4.2	0.52 ⁺	-	-0.038	5.2	0.001	R
33.5	8.2	8.2	-	-0.0002	0.15	0.89	NC
33.5	15.6	11.5	-	-0.001	0.8	0.4	NC
33.5	20.6	26.5	-	+0.00002	0.02	0.98	NC
33.5	22.4	18.8	-	-0.001	0.02	0.98	NC

^a "t" and p values calculated from regression analysis.

* denotes that therapy has been prematurely terminated due to progression and figures quoted are for tumour diameters at the end of endocrine therapy.

~ details when regression analysis not possible between weeks 4 and 12 because regression so rapid that no clinical residual tumour by 4 weeks.

^ although regression analysis was significant this patient developed a new mammographic lesion during therapy which was confirmed to be malignant on histology.

+ patients treatment terminated after 3 cycles due to iliofemoral thrombosis.

Mammographic Regression

Examples of the changes in mammographic tumour size with systemic therapy are shown in Figures 2, 4, 6 and 8. Statistical analysis of mammographic changes in tumour volume was not practicable since measurements were only available for 3 time points i.e. 4, 8 and 12 weeks. In order to compare clinical with mammographic changes an arbitrary value of 50% reduction in tumour volume between treatment weeks 4 and 12 has been taken and the results tabulated for those tumours achieving statistical regression and those demonstrating no change (Tables 10 and 11). In 7 patients, mammograms showed diffuse changes only and the architectural abnormality was not

measurable. In general there was good agreement between clinical and mammographic estimation of volume changes. Of the 13 patients who achieved complete clinical regression all demonstrated a marked reduction in mammographic tumour size but 7 had some form of residual mammographic abnormality although most measured less than a centimetre. This did not relate to the presence of residual tumour on histological examination.

Table 10

Relationship between a demonstrated reduction in clinical and mammographic volume between treatment weeks 4 and 12 in 58 patients with large operable breast tumours who on statistical analysis of changes in tumour volume demonstrated significant regression.

Clinical volume reduction	Mammographic volume reduction	
	<50 %	≥50 %
<50 %	3	2
≥50 %	7	40

6 patients had no definite mammographic abnormality.

Table 11

Relationship between a demonstrated reduction in clinical and mammographic volume between treatment weeks 4 and 12 in 33 patients with large operable breast tumours who on statistical analysis of changes in tumour volume demonstrated no significant regression and clinically did not demonstrate progression.

Clinical volume reduction	Mammographic volume reduction	
	<50 %	≥50 %
<50 %	27	2
≥50 %	2	0

1 patients had no definite mammographic abnormality.

DISCUSSION

A semiquantitative method of assessing tumour response has been reported. This is based on statistical analysis of changes in tumour volume during systemic therapy. Evidence would suggest that given a single experienced operator measurement of clinical diameter by estimating the mean of 8 caliper measured clinical diameters gives reproducible results. This method of measurement has been integral to assessment of response. The reproducibility studies were however performed on large tumours (mean diameter 5cms) prior to initiating systemic therapy and the reproducibility of measurement has been previously documented to be higher in tumours measuring greater than 3.2 cm (Warr, Mckinney & Tannock, 1985). As these large tumours regressed on systemic therapy, exact measurement of clinical size became more difficult, especially when tumours measured less than 2cm since there was often residual thickening around the central tumour nidus. This potential increase in the error rate with response to therapy may in part explain why in 2 cases the slope of the regression line failed to reach statistical significance at the 5% level despite over a 50% reduction in tumour volume between treatment weeks 4 and 12. In the remaining case where there was a significant difference between clinical measurements and eventual outcome, this was due to clinical evidence of progression with de novo presentation of breast lymphoedema.

In order to reduce the measurement errors introduced by pretreatment surgical biopsy of the tumour, clinical diameters taken during the first 4 weeks on treatment were not included in the statistical analysis of response and the biopsy was performed by a single committed surgeon in an attempt to standardise the amount of tumour removed from the tumour core. Although sensitive tumours may have shown a reduction in volume during this first 4 week period, if clinically relevant then a reduction in volume should continue. The exception to this were those exquisitely chemosensitive tumours which had become clinically impalpable by 4 weeks.

Changes in the radiological size of the tumour were not used to define response but rather to confirm clinical observations. In general there was close but not complete agreement between the two parameters. Clinical measurement was more likely to overestimated the degree of reduction ($n = 9$) than mammography ($n = 4$). The reasons for this discrepancy remain obscure. It could be argued that mammography is more objective than clinical examination but the employment of multiple clinical

measurements significantly reduces the intraobserver error. Also it is not possible by mammography to distinguish between a malignant and benign process hence making it difficult to differentiate between residual tumour and desmoplastic reaction.

Multiple measurements reduce the false positive error associated with basing categorisation of response on only two measurements and so the described method is more sensitive than the routinely used UICC criteria, which demands over an 80% reduction in tumour volume before regression can be defined (Hayward et al. 1977 Ward et al. 1977). In addition it allows assessment of the rate of regression although as yet this is of undefined biological significance. If the results are to be meaningful the method does require multiple regular attendance by the patient to the clinic in addition to the measurements being performed by a single highly committed clinician.

CHAPTER 8

ASSESSMENT OF RESPONSE

Clinical Assessment of Response

Response to Endocrine Therapy

Twenty-four of the 61 (39.3%) patients treated by primary endocrine therapy demonstrated significant regression of their tumours at 12 weeks (Table 12). The overall response rate appeared to be independent of menopausal status or endocrine therapy used (Table 13), with the exception that none of the 3 postmenopausal patients who received goserelin therapy achieved significant regression.

Rate of Regression with Endocrine Therapy

The rate of reduction in tumour size which followed endocrine therapy was variable but was in general slow (Figure 9). Although overlap did exist in relation to the gradient of the regression slope between some patients who achieved regression on therapy and others who were categorised as no change, eventual determination of response was a function of the confidence of the regression slope, some tumours being more difficult to measure consistently .

The median time taken to achieve half volume (T1/2) in those patients who showed significant regression with endocrine therapy was 44 days (range 3 - 150 days, Figure 11). The rates of regression have been related to the specific endocrine therapy (Table 13). Premenopausal women treated by goserelin appeared to have a slightly faster rate of regression (median 25 days, range 10-50 days) than other patients but the numbers within each treatment group are small. No correlation was demonstrated between the time taken to achieve half volume and the ER concentration of the tumour (Figure 10). The median T1/2 for premenopausal women was 33 days (range 10 - 150 days) while for postmenopausal women it was 50 days (range 3 - 150 days). the difference is not significant (Mann -Whitney U Test, $U = 43.5$, $p < 0.05$). Only one tumour (treated with tamoxifen) showed complete clinical regression within the time period of the study. For various reasons the duration of preoperative antioestrogen therapy was prolonged beyond 12 weeks in 8 patients. Seven tumours continued to regress at the same rate, but one tumour previously regarded as static, underwent a rapid reduction in size at 5 months.

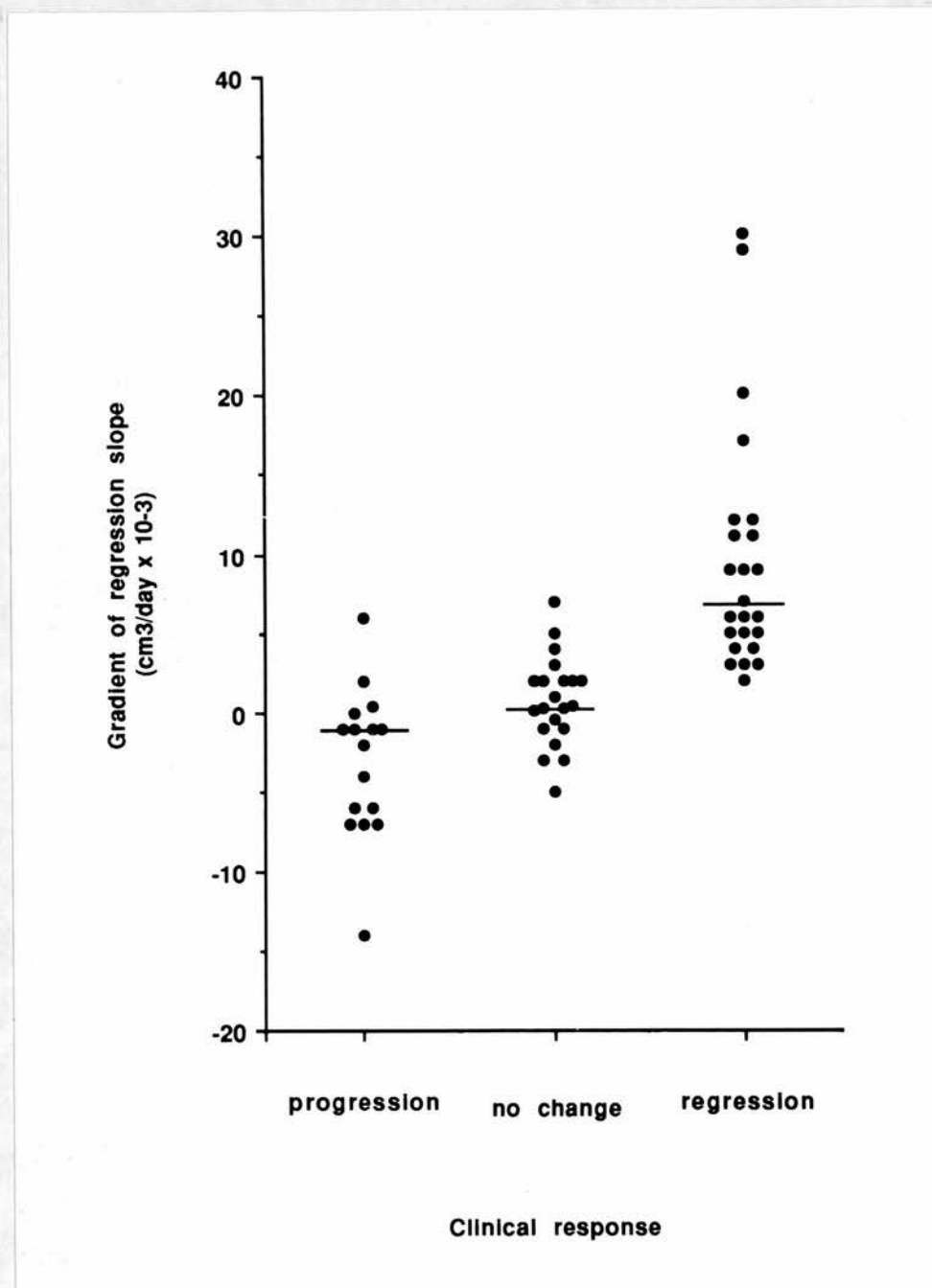


Figure 9
 Relationship between the rate of regression as measure by gradient of regression slope and response of the primary tumour to endocrine therapy in 61 patient with large operable breast cancers. The rate of regression was analysed from linear regression analysis of changes in tumour volume between treatment weeks 4 and 12. The bars denote median values.

Table 12

Relationship between response to hormonal therapy and oestrogen receptor concentration of the primary tumour as determined by the dextran-coated charcoal adsorption method in 61 patients with large operable breast cancer.

	NO. OF PATIENTS WITH SIGNIFICANT REGRESSION/ TOTAL		
	ER<20*	ER>20*	TOTAL
PREMENOPAUSAL			
oophorectomy	0/2	2/3	2/5
goserelin	0/3	7/13	7/16
POSTMENOPAUSAL			
tamoxifen	0/5	4/6	4/11
aminoglutethimide	0/4	4/6	4/10
4-hydroxyandrostenedione	-	7/16	7/16
goserelin	0/1	0/2	0/3
TOTAL	0/15	24/46	24/61

* fmol/mg cytosol protein

Table 13

The relative rates of regression as measure by time to half volume in relation to type of endocrine therapy employed in the 24 patients who achieved significant regression on endocrine therapy.

Premenopausal	
Oophorectomy (n = 2)	- (44 - 150 days)
Goserelin (n = 7)	25 (10 - 50 days)
Postmenopausal	
Tamoxifen (n = 4)	101 (3 - 102 days)
Aminoglutethimide (n = 4)	60 (43 - 100 days)
4-Hydroxyandrostenedione (n = 7)	50 (27 - 75 days)
Total (n = 24)	44 (3 - 150 days)

Response to Cytotoxic Therapy

A significant reduction in tumour volume was observed in 34 of the 47 patients (72.3%) who received cytotoxic therapy (Table 14). Thirteen patients (27.6%) had complete clinical regression of their tumour. No patient showed evidence of tumour progression during treatment with chemotherapy.

Table 14

Response rates in 47 patients with large operable cancers of the breast treated with 4 cycles of the chemotherapeutic regime CHOP before definitive locoregional surgery.

	No. with significant regression / total	No. with complete clinical regression
Primary cytotoxic therapy		
ER <20*	23/27	8
Following failed endocrine therapy		
ER < 20*	8/10	4
ER ≥ 20*	3/10	1
Total	34/47	13

* fmol/mg cytosol protein

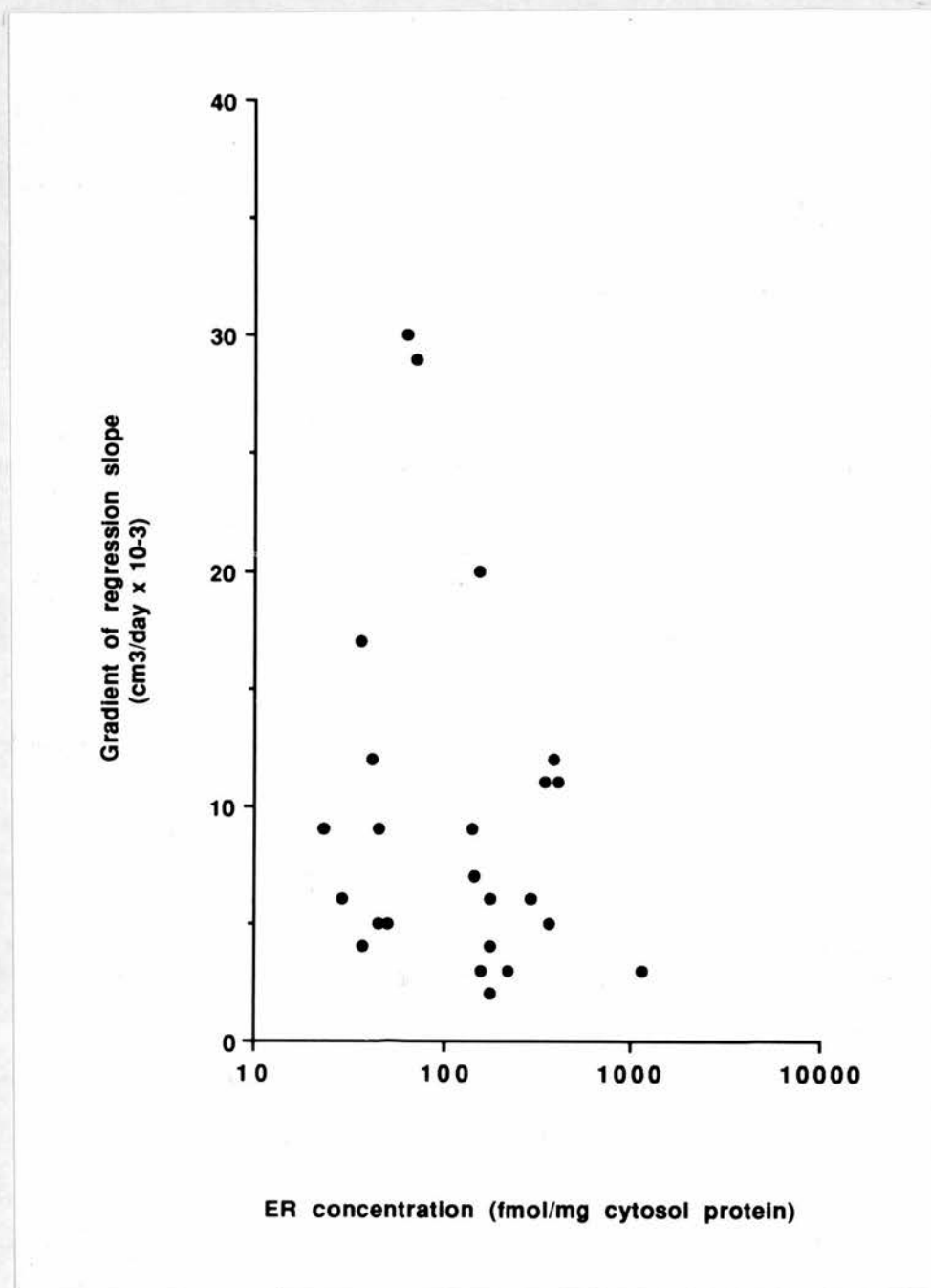


Figure 10
 Relationship between the gradient of the regression slope and ER concentration in 24 patients who demonstrated significant regression of their primary tumour to endocrine therapy. The relationship is not statistically significant ($R = 0.22$, Spearman Rank Test)

Rate of Regression with Cytotoxic Therapy

There was a wide variation in the rate at which individual tumours responded to chemotherapy (Figure 12). The rate of regression was on average more rapid than that achieved with endocrine therapy, with a median T1/2 of 20.2 days (range 2.6 - 77.3 days, Figure 11). The correlation between the gradient of the regression slope and the clinical response as assessed at three months was high . Those patients with steeper regression slopes were more likely to achieve complete clinical response. The median time taken to reach 50% of the original pretreatment volume in those patients achieving complete clinical response was 9.2 days (range of 2.6 - 22.2 days).

Response to therapy measured at 42 days, i.e. after two cycles of chemotherapy, gave a strong indication of the likelihood of response at 84 days. The rate of regression was independent of ER content (Figure 13) of the primary tumour.

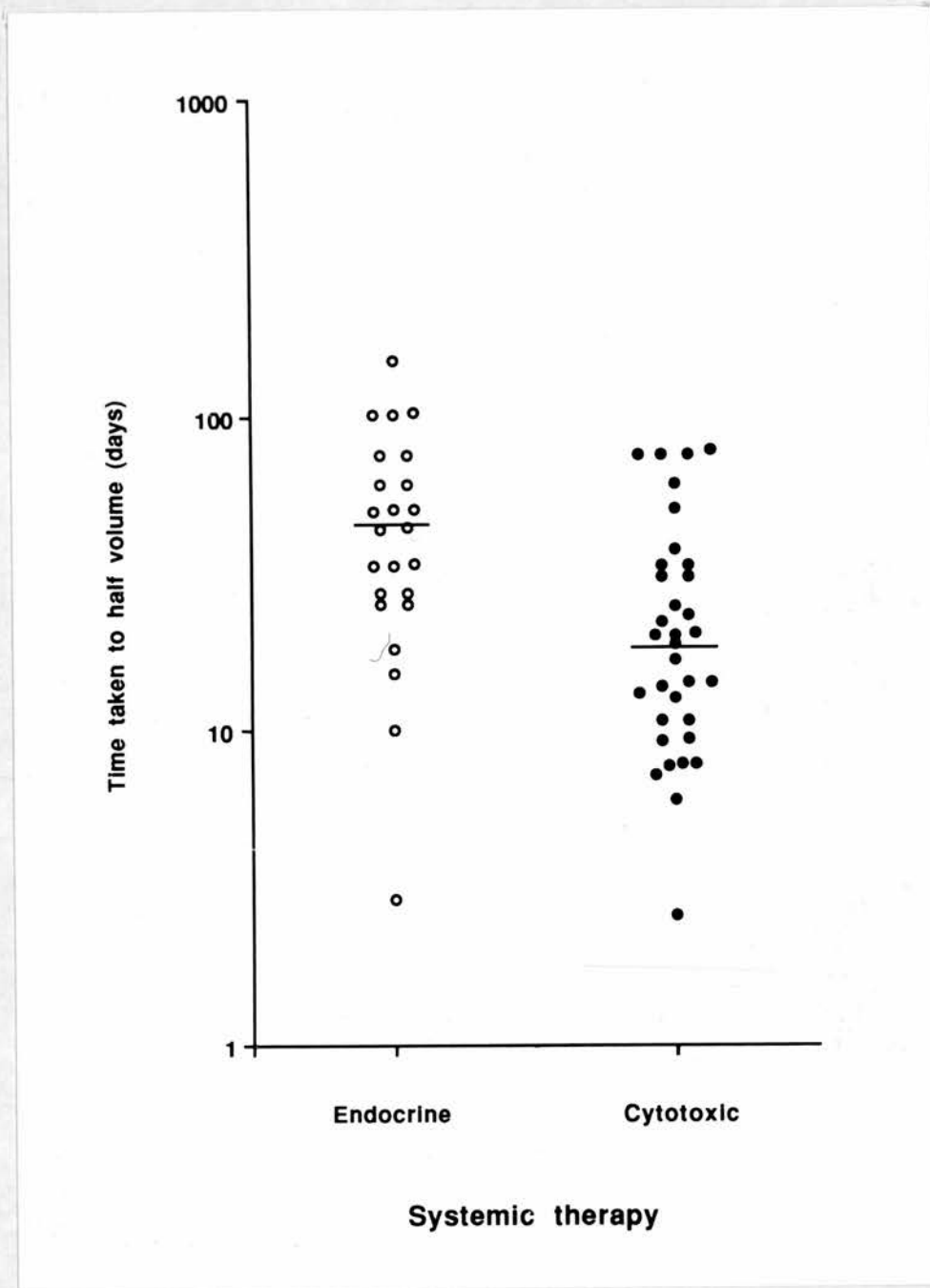


Figure 11
 Comparison of the time taken to achieve half volume in patients demonstration significant regression in endocrine (n = 24) and cytotoxic therapy (n = 34). The time taken to half volume was extrapolated from linear regression analysis of changes in actual tumour volume between weeks 4 and 12. The median values are shown by bars in each group.

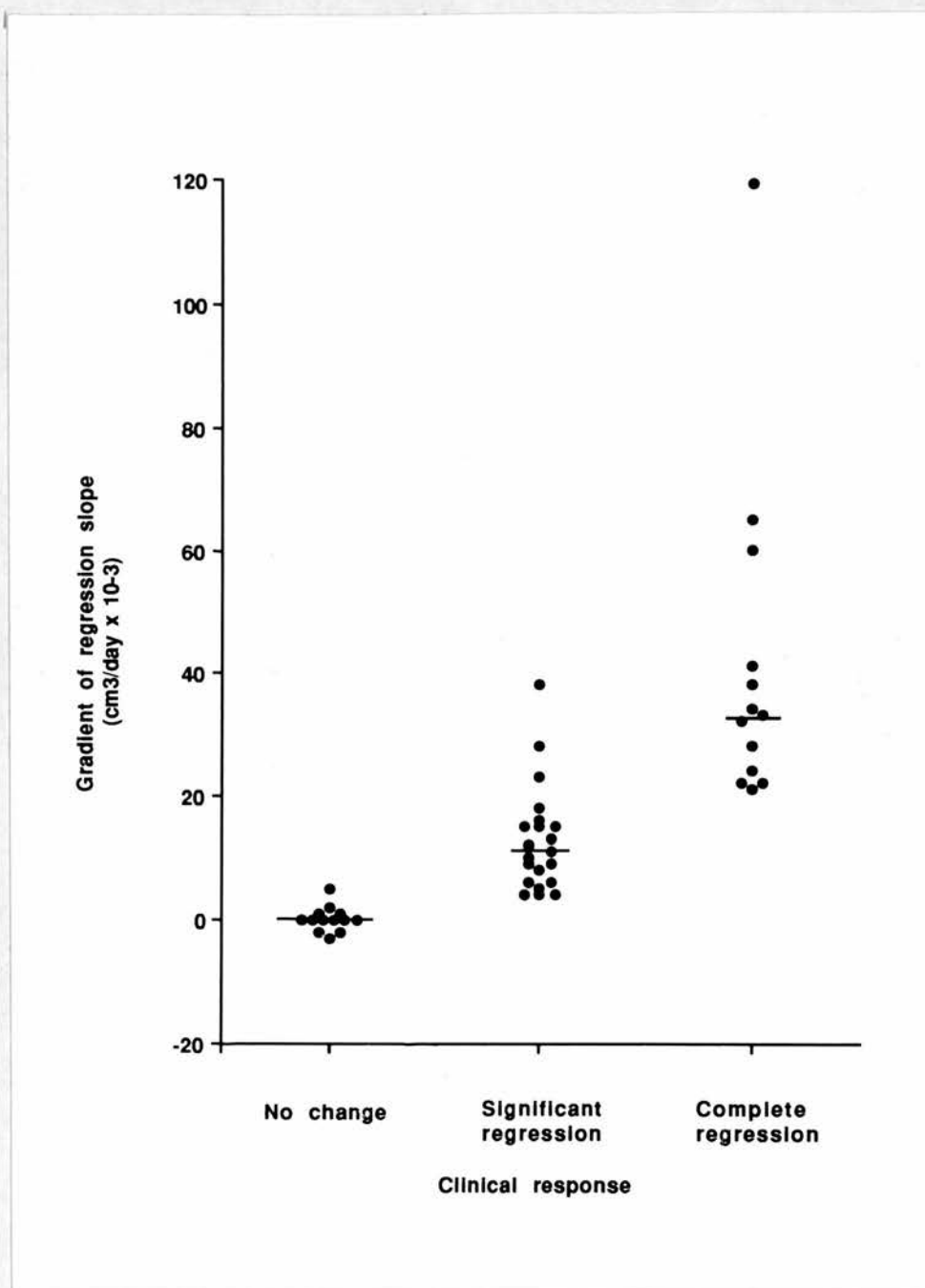


Figure 12
 Relationship between the rate of regression as measured by the gradient of the regression slope and response of the primary tumour to cytotoxic therapy, in 47 patients with large operable breast cancers. The regression was assessed from linear regression analysis of changes in tumour volume between treatment weeks 4 and 12. The median values are denoted by bars.

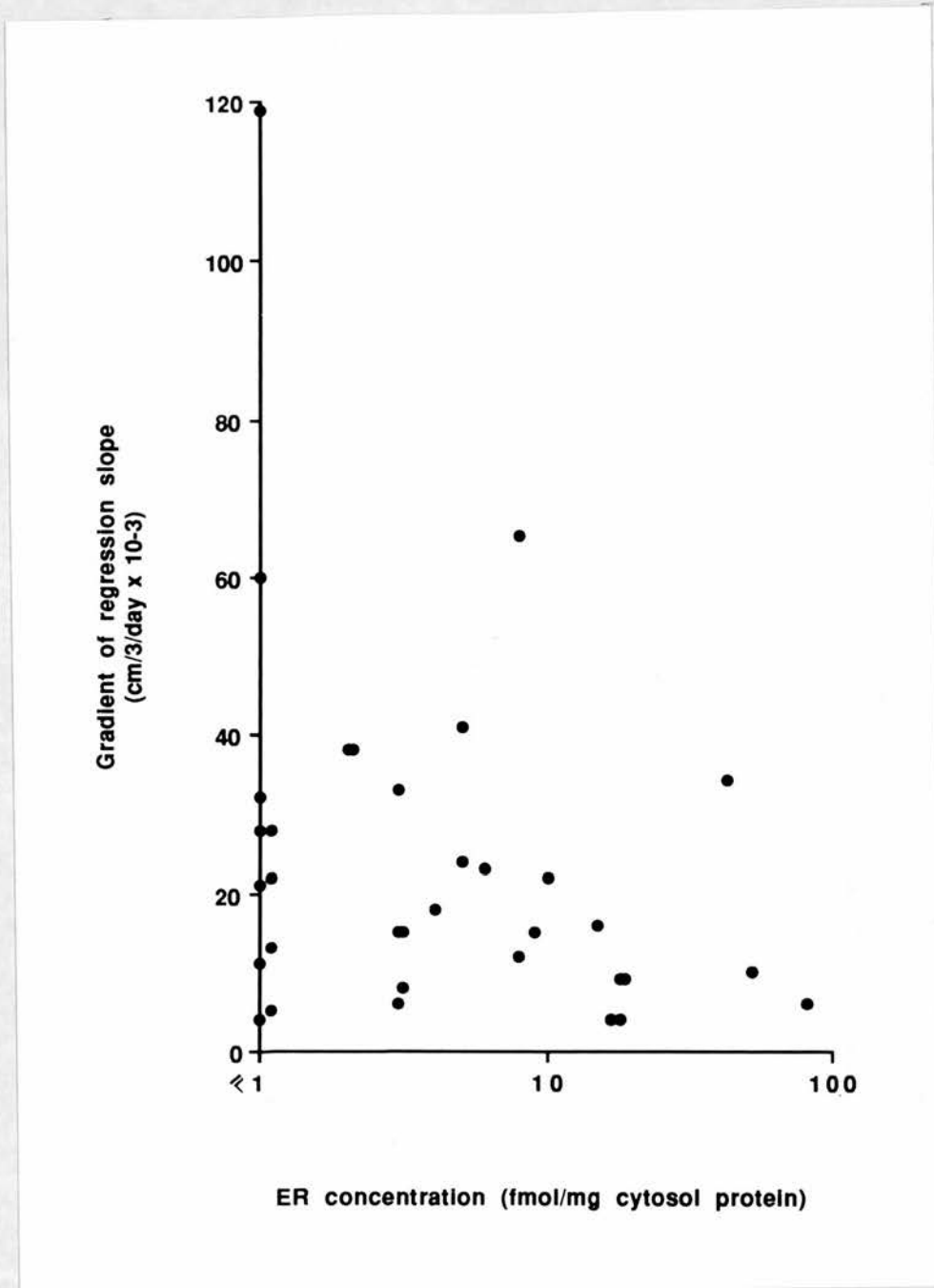


Figure 13
 Relationship between the rate of regression to cytotoxic therapy as measured by the gradient of the regression slope and the ER concentration of the primary tumour in the 34 patients who demonstrated significant regression to cytotoxic therapy. The relationship between the two parameters is not significant ($R = 0.255$, Spearman Rank Test).

Histological Assessment of Response

All tumours were ductal carcinoma of no special type although several showed features of differentiation, these were not thought to be present in sufficient quantity (>90%) to describe them as being of special type.

All patients, following 12 weeks treatment with endocrine therapy had residual histopathological disease in their mastectomy specimen. In 8 (17%) of the 13 patients who achieved complete clinical remission following 4 cycles of CHOP, there was no histological evidence of residual invasive carcinoma in the post-treatment specimen although in one of these there was a small focus of intraduct carcinoma. In the remaining 5 patients there was microscopic evidence of residual invasive carcinoma. For those patients with residual gross tumour there was little change in the overall histological pattern .

Axillary Lymph Node Status

Histological assessment of the axillary lymph node status was performed in 46 patients (51%) before and in 86 patients (98%) on completion of systemic therapy. Metastatic carcinoma was detected in 33 (72%) and 42 cases (49%) respectively. Overall 56 patients (64%) had axillary metastases detectable at some stage in their management.

Table 15.

Relationship between the presence or absence of axillary node metastases before and after primary systemic therapy in 43 patients with large operable breast cancer.

Post-treatment	Pre-treatment	
	metastasis	no metastasis
metastasis	19	1
no metastasis	14	9

Comparison of pre- and post-treatment axillary node status was possible in 43 patients (Table 15). Of the 33 patients in whom axillary node metastasis were detected pretreatment, 14 had no evidence of metastases following systemic therapy. Of these 8 had shown significant regression during systemic therapy of which 5 were clinically complete. Axillary node metastases were found in only one of the 10 patients in whom the pretreatment axillary node sample had failed to demonstrate metastases. This

patient did not response to 4-hydroxyandrostenedione or proceed to chemotherapy and so may represent a true progression of axillary node status.

Survival

The median time from initiation of systemic therapy to the date of survival analysis was 42 months (range 16 - 72 months); overall and relapse-free survival for all patients within the study is shown in Figure 14. Twenty-three patients have represented with recurrent disease while 13 patients have died from their disease. The cumulative overall survival of those patients surviving greater than 4 years ($n = 27$) was 77% (+/- 5%), with 67.2% (+/- 6%) of patients remaining disease free. The site of first tumour recurrence is shown in Table 16.

A total of 14 patients had pathologically proven local recurrence at the time of analysis although 6 occurred coincidentally with distant metastasis. The local recurrence rate is shown in Figure 14 and shows that at 4 years 83% (+/- 4%) of the patients had no evidence of local relapse. Following treatment with radiotherapy and further systemic therapy in one patient local disease remained uncontrolled at the patients death.

Survival and Prognostic Factors and Response to Therapy

Survival, distant disease-free and relapse-free survival has been related to the presence or absence of axillary node metastasis (Figure 15) and ER concentration of the primary tumour (Figure 16).

Overall and relapse-free and local recurrence free survival were all significantly better in that group of patients who had never demonstrated the presence of axillary node metastases in either their pretreatment or posttreatment axillary node specimen.

Within this series no significant relationship between survival and pretreatment tumour ER concentration was observed (Figure 16). However in general patients with a tumour of ER concentration of ≥ 20 fmol/mg cytosol protein fared better especially if overall survival was considered. In particular patients with endocrine-sensitive tumours, as a subgroup, exhibited a particularly good prognosis (Figure 17). To date within this group of 24 patients there have been no deaths although 6 patients have shown disease relapse. When compared to patients who did not exhibit significant regression with endocrine therapy, overall survival is significantly improved ($p = 0.01$), while both relapse-free survival ($p = 0.07$) and local disease free survival ($p = 0.08$) just fail to reach statistical significance.

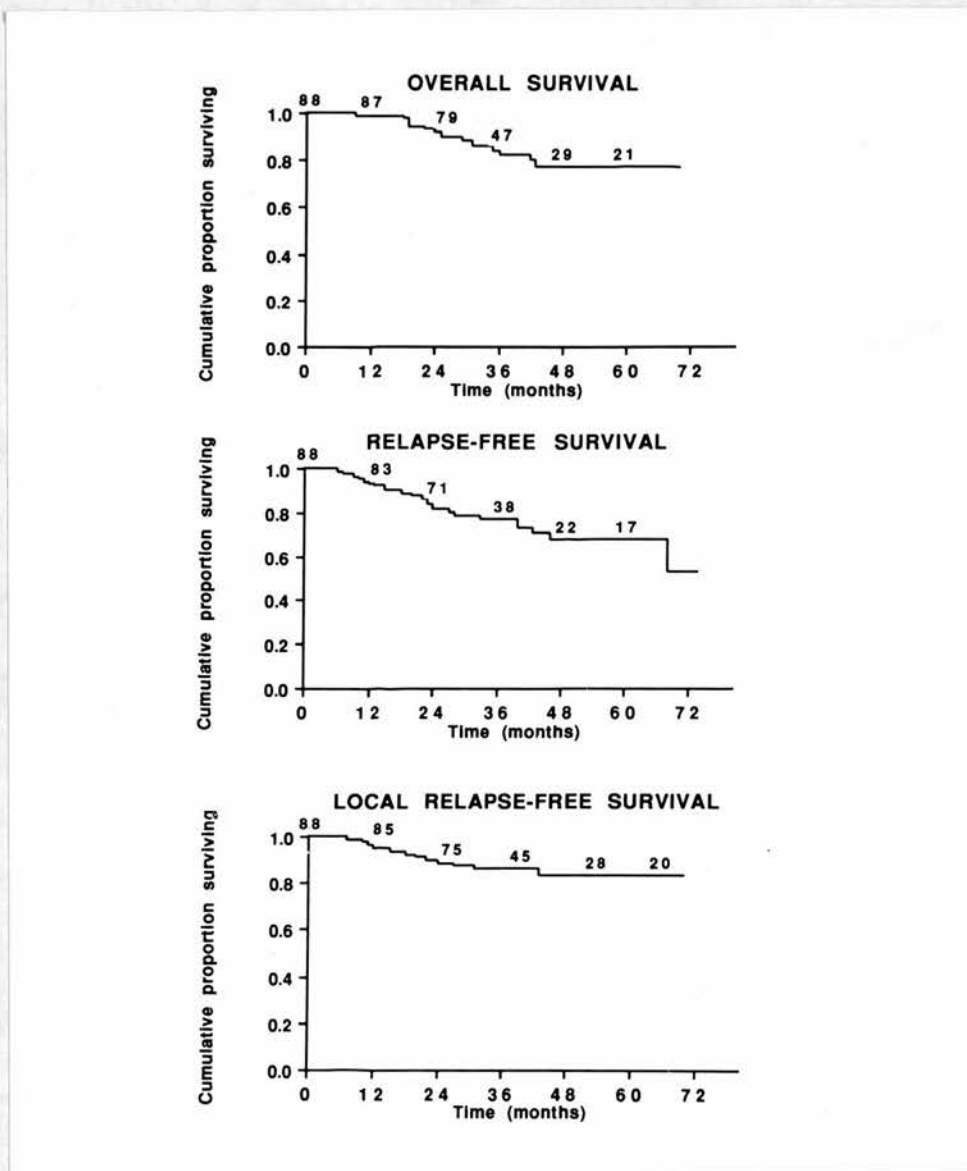


Figure 14
 Overall, relapse-free and local relapse-free survival for the 88 patients with large operable breast cancers who underwent primary systemic therapy. The median time to survival analysis was 42 months (range 16 - 72 months). The number remaining available for analysis is shown at yearly intervals.

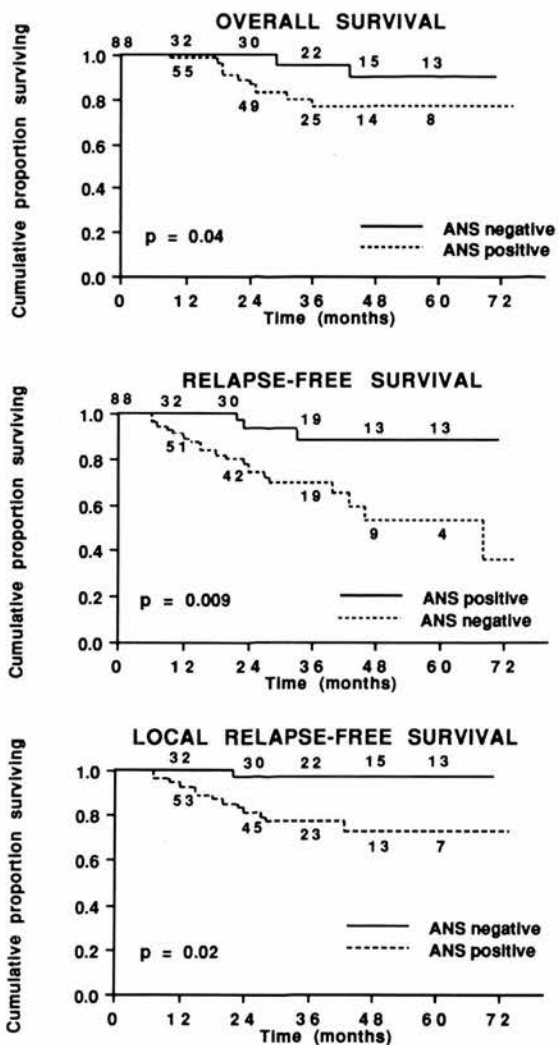


Figure 15
 Overall, relapse-free and local relapse-free survival following primary systemic therapy analysed in relation to axillary node status in the 86 patients for which it was available. Analysis was on an ever axillary node positive or never axillary node positive basis. The difference between the two groups was analysed by the General Wilcoxon Test. The number of patients remaining for analysis is shown at yearly intervals.

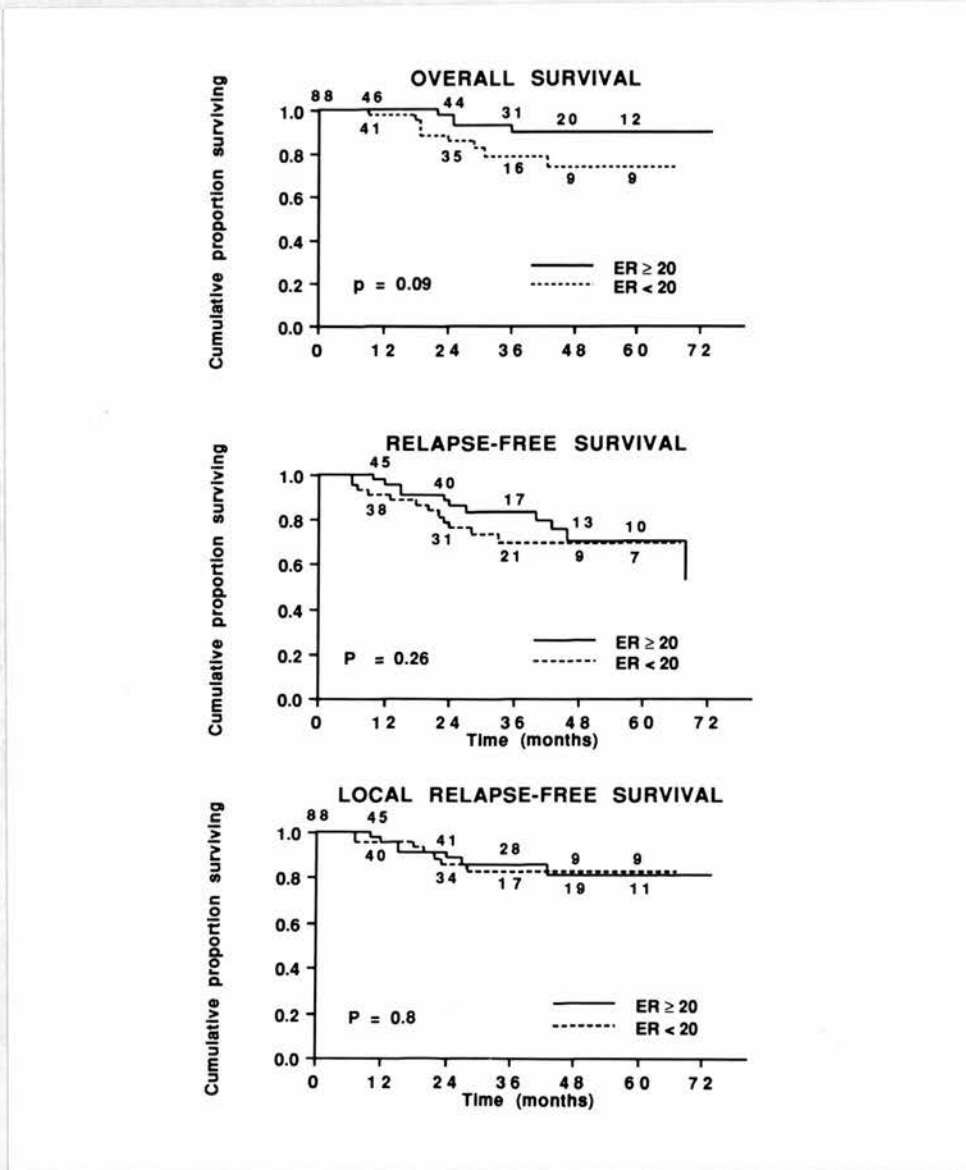


Figure 16
 Overall, relapse-free and local relapse-free survival following primary systemic therapy analysed in relation to oestrogen status of the primary tumour. A cut of value of 20 fmol/mg cytosol protein has been taken to differentiate into those patients with ER negative/poor tumours and those with ER moderate/rich tumours. The difference between the two group was analysed by the Generalised Wilcoxon Test. The number of patients remaining for analysis is shown at yearly intervals.

Table 16.

Site of first tumour recurrence in 23 of the 88 patients with large operable breast cancer treated by primary systemic therapy who have demonstrated relapse (median follow-up period 42 months, range 16 - 72 months) subdivided for axillary node status.

Site of relapse	Axillary Node Status		Total
	Ever AN+	Never AN+	
Loco-regional alone	7	1	8
Distant alone	7	2*	9
Both Distant and Local	4	2	6
TOTAL	18	5	23

*post-treatment axillary node status

No significant relationship could be demonstrated between clinical response to cytotoxic therapy and either overall survival ($p = 0.37$), distant disease free survival ($p = 0.64$) or relapse free survival ($p = 0.37$) (Figure 18) although again as a subgroup those patients who achieved a complete clinical response fared best. However if the survival data is reanalysed in relation to those who demonstrated marked chemosensitivity, that is a time to half volume less than 20 days, a more obvious difference can be shown between those patients who had a rapid response to chemotherapy and those who did not, overall survival (0.08) distant disease free survival ($p = 0.05$) relapse free survival ($p = 0.3$) and local survival ($p = 1.00$) (Figure 19).

Within this small series no significant relationship to menopausal status was demonstrated.

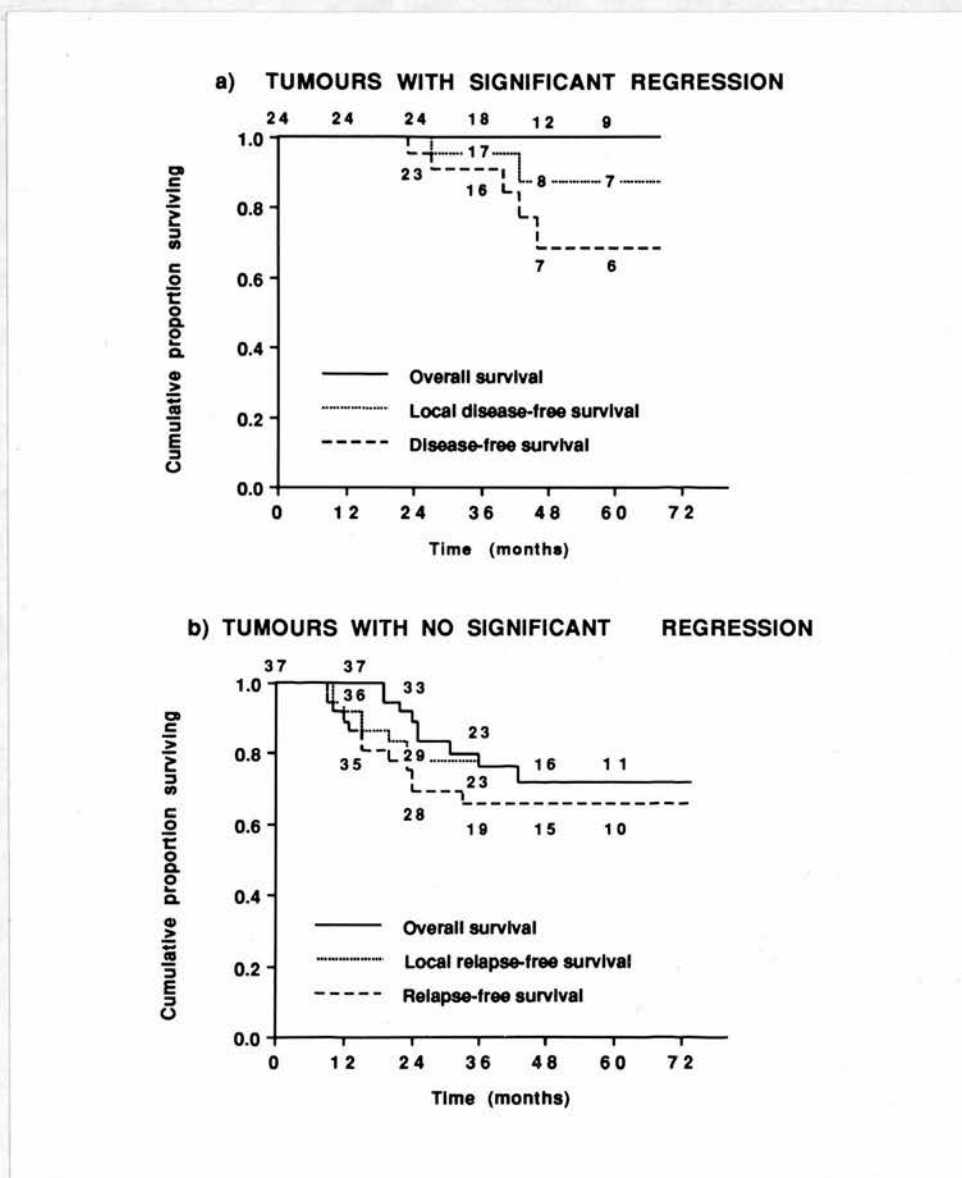


Figure 17
 Overall, relapse-free and local relapse-free survival is shown for the 24 patients who achieved significant regression (a) with endocrine therapy and the 37 who did not (b). The difference between the two response categories is statistically significant for overall survival ($p = 0.01$), but just fails to reach significance for relapse-free (0.07) and local relapse-free (0.08) survival when analysed by General Wilcoxon Testing. The numbers remaining for analysis are shown at yearly intervals.

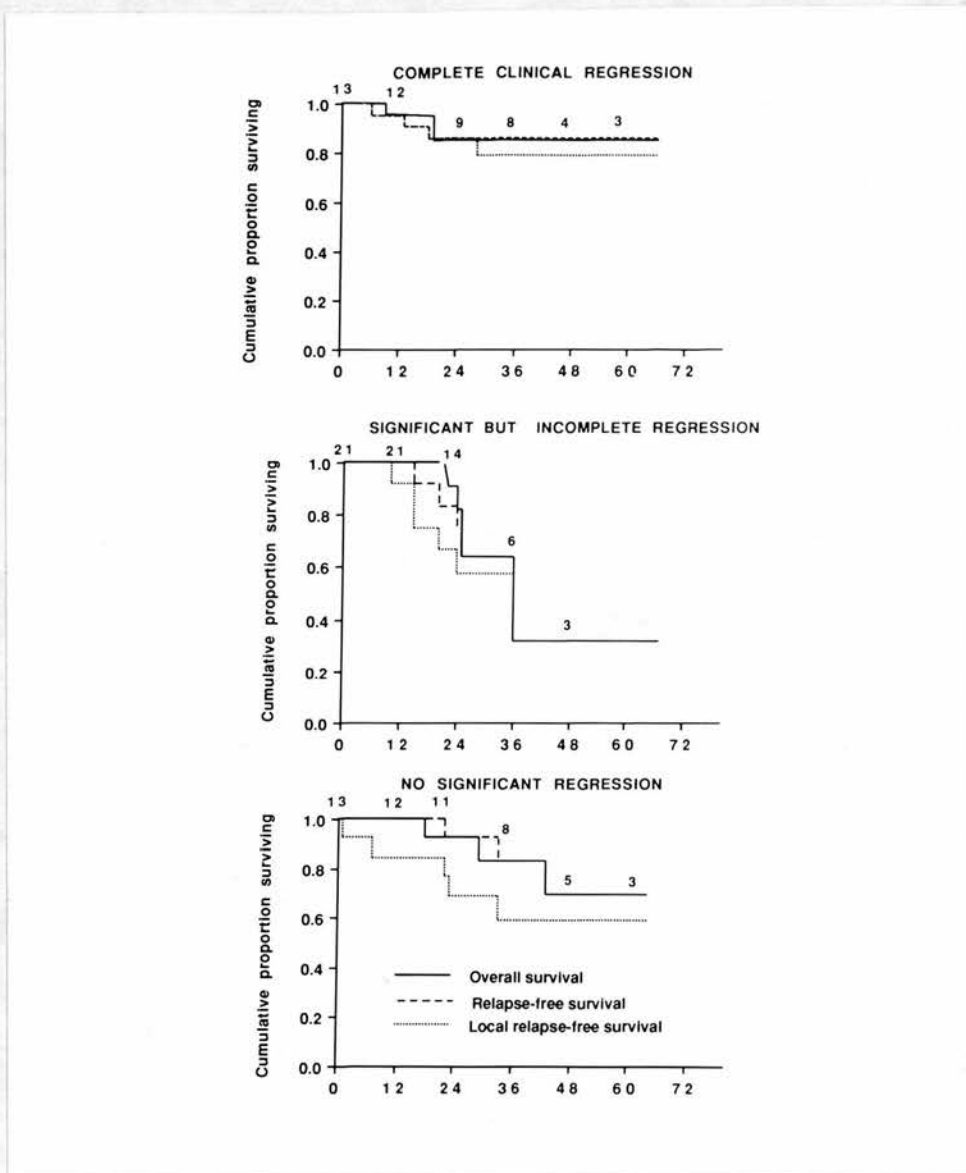


Figure 18

Overall, relapse-free and local relapse-free survival is shown in relation to response to cytotoxic therapy. Three response categories have been defined, those achieving complete clinical regression (n = 13), those achieving a significant but clinically incomplete regression (n = 21) and those showing no change (n = 13). No significant relationship was demonstrated between these three response groups and overall (p = 0.37), relapse-free (p = 0.65) survival although the relationship with local relapse-free survival just fails to reach statistical significance (p = 0.06; Generalised Wilcoxon Test). The number remaining available for analysis is shown at yearly intervals.

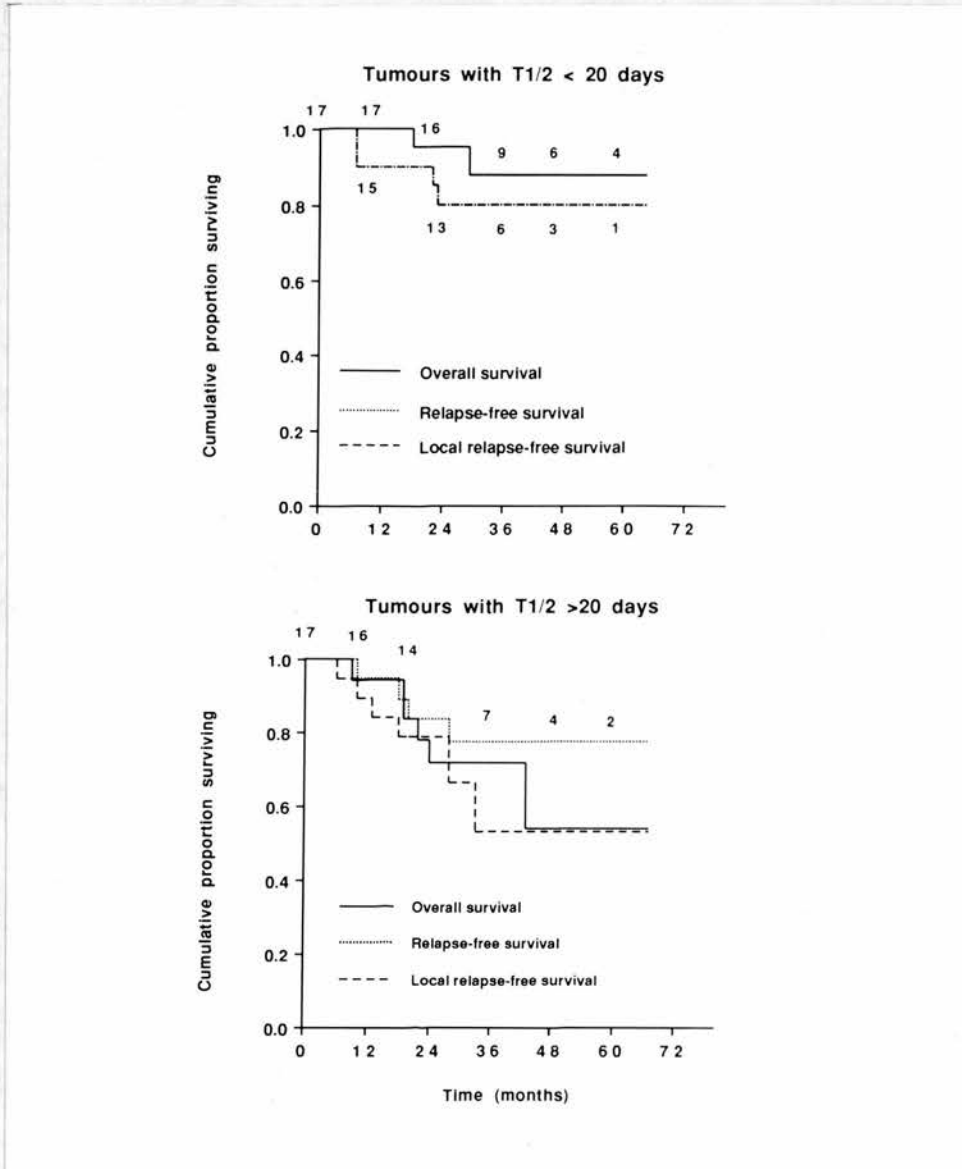


Figure 19 Overall, relapse-free and local relapse-free survival is shown in relation to the rate of response to cytotoxic therapy. The median $T_{1/2}$ (20days) has been used to define two groups, those with a more rapid ($T_{1/2} < 20$ days) and those with a slower ($T_{1/2} > 20$ days) rate of regression. The statistical difference between the two groups as analysed by the Generalised Wilcoxon test just fails to reach statistical significance for overall survival ($p = 0.08$) but is insignificant for relapse-free survival ($p = 0.34$) and local relapse-free ($p = 1.00$) survival. The number remaining available for analysis is shown at yearly intervals.

Assessment of Endocrinological Effects of Hormonal Therapy

Endocrinological Effects of Aromatase Inhibitors

The mean serum levels of oestrone before and during treatment with 4-hydroxyandrostenedione for 8 postmenopausal women are shown in Table 17. The level of suppression was variable during the first 6 weeks but appeared more stable at around 50% of the pretreatment level after this time. The levels for individual patients are shown in Figure 20. Although several patients show high breakthrough peaks of serum oestrone levels, there was no relationship between clinical response and the level of suppression of serum oestrone (Figure 20).

Table 17.

Effect of 250mg intramuscular 4-hydroxyandrostenedione given at 14 day intervals on serum oestrone levels in 8 postmenopausal women with breast cancer. Serum oestrone levels are expressed as the mean for the 8 patients (+/- standard error of the mean).

Treatment Day	Serum oestrone level pmol/litre
0	62.0 +/-18.2
14	28.2 +/- 7.3
28	51.0 +/- 16.2
42	28.4 +/- 6.0
56	22.9 +/- 5.3
70	20.3 +/- 4.9
84	18.6 +/- 4.1

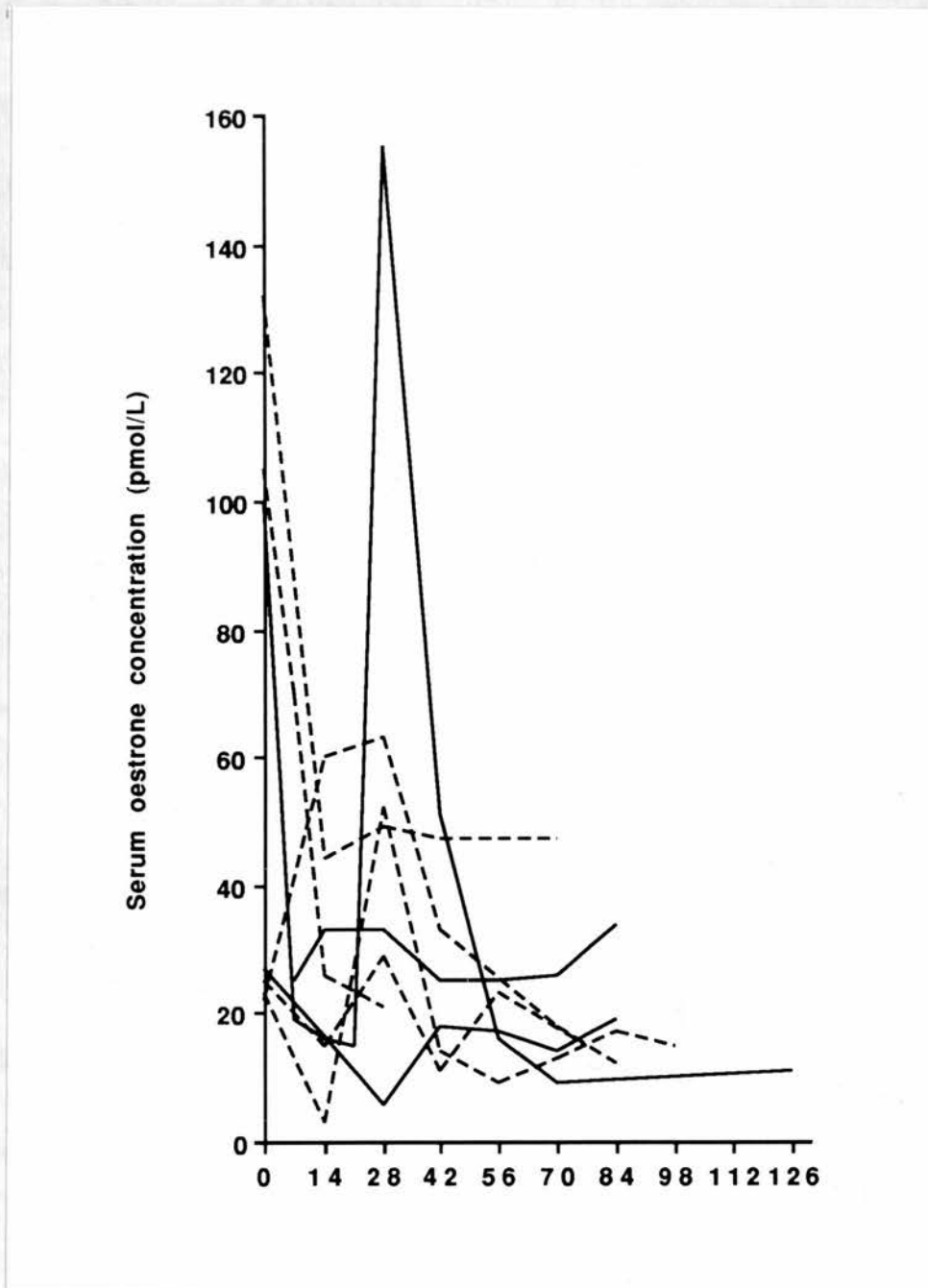


Figure 20

Effect of 4-hydroxyandrostenedione (250mg i.m. at 14 day intervals) on serum oestrone levels in 8 postmenopausal women with large operable breast cancers. Those patients who achieved significant regression are shown by _____, those who did not achieve significant regression are shown by -----.

Endocrinological Effects of the LHRH Analogue Goserelin

The effect of goserelin (3.6mg s.c. at 28 day intervals) on serum oestradiol and progesterone were evaluated in 6 premenopausal women (Figure 21 and 22) . In addition the effect on serum gonadotrophin levels were measure for 2 premenopausal and 2 postmenopausal patients (Figures 23 and 24). Levels of LH were dramatically reduced by 14 days and by 28 days in all 4 cases had reached low levels. Consistently low concentrations were found in all subsequent serum samples. FSH levels in postmenopausal women were similarly reduced, but in the 2 premenopausal patient studied suppression was not so conspicuous although levels were always below 10 U/l. In premenopausal women castration levels of both serum oestradiol and progesterone were reached by the fourteenth day of treatment although in several cases a dramatic rise initially occurred. These low levels were maintained while therapy continued except in three cases where a small transient rises in serum oestradiol was shown. Despite this rise one of the three patients achieved significant regression. Levels of serum oestradiol and progesterone within the 2 postmenopausal patients studied were within the normal postmenopausal range.

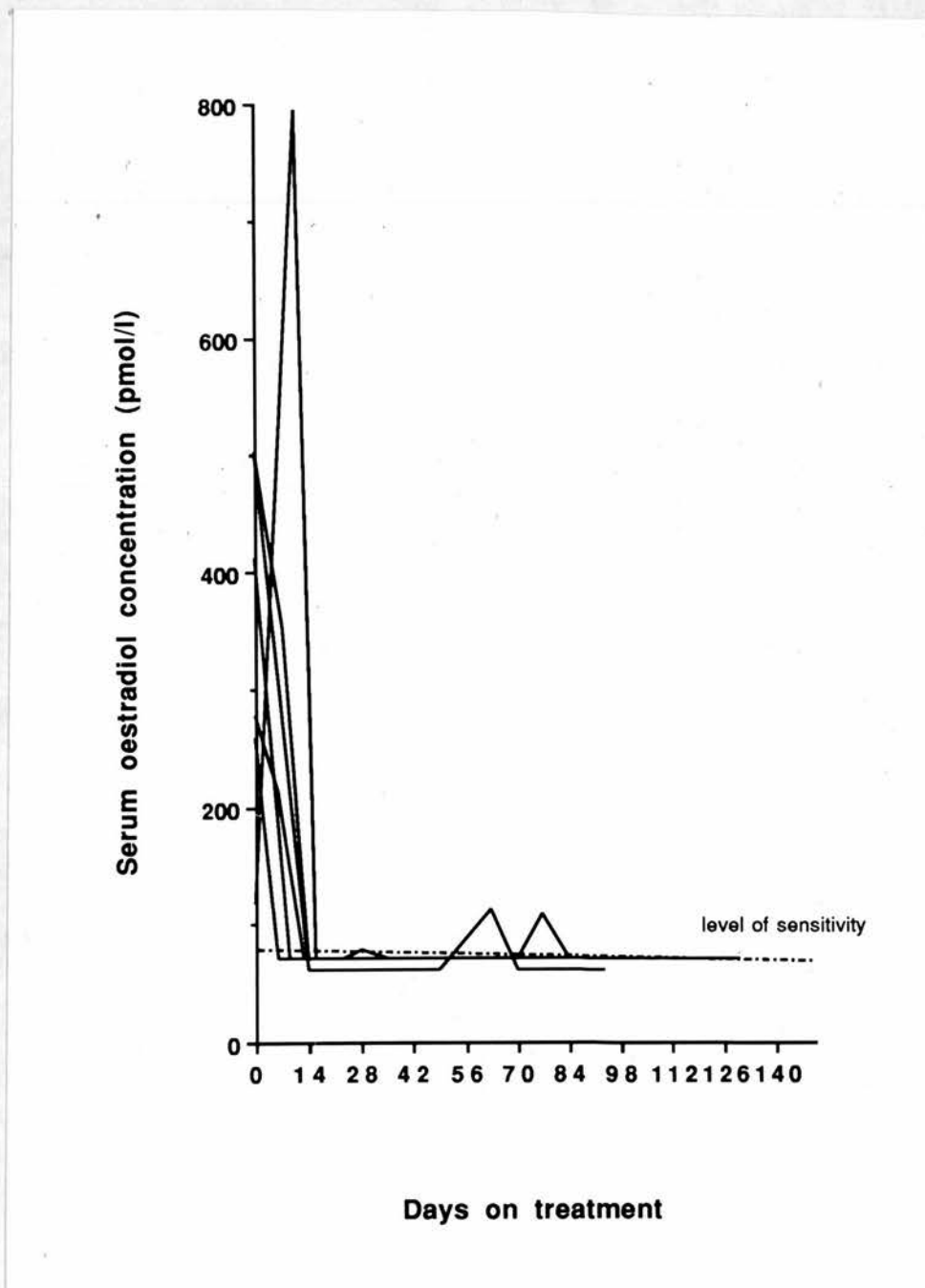


Figure 21
 Effect of goserelin (3.6mg s.c. at 28 day intervals) on serum oestradiol levels in 6 premenopausal women with large operable breast cancers.

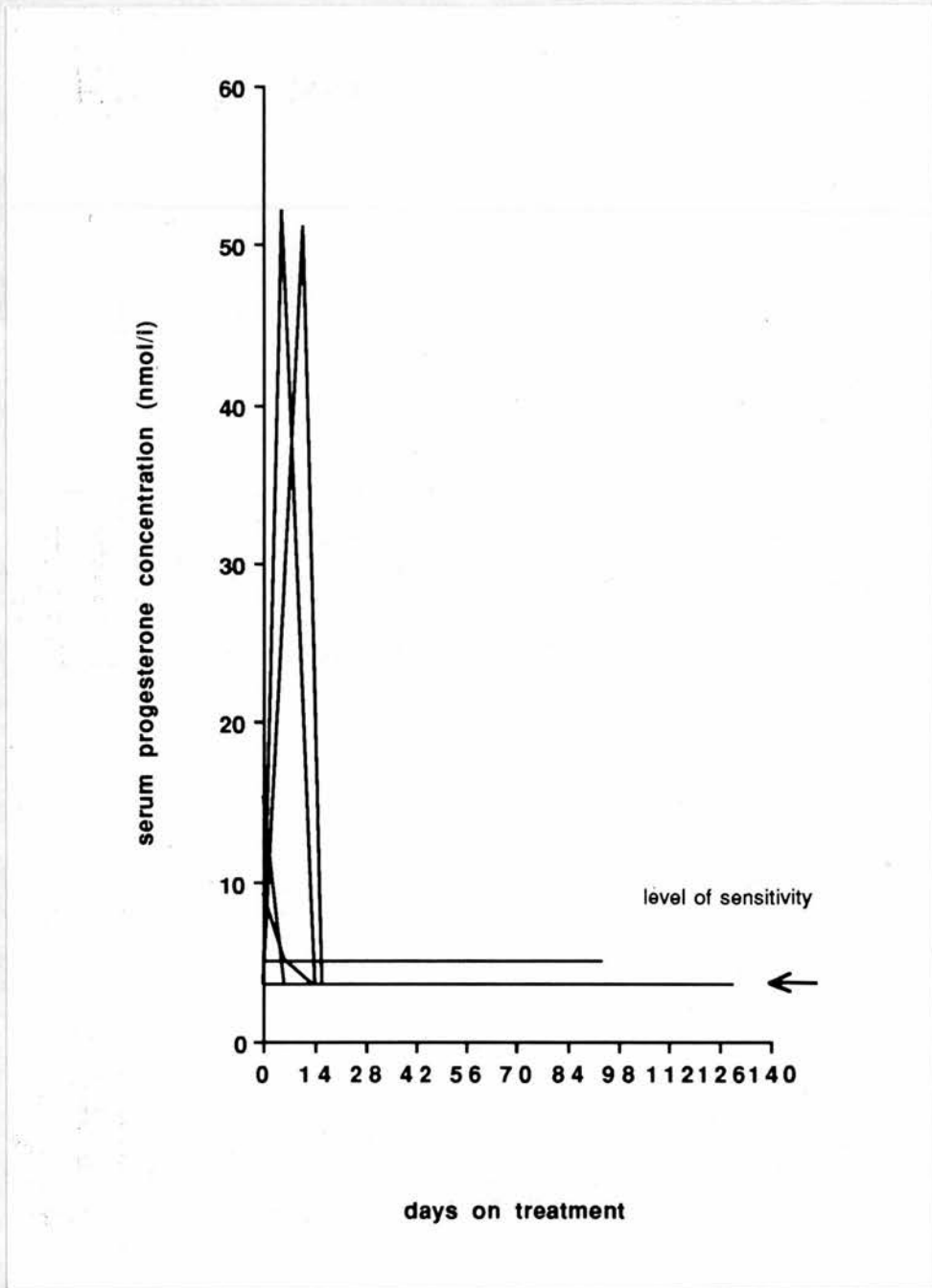


Figure 22
 Effect of goserelin (3.6mg s.c. at 28 day intervals) on serum progesterone levels in 6 premenopausal women with large operable breast cancers

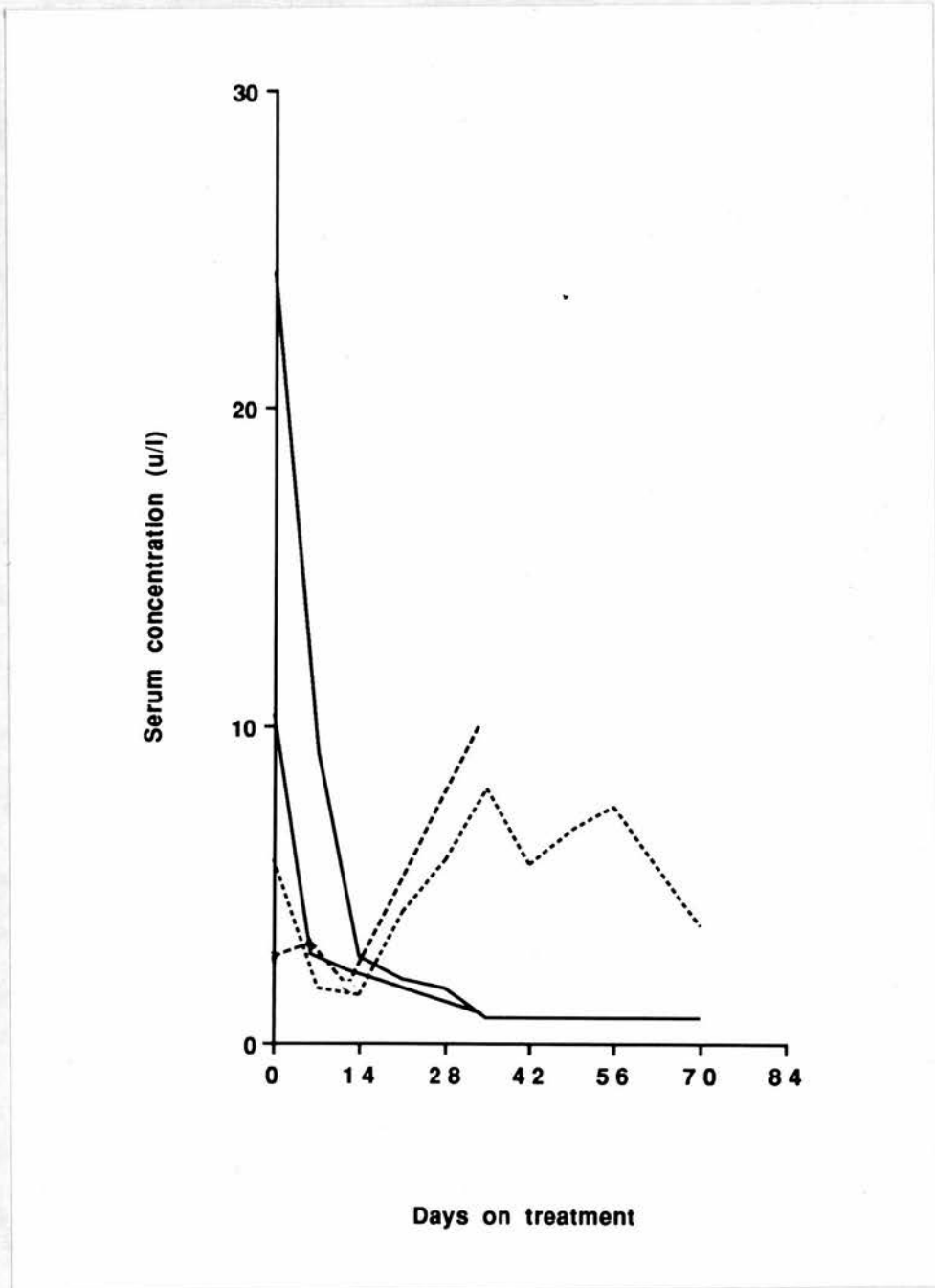


Figure 23
 Effect of goserelin (3.6mg s.c. at 28 day intervals) on serum lutenising hormone levels (____) and follicule stimulating hormone (- - -) in 2 premenopausal women with large operable breast cancers.

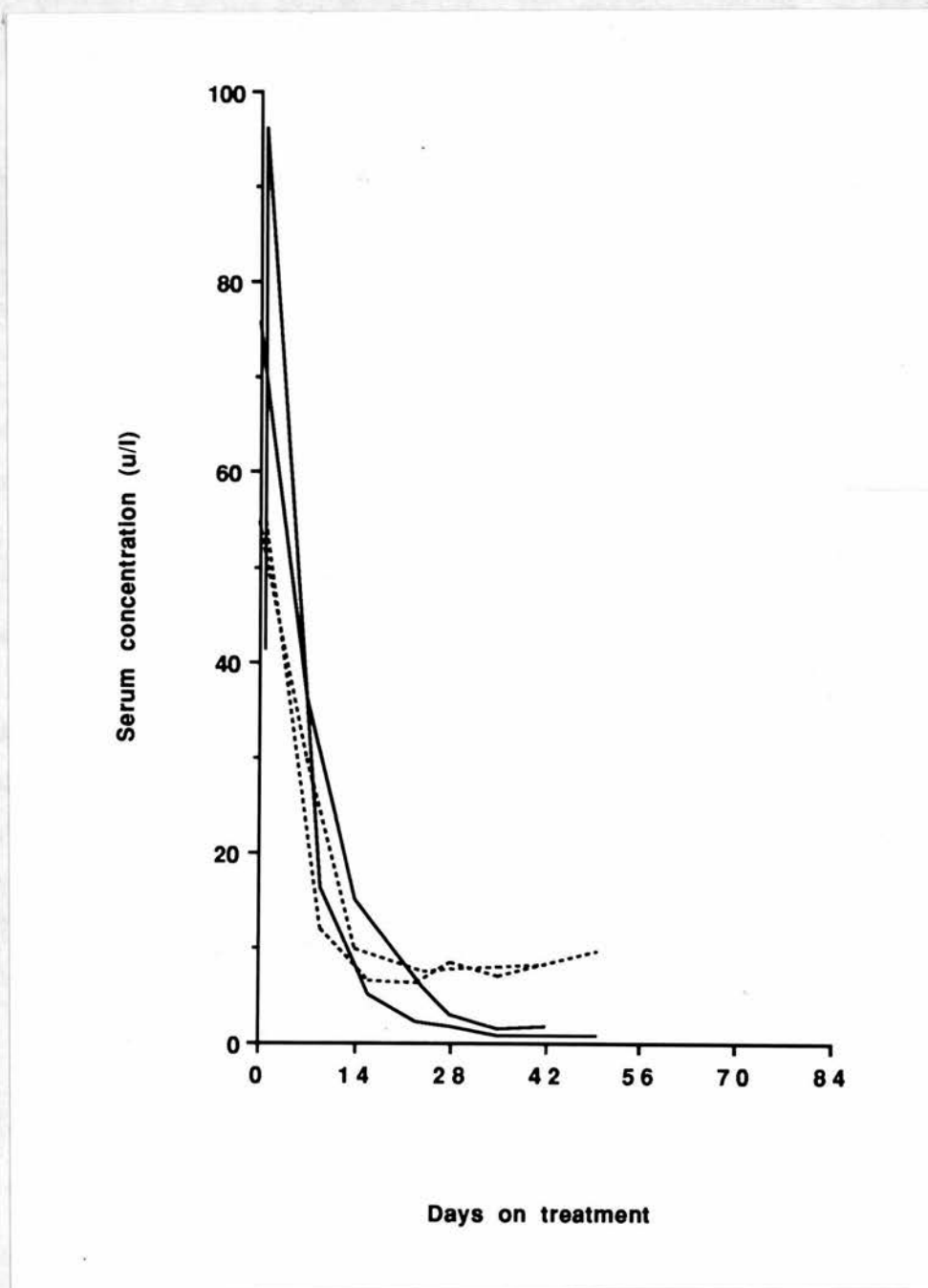


Figure 24
 Effect of goserelin (3.6mg s.c. at 28 day intervals) on serum lutenising hormone levels (—) and follicle stimulating hormone (---) in 2 postmenopausal women with large operable breast cancers.

Assessment of Toxicity

Endocrine Therapy

The side-effects experienced during treatment with endocrine agents were minimal.

Goserelin

All side-effects noted with goserelin were attributable to hypo-oestrogenism. In premenopausal patients menstruation ceased within 4 weeks of initiating therapy. Hot flushings were noted by 7 patients (37%) and were moderately severe in 2. Vaginal dryness was a problem with one patient but headaches or mood changes were not described. Local or general allergic reactions were not noted. There was no clinically relevant change in any of the haematological or biochemical parameters tested during the therapeutic period and no patient required to be withdrawn from the study because of pharmacological side-effects..

4-hydroxyandrostenedione

Of the 16 patients who received 4-hydroxyandrostenedione 8 (50%) experienced some adverse effect. One patient developed a self-limiting erythematous skin rash on the buttocks and 4 (25%) complained of a tender lump at the site of intramuscular injection although in the majority of cases this had settled within a week. Hot flushings were reported by 2 patients. A mild self limiting abnormality of liver function tests was noted in four patient but this was not clinically significant and did not require cessation of the drug; it is unclear if this was due to true toxicity or an incidental finding. Haemotoxicity was not noted and treatment was not discontinued in any patient because of toxicity.

Tamoxifen

Tamoxifen was well tolerate by all patients. One patient however complained of moderately severe hot flushings.

Aminoglutethimide

In 6 of the 10 patients no significant side-effect to aminoglutethimide therapy was noted. Four patients complained of nausea and lethargy on initiation of therapy. No allergic or haematological reactions were observed.

Cytotoxic Therapy

The incidence and severity of side effects experienced during treatment with this combination regimen are shown in Table 18. Alopecia, although transient was noted by all patients and was severe in 89%. Other principle side effects included nausea and vomiting (91% WHO grade 2 or greater), stomatitis (68% WHO grade 2) and neutropenia 26% WHO grade 2 or greater). In addition one fifth of patients developed symptoms of dyspepsia, 3 requiring H² antagonists for symptomatic control. Mild sterile dysuria was noted by 13% of patients. One person developed a dystonic reaction to the high dose (40mg) metoclopramide anti-emetic regime.

In 2 patients dose adjustment was necessary due to neutropenia. In a further 2 patients vincristine was omitted from the third and fourth cycles because of autonomic neuropathy. Premature termination of therapy was required because of nonspecific toxicity in 2 patients and iliofemoral thrombosis in one patient. Of the 25 patients who were premenopausal, cessation of menstruation occurred in 18.

There were no treatment related deaths and no demonstrable increased morbidity associated with definitive surgery.

Table 18.

Toxicity associated with the preoperative administration of 4 cycles of adriamycin, vincristine cyclophosphamide and prednisolone in 47 patient with large operable breast cancer as defined by the WHO toxicity grading (Miller et al. 1981). The figures given are the percentage of patients suffering from the given toxicity in at least one course of therapy.

	WHO grade			
	1	2	3	4
Anaemia	15	-	-	-
Leukopenia	15	15	9	2
Altered transaminases	2	-	-	-
Nausea/vomiting	9	68	21	2
Stomatitis	11	68	-	-
Diarrhoea	11	6	-	-
Constipation	6	-	-	2
Infection	28	4	2	-
Alopecia	-	11	89	-
Peripheral neurotoxicity	38	2	-	2

DISCUSSION

In this study we have been able to observe directly the effect of systemic therapy on primary breast cancers. Both endocrine and cytotoxic therapies have been used. Although a variety of endocrine agents were initially tried in the pilot study, these were found to be of comparable effect and the proportion of patients achieving regression was similar to that documented using the same agents in advanced disease (Hawkins, 1985; Coombes, Stein & Dowsett, 1989; Nicholson & Walker, 1989). In the more formalised protocol interest was centred on the effects of the gonadotrophin-releasing hormone agonist goserelin in premenopausal women and the peripheral aromatase inhibitor 4-hydroxyandrostenedione in postmenopausal women with tumour of ER concentration greater than 20fmol/mg cytosol protein. The cytotoxic regime remained unchanged at 4 cycles of CHOP.

A study of the endocrinological efficacy of goserelin within this group of patients mirrors those reported by other workers (Nicholson et al. 1984, 1987) in that low levels of gonadotrophins, oestradiol and progesterone were reached within 28 days of initiating therapy, and in the majority of cases these were maintained while treatment continued. While the rate of decline in serum sex steroids was not as rapid as that which would be expected following surgical oophorectomy, assessment for response did not start until 28 days at which time therapeutic hypoestrogenism was achieved. In some patients an initial transient rise in oestradiol occurred but this did not appear to produce an important clinical effect. Goserelin caused regression of primary disease in 7 of the 16 (43%) premenopausal women with ER-moderate/-rich tumours. This closely parallels the response rates achieved using surgical or radiation induced oophorectomy in premenopausal women with advanced ER-positive breast cancers. However it should be noted that the response was achieved without the morbidity associated with surgical or radiotherapeutic intervention; side effects being minimal and mainly related to hypo-oestrogenism. In addition to the drug being well tolerated it had the added advantage that treatment could be readily discontinued should therapy prove ineffective. It would therefore appear more suitable for the primary treatment of premenopausal women with ER-positive tumours, however once response has been assessed it may be more convenient for economic reasons to offer surgical bilateral oophorectomy as continuing adjuvant therapy.

Three postmenopausal women were treated primarily by goserelin but none achieved significant regression. Although tumour remission has been recorded in 10 % of postmenopausal women with advanced disease receiving gonadotrophin-releasing hormone agonists (Nicholson & Walker, 1989), this regression rate is too low to accept for primary treatment especially with the availability of other drugs, for example tamoxifen with known higher efficacy.

In postmenopausal women aromatase inhibitors were originally tested because of the interest in correlation of response with the activity of enzymes responsible for endogenous oestrogens production. Initially aminoglutethimide was the only aromatase inhibitor available, but during the study 4-hydroxyandrostenedione became available for clinical practice as part of a phase 2 clinical trial. Since initial trials had shown 4-hydroxyandrostenedione to be pharmacologically more active (Brodie & Santen 1976) and to be associated with fewer side-effects it was decided to use it as the preferred drug. The optimal clinical dose had yet to be confirmed and a dose of 250mg intramuscular at 14 day intervals was chosen. Seven of the 16 postmenopausal women (44%) who received 4-hydroxyandrostenedione achieved significant regression. The proportion regressing is similar to that demonstrated with other endocrine agents within this study (Table 12) and that documented by other workers in postmenopausal patients with advanced ER-positive breast cancers (49-58%; Hawkins 1985). Indeed Coombes and his colleagues (Coombes, Stein & Dowsett 1989) have demonstrated that increasing the dose over 250mg i.m. at 14 day intervals does not improve the proportion responding but does increase the incidence of side-effects.

The major endocrine effect of 4-hydroxyandrostenedione administration is a reduction in plasma oestradiol concentration (Dowsett et al. 1989). When using a dose of 250mg at 14 day intervals marginally suboptimal aromatase inhibition had been suggested during the last few days of the two-week cycle. (Dowsett et al. 1989). In the 8 patients investigated within this study a fall in plasma oestradiol was demonstrated in all patients following sustained treatment of greater than 6 weeks but varied between 11 and 82% of the pretreatment value (mean 50.1 +/- 6.5%). Escape from oestradiol suppression was more common during the first 6 weeks but did not appear to affect clinical response. Despite this submaximal suppression of serum oestradiol, in the light of the maintained clinical efficacy and low incidence of side-effects, 250mg i.m. every 14 days would appear to be an acceptable parenteral dose. Although reports of successful regression (Coombes, Stein & Dowsett 1989) have been made following treatment

with oral 4-OHA, the recommended method of administration of 4-OHA is still by deep intramuscular injection.

Systemic side-effects were not a major problem with 4-OHA but the requirement for intramuscular injection is a disadvantage and tamoxifen would appear to be preferable for primary therapy in postmenopausal women.

Within this study cytotoxic therapy with its greater associated toxicity was reserved for patients in whom endocrine therapy had failed or the likelihood of response to endocrine therapy was minimal (i.e. patients with ER-poor or ER-negative tumours). The proportion of such patients with tumours which were chemosensitive was high and lies within the observed range of 70-93% described with 'neoadjuvant' chemotherapy in more locally advanced breast cancers (Delena et al. 1981; Jacquillat et al., 1988; Hortobagyi et al., 1988; Swain et al., 1987). The objective regression rate of combination chemotherapy in metastatic disease is around 50% (maximum 70%) (Carter 1972). The response rates achieved with primary cytotoxic therapy are therefore on average higher than those recorded for treatment of advanced disease. It is difficult to establish whether this is a function of the more precise method of evaluating response or whether there is a difference in the response rates of early and metastatic tumours. The response rates achieved with induction chemotherapy in more locally advanced disease would suggest that the latter may be so (Rubens et al. 1980), De Lena et al. 1981, Hortobagyi et al. 1983, Sorace et al. 1985; Swain et al. 1987). There also exists mathematically (Goldie and Coldman 1979 and experimentally (Skipper 1960, Skipper et al. 1964, Ling 1978) evidence to suggest that the likelihood of a resistant line developing appears closely related to the number of previous cell divisions. Cytotoxic therapy is therefore likely to be more effective the earlier it is given in the life history of the tumour. The number of patients with non-endocrine responsive tumours (i.e. ER-poor/negative tumours) who achieved complete clinical (27.6%) and complete pathological (17%) remission was also impressive. This coincides well with the complete clinical regression rates (4 - 23%) observed using induction chemotherapy in locally advanced breast cancer (Rubens et al., 1980; De Lena et al., 1981; Hortobagyi et al., 1988; Kantarjian et al., 1984).

The rate of regression achieved with systemic therapy was highly variable and appeared to be a function of the individual tumour responsiveness rather than the systemic therapy used except that regression on endocrine therapy was on average, slower than that achieved with cytotoxic therapy. This may reflect the cytostatic as

opposed to cytotoxic effect of endocrine therapy. Within the period of the study only one patient achieved complete clinical regression during endocrine therapy and in no patient was remission pathologically complete. In contrast the response to cytotoxic therapy was occasionally rapid and in those patients with such a rapid response, complete clinical (27.6%) and even complete pathological response (17%) was observed. None of the parameters studied, including tumour size, axillary node status, patients age or menopausal status were able to define which patients were more likely to achieve complete remission. The biological significance of the rate of regression to systemic therapy is as yet uncertain. In a study of the long term follow-up of elderly patients with locoregional breast cancer treated with tamoxifen alone, survival could be related directly to the response achieved to tamoxifen, with patients achieving a complete remission faring best (Horobin et al. 1991). Within the present study the demonstration of a significant response to endocrine therapy would also appear to identify a group of patients who have a very good prognosis. It is impossible to tell from this study whether the improved prognosis is due to biological preselection or benefit gained from adjuvant endocrine therapy. When survival was analysed against response for patients receiving cytotoxic therapy, patients achieving a rapid reduction in tumour volume had a statistically better prognosis. In particular those achieving the most rapid tumour reduction that is those with complete clinical response did very well although the difference failed to reach statistical significance and the numbers within the subgroup were small. It certainly would seem conceivable that if the tumour was highly sensitive to chemotherapy, there would be less likelihood of residual viable tumour at the end of therapy. Cure may only be possible in this small subsection on the patients with very sensitive tumours. However as yet it is not possible to gauge either the optimum dose or number of cycles of chemotherapy which should be administered to effect cure. Since the primary tumour is an exquisite marker of responsiveness, one possible method would be to persue induction treatment to maximal response. In advanced disease the initial enthusiasm associated with combination chemotherapy for breast cancer giving complete remission rates in the 20% range, turned to pessimism as the complete remission rates refused to rise (until recently) and the patients who attained a complete remission almost invariably relapsed (Bonadonna 1987). These disappointing results may be due to inadequate exploration of the dose-response curve. As in lymphomas, high dose regimes in advanced breast cancers with autologous bone marrow transplant programs have finally increased complete remission rates (up to 49%) with some appearing durable (Peters et al. 1989; Swain et al. 1987). Recent data from Norton (Norton 1988) suggests that we may have come closer than we know to to eradicating the last cancer cell in breast cancer in

adjuvant trials, but our assay systems are too insensitive to detect it. Differences in survival at 5 years cannot be distinguished between patients whose residual tumour cell number was reduced to one and patients whose cell number was reduced to a million, because of differing regrowth characteristics of the remaining cancer cells at different residual volumes. Primary chemotherapy studies should be designed to offer the opportunity to overcome these obstacles and take advantage of the near miss while also identifying those patients in whom further aggressive chemotherapy is unlikely to produce an improved effect.

Within this study the 4 year local recurrence rate for what is a high risk population was 17 (+/-4)%, with only one case being uncontrolled by subsequent therapy. Historical series of staged mastectomies undergoing a radical or modified radical mastectomy quote the incidence of local recurrence to vary between 24 and 29% at five years in tumours measuring ≥ 4 cm at the time of diagnosis (Donegan 1979). It would therefore appear that leaving the tumour in situ while response is being observed does not compromise local control. Indeed primary systemic therapy may permit conservative surgery in patients with large operable tumours which would otherwise require mastectomy and this is now being explored in several centres (Mansi et al., 1989; Bonadonna et al., 1990). Both studies are immature and although significant downstaging of local disease with systemic therapy was achieved in 51% (29/57) and 81% (127/157) patients respectively, the follow-up periods are too short to be sure of the impact of primary systemic therapy on local control when radical surgery is not eventually performed. Overall within this study a significant reduction in tumour size making conservation surgery theoretically possible was produced in 66% (58/88) of patients. Primary systemic therapy however is not yet orthodox for operable disease and it was not thought justified to perform less than a mastectomy in the majority of patients. In 6 patients with complete clinical response however, wide local excision of the previous tumour site was performed and supplemented, in 5 cases, by radiotherapy. With a median follow-up of 42 months only one of the 6 patients has developed local recurrence, this being adequately controlled by subsequent mastectomy. In future studies a more conservative approach would be worthy of trial, since the possibility of being able to maintain an intact breast after aggressive treatment may provide an important psychological incentive in a population of patients, some of whom initially delayed seeking medical attention for fear of losing their breast.

There is theoretical (Goldie and Coldman, 1979; Skipper 1980), experimental (Schabel et al., 1979; Fisher, Gundez, Saffer 1983) and clinical (Nissen-Meyer et al., 1986;

Ragaz 1986) evidence, that early administration of systemic therapy in the treatment schedule of patients with breast cancer may improve survival. The 4 year cumulative survival rate within this study was 77 (+/-5)%, with 83 (+/-4) % of patients remaining free of local recurrence. Comparison with historical controls series is fraught with error but it would appear that the survival rates within this present series compare favourably to 5 year survival rates achieved with orthodox treatment of large tumours of similar stage (Table 1) even if the figures are corrected for the beneficial effect of systemic therapy.

It is possible that the good survival within the present series may simply be a function of the selection bias and so the prevalence and importance of other prognostic indices have been studied. The most important biological predictor for tumour recurrence and survival is the presence of axillary node metastases. Initially axillary node sampling was not an integral part of the pretreatment assessment. Analysis of the pathological post-treatment axillary node staging of the first 43 patients demonstrated a lower incidence (51%) of positivity than would be expected. Since clinical axillary node staging is notoriously unreliable (Wallace & Champion 1972; Fisher et al. 1975) it was felt that pathological staging of the axillary nodes should be included in the preoperative assessment if survival data was to be assessed. Of the 45 patients in whom pretreatment axillary node status was known there was a higher incidence of lymph node metastases (73%) which is more in keeping with previous studies for tumours of similar stage (Carter Allen & Henson 1989). Following therapy however only 20 (44%) of this subgroup of 45 patients had detectable node metastases. Overall 56 patients (64%) had axillary metastases detected at some stage in their management, although this figure may be lower than the true pretreatment figure and suggests that sample bias for the study is not a major feature, also within the series axillary node status persists as a strong indicator of prognosis for both overall, relapse-free and local relapse-free survival.

The value of ER status as a prognostic index has been argued (Spyratos et al. 1989; Sutton et al. 1987; Hawkins et al. 1987; Chevalier et al. 1988; Shek & Godolphin 1989; Raemaekers et al. 1985; Knight et al. 1977; Parl et al. 1984; Godolphin, Elwood & Spinelli 1981; Mason et al. 1983; Todd et al. 1987; Howat et al. 1983; Williams et al 1987; Alexieva-Figusch et al. 1988; Maki & Hoehn 1989) but survival results, particularly following adjuvant endocrine therapy may have been scewed if the distribution of ER status was abnormal. Within the reported literature approximately 70% of primary breast tumours can be expected to be ER-positive (Kvinnslund 1986);

the proportion of patients with ER-positive tumours increasing with age (Elwood & Godolphin 1980; Helin et al., 1988; Cooper et al., 1989) such that around 80% of patients over the age of 60 have ER-positive tumours, whilst less than 60% of patients under 40 have ER-positive tumours (Elwood & Godolphin 1980). Within this present study 52% (46/88) of patients (60% [30/50] postmenopausal patients and 42% [16/38] premenopausal women) had ER-positive tumours. This is at the lower limits of previously reported series which is probably a function of the higher cut off value as the criteria for positivity (≥ 20 as opposed to 5 fmol/mg cytosol protein). Although ER status did not provide a significant prognostic index within the present series patients with ER-positive tumours tended to survive longer than patients with ER-negative tumours. This may well have been a function of the subgroup of patients with ER-positive tumours who regressed on endocrine therapy and demonstrated a remarkably good prognosis.

This study was undertaken to ascertain whether appropriate long-term systemic therapy could be selected in the individual patient by direct assessment of primary tumour response before surgical excision. It has been suggested that in some metastases the cancer cells are functionally different to those in the primary cancer from which they arose. Differences in phenotype eg. ER status (Hull et al., 1983; Klinga et al., 1982; Lee 1982; Kamby, Rasmussen & Kristensen 1989), have been demonstrated. Ninety per cent of lymph node metastases have the same ER status as the primary tumour, while corresponding values for liver and bone metastases were 75 and 58% respectively (Kamby, Rasmussen & Kristensen 1989). Also in general the doubling times of metastases are more rapid than the primary tumour (Scarckney, McCirmack & Cuchural 1978). Since all metastases originate from the primary tumour, this may arise as a result of cloning of tumour cells with a higher metastatic potential (and potentially more aggressive biology) or a change in biological characteristics of the metastasised cells as a result of local environmental growth conditions. Differing sensitivities to chemotherapeutic drugs have also been demonstrated between some primary cancers and their metastases (Donelli et al. 1977; Fugmann et al. 1977; Slack & Bross 1975; Trope 1975). Studies on the differential distribution of chemotherapeutic agents in vivo suggested that part of this difference in chemosensitivity between primary tumour and its metastases however may reflect on blood supply and hence availability of drug to the tumour cells (Donelli et al. 1977).

Within the present series it has not been possible to directly compare the therapeutic effect on primary and metastatic disease since a prerequisite for entry into the study

was freedom from overt systemic metastases. However it was possible to compare axillary node status before and after therapy. A much higher proportion of patients had axillary node metastases before (72%) compared to after (49%) therapy. No quantitative relationship however could be demonstrated between response to systemic therapy and disappearance of axillary node metastases in the 14 patients who were demonstrated to convert from axillary node positive to axillary node negative. While it is conceivable that axillary node sampling had simply removed the few lymph nodes with metastatic disease it is also possible that the systemic therapy has been effective in eradicating axillary metastases. Certainly all patients who were axillary node positive prior to chemotherapy and achieved complete clinical regression also had no demonstrable tumour remaining within their axillary lymph nodes, suggesting a therapeutic effect on both primary and metastatic tumour in these highly sensitive tumours.

More impressive is the demonstrable relationship between response of the primary tumour to systemic therapy and overall survival for both endocrine and cytotoxic therapy. Overall survival is related to the behaviour of the metastases and so a direct relationship can be demonstrated between function of the primary tumour and subsequent behaviour of its metastases. While it is possible therefore that in a small number of cases the response of the primary tumour may not reflect the effect on metastatic disease, overall the relationship appears to apply.

CHAPTER 9

RESPONSE AS A FUNCTION OF POTENTIAL PREDICTIVE INDICES

BIOCHEMICAL DETECTION OF OESTROGEN RECEPTOR ACTIVITY

Reproducibility of Measurement

Quality controls consisting of pools of minced human uterus or lyophilised powders were processed at least twice per week. The overall intra-assay precision on a pool of minced uterine tissue was 15.4% (n = 5). Inter-assay precision on lyophilised powders (no homogenation step) was 17.8% (n = 10) at low levels (mean 27 fmol/mg cytosol protein) and 11.7% at high levels (mean 90 fmol/mg cytosol protein); on two pools of minced uterine tissue (including homogenisation) it was 25.5% (n = 144) at low levels (mean 48 fmol/mg cytosol protein) and 17.0% (n = 48) at a higher level (mean 111 fmol/mg cytosol protein).

Biochemical Assay of Oestrogen Receptor Activity and Response to Endocrine Therapy

Material for biochemical analysis of ER activity was available for all 88 patients. Forty-six (53%) had an ER concentration of ≥ 20 fmol/mg cytosol protein. The proportion of postmenopausal women with ER moderate-rich tumours was higher (30/50, 60%) than in premenopausal women (16/38, 42%). The relationship between ER status of the primary tumour and patient menopausal status for those patients undergoing endocrine therapy is shown in Table 19.

The relationship between response to endocrine therapy and ER concentration as measured by the dextran-coated charcoal adsorption method is given in Figure 25 and Table 20. All responding tumours had an ER concentration of ≥ 20 fmol/mg cytosol protein. The overall response rate for patients with tumours of ER concentration of ≥ 20 fmol/mg cytosol protein was 52.2% (24/46) and appeared independent of menopausal status ($p = 0.76$, Fishers exact test), relative concentration of ER (i.e. 20-100 vs >100 fmol/mg cytosol protein; $\chi^2 0.32$, $p = 0.57$) or endocrine therapy used. Of note is that two third (10/15) of all ER-poor/negative tumours progressed during endocrine treatment compared to only 13% (6/46) of tumours with an ER concentration of ≥ 20 fmol/mg cytosol protein.

Table 19

Relationship between ER status of the primary tumour as determined by dextran-coated charcoal assay and patient menopausal status in relation to the proportion of patients demonstrating significant regression for 61 patients with large operable breast cancers treated by primary endocrine therapy. The median ER concentration was higher in postmenopausal (median 130, range 0-1151 fmol/mg cytosol protein) than premenopausal (median 30, range 0-174 fmol/mg cytosol protein) women.

ER Subgroup*	Premenopausal	Postmenopausal	Total
< 5	0/2	0/6	0/8
5 - 19	0/3	0/4	0/7
20 - 99	7/14	3/7	10/21
≥100	2/2	12/23	14/25
Total	9/21	15/40	24/61

* fmol/mg cytosol protein

Table 20

Response to endocrine therapy in 61 patients with large operable breast cancer subdivided for oestrogen receptor status as measured by the dextran coated charcoal adsorption method.

ER status	total no.	significant regression	no change	progression
ER - poor <20*	15	0	5	10
ER - rich ≥20*	46	24	16	6
Total	61	24	21	16

* fmol/mg cytosol protein

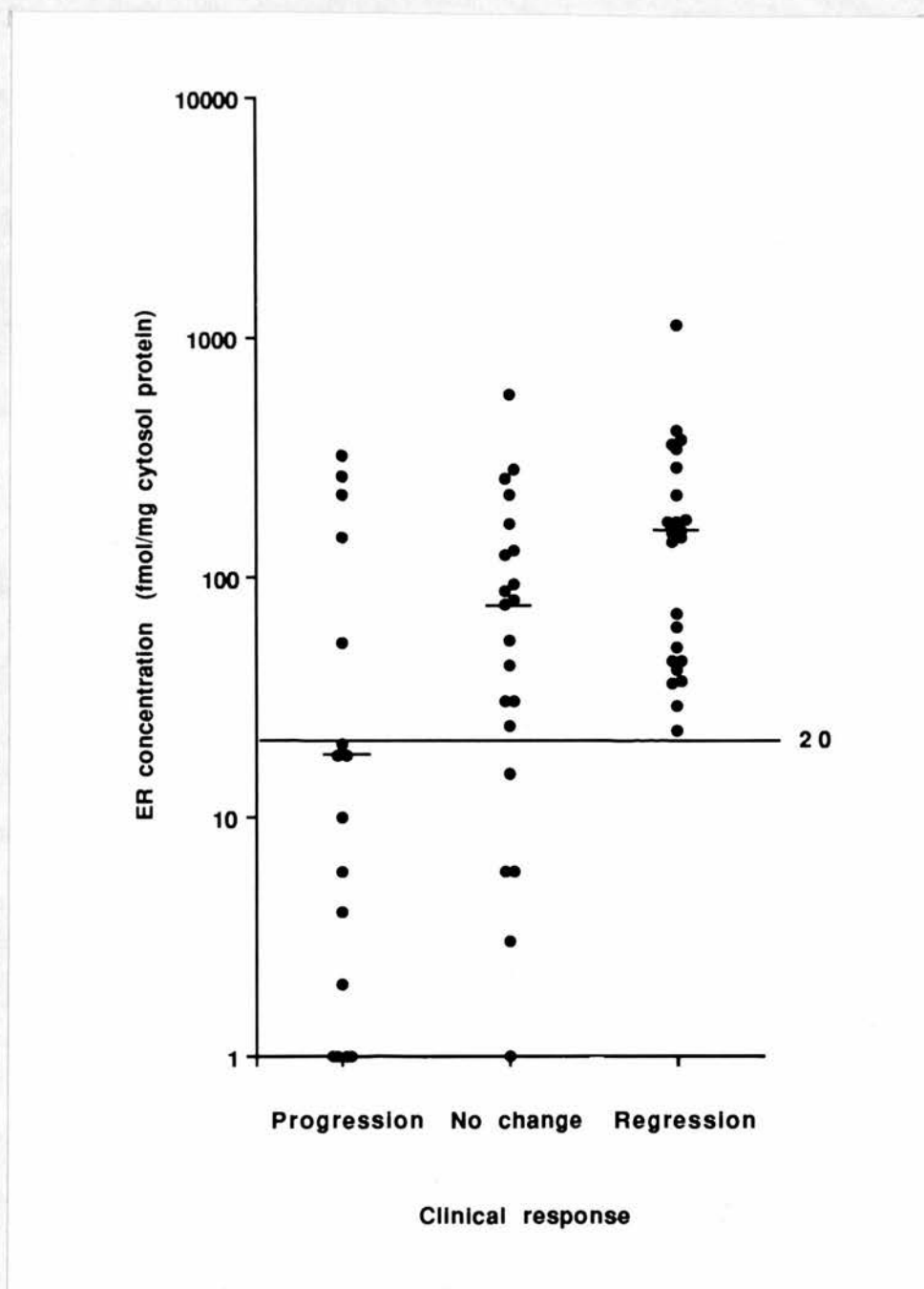


Figure 25
 Relationship between ER concentration of the primary tumour as determined by dextran-coated charcoal assay and response to primary endocrine therapy in 61 patients with large operable breast cancer. The horizontal line marks the level of 20 fmol/mg cytosol protein. The median values for each response group is shown by a bar.

Relationship Between Response to Cytotoxic Therapy and Oestrogen Receptor Status

There was no statistically significant relationship between response to chemotherapy and the pretreatment variables of age, menopausal status, initial tumour size (Table 21). Of the tumours which had failed to respond to endocrine therapy those with an ER concentration of ≥ 20 fmol/mg cytosol protein were significantly less likely to respond to chemotherapy when compared to those tumours with an ER concentration of < 20 fmol/mg cytosol protein (30% vs 80%, $p = 0.023$, Fishers exact test, 2 tails). The proportional response of ER poor/negative tumours to cytotoxic therapy was similar irrespective of prior, failed endocrine therapy or primary therapy (80% vs 83.8%).

Table 21.

Relationship between pretreatment variables and significant tumour regression following 4 cycles of preoperative cytotoxic therapy (CHOP). Patients were designated postmenopausal if it was greater than one year since their last menstrual period. Pretreatment axillary node status as determined from histological examination of an axillary node sample was available for 29 patients

	No. significant regression/ total no.	x ²	p value
TUMOUR DIAMETER			
< 5 cm	19/26		
5 - < 6 cm	10/14	0.813	0.67
≥ 6 cm	7/8		
AGE			
30-39 years	3/5		
40-49 years	14/18		
50-59 years	13/19	0.939	0.82
>60 years	4/5		
MENSTRUAL STATUS			
premenopausal	19/25		
postmenopausal	15/22	0.357	0.55
OESTROGEN RECEPTOR CONCENTRATION			
< 20 fmol/mg cytosol protein	31/37		
≥ 20 fmol/mg cytosol protein	3/10	11.38	0.0007
AXILLARY LYMPH NODE STATUS			
metastasis	15/23		
no metastasis	6/6	2.88	0.09
unavailable	5/13		

DISCUSSION

Statistical analyses of large controlled randomised trials have shown that survival of patients with operable breast cancer can be improved by systemic endocrine or cytotoxic therapy (Early Breast Cancer Trialists' Collaborative Group 1988). These trials however have not defined which therapy is most suitable for an individual patient. Given the morbidity of cytotoxic therapy (Glass et al. 1981, Valagussa et al. 1983) an unselective policy is not ideal but as yet here exists no ideal method of matching individual patients with appropriate adjuvant systemic therapy. Furthermore the value of tumour oestrogen receptor status (ER) in selecting patients for adjuvant hormonal therapy remains controversial (Palshof et al. 1985, Rose et al. 1985, Fisher et al. 1986 Rutquist et al. 1987, Bianco et al. 1988, Scottish Breast Cancer Trials Committee 1987, Nolvadex Adjuvant Trial Organisation 1988). By removing a small portion of tumor prior to initiating primary systemic therapy and direct observation of individual tumour response it has been possible to examine the value of specific histological and biochemical indices as predictive indices for response to both endocrine and cytotoxic therapies.

Since endocrine therapy is associated with low morbidity it was decided that all patients should initially receive primary endocrine therapy. Cytotoxic therapy with its greater morbidity, was reserved for those who failed to achieve significant regression with endocrine therapy. Analysis of the results from the initial pilot group of 36 patients demonstrated that none of the patients with tumours of ER concentration less than 20 fmol/mg cytosol protein showed significant regression and indeed two-thirds showed evidence of progression. With the direct demonstration of the lack of efficacy of endocrine therapy in ER-poor/negative tumours, it was no longer considered justifiable to use an indiscriminant policy and primary endocrine therapy was then reserved only for those patients with tumours of ER concentration ≥ 20 fmol/mg cytosol protein. Cytotoxic therapy was administered as primary therapy to those with ER-negative/-poor tumours or to patients with ER rich tumours when endocrine therapy had proved ineffective.

This correlation of endocrine sensitivity and ER status of the primary tumour parallels the situation in advanced disease (Hawkins, Roberts & Forrest 1980; Oriana et al. 1986; Coombes et al. 1986; 1987; Nicholson et al. 1987) and would support the theory that the ER is central to determining hormone sensitivity in the primary tumour as well as in the advanced disease situation. Many theories have thus been devised to explain

the discordance between the value of the ER in predicting efficacy in the adjuvant and therapeutic trials, these include inadequate quality control in the assay of ER and subgroup analysis of small sub-populations in adjuvant studies not stratified for ER. However within the NATO trial, the observed to expected biological value of ER status and histological grade as prognostic indicators was maintained, suggesting further explanation is required (Nolvadex Adjuvant Trial Organisation 1988).

Analysis of the relationship between ER status and benefit from adjuvant endocrine therapy have been based totally on utilising tamoxifen as the therapeutic agent. There is now increasing evidence that tamoxifen has anti-tumour actions which are independent of the ER. Two distinct mechanisms of growth inhibition have been demonstrated in human breast cancer cell lines (Sutherland, Watt & Ruenitz 1986; Etienne et al. 1989); both effects appear confined to the G1 phase of the cell cycle. In addition to the ER-mediated oestrogen-reversible growth inhibition, high concentrations of tamoxifen produce an oestrogen-irreversible cytotoxic effect which can be distinguished from nonspecific toxicity by its cell cycle specificity (Sutherland et al. 1983; Etienne et al. 1989). Several potential sites of action include inhibition of protein kinase C (O'Brian et al. 1986) or calmodulin a substance known to play an important role in cell cycle progression (Lam 1984). Tamoxifen also stimulates the secretion of TGF-B by the ER-positive cell line MCF7 (Knabbe et al. 1987) and fibroblasts (Baum et al. 1989). This may act by paracrine or autocrine action to inhibit the growth of surrounding tumour cells irrespective of their ER content (Knabbe et al 1987). It is uncertain how significant these non-oestrogen receptor dependent inhibitory actions are in vivo but because of these findings the relationship between the predictive value of ER status and benefit from adjuvant tamoxifen cannot necessarily be extrapolated to other forms of endocrine therapy.

Within this study, response to preoperative chemotherapy has shown that chemosensitivity was independent of the tumour size, axillary node status or menopausal status or age of the patient. This suggests that the inherent chemosensitivity of the tumour may well be more important than these factors in determining response.

Of those individual tumours directly demonstrated as endocrine-resistant the proportion of ER-poor/negative tumours regressing with chemotherapy paralleled that of primary chemotherapy (~80%) but the efficacy in ER-rich tumours was much lower (30%). This may reflect either preselection of a subgroup of patients with biological

nonresponsive tumours or an inherently chemoresistance of ER-positive tumours. A positive correlation is known to exist between proliferation rate and efficacy of cytotoxic therapy (Valeriote & van Putten 1975) while tumours with significant amounts of the ER tend to have lower proliferating indices (Meyer & Lee 1980, Olszewski et al. 1981, Kallioniemi et al. 1987, Dressler et al. 1988). ER-positive tumours would in theory therefore be expected to be more chemoresistant to cytotoxic agents. This has not been uniformly held up in clinical practice for cytotoxic therapy in advanced breast cancer (Rubens et al. 1980; Jonat et al. 1980; Bonadonna et al. 1980; Stewart et al. 1983).

It has been argued (Padmanabhan, Howell & Rubens 1986) that the principal beneficial effect of adjuvant cytotoxic therapy is through its ability to produce a medical oophorectomy. This study has directly demonstrated that chemotherapy is effective in postmenopausal patients, in patients with ER-poor/negative tumours and ER-positive tumours which have been directly shown to be endocrine resistant. The effect is rapid and occurs before the onset of full suppression of ovarian function. The recent demonstration that a significant improvement in disease-free survival can be obtained in ER-negative tumours (Bonadonna et al. 1987) following adjuvant chemotherapy supports the argument that the principal effect, particularly in ER-negative tumours is by direct cell kill. It is difficult to determine the clinical implication of ovarian suppression in the long term for premenopausal patients with ER-positive tumours, when it is possible that ovarian suppression may have an additional effect. It does however seem suboptimal to use cytotoxic therapy as a method of inducing ovarian ablation.

In summary the ER of the primary tumour can be used to differentiate a group of patients for whom initially treatment with endocrine therapy is futile, namely those with an ER concentration of less than 20 fmol/mg cytosol protein. Such patients should receive primary cytotoxic therapy. Response to endocrine therapy within patients with ER-moderate/rich tumours can be expected in around 50% patients. Response in the individual patient however can only reliably be determined by direct observation of the effect of therapy. In this way those patients in whom continuing endocrine systemic therapy is appropriate have been selected and survival data would suggest that such patients have an excellent prognosis. Similarly direct objective assessment of tumour response to systemic therapy allows cessation of endocrine therapy where it has been demonstrated to be of no value with initiation of chemotherapy if desired.

The effect of cytotoxic therapy is not dependent on the presence of the ER and indeed a subpopulation of patients with endocrine-resistant, ER-positive tumours can be identified which are less likely to be sensitive to cytotoxic agents.

CYTOCHEMICAL ASSAY OF OESTROGEN RECEPTOR ACTIVITY

Material for cytochemical analysis of ER activity was available for 50 patients. Of these, thirty patients (ER < 20 fmol/mg cytosol protein n = 5, ER ≥ 20 fmol/mg cytosol protein n = 25) received primary hormonal therapy (goserelin n = 9, tamoxifen n = 3, aminoglutethimide n = 9, 4-hydroxyandrostenedione n = 9) and twenty patients, all with ER-poor/negative (< 20 fmol/mg cytosol protein) tumours received primary cytotoxic therapy.

Reproducibility of Cytochemical Staining and Correlation with Biochemical Oestrogen Receptor Activity

Biochemical ER activity was determined in all 50 patients. In 13 patients (26.0%), the ERICA was not assessable either because the aspirates were acellular (n = 10) or the cells were too badly damaged during processing (n = 3). Comparison of biochemical and cytochemical ER assays was therefore possible in only 37 tumours.

The range of ER concentration using the DCC adsorption method was 0 - 584 fmol/mg cytosol protein; 28 of the 37 tumours (75.7%) had an ER concentration of > 5 fmol/mg cytosol protein. Using ERICA, 62.2% of tumours had positive staining (range of "staining index" 0.04 - 2.9).

The correlation between two observers scoring the same ERICA specimen independently was high for all three measured parameters, that is % cell staining (r = 0.98, Figure 26), staining intensity (r = 0.95, Figure 27) and staining index (r = 0.97, Figure 28).

The biochemical ER concentration was related to the cytochemical percent cells staining (r = 0.90, Figure 29a), and staining intensity (r = 0.90, Figure 29b). Integrating these values as a "staining index" did not improve this already high correlation (r = 0.89, Figure 30). No tumour with an ER concentration of < 5 fmol/mg cytosol protein had significant cytochemical staining while all tumours with an ER value of >20 fmol/mg cytosol protein by DCC showed cytochemical staining. Three tumours had an ER concentration of between 5 and 10 fmol/mg cytosol protein but only one (ER 8 fmol/mg cytosol protein) showed any staining and even then at a very low levels ("staining index" 0.04).

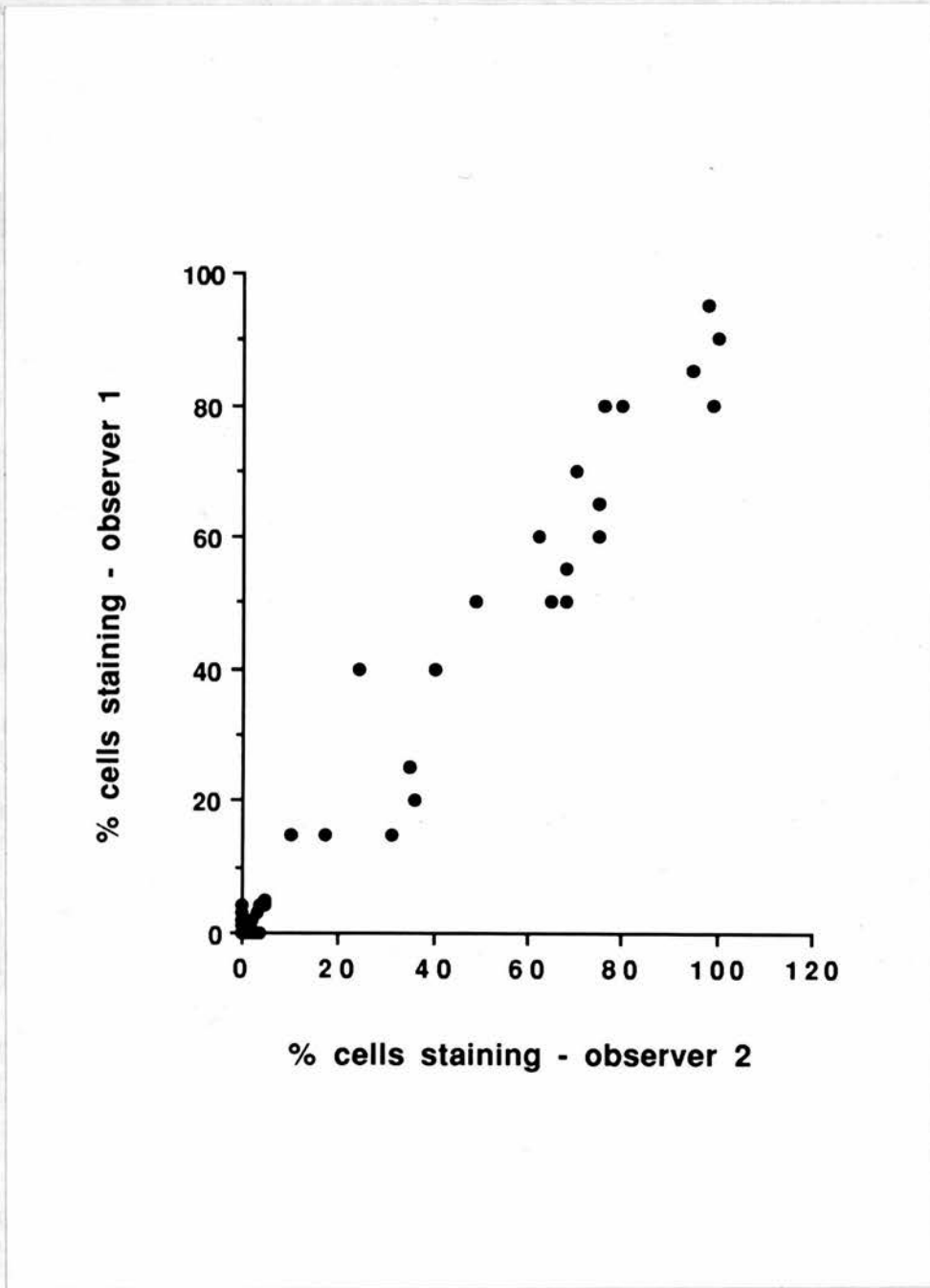


Figure 26
 Correlation of interobserver scoring of % cells staining on fine-needle aspirates with the ERICA kit in 37 patients with large operable breast cancers. The Spearman's Rank Correlation Coefficient was 0.98.

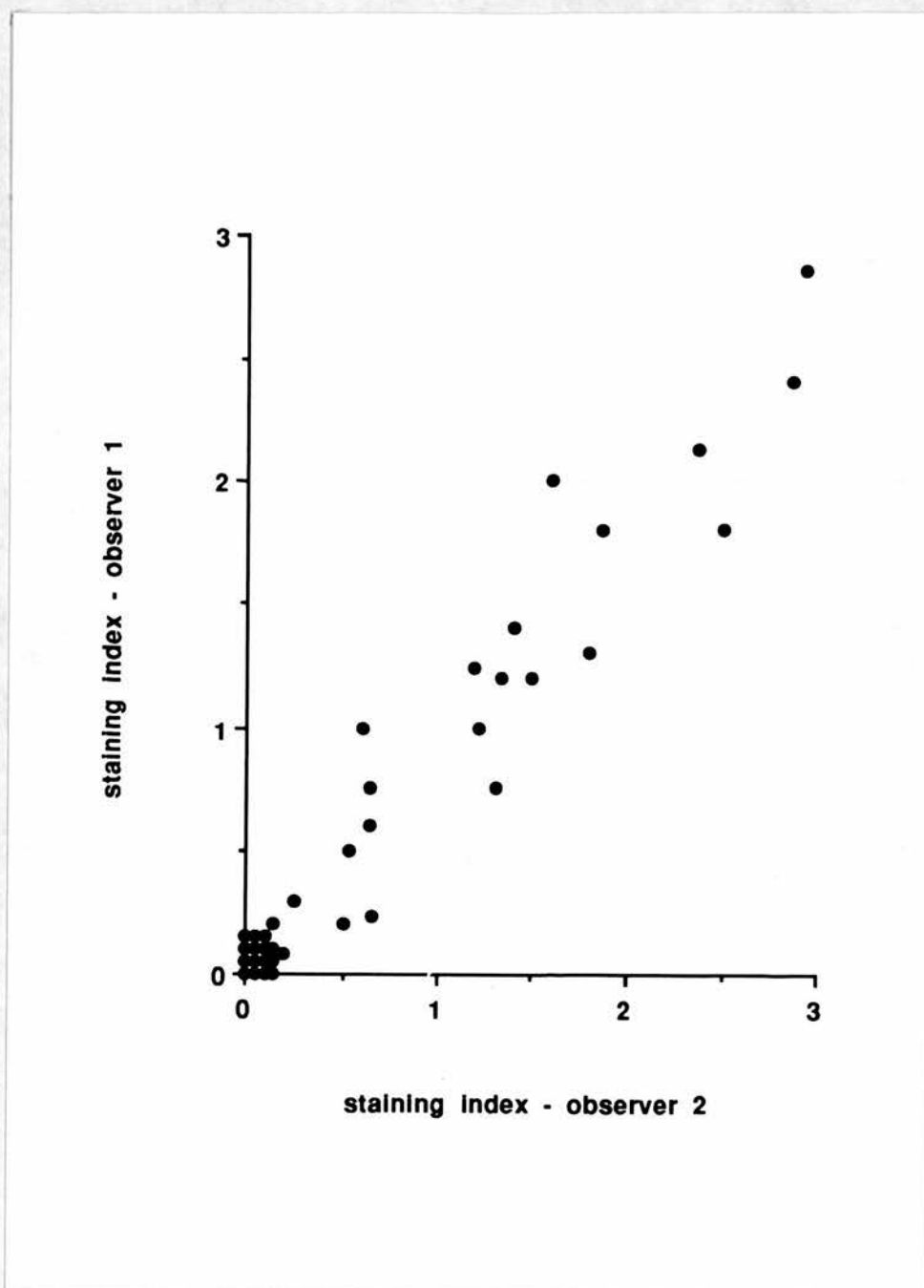


Figure 28
 Correlation of interobserver scoring of staining index on fine-needle aspirates stained with the ERICA kit in 37 patients with large operable breast cancers. The Spearman's Rank Correlation Coefficient was 0.95.

Relationship Between Biochemical and Cytochemical ER Status and Response to Endocrine Therapy

Thirty patients received endocrine therapy. Although response data was available for all patients, assessment of ERICA activity was only possible for 20 tumours. Of these, 11 showed regression, 6 exhibited no change, and in 3, there was evidence of progression.

Biochemical ER concentration was related to response (Figure 31) and showed a statistically significant correlation ($r = 0.48$, $p < 0.05$). The cytochemical "staining index" also related significantly to hormone responsiveness ($r = 0.54$, $p < 0.02$, Figure 31). When however subdivided into its constituent parts, this correlation appeared primarily to be a function of the percent cells staining (Figure 33a, $r = 0.55$, $p < 0.02$); mean staining intensity showed a poor association with subsequent response to endocrine therapy (Figure 33b, $R = 0.38$ $p > 0.05$) and did not improve the predictive value over that obtained from analysis of the proportion of cells staining alone. This was evaluated by considering the association between response and staining intensity only in those tumours in which staining was present ($r = 0.05$, $p > 0.10$).

All tumours responding to therapy had greater than 20% cells staining for ER, or a "staining index" in excess of 0.3. Although there was overlap between the response categories, a gradual step-wise increase in the median "staining index" and the percent cells staining was demonstrated between those tumours which progressed (0.00, 0% cells staining), those remaining static (0.7, 32% cells staining), and those which regressed (1.35, 68% cells staining) (Figures 32 and 33). In general, the higher the staining index or percent cells staining, the greater the likelihood that the patient would respond to therapy (<20% cells staining 0% response, 20-50% staining 50% response, >50% staining 82% response).

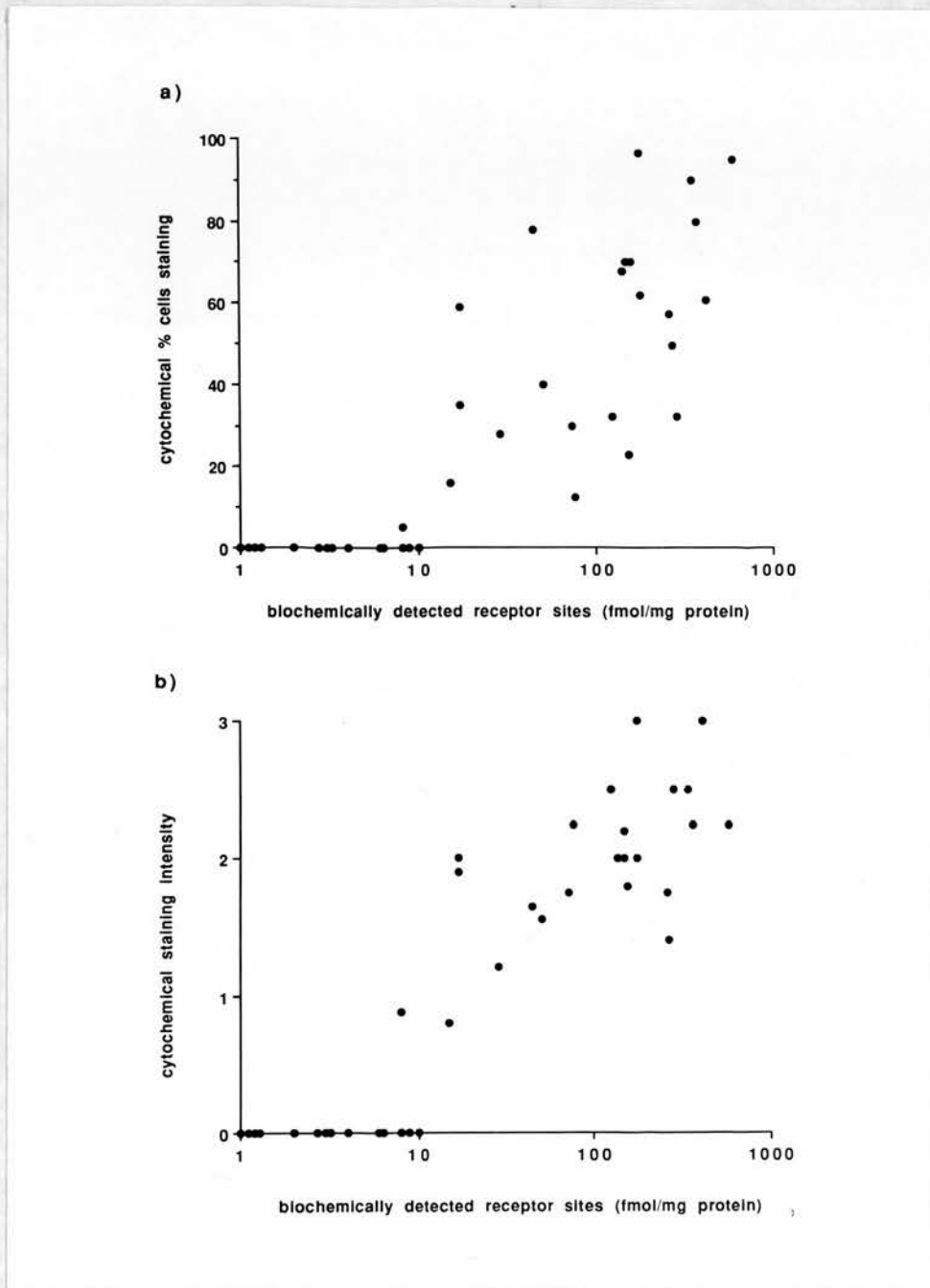


Figure 29

Relationship between the two indices a) % cells staining and b) average staining intensity of immunocytochemical staining for ER in a fine needle aspirate and ER concentration, as determined by DCC assay, in 37 large, operable, primary breast cancers. The Spearman Rank Correlation Coefficient of both graphs was 0.90, giving a p value < 0.001.

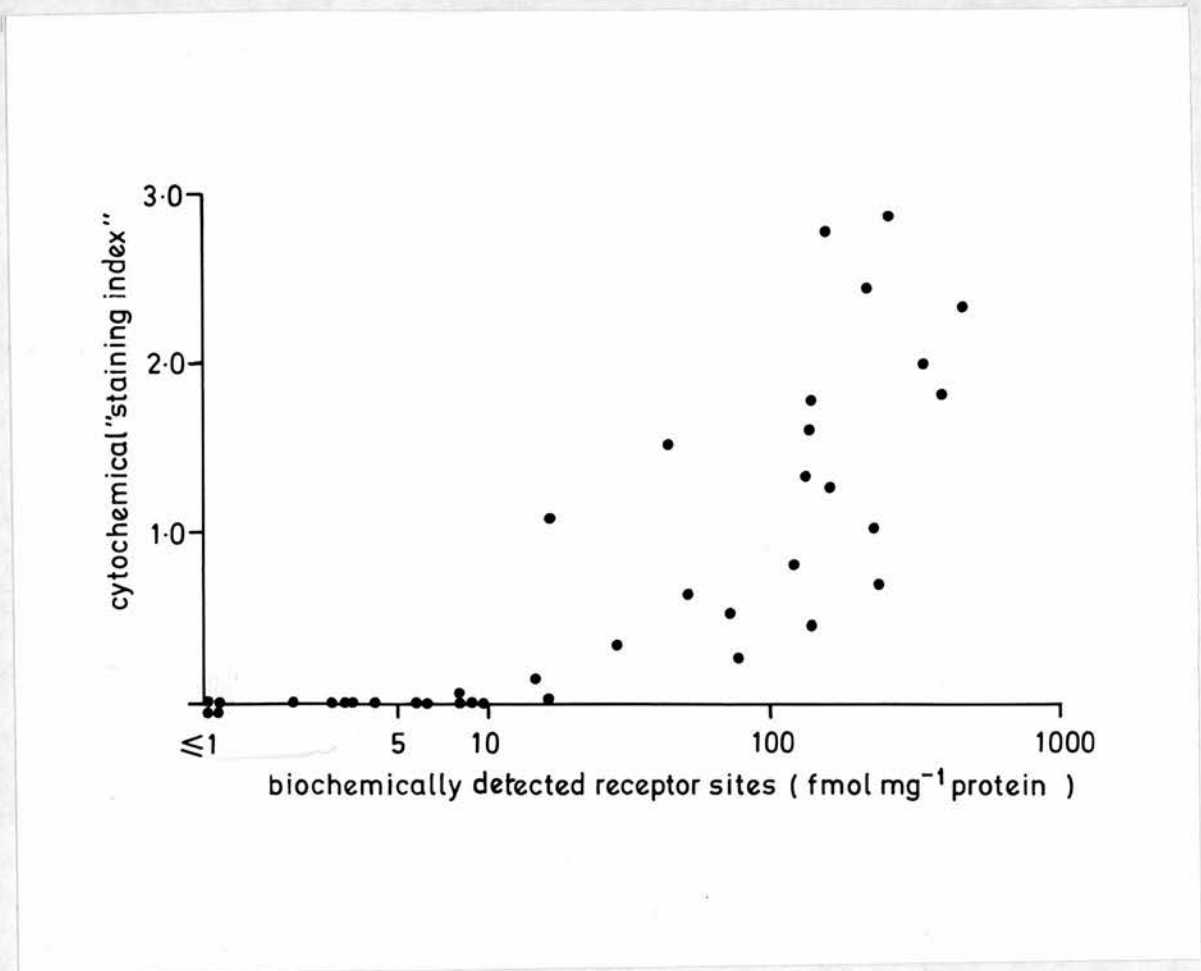


Figure 30
 Relationship between the staining index of immunocytochemical staining for ER in a fine needle aspirate and ER concentration, as determined by DCC assay, in 37 large, operable, primary breast cancers. The Spearman's Rank Correlation Coefficient was 0.89, giving a p value < 0.001.

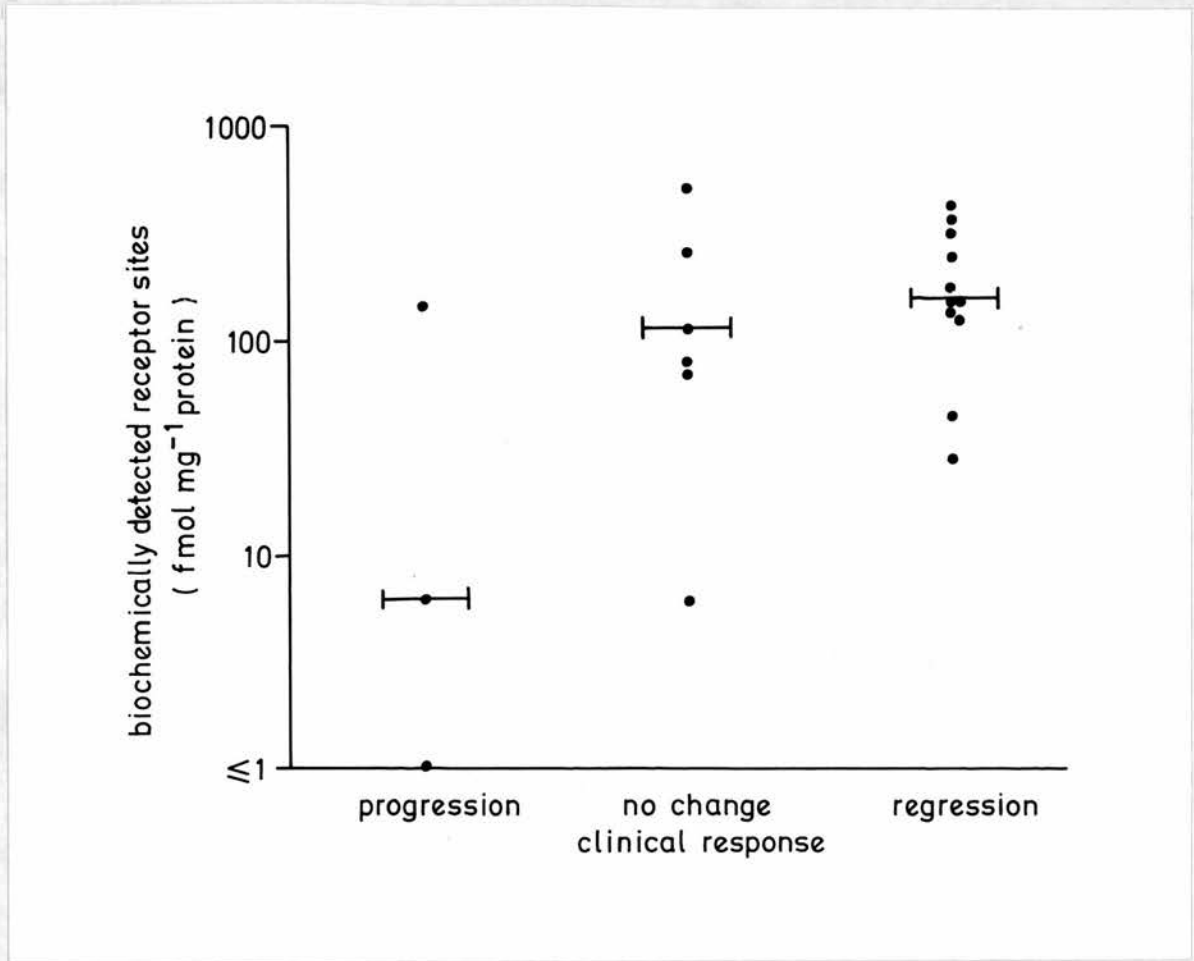


Figure 31
 Relationship between ER concentration, determined by DCC assay and response of the primary tumour to endocrine therapy, in 20 patients with operable breast cancer. The Spearman's Rank Correlation Coefficient was 0.48, giving a p value of <math>< 0.05</math>. The median value within each group is shown by a horizontal bar.

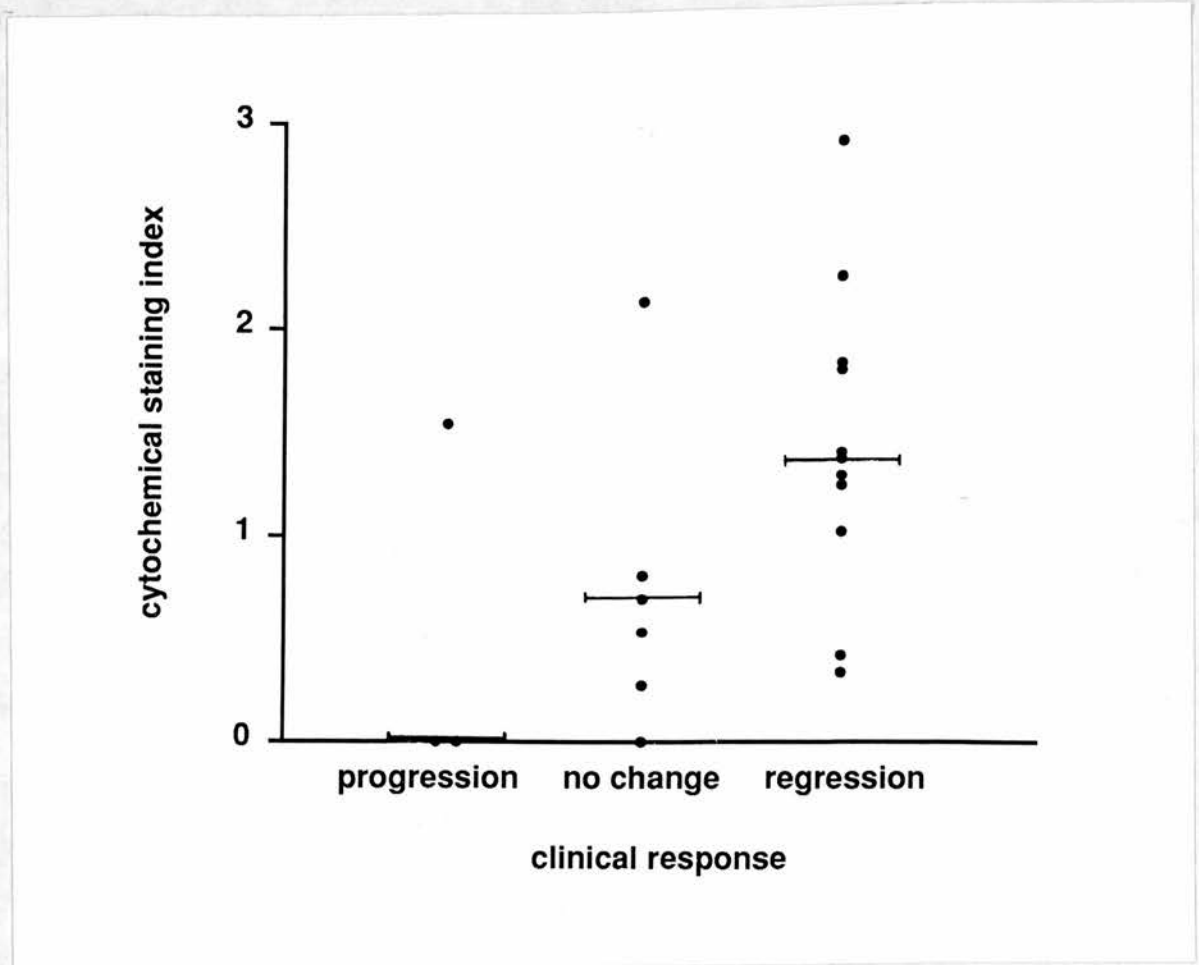


Figure 32
 Relationship between the "staining index "(ERICA) of a fine needle aspirate and response of the primary tumour to endocrine therapy, in 20 patients with operable breast cancer. The Spearman Rank Correlation Coefficient was 0.54, giving a p value of < 0.02. The median value within each group is shown by a horizontal bar.

DISCUSSION

The development of an antibody to the oestrogen receptor (Greene, Fitch & Jensen 1980; Greene & Jensen 1982) has led to the introduction of a commercially available immunocytochemical assay for the ER protein (ERICA, Abbott laboratories). This cytochemical assay offers several advantages over the conventional steroid-binding biochemical assay currently employed in clinical practice since it is rapid, does not require complex laboratory materials and can be performed on small tissue samples obtained by fine-needle aspiration (FNA, Flowers et al. 1986; Hawkins et al. 1988). Several previous studies have examined the relative sensitivities and specificities of the biochemical and immunocytochemical assay methods. Although in general these have demonstrated a good correlation, several differences have been noted (King et al. 1985; McCarty et al. 1985; McLelland et al. 1985, 1986; Hawkins et al. 198; Charpin et al. 1988).

Biochemical assay of ER is performed on homogenised tumour, and while measuring the total receptor content, fails to demonstrate either the proportion of cells which contain ER or the amount of ER contained within individual cells. Cytochemical immunoassay allows assessment of this heterogeneity and in our hands gives consistent and reproducible results with a high interobserver correlation ($r = >0.95$). This is consistent with the reproducibility demonstrated by Kinsel and her colleagues who report an intraobserver correlation of 0.94 and an interobserver correlation of 0.91 when comparing ER activity measured biochemically to ERICA staining in fixed tissue sections (Kinsel et al. 1989).

This study also confirmed the reports of others who have suggested that cytochemical staining of aspirates by the ERICA kit accurately reflects the ER content of the tumour since there was a good correlation between cytochemical staining of both the proportion of cells staining, and their relative staining intensities with biochemical assay. Integrating the cytochemical scores as a "staining index", did not improve this already high correlation. A few tumours of moderate/high biochemically measured receptor value were identified which showed a low staining index (<1.0). The reasons for the low degree of staining within these aspirates have not fully realised since these specimens were not of lower cellularity or higher blood content than those showing a stronger correlation between cytochemistry and biochemistry. Nevertheless the finding

of a correlation coefficient of 0.9 ($n = 37$) is in fact higher than those reported by other workers when comparing ERICA staining on aspirates and receptor concentration in biopsies (Flowers et al. 1985, $r = 0.74$, $n = 33$; Cavailles et al. 1987, $r = 0.76$, $n = 35$). These correlations are higher than that of 0.48 ($n = 95$) found by McLelland et al. (1987) but their series included some comparisons between ERICA staining and DCC assays on different tumour deposits. A similar order of magnitude of correlation has also been found when comparing ERICA staining in frozen or paraffin sections with biochemically determined receptor concentration (King et al. 1985, $r = 0.76$, $n = 38$; McCarty et al. 1985, $r = 0.79$, $n = 38$; Andersen, Orntoft, Skougaard Poulsen 1986, $r = 0.91$, $n = 35$; Hawkins, Sangster & Krajewski 1986, $r = 0.87$, $n = 34$; Kinsel et al. 1989, $r = 0.63$, $n = 257$). Ultimately however, validity of the immunoassay system must be based on a direct comparison with its predictive power for response to endocrine therapy.

A positive association was demonstrated between ERICA staining and response to endocrine therapy such that tumours with a high proportion of cells staining for ER ($> 20\%$) were more likely to be hormone responsive than those with a lower score. Consideration of the semiquantitative estimation of mean staining intensity seemed to add little to the information gained from assessment of % cells staining alone. However only a small series of patients were available for analysis and due to a necessary change in treatment protocol a high proportion of these patients had ER positive tumours. Similar results have however been demonstrated in a cohort of 52 elderly patients treated primarily with tamoxifen (Gaskell et al. 1989) in whom 40% had ER-poor/negative tumours. This study also indicated that the best predictor of regression was the proportion of tumour cells which stained for ER (Gaskell et al. 1989). In 9 patients without demonstrable activity the tumour rapidly progressed whilst on tamoxifen. Coombes et al. (1987) have similarly reported a positive association between cytochemical staining for ER and hormone-sensitivity in advanced breast cancer.

It is interesting to speculate why intensity of staining may not add to % cell staining in prediction of response to hormonal therapy. Although the technical assessment of intensity was more subjective than that of percent cells staining, both parameters related equally well to the biochemical ER concentration and so an alternative explanation must be sought. In another hormonally controlled organ, the testis, it is well documented that response to luteinising hormone (LH) can be elicited with occupancy of only 1% of cellular LH receptors and is maximal when 10% of receptors are occupied (Genuth

1983). If the threshold for the cytochemical detection of ER within a cell corresponded to that required for hormone responsiveness, a further increase in the number of ER within the cell and hence intensity of staining for ER, would be of no biological importance. One could then expect a direct relationship between the proportion of cells staining and the likelihood of response to exist. Although the numbers are small within this study, the pattern of response is in keeping with this hypothesis.

The discrepancy in sensitivity between cytochemical and biochemical detection of ER, at low levels of ER (5-20 fmol/mg cytosol protein) may be of scientific concern, but previous studies (Jensen 1975, McGuire et al. 1974) as well as the present data have shown that a tumour is unlikely to respond to endocrine therapy if the ER concentration is < 20 fmol/mg cytosol protein. An ER activity of 20 fmol/mg cytosol protein would appear to be within the limits of detection of the cytochemical assay. The proportion of tumour cells staining for ER is at least as good as biochemically determined ER status, in predicting hormone responsiveness.

Despite the potential value of the staining of breast aspirates by ERICA, it is important to remember several limitations. Measurement of ER was only possible in 74% of fine-needle aspirates within this study. This proportion may be improved by performing a second aspiration (which is still less invasive than surgical biopsy) but the figure is lower than would be expected given an experienced aspirator. This may relate to the minimum number of tumour cells (around 20) required before the specimen was considered adequate for assessment. Although staining may be visible in a lesser number of cells, a cell count of less than 20 was thought to be insufficient to take account of possible tumour heterogeneity. This is to be contrasted with Flowers et al. (1985) who consider 5 cells as adequate. Secondly the method of cell staining produces some morphological damage to the aspirated cells but interpretation of cell type can be obtained from the concomitant smear which has been fixed directly.

In summary immunocytochemical determination of the proportion of tumour cells staining for ER is a simple, reliable and reproducible technique which can be performed without surgical intervention and the results correlate well with hormone responsiveness. The technique thus has a potential role in determining the value of hormone therapy in those patients in whom systemic therapy is the preferred treatment option i.e. the elderly patient or those with advanced local or metastatic disease. In those patients with operable disease, early determination of the ER status of their

tumour may in the future facilitate preoperative counselling of the patient in relation to appropriate systemic therapy.

RELATIONSHIP BETWEEN LOCAL STEROIDOGENESIS AND RESPONSE TO SYSTEMIC THERAPY

TUMOUR AROMATASE ACTIVITY

Aromatase activity was measured in a total of 62 tumours; 32 tumours before starting systemic therapy and 30 tumours on completion of 12 weeks (or if both endocrine and cytotoxic therapy was given 24 weeks) systemic therapy. All tumours were from postmenopausal women. Significant aromatase activity (≥ 0.5 pmol E₂ produced/g/hr = $\geq 0.02\%$ conversion) was detected in 21 of the 32 pretreatment tumours (65%). Levels varied from < 0.5 to 19.77 pmol E₂ produced/g/hr (median 0.62 pmol E₂ produced/g/hr). No significant relationship was observed between tumour aromatase and ER activity (Table 22).

Reliability of Tumour Aromatase Activity

Due to the large amount of tumour required for assay of tumour aromatase it was not possible to obtain formal measurement of tumour aromatase activity in multiple samples from the same tumour.

Tumour Aromatase Activity and Response to Systemic Therapy

Pretreatment tumour aromatase activity was directly related to clinical response in 32 postmenopausal patients; 25 patients received an aromatase inhibitor (4-hydroxyandrostenedione (n = 12), 4-hydroxyandrostenedione and CHOP (n = 3), aminoglutethimide and hydrocortisone (n = 7), aminoglutethimide and hydrocortisone plus CHOP (n = 3); 6 patients received the chemotherapeutic regime CHOP alone and one patient was given tamoxifen. All patients who received 4-hydroxyandrostenedione had tumours with an ER concentration of ≥ 20 fmols/mg cytosol protein, 4 of the patient who were treated with aminoglutethimide however had ER-poor tumours. The relationship between tumour aromatase activity and clinical response is shown in Table 23.

When response to aromatase inhibition was analysed in relation to the presence or absence of detectable levels of tumour aromatase activity (cut of level 0.5 pmol E₂ produced/g/hr), no significant relationship was demonstrated (Table 23)

Table 22

Relationship between the oestrogen receptor concentration of the primary tumour as measured by the dextran coated charcoal method and tumour aromatase activity in 32 patients with large operable breast cancers. ($p = 0.67$, Fishers exact test).

ER status	Tumour aromatase	
	negative <0.5*	positive ≥0.5*
<5 fmol/mg protein	3	4
≥5 fmol/mg protein	8	17

If however a higher level of activity at 0.85 pmol E2 produced/g/hr is taken, then although not absolute, a statistically significant relationship between response to aromatase inhibition and the presence of high levels of tumour aromatase activity was detected (Table 24). Significant regression was seen in 7 out of 10 tumours with higher activity (≥ 0.85 E2 produced/g/hr) whereas only 4 of 15 tumours with lower levels demonstrated significant regression. There was no relationship between response to chemotherapy and aromatase activity of the primary tumour (Table 24).

Table 23

Relationship between clinical response to aromatase inhibitors and tumour aromatase activity in 32 large operable breast cancers. The relationship has been analysed using the Fishers exact test.

	Tumour Aromatase Activity		p value
	negative <0.5*	positive ≥0.5*	
Aminoglutethimide			
Significant regression	1	3	
No significant regression	3	3	0.57
4-hydroxyandrostenedione			
Significant regression	3	4	
No significant regression	3	5	1.0
Aromatase Inhibitor - Total			
Significant regression	4	7	
Non significant regression	6	8	1.0
Chemotherapy (CHOP)			
Significant regression	1	3	
No significant regression	1	1	1.0
Tamoxifen			
No significant regression	1	0	-

*pmol E² produced/g/hr

Table 24

Relationship between clinical response to aromatase inhibitors and tumour aromatase activity in 32 large operable breast cancers. The relationship has been analysed using the Fisher exact test.

	Tumour Aromatase Activity		p value
	<0.85*	≥0.85*	
Aminoglutethimide			
Significant regression	1	3	0.19
No significant regression	5	1	
4-hydroxyandrostenedione			
Significant regression	3	4	0.32
No significant regression	6	2	
Aromatase Inhibitor - Total			
Significant regression	4	7	0.048
Non significant regression	11	3	
Chemotherapy (CHOP)			
Significant regression	2	2	1.00
No significant regression	1	1	
Tamoxifen			
No significant regression	1	0	-

*pmol E² produced/g/hr

17B-HYDROXYSTEROID DEHYDROGENASE ACTIVITY

17B-hydroxysteroid dehydrogenase activity was measured before therapy in 24 patients; 10 were premenopausal and 14 were postmenopausal. Thirteen patients had tumours with an ER concentration of ≥ 20 fmol/mg cytosol protein. Systemic therapy within the 24 patients was as follows, 6 received 4-hydroxyandrostenedione therapy alone, two patient underwent therapy with goserelin followed by surgical oophorectomy, 11 patients received combination chemotherapy (CHOP), and 5 patients received both endocrine (4-hydroxyandrostenedione n = 2, goserelin n = 3) and chemotherapy (CHOP).

Relationship Between 17B-hydroxysteroid Dehydrogenase and Patient and Tumour Characteristics

Enzyme activity was detected in all samples assayed but there was a wide variation between individual patients (range 0.27 - 21.7; median 2.9 pmol/mg protein/hr). No relationship could be demonstrated between 17B hydroxysteroid dehydrogenase activity and menopausal status of the patient (Mann-Whitney U Test; Figure 34) or the oestrogen receptor status of the tumour (Spearman Rank Test; Figure 35). Comparison of pretreatment 17B-hydroxysteroid dehydrogenase activity and pathological axillary node status was only possible for 12 patients, no obvious relationship could be demonstrated (Mann-Whitney U Test; Figure 36).

Relationship Between 17B-Hydroxysteroid dehydrogenase Activity and Response to Systemic Therapy

The relationship between pretreatment peritumour fat 17B-hydroxysteroid dehydrogenase activity and response to systemic therapy is shown in Figure 37. There was no significant difference in the mean activity of those patients who did (mean 1.95, range 0.28-5.42 pmol T/mg protein/hr) and did not (mean 1.39, range 0.27 - 2.88 pmol T/mg protein/hr) achieve significant regression with systemic therapy (Mann-Whitney U Test). The lack of correlation was obvious irrespective of whether endocrine, cytotoxic or both methods of treatment were considered.

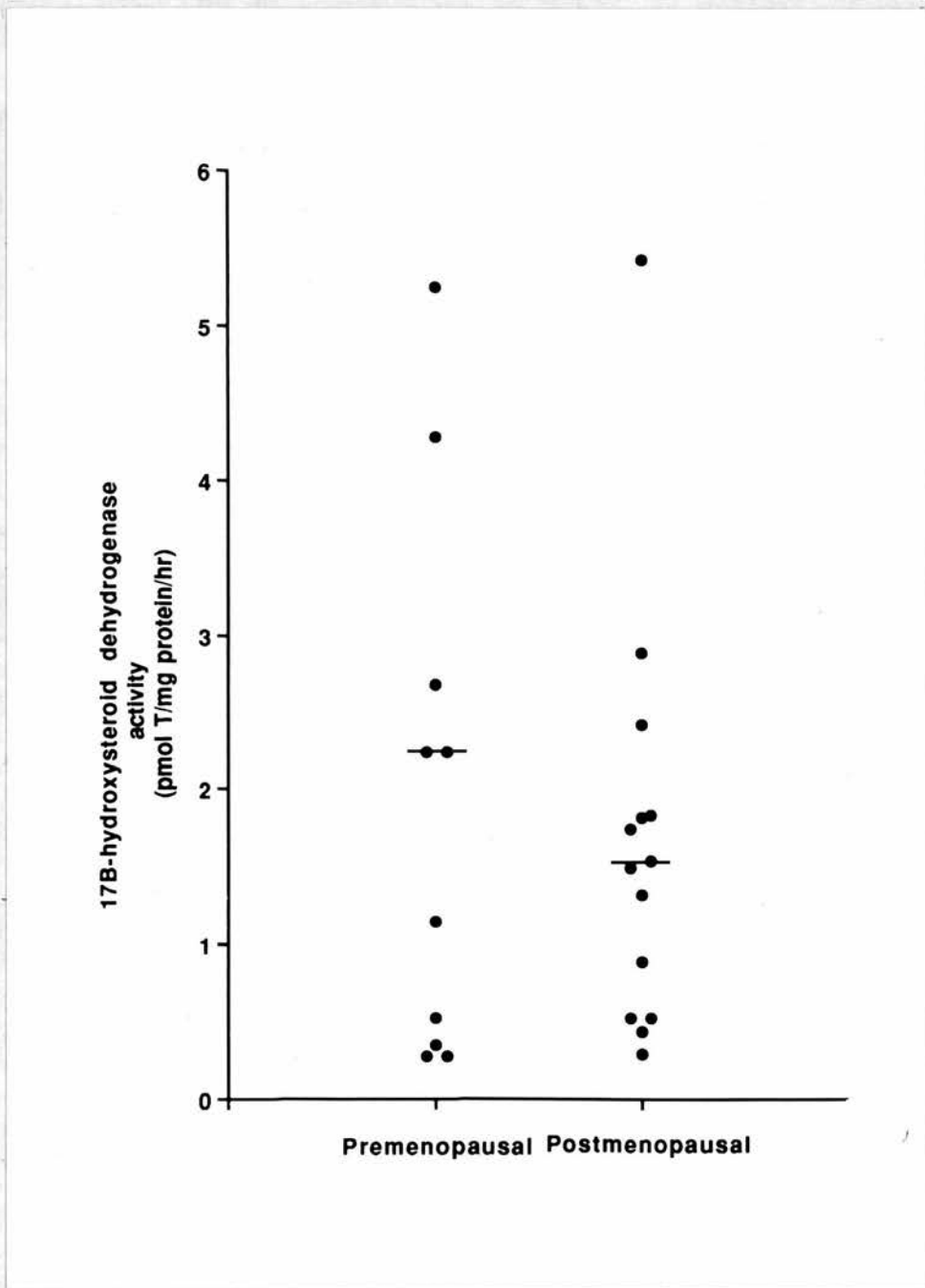


Figure 34
 Relationship between 17B-hydroxysteroid dehydrogenase activity in peritumour fat and menopausal status in 24 patients with large operable breast cancers. Bars denote median values. There is no statistical difference between the two groups using the Mann-Whitney U Test.

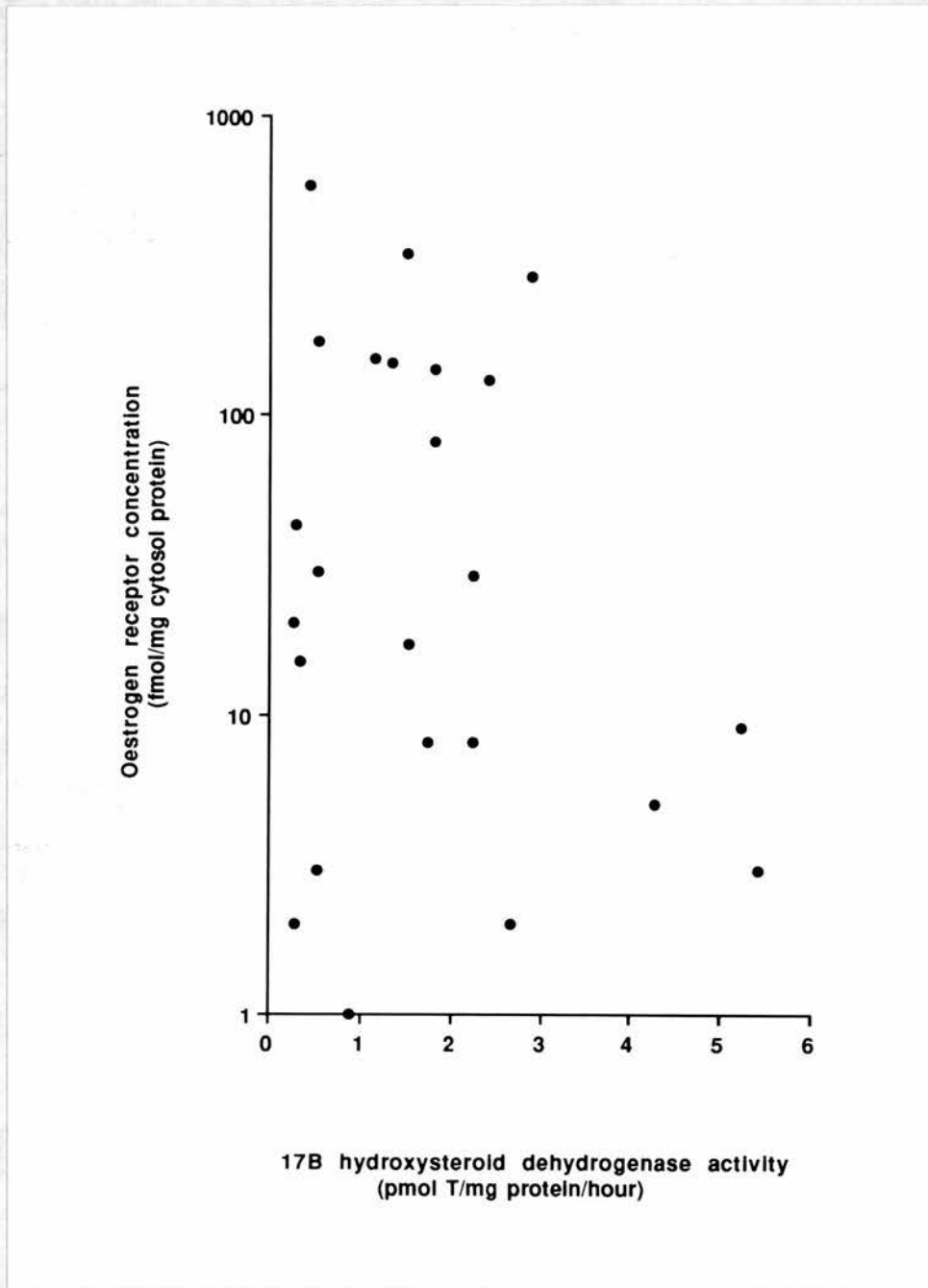


Figure 35
 Relationship between the oestrogen receptor concentration of the primary tumour and the 17B-hydroxysteroid dehydrogenase activity of the peritumour fat in 24 operable breast cancers. No significant correlation was demonstrated (Spearman Rank Test, $R = -0.122$, $p > 0.05$).

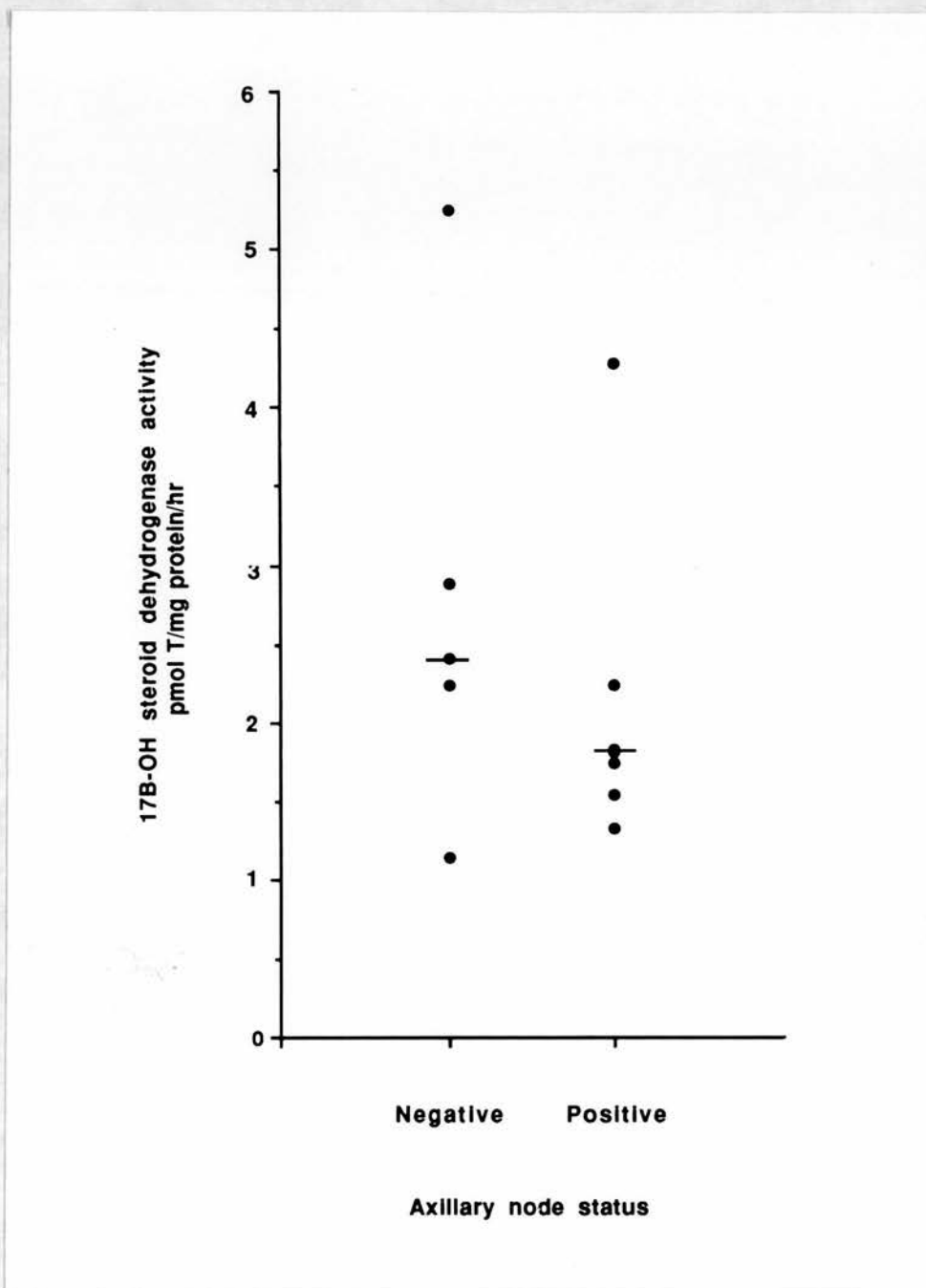


Figure 36
 Relationship between 17B-hydroxysteroid dehydrogenase activity in peritumour fat and axillary node status in 12 patients with large operable breast cancers. Bars denote median values. There is no statistical difference between the two groups using the Mann-Whitney U Test.

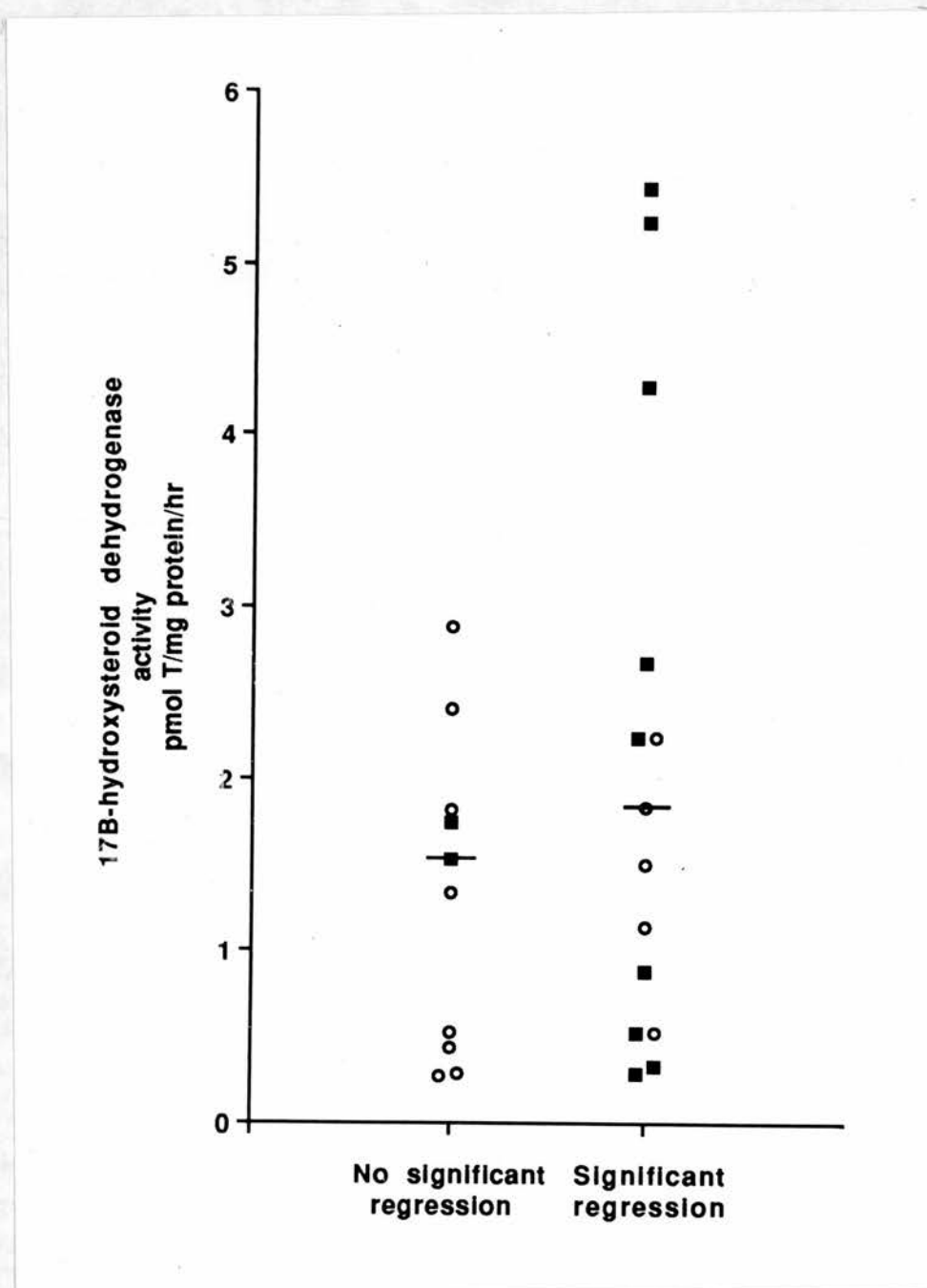


Figure 37
 Relationship between 17B-hydroxysteroid dehydrogenase in peritumour fat and clinical response to systemic therapy in 24 operable breast cancers. Patients treated with endocrine therapy are shown by open circles, and those treated with primary chemotherapy by solid squares. The horizontal bars denote median values. The difference between the two response categories is not significant by the Mann-Whitney U test.

DISCUSSION

The involvement of steroid hormones in the natural history of breast cancer has long been recognised. Although the level of circulating hormones has been extensively investigated no specific abnormality has been related to an increased risk of developing breast cancer, castration at an early age however is associated with a reduced risk of breast cancer (Feinleib 1968). The circulating levels of oestrogens and their precursors do not accurately reflect levels within breast cancers (Van Landeghem et al. 1985; Vermeulen et al. 1986) and local steroidogenesis may have considerable biological significance. The relationship between hormone responsiveness and the activity of two of these enzymes, namely aromatase and 17 β -hydroxysteroid dehydrogenase has been studied.

A positive association has been demonstrated between the presence of high aromatase activity in a tumour and an increased likelihood of response to anti-aromatase therapy. The addition of an aromatase activity of greater than 0.85 pmol E₂ produced/g/hr to ER concentration improved the prediction for response to aromatase inhibition from 50% (11/22) to 70% (7/10). Miller (personal communication) has not been able to demonstrate any relationship between tumour aromatase activity and clinical response in either premenopausal women treated by surgical castration or postmenopausal women treated with tamoxifen. This suggests that tumour aromatase has a particular predictive association with anti-aromatase agents. The absence of significant levels of tumour aromatase was not synonymous with resistance to aromatase inhibitors presumably because these tumours derived their oestrogen as a result of aromatase activity in other tissues e.g. fat.

Tumour aromatase activity can be regulated by autocrine and paracrine hormones produced by the tumour itself (Lippman et al. 1986, Knabbe et al. 1987) and this represents a possible mechanism of growth control for hormone responsive tumours. Although no relationship was demonstrated in this study between ER-status and tumour aromatase activity, tumour aromatase activity may only be of clinical importance in ER-positive tumours.

17 β hydroxysteroid dehydrogenase is a pivotal enzyme in the interconversion of both androgens and oestrogens from the relatively inactive precursors androstenedione and

oestrone to the biologically active testosterone and oestradiol. Within this study 17B hydroxysteroid dehydrogenase activity has been assessed by measuring the conversion of androstenedione to testosterone. It has been established however that essentially the same order of enzyme activity can be detected whether 1B 3H androstenedione or 2,4,6,7 3H oestrone is used as a substrate although interconversion of androgenstends to be greater than that of oestrogens (Miller & O'Neill 1987) .

The levels of 17B -hydroxysteroid dehydrogenase activity detected within the presented group of patients was similar to that described by other authors for breast cancers of greater than 3 cm in diameter using a similar methodology (median 1.9, range 0.3 - 7.8 pmol testosterone/mg protein/hour; Miller & O'Neill 1987). The spectrum of activity was similar irrespective of patient menopausal status confirming previous published work (Santen et al. 1986).

Although no direct evidence is yet available, it has been postulated that tumours with a higher level of endogenous oestradiol production are more likely to be hormone sensitive. It has been suggested that ER-positive tumours (indirect measure of hormone sensitivity) have a significantly higher 17B-hydroxysteroid dehydrogenase activity than ER-negative tumours (Vermeulen and Deslypere, 1989). Despite an equivalent sample size this relationship has not been reproduced in this study. Similarly no direct relationship between 17B-hydroxysteroid dehydrogenase activity and tumour response to endocrine therapy was demonstrated. Results from only 13 patients were available for analysis and the failure to demonstrate any relationship may be a function of small population size. As a discriminating index for hormone sensitivity however 17B hydroxysteroid dehydrogenase activity would appear to have little value in routine practice.

In summary, tumor aromatase activity ≥ 0.85 pmol E2 produced/g/hr has a predictive association for response to anti-aromatase agents but the relationship is not absolute. No relationship was apparent between the endogenous ability to produce oestradiol and response to either endocrine or cytotoxic therapy.

Relationship Between Response and Histological Grade

Consistency of Grading

Grading was available for a total of 147 tumour specimens; 80 pretreatment and 67 post-systemic treatment. When the specimen were regraded blind by the same pathologist, in only 9 of the 147 tumours was a discrepancy in tumour grade noted giving an overall 92.5% concordance. When a discrepancy occurred the specimens were regraded a third time.

Histological grading of the pretreatment biopsy was available for 80 of the 88 patients within this study, of these 7(9%) tumours were classified as grade I, 26(32%) as grade II and 47(59%) as grade III.

Relationship Between Histological Grade and Other Prognostic Parameters

An inverse relationship between histological grade and ER concentration was noted within this cohort of patients (Table 25). This trend however failed to reach statistical significance. The relationships between histological grade, menopausal status and axillary node status of the patient are shown in Table 26 and 27 respectively. No significant correlation was demonstrated.

Table 25.

Relationship between histological grade and oestrogen receptor status of the primary tumour as determined by dextran-coated charcoal adsorption method, in 88 large operable breast cancers. The relationship does not reach statistical significance ($\chi^2 = 3.71$, $p = 0.16$).

Histological grade	Proportion of tumours with ER concentration $\geq 20^*$
I	5/7 (72%)
II	16/26 (62%)
III	19/47 (40%)
unknown	6/8 (75%)

TOTAL	46/88 (52%)

* fmol/mg cytosol protein

Table 26.

Relationship between histological grade as defined by Bloom and Richardson criteria and menopausal status in 88 patients with large operable breast cancers. The relationship is not significant ($\chi^2_2 = 3.89$, $p = 0.143$)

Histological grade	Menopausal status	
	premenopausal	postmenopausal
I	5	2
II	12	14
III	16	31
unknown	5	3

TOTAL	38	50

Table 27.

Relationship between pretreatment histological grade and axillary lymph node status in 88 patients with large operable breast tumours. The relationship is not significant ($\chi^2_2 = 0.364$, $p = 0.833$).

Histological grade	Axillary node status		
	positive	negative	unknown
I	4	1	2
II	10	3	13
III	16	7	24
unknown	3	1	4

TOTAL	33	12	43

Relationship Between Pretreatment Histological Grade and Response to Systemic Treatment

Direct comparison of pretreatment histological grade and response was available for 55 patients receiving endocrine and 25 patients receiving primary cytotoxic therapy. No significant relationship was demonstrated between response to endocrine therapy and histological grade but patients with grade III tumours appeared less likely to respond to endocrine therapy (Table 28). This trend was no longer obvious when only those patients with tumours of ER concentration of ≥ 20 fmol/mg cytosol protein were considered (Table 28).

There was also no correlation between grade and response to primary cytotoxic therapy (Table 29). The proportion of tumours within grades II and III which achieved complete response following cytotoxic therapy was similar at 29% and 30% respectively.

Table 28.

Relationship between histological grade of the primary tumour as judged by Bloom and Richardson criteria, oestrogen receptor status (dextran-coated charcoal adsorption assay) and response to endocrine therapy in 61 large operable breast cancer. No significant relationship exist either when all patients receiving endocrine therapy are considered ($\chi^2_2 = 0.778$, $p = 0.68$) or only those with tumours of ER concentration ≥ 20 fmol/mg cytosol protein ($\chi^2_2 = 0.153$, $p = 0.93$).

Histological grade	TOTAL Proportion of patients with significant response	ER $\geq 20^*$ Proportion of patients with significant response
I	3/6	3/5
II	8/19	8/16
III	10/30	10/19
unknown	3/6	3/6
<hr/>		
TOTAL	24/61	24/46

* fmol/mg cytosol protein

Table 29

Relationship between histological grade of the primary tumour as judged by Bloom and Richardson criteria and response to primary cytotoxic therapy in 27 patients with large operable breast cancer. No significant relationship exists ($\chi^2_2 = 0.24$ $p = 0.89$).

Histological grade	Proportion of patients with significant response
I	1/1
II	6/7
III	14/17
unknown	2/2
<hr/>	
TOTAL	23/27

DISCUSSION

Histological grading is based on a subjective assessment of microscopic appearances. Difficulties in consistency and reproducibility are possible. Within this series a high degree of consistency was achieved on the first assessment. The figure of 92.5% compares very favourably with that reported by the Nottingham/Tenovus study (Elston 1988) who report a 90% agreement on first assessment when sections were graded independently by two experienced histopathologists, and by Fisher (Fisher et al. 1975) who report a 94% agreement by the same reviewer on different occasions.

Within this series of patients the distribution of tumours with respect to histological grade was within the limits of previously reported series (Table 6) although the number of patients with grade I tumours at 9% was within the lower limits of the range. This has acted to reduce the statistical power of the results in relation to response.

Within this study an inverse relationship between histological grade and response to endocrine therapy was observed (50% grade I, 42% grade II and 33% grade III), although this failed to reach statistical significance. Previously reported studies have demonstrated a significant relationship (Millis et al. 1981, Masters et al. 1987, Williams et al. 1986) and the failure to do so within this study may be a function of the smaller number of patients particularly those with grade I tumours.

A positive correlation between tumour grade and ER concentration has previously been demonstrated such that the more differentiated, lower grade tumours are more likely to be ER-positive (Fisher et al. 1987; Kamby et al. 1988; Singh et al. 1988). Response to endocrine therapy is known to occur more frequently in ER-positive tumours (McGuire et al. 1975; Hawkins Roberts & Forrest 1980). The relationship observed within previous studies between grade and response to endocrine therapy may therefore simply be an indirect function of the relationship between grade and ER concentration although an additional predictive effect of grade to ER status has been suggested (Williams et al. 1986). The proportion of ER-positive tumours responding within this latter series was however low at 32%. Most other series report response rates of 49-58% for patients with ER-positive tumours (McGuire et al. 1975, King et al. 1982; Hawkins, Roberts & Forrest 1980). Had these higher rates been found then the small additional effect of grade may not have been obvious. Certainly when the response rates were recalculated within this study to consider only those patients with tumours of ER concentration ≥ 20 fmol/mg cytosol protein (overall response rate 52%) the

predictive effect of grade was diminished (60% grade I, 50% grade II and 53% grade III).

Well differentiated tumours with the exceptions of lymphomas and chronic lymphocytic leukemia tend to be relatively resistant to chemotherapy with poorly-differentiated tumours more likely to respond (Whitehouse 1984). This study has suggested that histological grade has no such predictive value in breast cancer and confirms the finding in advanced breast cancer of other authors (Masters et al. 1987). The overall response rate to primary chemotherapy within this study was high at 85% and given the small number of patients it was unlikely that anything other than a dramatic difference would have been obvious.

Within this study estimation of tumour grade did not give any useful additional information in relation to hormone-responsiveness over that provided by measurement of the ER concentration alone. Similarly tumour grade was not a useful predictor of response for cytotoxic therapy.

RESPONSE TO SYSTEMIC THERAPY AND DNA ANALYSIS

Fine-needle aspiration as a Method of Cell Yield for Flow Cytometry

To determine the qualitative reproducibility of flow cytometric DNA analysis, using fine-needle aspiration as the method of biopsy, 4 FNAs were taken from each of 20 excised tumour specimens. On histological examination, all tumours were primary breast adenocarcinomas of no special type. From the total of 80 FNA specimens, 12 (15%) were acellular and 2 (2.5%) were unassessable; DNA histograms were therefore evaluable in a total of 66 FNAs (82.5%). Since in one tumour, all 4 FNAs were acellular, DNA analysis was eventually possible in 19 tumours (95%), although in only 17 was a comparison between FNAs from the same tumour possible. Three tumours (15.7%) were diploid, the remaining 16 (84.3%) were aneuploid (hypodiploid 1(6.2%), hyperdiploid 7, multiploid 1, tetraploid 6, hypertetraploid 2).

All three diploid tumours maintained their diploid character in all FNAs examined. Similarly all aneuploid tumours conserved their aneuploid status and there was good concordance between the DIs of the aneuploid cell line(s) within specimens from the same tumour (pooled standard deviation 0.078, range 0.017 - 0.19, Table 30). There was however a discrepancy in the aneuploid subclassification of 3 of the 16 aneuploid tumours. These tumours remained consistently aneuploid, but in two a second aneuploid cell line appeared in one of the 4 aspirates, while in the other, two aneuploid cell lines were detected in 3 of the 4 aspirates. In all three cases, the deviation occurred in only one of the 4 specimens available for analysis and in each the abnormal cell line constituted only approximately 11% of the total cell count.

There was a discrepancy in ploidy status in 5 of the 19 tumours when the FNA sample was compared to paraffin tissue sections of the same tumour specimen (Table 32). In 4 cases the FNA reproducibly detected an aneuploid cell line not picked up on the paraffin sections. The reverse being true for only one case. The presence of tumour cells was confirmed cytologically in the FNA specimens.

For any given tumour however, the difference between the relative proportion of cells with diploid and aneuploid DI was much greater (Figure 31). The pooled standard deviation within the aneuploid population was 14.6% (range 1.56% - 39.1% total cell counts).

Table 30

Variability of the DNA index of 17 aneuploid cell lines within 4 separate FNAs taken from the same tumour.

1	DNA Index	3	4	Mean	Standard deviation	CV	Variance
1.31	1.31	1.29	1.34	1.31	0.021	1.57	0.00043
1.29	1.27	1.41	1.29	1.31	0.064	4.87	0.0041
1.26	1.42	1.39	-	1.35	0.085	6.30	0.007
1.55	1.57	1.49	1.43	1.51	0.063	4.19	0.004
1.62	1.93	1.57	-	1.71	0.19	11.43	0.038
1.69	1.72	1.56	1.73	1.67	0.079	4.69	0.006
1.76	1.74	1.73	1.72	1.74	0.017	0.98	0.00029
1.81	1.84	-	-	1.83	0.021	1.16	0.00045
1.96	1.91	1.90	1.91	1.92	0.027	1.41	0.0007
1.91	1.94	1.98	1.92	1.94	0.031	1.598	0.0091
1.84	1.93	2.07	-	1.95	0.116	5.95	0.013
2.03	2.10	1.92	1.96	2.0	0.080	3.96	0.006
1.98	1.99	2.12	-	2.03	0.078	3.847	0.0061
1.92	2.20	2.03	2.03	2.04	0.120	5.65	0.013
2.38	2.33	2.39	2.37	2.37	0.026	1.11	0.0007
2.45	2.54	2.54	2.58	2.53	0.056	2.1	0.003
2.9	2.83	2.83	2.91	2.87	0.043	1.52	0.0019

Mean

3.67

Pooled variance = 0.0062

Pooled standard deviation = 0.079

Table 31

Variability of the proportion of aneuploid cells of 17 aneuploid cell lines within 4 FNAs taken from the same tumour.

1	DNA Index			Mean	Standard deviation	CV	Variance
	2	3	4				
47.2	37.7	47.4	44.6	44.2	4.53	10.25	20.55
19.1	14.9	18.5	16.0	17.12	2.00	11.68	4.00
35.2	36.7	52.4	33.4	39.42	8.78	22.20	76.64
26.6	27.4	11.6	44.0	27.40	13.24	48.30	175.28
68.8	61.3	73.2	56.6	64.40	7.44	11.45	55.31
11.1	9.8	12.9	-	11.27	1.56	13.82	2.42
47.3	45.4	52.7	58.7	51.00	5.98	11.72	35.74
51.5	61.1	65.3	64.3	60.55	6.29	10.39	39.61
51.3	25.6	21.0	39.1	34.25	13.72	40.06	188.20
19.0	58.8	64.6	64.5	51.72	21.98	42.50	483.32
87.4	24.2	90.8	-	67.47	37.51	55.60	1406.80
18.9	87.7	21.1	-	42.57	39.10	91.80	1528.97
59.8	53.4	47.2	50.7	52.78	5.33	10.09	28.38
77.5	70.1	72.6	69.6	72.4	3.61	4.99	13.06
58.6	41.1	67.7	-	55.8	13.52	24.23	182.77
21.9	24.6	-	-	23.25	1.91	8.21	3.64
92.9	88.4	89.5	91.5	90.57	2.01	2.22	4.05
Mean						24.68	

Pooled variance = 213.7

Pooled standard deviation = 15.81

Table 32

The relationship between the detection of an aneuploid cell line in FNA compared to the same tumour on histological tissue section is shown for 19 tumours in which reproducibility was measured.

Fixed Tissue	FNA	
	Diploid	Aneuploid
Diploid	2	4
Aneuploid	1	12

Of the three diploid tumours, only two had more than one specimen available for analysis. The standard deviation for % S-phase was 6.3% (range 8.3% - 22.5% total cell counts).

Relationship Between Ploidy, DNA Index and Response to Systemic Therapy

FNA material from the primary tumour before treatment was available in 53 patients, 29 received endocrine therapy and 24 primary cytotoxic therapy. Of the 29 aspirates from patients who received endocrine therapy 3 (10%) were acellular. Of the remaining 26, 18 (69%) were aneuploid and 8 (31%) were diploid. Significant regression was noted in 5 of the 8 patients with diploid tumours (62.5%) but in only 6 of the 18 patients with aneuploid tumour (33.3%). With the small numbers of patients studied the relationship does not reach statistical significance (Table 33). The proportion of tumours showing significant regression was similar for both tetraploid and hyperdiploid tumour (Table 34). All patients who received endocrine therapy within this subgroup had tumours with an ER \geq 20 fmol/mg cytosol protein.

Twenty of the 24 patients who received primary cytotoxic therapy had sufficient cells to allow DNA analysis on the FNA. Of these 16 (80%) had aneuploid tumours and 4(20%) had diploid tumours. The relationship between ploidy status, DNA index and response to cytotoxic therapy is shown in Tables 35 and 36 respectively. No obvious relationship between regression with cytotoxic therapy and either ploidy status and DNA index was demonstrated. All patients who received cytotoxic therapy within this subgroup had tumours with an ER concentration of <20 fmol/mg cytosol protein.

Table 33

Relationship between ploidy measured by flow cytometric analysis of FNA biopsy of the primary tumour before therapy and response to endocrine therapy in 26 patients with large operable breast cancers. The relationship is not significant ($p = 0.22$, Fishers exact test).

	Diploid	Aneuploid
Significant regression	5	6
No significant regression	3	12

Table 34

Relationship between DNA index of the 18 patients with aneuploid tumours as measured by flow cytometric analysis of FNA biopsy of the primary tumour before therapy and response to endocrine therapy.

	Significant regression	No significant regression
Hypodiploid (DI<0.9)	-	1
Hyperdiploid (1.1 >DI<1.9)	3	6
Tetraploid (1.9 >DI<2.1)	2	4
Hypertetraploid (DI >2.1)	-	1
Multiploid	1	-

Table 35

Relationship between ploidy measured by flow cytometric analysis of FNA biopsy of the primary tumour before therapy and response to cytotoxic therapy in 20 patients with large operable breast cancers. The relationship is not significant ($p = 1.00$, Fishers exact test).

	Diploid	Aneuploid
Significant regression	3	11
No significant regression	1	5

Table 36

Relationship between DNA index of the 17 patients with aneuploid tumours as measured by flow cytometric analysis of FNA biopsy of the primary tumour before therapy and response to cytotoxic therapy.

	Significant regression	No significant regression
Hypodiploid (DI<0.9)	1	1
Hyperdiploid (1.1 >DI<1.9)	3	2
Tetraploid (1.9 >DI<2.1)	2	2
Hypertetraploid (DI >2.1)	2	1
Multiploid	2	1

DISCUSSION

Fine-needle aspiration biopsy was first introduced in 1930 (Martin & Ellis 1930) but its potential as a method for harvesting cells for both diagnostic cytopathology (Zajdela et al. 1975; Dixon et al. 1983, 1984) and flow cytometric analysis (Levack et al. 1987; Remvikos et al. 1988) has only been realised more recently. The method has several obvious advantages over other biopsy methods in that it is relatively atraumatic and easily performed as an outpatient thus potentially allowing sequential tumour biopsies. There are however more subtle advantages. When different paraffin block sections from the same tumour are assessed intratumour variation in DNA ploidy and index have been observed in 21% of ovarian, 24% of breast cancers with still higher rates reported for colorectal (Quirke et al. 1985; Hiddeman et al. 1986; Petersen, Lorentzen & Bichel 1980) and renal cell carcinoma (Ljungberg, Stenling & Roos 1985). The usual technique of multiple (X10) passages through the tumour in varying directions ensures that the sample contains cells from a wide area of the tumour. It may therefore represent a potentially more reliable method for harvesting cells for DNA analysis (Hartley et al. 1988).

This study confirms previous reports that FNA will in the majority of cases (82.5%), provide sufficient tumour cells to allow good quality flow cytometric DNA histograms (Levack et al. 1987). The technique therefore compares favourably in this respect to the results obtained when fresh homogenised solid tissue is used as the tissue source (Dressler et al. 1988). The majority of acellular aspirates were from 3 tumours and this may reflect the cellularity and structure of those tumours. The frequency of aneuploidy and the distribution of aneuploidy subclassification, both fall within previously observed ranges (Dressler et al. 1988; Harvey et al. 1987; Barlogie et al. 1983).

The high reproducibility of ploidy within this study means that by using FNA as a biopsy technique one would be unlikely to misclassify ploidy status. Although there was a discrepancy in the detection of a cell population in 2 of the 66 evaluable specimens this did not ultimately affect the classification to either a diploid or aneuploid tumour type. The conflict occurred when the cell line constituted only 11% of the total cell population and may therefore be at the lower limits of detection for an aneuploid cell line. If only a simple estimate of ploidy status is required, FNA can be employed as a biopsy technique.

For a specific tumour however the proportion of cells within any given aneuploid population was not so reproducible suggesting that changes in the relative proportions of cell lines may be difficult to detect reliably. Serial FNA would therefore not appear to be an acceptable biopsy technique by which to follow the response of cell lines to therapy. Tumours may also be genetically unstable and undergo spontaneous loss or acquisition of new cell clone populations unrelated to therapy and so such studies may have their own intrinsic difficulty irrespective of the method of measurement of DNA indices.

Within the two diploid tumours for which multiple FNA samples were available examined no direct comment can be made on the reproducibility of FNA in measuring %S phase except to say that a wide range was observed. Significant intratumour variation in cell proliferation of diploid tumours has been documented by other workers using TLI (Lambert 1986; Norton 1985; Smallwood, Cooper & Taylor 1983) and %S-phase (Kallioniemi 1988). For such diploid tumours, the variation may be related to the inability to discriminate by flow cytometry between diploid tumour and nontumour cells. Calculation of %S phase in aneuploid tumours is complex and so with the software available no attempt has been made to look directly at this variable in aneuploid cell lines.

In summary FNA would appear to be a reliable method for determining ploidy and DI of aneuploid cell lines although cell lines which constitute a low proportion of the total cell population may be difficult to identify consistently. FNA would not appear to be a reliable method by which quantitative changes in specific cell populations can be detected by flow cytometric analysis. Within this small study no attempt has been made to distinguish the major source of error whether it be tumour heterogeneity, sampling technique, method of assay or a combination of all three.

Previous reports on the relationship between ploidy and response to systemic therapy have suggested that tetraploid tumours may represent a specific subgroup of endocrine responsive tumours in which response could be expected in as high as 74% (34/46) compared to 50% (26/52) in diploid tumours and 39% (15/38) in aneuploid tumours (Baildam et al. 1987b). Patients with diploid or tetraploid tumours have also been shown to survive longer and stay in remission longer following treatment with endocrine therapy than other tumours (Baildam et al. 1987a, 1987b; Stuart-Harris et al. 1985). Closer analysis of these results however showed that over 85% of the tetraploid tumour were ER-positive tumours compared to a significantly lower proportion in the

other tumour types and this may account for the difference observed. Within the present study all tumours had an ER concentration of ≥ 20 fmol/mg cytosol protein. Although the numbers are small the response rate for diploid tumours did appear higher (5/8) when compared to aneuploid tumours (6/18). When response of the aneuploid tumours was analysed by DNA index the proportion of tumours regression within the tetraploid group was similar to that within the hyperdiploid group at approximately one third. This study would suggest that when the confounding factor of ER status is controlled, response can be observed in about 62% of diploid tumours. Response in aneuploid tumours is lower with about one third achieving significant regression irrespective of the DNA index of the aneuploid cell line. The reason for this difference remains unclear.

Within this study no relationship was demonstrated between DNA ploidy of the primary tumour and response of the primary tumour to chemotherapy with equivalent response rates of approximately 75% being observed for diploid and aneuploid tumours. Similarly no relationship between response and DNA index was observed. Other workers (Masters et al. 1987) have also failed to demonstrate any positive correlation although a trend toward better response in diploid tumours was noted. Selection of patients for cytotoxic therapy will not therefore be assisted significantly by assessment of DNA ploidy in the primary tumour.

Cytotoxic therapy has a direct effect on mitosing cells. There is some clinical evidence to suggest that cytotoxic therapy is likely to be more effective in tumours with high mitotic activity (Zittoun et al. 1975; Hart et al. 1977; Sulkes, Livingston & Murphy 1979; Zhang, Kennedy & Kiang 1984). Although ploidy and grade do relate to indices of proliferation (Steel 1977; Meyer & Connor 1977; Haag 1984; Dressler et al. 1988), it may be that they are too insensitive a measure of tumour kinetics. A more direct measurement of cell kinetics may be required to further investigate any possible association. The proportion of cells present within the S-phase can be directly computed from quantitative analysis of a flow cytometric DNA histogram but a practical problem exists for aneuploid tumours. Several computer programs have now been developed to circumvent this problem. Recently specific methods have been described using antigens incorporated or present within cycling cells and detected by simple immunocytochemical techniques. Such techniques include the use of bromodeoxyuridine (Gratzner 1982; Sasaki et al. 1988; Khochbin et al. 1988; Lacombe et al. 1988; Wilson et al. 1988) and Ki 67 (Gerdes et al. 1984; 1986; Lelle et al. 1987;

Barnaud et al. 1987; Charpin et al. 1988; Walker & Camplejohn 1988; Sasaki et al. 1988).

CHAPTER 10

EFFECTS OF SYSTEMIC THERAPY ON PREDICTIVE INDICES

Biochemical Assay of Oestrogen Receptor

ER concentration was measured before and after systemic therapy. Of the 88 patients within this study, one had bilateral tumours and in twenty-eight patients the post-treatment specimen (as assessed by the pathologist) contained <10% tumour. Thus 60 patients (with 61 tumours) were available for study.

Changes in ER Concentration According to Type of Systemic Therapy

The changes in ER concentration following therapy are shown for the 60 patients according to the form of systemic therapy in Figure 38. Although the changes in individual tumours varied considerably, even within one treatment group, when analysed as a treatment group, no consistent change in the direction of the ER concentration was seen (Table 37). Only the three patients treated with tamoxifen alone showed a significant (99%) fall in ER concentration after 3 months.

Changes in ER concentration According to Response to Therapy

If the 5 patients receiving tamoxifen are excluded (tamoxifen only $n = 3$, tamoxifen & chemotherapy $n = 2$), no significant difference between pre- and post-treatment ER concentration can be demonstrated irrespective of response to systemic therapy (Table 38). Examination of the relationship between changes in ER and changes in tumour volume in individual patients did not reveal any consistent pattern.

Changes in ER Concentration in Relation to Tumour Morphology

Although, on average, most treatments were without significant effect on ER concentration, in some individual patients there were large changes in tumour ER. In order to see if these related to tumour heterogeneity and sampling, the histological sections from 12 paired (pre- and post-treatment) tumour specimens were examined by a pathologist in the absence of any knowledge of the ER concentration.

Of 6 paired specimens showing a 'large' change in receptor concentration, four showed major differences in morphology between the pre- and post-treatment specimens. By contrast, none of the six paired specimens from patients showing little or no change in ER concentration exhibited any striking difference in histopathological appearance.

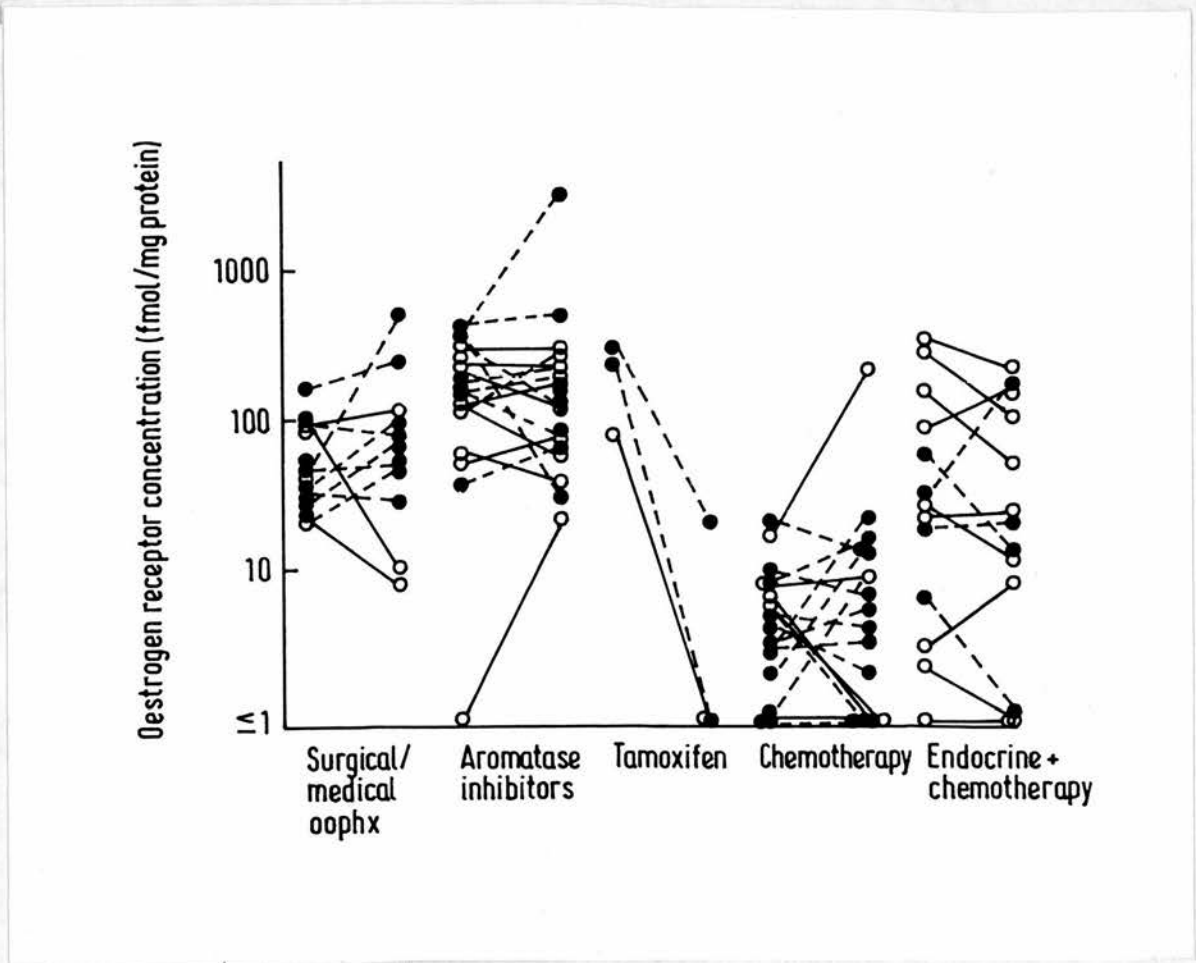


Figure 38
 Relationship between ER concentration as measured by the dextran-coated charcoal method, before and after systemic therapy with surgical/medical oophorectomy (n = 11), aromatase inhibitor (n = 17), tamoxifen (n = 3), cytotoxic therapy (CHOP, n = 17), or combined endocrine and cytotoxic therapy (n = 13). Patients who achieved significant regression are shown as - - -, patients who did not achieve significant regression are shown by _____. The lines join pre- and post-treatment samples for the same patient.

Table 37
Changes in oestrogen receptor concentration in 61 large primary breast cancers with systemic therapy.

	Oestrogen receptor conc. ^a			Sig ^b
	Pre-	Post-	Difference	
Surgical/medical oophorectomy (n = 11)	49	60	0.92	n.s.
Aromatase inhibitors (n = 17)	163	163	0.98	n.s.
Tamoxifen (n = 3)	186	2	6.3	p<0.05
Chemotherapy (n = 17)	4	4	0.98	n.s
Endocrine + Chemotherapy (n = 13)	24	18	1.02	n.s

^a Geometric mean calculated after logarithmic transformation of receptor concentration + 1.

^b Significance calculated from the paired 't' test, n.s. = not significant.

Table 38
Changes in receptor concentration and tumour volume in 56 large primary tumours according to response to systemic therapy.

	Oestrogen receptor conc. ^a			Sig ^b
	Pre-	Post-	Difference	
Regression (n = 33)				
Endocrine ^d (n = 17)	102	127	0.90	n.s.
Chemotherapy (n = 13)	3	4	0.94	n.s.
Endocrine + chemotherapy (n = 3)	43	34	1.1	n.s.
No significant regression (n = 23)				
Endocrine ^d (n = 11)	79	70	1.05	n.s.
Chemotherapy (n = 4)	8	6	1.14	n.s.
Endocrine ^d + chemotherapy (n = 8)	33	24	1.14	n.s.

^a Geometric mean calculated after logarithmic transformation of receptor concentration

^b Significance calculated from the paired 't' test, n.s. = not significant.

^d Patients on tamoxifen have been excluded.

DISCUSSION

Studies on the effect of systemic therapy on the oestrogen receptor (ER) concentration of breast cancer have previously relied upon examination of different tumour deposits (Taylor et al. 1982; Hamm & Allegra 1988). Since these deposits may differ in biological characteristics, including the concentration of ER (Hoehn, Plotka & Dickson 1979; Hawkins et al. 1981), this may lead to erroneous conclusions. The treatment of patient with large operable breast cancer by primary systemic therapy, with direct observation of response and eradication of local disease by planned locoregional surgery after 3-6 months of systemic therapy has allowed the study of the concentration of ER, both before and following systemic therapy within the same tumour mass.

This study has demonstrated that, on average, tumour ER concentration is little changed by most forms of systemic therapy. Large changes in tumour ER concentration in individual patients were probably related to tumour heterogeneity (Hawkins et al. 1977a; Van Netten 1985; Senbanjo et al. 1986). Patients on tamoxifen, however did show a marked fall in receptor concentration during therapy; this was almost certainly due to interference by tamoxifen or its metabolites in the ligand-binding assay, as noted by Hull et al. (Hull et al. 1983). In the present study, patients treated by medical or surgical oophorectomy showed only a slight but insignificant rise in tumour ER concentration. In a larger number of patients with fibroids, treated with the LHRH agonist, zoladex, however a similar but significant rise in concentration of ER in uterine tissues has been observed (Lumsden et al. 1989).

Previous studies in patients with breast cancer (Taylor et al. 1982; Hamm & Allegra 1988; Toma et al. 1986) and in experimental animals (Vignon & Rochefort 1976; Hawkins et al. 1977b; Cho-Chung et al. 1978) have shown a decrease in receptor concentration after endocrine manipulation or as in the present study no consistent change (Hull et al 1983; Mobbs et al. 1987). The conflicting results in human breast cancer may derive from the inclusion of patients on tamoxifen (Taylor et al. 1982) which as discussed previously cause an apparent reduction in ER concentration or from the difficulties of comparing different tumour deposits (Taylor et al 1982; Hamm & Allegra 1988).

In summary, ER concentration in breast tumours changed little after most forms of systemic therapy, even in regressing tumours. Thus a marked change in ER

concentration does not appear to be a component of the mechanism by which tumours are initially influenced by systemic therapy and in particular does not seem to be due to a relative reduction in the ER-positive cell population. In order to confirm this directly the study would need to be repeated using immunocytochemical techniques.

Effect of Systemic Therapy on Enzymes Involved in Local Steroidogenesis

Effect of Systemic Therapy on Tumour Aromatase Activity

The effect of systemic therapy on tumour aromatase activity is shown in Figure 39 and Table 39. Of the 12 patients treated with 4-hydroxyandrostenedione, 8 had demonstrable pretreatment levels of aromatase activity. Following 3 months of treatment, 7 showed a marked decrease and 4 demonstrated no change in the aromatase activity of their tumour. Overall there was a significant fall in tumour aromatase activity irrespective of the response to therapy. In one patient however, with no demonstrable pretreatment activity a marked rise in activity occurred during treatment with 4-hydroxyandrostenedione.

The general trend of aromatase activity in patients who had received aminoglutethimide and hydrocortisone was paradoxically upwards with 6 of the 7 patients showing a rise in aromatase activity *in vitro*: this occurred irrespective of patient response. One patient exhibited a marked fall in activity following treatment. Of the patients who received chemotherapy, marked variations in aromatase activity were demonstrated during therapy but no specific trend was obvious.

17B-Hydroxysteroid Dehydrogenase Activity Before and During Systemic Therapy

The effect of systemic therapy on 17B-hydroxysteroid dehydrogenase activity is interesting (Figure 40 and Table 40). There was on average a 70% rise in the 17B hydroxysteroid dehydrogenase activity following systemic therapy ($p < 0.01$) although changes in activity did vary between individual cases. The elevation in 17B-hydroxysteroid dehydrogenase activity was most significant in the group of patients treated with CHOP. Within the other treatment groups although the mean level of activity following treatment was higher than the pretreatment value, the difference was not statistically significant. The rise in 17B-hydroxysteroid activity appears to occur independent of tumour response to therapy.

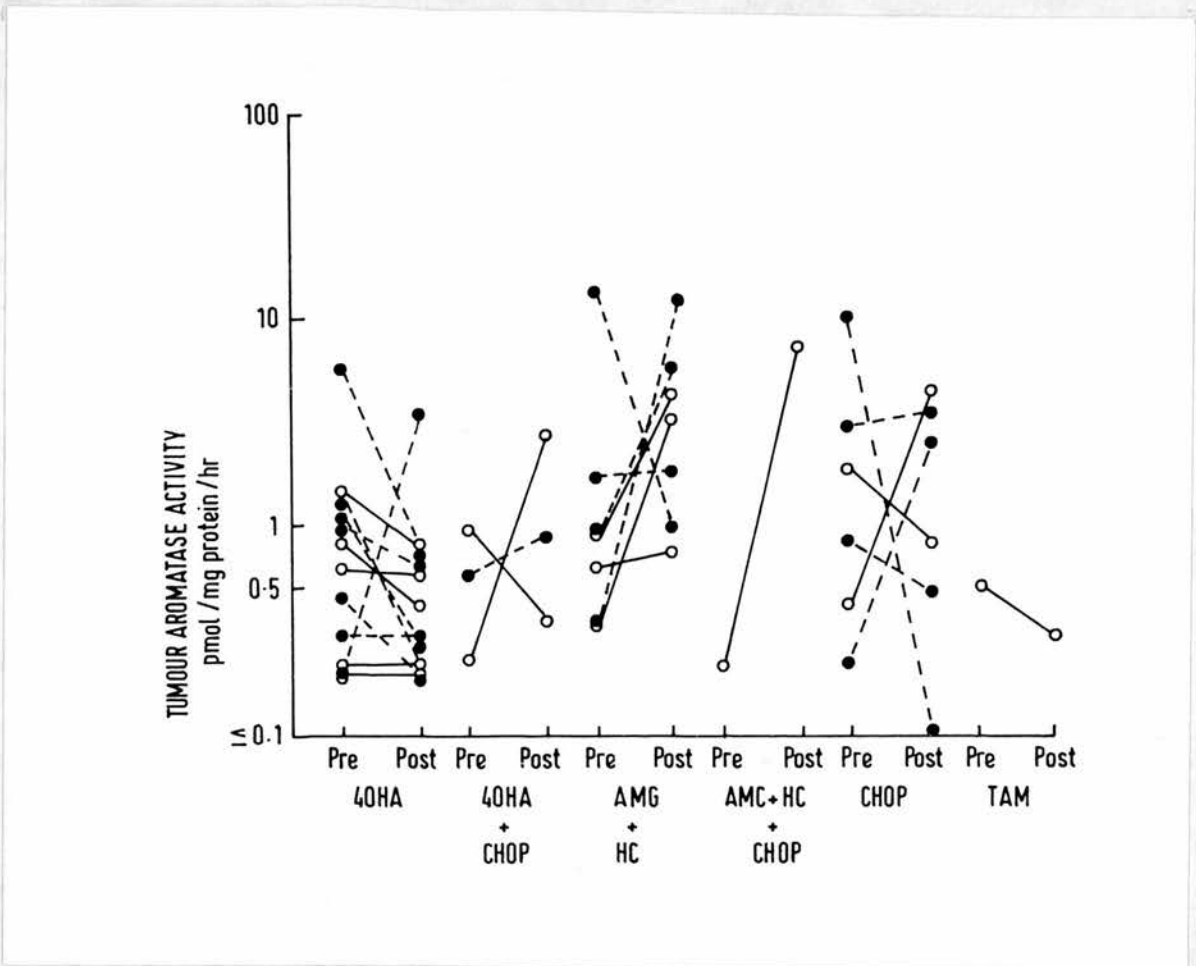


Figure 39

Relationship between tumour aromatase activity before and after systemic therapy with 4-hydroxyandrostenedione (4 OHA, n = 12), 4-hydroxyandrostenedione and cytotoxic therapy (4 OHA + CHOP, n = 3), aminoglutethimide and hydrocortisone (AMG + HC, n = 7), aminoglutethimide and hydrocortisone and cytotoxic therapy (AMG + HC + CHOP, n = 1), cytotoxic therapy (CHOP, n = 6) and tamoxifen (TAM, n = 1). Patients who achieved significant regression with therapy are shown as - - -, patients who did not achieve significant regression are shown by _____. The lines join pre and post-treatment samples from the same patient.

Table 40
Changes in tumour aromatase activity in 30 large operable breast cancer during systemic therapy.

	Tumour aromatase activity ^a *			Sig. ^b
	Pre-treatment	Post-treatment	Difference	
4-OHA (n = 12)	0.59	0.41	1.25	0.01
significant regression (n = 7)	0.74	0.48	1.22	n.s.
no regression (n = 5)	0.43	0.33	1.28	n.s.
4-OHA + CHOP (n = 3)	0.49	0.89	0.70	-
Significant regression (n = 1)				
no regression (n = 2)				
AMG + HC (n = 7)	0.91	2.63	0.69	n.s.
significant regression (n = 4)	1.36	3.12	0.69	-
no regression (n = 3)	0.53	2.09	0.55	-
AMG + HC + CHOP no regression (n = 1)	0.22	6.98	-	-
CHOP (n = 6)	1.06	0.99	1.08	n.s.
significant regression (n = 4)	1.2	0.76	1.32	-
no regression (n = 2)	0.83	1.07	0.72	-
TAM no regression (n = 1)	0.49	0.27	-	-

^a figures quoted are the geometric means of log transformed data in pmol/mg protein/hr

^b significance has been calculated using the Wilcoxon signed rank test, n.s. = not significant

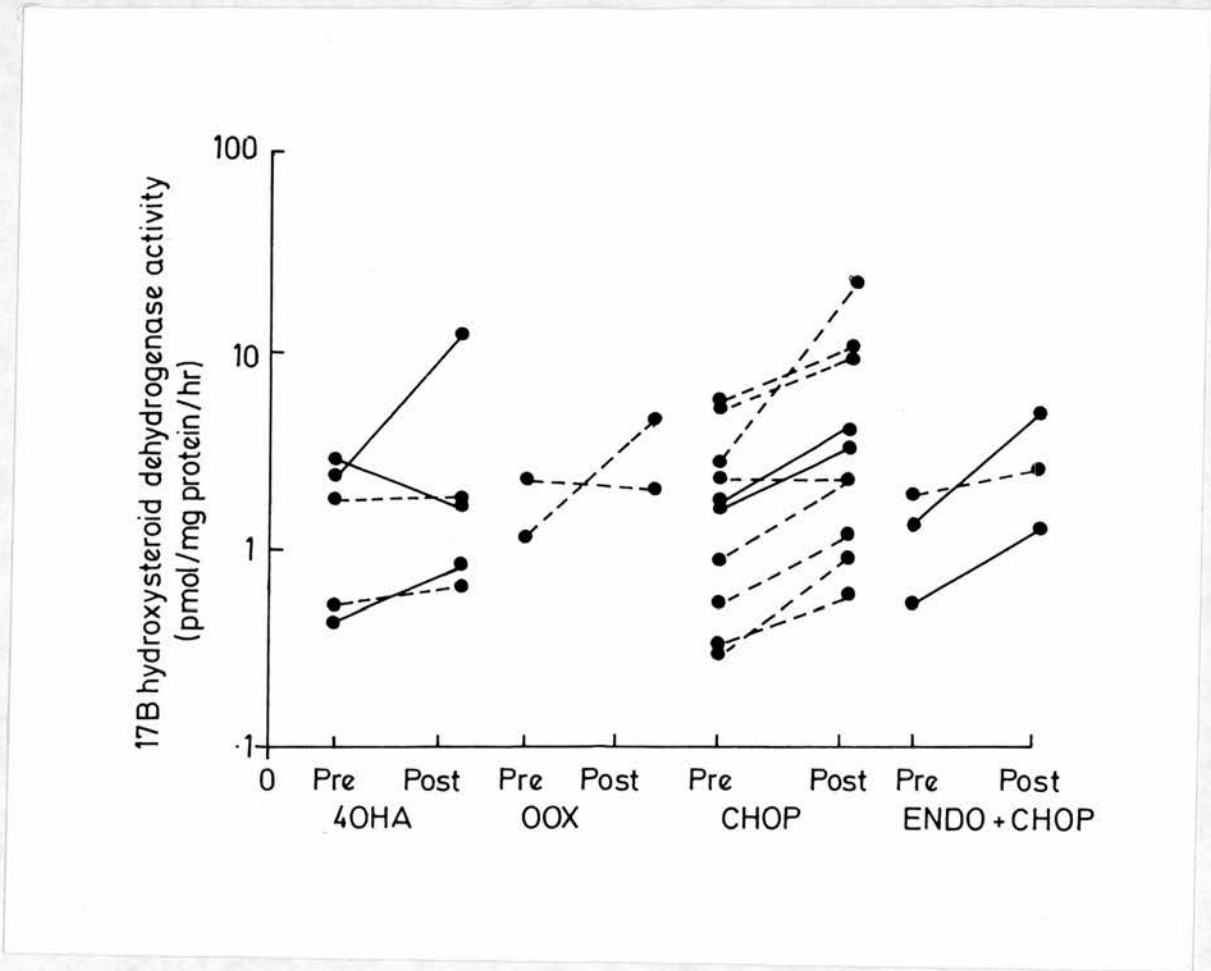


Figure 40
 Relationship between peritumour 17B-hydroxysteroid dehydrogenase activity before and after systemic therapy with 4-hydroxyandrostenedione (4 OHA, n = 5), oophorectomy (OOX, n = 2), cytotoxic therapy (CHOP, n = 9) and endocrine and cytotoxic therapy (endocrine + CHOP, n = 4). Patients who achieved significant regression with therapy are shown as - - -, patients who did not are shown as _____. The lines join pre- and post-treatment values for the same tumour.

Table 40
 Changes in 17B-hydroxysteroid dehydrogenase activity of peritumour fat in 20 patients with large operable breast cancers following systemic therapy.

	17B-hydroxysteroid dehydrogenase activity ^a			Sig. ^b
	Pre-treatment	Post-treatment	Difference	
4OHA (n = 5)	1.23	1.86	0.84	n.s.
significant regression (n = 2)	0.97	1.12	0.95	-
no regression (n = 3)	1.45	2.60	0.78	-
Oophorectomy (n = 2)				
significant regression	1.60	3.02	0.75	-
CHOP (n = 10)	1.60	3.53.	0.70	< 0.02
significant regression (n = 7)	1.58	3.35	0.69	< 0.25
no regression (n = 2)	1.63	3.54	0.72	-
Endocrine + CHOP (n = 3)	0.84	2.38	0.66	-
significant regression (n = 1)	0.73	1.48	-	-
no regression (n = 2)	1.58	3.02	0.62	-
<hr/>				
Total	1.30	2.61	0.73	< 0.01

^a figures quoted are the geometric means of log transformed data in pmol/mg protein/hr

^b significance has been calculated using the Wilcoxon signed rank test, n.s. = not significant.

DISCUSSION

Suppression of plasma oestrogen levels is a function of the inhibition of aromatase activity in all the peripheral tissues but little information exists concerning the direct effect of aromatase inhibitors on local oestrogen biosynthesis within breast cancers *in vivo*. By obtaining tumour tissue both before and during systemic therapy we have been able to study this. In general treatment with 4-hydroxandrostenedione was associated with reduced tumour aromatase activity. This reduction was more marked in patients who responded to therapy but the trend did not reach statistical significance, probably due to the small number of patients involved. These findings are consistent with the demonstrated effect of 4-hydroxandrostenedione on aromatase activity in *in vitro* studies (Miller & O'Neill 1987). James and colleagues (James et al. 1989) have also reported the effect of 4-hydroxyandrostenedione on tumour aromatase and DNA-polymerase activity in 6 postmenopausal patients with breast cancer. They report a marked reduction in tumour activity in 5 of the 6 patients.

Within this study for one patient no aromatase activity was detected in the pretreatment sample but a high level of activity was detected in the post-treatment sample. Failure of 4-hydroxyandrostenedione to produce a fall in the aromatase activity of tumours with significant pre-treatment activity has been noted *in vitro* (Miller & O'Neill 1987) and indeed in one of the six patients in James's series a paradoxical rise in tumour aromatase and DNA polymerase activity was also noted following treatment with 4-hydroxyandrostenedione (James et al. 1989). The reason for this rise remains obscure but it has been suggested that there exists a subset of tumour with significant levels of aromatase activity which are intrinsically resistant to the effect of 4-hydroxyandrostenedione. This patient within this series however achieved a highly significant reduction in tumour volume with therapy and serum oestradiol levels fell to a mean of 54.6% (SEM +/- 8.9%) of the pretreatment values suggesting that the drug was functional. The reason for this exception is not evident since tumour was histologically present in the material incubated from both the pretreatment and post-treatment specimens.

The increase in tumour aromatase activity observed in patients treated with aminoglutethimide and hydrocortisone is clearly incongruous for a drug which when incubated with breast tumour *in vitro* markedly inhibits aromatase activity (Miller, Smith & Telford 1987). This rise occurred irrespective of the patients clinical response to therapy and may be due to the concomitant effects of hydrocortisone. *In vitro*

studies in MCF7 breast cancer cell lines have shown that cortisol at a concentration of 10^{-6} M induces aromatase production (Killinger et al. 1987; Simpson et al. 1981). Treatment with aminoglutethimide and hydrocortisone is associated in vivo with depression of plasma oestrone, oestradiol and oestrone sulphate to between 30 - 55% of their pretreatment values (Harris et al. 1983; Santen et al. 1982; Vermeulen, Paridaens & Heuson 1983; Dowsett et al. 1985; Lønning, Johannessen & Thorsen 1989). Urinary excretion of oestrogen metabolites is increased in patients receiving aminoglutethimide due to the increased activity of hepatic mixed function oxidases (Lønning & Skulstad 1989). Both inhibition of oestrogen production and an increase in plasma clearance of oestrone and oestrone sulphate can be demonstrated in vivo (Lønning, Johannessen & Thorsen 1989). Aminoglutethimide can therefore influence oestrogen disposition by mechanisms unrelated to aromatase inhibition and such effects might be at least in part responsible for its mechanism of action in breast cancer.

Dramatic differences in aromatase activity between sequential tumour samples taken before and during treatment could be detected in patients who received the chemotherapeutic regime CHOP. No definite pattern emerged however and why this should be so remains unclear. Although the chemotherapeutic regimen did contain prednisolone this was only administered during the first 5 days of every cytotoxic cycle. Since surgery was not performed until 4 weeks from the last cytotoxic cycle it is unlikely that the steroids would have had any effect on the post-treatment aromatase activity unless the half life of the enzyme was long.

The increase in activity of 17 β -hydroxysteroid dehydrogenase activity following treatment with systemic therapy was highly significant and unlikely to be a spurious finding. None of the agents used are known to directly affect the activity of this enzyme and the increase in 17 β -hydroxysteroid dehydrogenase activity occurred independent of clinical response to systemic therapy. There was in general a reduction in tumour size post-treatment. Smaller tumours are associated with a lower peritumour fat 17 β -hydroxysteroid dehydrogenase activity (Beranek et al. 1985) but the findings are paradoxical to what would be expected if the difference in activity was simply a function of the change in tumour size. Homogenates from breast cancer cells have been demonstrated to alter 17 β -hydroxysteroid dehydrogenase activity in favour of reduction (McNeill et al. 1986; James et al. 1989). Interference with tumour cell growth equilibrium may cause the tumour cells to release nonspecific growth substances into its surrounding milieu and so effect changes in 17 β -hydroxysteroid dehydrogenase activity. A reduction in tumour size is a complex balance between the

rate of cellular regeneration and cell death, increased cell death may still occur without an obvious reduction in tumour size. It would therefore be possible to affect changes in 17B-hydroxysteroid dehydrogenase activity without being able to demonstrate a significant change in tumour size.

In summary, tumour aromatase activity is significantly reduced following the *in vivo* administration of 4-hydroxyandrostenedione. This reduction in activity is most marked in tumours which demonstrated significant regression suggesting a direct causal relationship. Tumour aromatase was paradoxically raised following the administration of aminoglutethimide and hydrocortisone although this did not affect clinical response. The effect of the chemotherapeutic regime CHOP on tumour aromatase was variable and no definite pattern emerged.

17B-hydroxysteroid dehydrogenase activity was significantly elevated following systemic therapy irrespective of type of therapy or tumour response. The reasons for this remain obscure but may relate to local growth control mechanisms.

Relationship Between Pre- and Post-treatment Histological Grade.

Sufficient residual tumour was available to allow grading in 67 of the 75 post-treatment specimens in which there was residual tumour. In 60 of these patients pretreatment grading was also available allowing the effect of therapy on grade to be assessed (Table 41, 42 and 43). In 44 of the 60 tumours studied (73%) no change in grade was noted with therapy. In those tumours in which a change was observed, the shift was small and did not relate to response.

Table 41.

Tumour histological grade, as judged by Bloom and Richardson criteria, before and after primary systemic therapy in 60 patients with large operable primary breast cancer.

Post-treatment grade	Clinical response		
	progression	no change	significant regression
reduced	-	2	9
unchanged	2	16	25
increased	-	1	5

Table 42.

Tumour histological grade, as judged by Bloom and Richardson criteria, before and after twelve weeks primary endocrine in 33 patients with large operable primary breast cancer.

Post-treatment grade	Clinical response		
	progression	no change	significant regression
reduced	-	1	6
unchanged	2	9	12
increased	-	-	3

Table 43.

Tumour histological grade, as judged by Bloom and Richardson criteria, before and after primary cytotoxic therapy in 27 patients with large operable primary breast cancer.

Post-treatment grade	Clinical response	
	no change	significant regression
reduced	1	3
unchanged	7	13
increased	1	2

DISCUSSION

In vitro cell cycle analysis of hormonally-responsive tumours has demonstrated that oestrogen deprivation reduces the number of cells undergoing mitosis with reduction of the cells within the S-phase and accumulation of cells in the growth arrest phase of G0/G1 (Sutherland et al. 1983, 1986; Lykkesfeldt et al. 1984; Bruno et al. 1988). Mitotic rate forms an integral part of assessment of grading and so it may have been expected that a reduction in grade would have occurred with those tumours which responded to endocrine therapy but no such change was observed in this study. It may be that mitotic counting and grading are too insensitive a parameter of cell proliferation to detect these subtle changes. Cytotoxic therapy directly kills cells undergoing division and as such may not be expected to cause change in the number of cells undergoing mitosis.

SUMMARY AND CONCLUSIONS

CHAPTER 11

SUMMARY AND CONCLUSIONS

This study has described a method for direct, in vivo assessment of primary human breast cancer in response to systemic therapy. By combining a measuring system based on the mean value of eight clinical diameters taken at weekly intervals for 12 weeks, a method of response analysis has been devised which was both sensitive and reproducible. The application of statistical analysis to the changes in tumours volume during therapy meant that not only could response be objectively assessed but also the rate of regression could be determined. Although this parameter is as yet of undetermined biological significance, initial survival analysis at least in relation to response to chemotherapy suggest that the most sensitive tumours may have a better survival. This would be of relevance in selecting out patients whose tumours are highly chemosensitive and for whom continuing cytotoxic therapy to the point of cytoeradication is legitimate. The method of assessment as presented is however time consuming and dependent on a single committed observer. A more arbitrary method of measurement of clinical size such as ultrasound would reduce interobserver error and therefore make the system more universally acceptable. This was not available at the outset of the study.

By using the developed 'human tumour model system' the predictive value for response of several pretreatment tumour indices has been explored. This was made possible by performing a biopsy of the tumour before initiating systemic therapy. It was however important that not too much tumour was removed at the time of biopsy so that sufficient tumour remained on which to assess response. This severely limited the number of parameters which could be assessed. Of those parameters studied in relation to endocrine therapy, the ER concentration proved to be of most value. It was possible to define a group of patients in which endocrine therapy was highly likely to fail ($ER < 20$ fmol/mg cytosol protein) and for whom primary cytotoxic therapy despite its higher morbidity should be recommended. Of those patients with tumours of ER concentration ≥ 20 fmol/mg cytosol protein approximately one half showed significant regression with endocrine therapy but response of the individual patient could only be assessed on a personal basis. The proportion of cells staining for ER as measured by immunocytochemical methods was at least as good as the biochemically determined ER status in predicting hormone responsiveness.

For those patients who received an aromatase inhibitor, measurement of tumour aromatase had some predictive merit, in that the absence of high levels (≥ 0.85 pmol E2 produced/g/hr) was associated with a low likelihood of response to the aromatase inhibitor (4/15, 27%). The presence of high levels of tumour aromatase was not synonymous with response to an aromatase inhibitor but around 70% (7/10) of those tumours did show significant regression. No significant relationship was demonstrated between tumour grade or peritumour fat 17 β hydroxysteroid dehydrogenase activity and response to endocrine therapy.

Of the indices of tumour grade, tumour size, axillary node status, menstrual status or patient age studied in relationship to regression with cytotoxic therapy none showed predictive potential. Those patients however with tumours of ER concentration ≥ 20 fmol/mg cytosol protein which had failed to regress on endocrine therapy also had a significantly higher failure rate on cytotoxic therapy. The reason for this remains obscure, it is unclear whether this is a function of the ER concentration alone or a preselection of tumours with a resistant phenotype.

In summary of the predictive indices examined within this study, only ER concentration and possibly tumour aromatase activity have been of any value in preselecting a group of patients with an increased likelihood of responding to endocrine therapy. The other parameters studied have been uniformly disappointing in their prophetic potential. For the individual patient however it is not yet possible to predict with a high degree of certainty those who will benefit from a specific form of systemic therapy. For this reason there is theoretical value in giving systemic therapy as the preferred first line treatment, since it would allow response to therapy to be assessed on an individual basis and hence determination of appropriate systemic therapy. Although there may be some evidence to suggest that the cells in metastases may differ functionally from the primary tumour, within this study results indicate that the response of the primary tumour to systemic therapy does reflect overall survival patterns.

By using this principal and the information gained in the pilot study in relation to value of ER in determining response to endocrine therapy a selective protocol for adjuvant systemic therapy was devised. Patients with an tumours which had an ER concentration < 20 fmol/mg cytosol protein were given primary cytotoxic therapy. Patients with tumours of ER concentration ≥ 20 fmol/mg cytosol protein received

primary endocrine therapy. Individual response could then be assessed, endocrine therapy being continued in those showing significant regression but stopped when it has been demonstrated to be of no value with initiation of chemotherapy if desired. Preoperative systemic therapy would also allow selection of those patients for whom chemotherapy is highly effective and in whom dose-intensification may increase the probability of cure while also demonstrating those in whom further cytotoxic therapy is unlikely to be of value in the adjuvant setting. Although determination of ER concentration was reliant on a wedge biopsy if the policy was to be more uniformly adopted the increasing acceptance of fine-needle aspiration techniques for diagnosis (Dixon et al., 1984) and more recently ER assay (Coombes et al., 1987; Hawkins et al., 1988; Gaskell et al., 1989) would lend feasibility to this approach avoiding the need for open biopsy.

The administration of systemic therapy as first line treatment has also the potential of reducing primary tumour size, such that breast conservation could become technically possible in patients with tumours previously requiring mastectomy because of their size at initial presentation. A similar approach, with some success, has been pioneered in locally advanced breast carcinoma (DeLena et al., 1978; Hortobagyi et al., 1985 ; 1988; Swain et al., 1987, Jacquillat et al., 1988), osteogenic sarcoma (Rosen, 1982; 1985; Eilber et al. 1987), squamous carcinomas of the head and neck (Schuller et al. 1984; Price and Hill, 1986). Certainly within this study a significant reduction in the size of the primary tumour was achieved in a high proportion of patients (66%; 58/88), making breast conservation theoretically possible. It remains uncertain whether such treatment compromises eventual local control since it assumes that tumour shrinkage is uniform and does not leave residual tumour scattered at the periphery. It is vital therefore that before such a policy of conservation becomes routine there is adequate investigation into the resulting local and overall survival. Such studies are at present ongoing but are as yet immature.

A disadvantage of preoperative systemic therapy is the potential psychological morbidity induced by leaving the tumour in situ while initial systemic therapy is undertaken. In general this did not prove to be a problem even in patients with nonresponsive disease. This was probably due to the fact that surgical removal of the tumour, regarded by many patients as the critical step in their management, was still possible. Formal examination of psychological morbidity was not however undertaken during this study, but should be part of any future work.

By obtaining a biopsy of tumour tissue both before and after systemic therapy, it was possible using this tumour model system to evaluate directly the effect of systemic therapy on potential indices of response within the same tumour. Although changes in the indices examined within this study would not appear to be of value as an early predictive index for response, the changes have been of some value in identifying the mode of action of the administered drugs.

Within this study the ER concentration of a primary breast cancer was changed little by systemic therapy irrespective of tumour response. This suggests that an alteration of tumour ER concentration is not integral to the mechanism of regression within responding tumours. The reduction in ER concentration as measured by the dextran-coated charcoal method in patients treated by tamoxifen, is likely to be artificial since tamoxifen or its metabolites interfere with the ligand-binding assay (Hull et al. 1983). For a true analysis of the effect of tamoxifen on ER concentration an alternative method of ER assay would have to be used.

This study has confirmed *in vivo*, that 4-hydroxyandrostenedione administration is associated, in the majority of patients with a reduction of tumour aromatase activity. This reduction was most marked in patients who achieved significant regression, suggesting a linked effect. In contrast patients treated with aminoglutethimide, a drug which *in vivo* markedly inhibits aromatase activity (Miller, Smith & Telford 1987), showed a paradoxical rise in tumour aromatase activity irrespective of tumour response. Patients receiving aminoglutethimide (and hydrocortisone) have been shown by other workers to have increased plasma clearance of oestrogens due to increased activity of hepatic mixed function oxidases (Lønning & Skulstad 1989) in addition to depressed plasma oestrogen levels (Harris et al. 1983; Santen et al. 1982; Vermeulen et al. 1983; Dowsett et al. 1985; Lønning, Johannessen & Thorsen 1989). *In vivo* therefore aminoglutethimide would appear to effect oestrogen metabolism by mechanisms unrelated to the inhibition of tumour aromatase activity. The rise in tumour aromatase activity observed *in vitro* following the *in vivo* administration of aminoglutethimide is thought to be due to the concomitant administration of hydrocortisone.

The increase in activity of 17 β -hydroxysteroid dehydrogenase activity following systemic therapy was highly significant and almost universal. The reason for this effect remains obscure since none of the agents used are known to directly effect the activity of the enzyme and the effect was independent of clinical response.

In the majority of patients (73%) studied no change in histological grade was observed following systemic therapy. When a change was observed, the shift was small and did not relate to response. Since it is known from *in vitro* studies that tamoxifen reduces the number of cells undergoing mitosis one might have expected to demonstrate a reduction of grade in responding tumours. However it is likely that because of *in vivo* heterogeneity, tumour grade is too insensitive a parameter to detect this phenomenon.

Finally there is theoretical (Goldie and Coldman, 1979; Skipper 1980), experimental (Schabel et al., 1979; Fisher, Gundez, Saffer 1983) and clinical (Nissen-Meyer et al., 1986; Ragaz 1986) evidence to suggest that systemic therapy when administered early in the treatment of patients with breast cancer improves survival. A direct comparison of pre- and post-operative chemotherapy in operable breast cancer is at present under trial in the USA (NSABP trial protocol B-19). Chemotherapy is toxic however and many patients appear to obtain an adequate adjuvant effect from endocrine therapy. Certainly using the selective policy for systemic therapy described above the overall survival, disease-free survival and local disease-free survival compares favourably with that achieved with orthodox treatment of tumours of similar stage in which the adjuvant therapy is given following definitive surgery. Comparison with historical control series is fraught with error and proof of the value of preoperative therapy requires a controlled randomised trial in which this selective approach is assessed against conventional treatment. Such a trial is currently underway in Edinburgh. The protocol is given in the appendix.

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APPENDIX

**Randomised Controlled Trial of Primary
Systemic Therapy versus Conventional
Therapy in Operable Breast Cancer**

**On behalf of the Longmore Breast Group and The
Scottish Cancer Trials Breast Committee**

SCTO/01.01.90

A. INTRODUCTION :

1. Analyses of data accrued from large controlled trials now indicate that the long-term survival of patients with operable breast cancer can be improved by the systemic administration of anti-oestrogens and, in the case of premenopausal women, by giving cytotoxic therapy^{1,2}. However, the benefit gained by these forms of therapy is not uniform. An unselected treatment policy, therefore is not cost effective and in the case of chemotherapy may lead to needless morbidity.
2. While the concentration of oestrogen receptor (ER) within the tumour is a valuable indicator of the likelihood of response to endocrine therapy in patients with metastatic disease^{3,4}, the role of ER determination in the planning of adjuvant treatment in patients with operable breast cancer whose primary tumour has been removed remains uncertain⁵⁻¹¹.
3. Studies conducted to date in the Longmore Breast Unit, Edinburgh, have shown that the prior administration of systemic therapy in 88 patients with large (diameter > 4cm) but operable breast cancer have allowed direct assessment of tumour response before mastectomy.

Of 15 patients with tumours having an ER concentration of <20 fmol/mg cytosol protein, all failed to benefit from anti-oestrogen therapy, whereas of those with tumours of higher ER concentration, 52% (24/46) showed evidence of tumour regression.

27 patients, all with ER poor tumours received cytotoxic therapy (4 cycles of CHOP; cyclophosphamide, adriamycin, vincristine and prednisone) as the primary therapy; a further 20 patients received cytotoxic therapy following failed endocrine therapy - 10 had ER poor tumours and 10 had ER \geq 20. 79% (37/47) of the patients receiving cytotoxic therapy showed significant regression of tumour size.

Preliminary analysis of survival data suggests that the overall survival rate (actuarial survival rate at 36 months = 86%) may be superior to that to be expected from the literature^{12,13,14}.

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B. AIMS OF TRIAL

1. To assess the value of selected preoperative systemic therapy by comparing the local recurrence rate, distant disease free interval and survival of patients with large but operable breast cancer, randomised to treatment by either primary selected systemic therapy followed by mastectomy, or mastectomy and unselected adjuvant systemic therapy.
2. To test feasibility by first conducting a pilot trial in the Longmore Breast Unit with a view to subsequent expansion into a multi-centre trial.

C. DESIGN OF TRIAL:

This is summarised in Figure 41.

D. PATIENTS TO BE INCLUDED

1. All patients under the age of 70 with operable breast cancer of clinical diameter greater than 4cm as measured by calliper.
2. Entry requires that neither the primary tumour nor any palpable lymph nodes be fixed deeply on clinical examination (ie neither partially to fascia or completely to muscle) and that the tumour does not directly involve overlying skin. (Paget's disease of the nipple or dimpling of the skin overlying the tumour is acceptable).
3. No evidence of distant metastases.

E. PATIENTS TO BE EXCLUDED:

1. Patients of 70 years of age or more.
2. Patients with tumours 4cm or less in diameter on clinical measurement.
3. Patients with T₄ lesions.
4. Patients with fixed axillary nodes (N₂).
5. Patients with bilateral primary breast cancer.
6. Patients with evidence suggestive of metastatic spread.
7. Patients with in-situ carcinoma only.
8. Pregnant, lactating or premenopausal patients who are anxious to become pregnant at a later date.
9. Patients with any other condition which precludes adequate surgery, adjuvant therapy or follow-up.
10. Patients for whom adequate follow-up is likely to be difficult owing to their residence being at a distance or abroad.
11. Previous malignant disease other than adequately treated basal- or squamous- cell carcinoma of the skin or carcinoma-in-situ of the cervix.

F. PRE-OPERATIVE INVESTIGATIONS:

1. Fine needle aspiration cytology to confirm the presence of carcinoma.
2. Sample of aspirate to be sent for oestrogen receptor measurement by the ERICA method.
3. Bilateral 2-view mammography.
4. Investigations to exclude metastatic disease:
 - a) FBC + ESR. Bone marrow if indicated by blood count.
 - b) Urea, serum electrolytes, serum calcium and liver function tests.
 - c) Chest X-ray, isotopic bone scan with X-rays of any suspicious areas of increased uptake and ultrasound or CAT scan of the liver if clinically indicated or if liver function tests abnormal.
5. Plasma oestradiol, oestrone, FSH and LH estimations in women of uncertain menstrual status (see para G.2c below).
6. Serum sample to be taken and stored frozen for future marker studies.

G. REGISTRATION, STRATIFICATION AND RANDOMIZATION:

1. All potentially eligible patients to be registered and the reason why those not randomised for therapy within the trial recorded (unsuitable or unwilling).
2. Patients will be classified as premenopausal or postmenopausal, according to the following definition:
 - a) postmenopausal patients are those whose last menstrual period was at least 12 months previously or who have had a previous unrelated bilateral oophorectomy.
 - b) premenopausal patients are those having regular periods and those whose most recent menstrual period was within the preceding 3 months.
 - c) patients under the age of 50 years who have had a hysterectomy without bilateral oophorectomy and those whose last menstrual period was more than 3 months but less than 12 months earlier will require to have

estimations of plasma oestradiol, oestrone, FSH and LH and a pregnancy test performed to define menstrual status.

3. Randomisation will be carried out at the Scottish Cancer Trials Office. The following will be required on all trial patients for the initial data base and should be forwarded to SCTO as soon as possible:
 - a) Completed registration form with, if indicated, the results of tests to determine menstrual status.
 - b) Completed surgical record form.
 - c) Copies of relevant pathology reports.
 - d) Oestrogen receptor results with methods of determination.
4. Patients will be randomised to one of the following two options:
 - a) Primary systemic therapy followed by mastectomy.
 - b) Conventional therapy by mastectomy followed by adjuvant systemic therapy.

H. PRIMARY SYSTEMIC THERAPY GROUP:

1. **WEDGE BIOPSY:** Patients randomised to this arm of the trial will first have a wedge biopsy of their tumour. This can be done under local anaesthetic though some might prefer a GA. The skin incision should be made transversely directly over the tumour and the incisional biopsy performed removing approximately 0.6 cm³ of the primary tumour. A liga-clip should be placed at the tumour site and the specimen sent on ice to the pathology laboratory for histological examination and ER assay by the research laboratory. Any remaining tumour tissue should be stored in liquid nitrogen.
2. **SYSTEMIC THERAPY:** This should commence within 10 days of the wedge biopsy and be according to the menstrual and receptor status as follows:
 - a) Premenopausal women with tumours of ER level 20 or more fmols/mg cytosol protein or equivalent should be commenced on goserelin (Zoladex ICI 118630) 3.6 mg by subcutaneous implant at 28 day intervals.

- b) Postmenopausal women with tumours of ER level 20 or more fmols/mg cytosol protein or equivalent should be commenced on tamoxifen 20 mg daily.
- c) All patients with tumours of ER level less than 20 fmols/mg cytosol protein or equivalent should be commenced on CAP chemotherapy, based on a 3 week cycle, as follows:

Cyclophosphamide 1gm/m² I.V.on day 1
 Adriamycin 50mg/m² I.V. on day 1
 Prednisolone 40mgs daily for 5 days orally.

- d) Those in categories “a” and “b” above in whom a significant reduction in tumour diameter (see para J below) is not achieved by 12 weeks of endocrine therapy should be changed to CAP chemotherapy as described in “c” above. (ie those classified as no change as well as those deemed to have progressed).

3. MASTECTOMY: Within 6 weeks of the 4th course of CAP or as soon as progression of disease on CAP takes place, whichever is earlier, or after 3 months when responding to endocrine therapy, ALL patients should have a mastectomy and axillary clearance. At least 3 cm of skin around the tumour should be removed. In cases where the tumour has regressed, skin removal should be planned to be according to the site and size of the original tumour mass. A latissimus dorsi myocutaneous flap can be used to provide the necessary skin cover and can usually be combined with breast reconstruction. Axillary surgery should be a full level 3 axillary dissection with division of the pectoralis minor muscle.

4. RADIOTHERAPY: Where adequate skin (as defined above) cannot be excised or when the tumour is found at surgery to be tethered to the pectoral fascia or muscle, breast reconstruction should not be performed and radiotherapy should be given to the skin flaps. (see appendix 1 for radiotherapy guide-lines).

5. POST-MASTECTOMY SYSTEMIC THERAPY: This should be carried out as follows:

- a) Premenopausal patients whose tumours responded to goserelin should have a bilateral oophorectomy as soon as is practicable after mastectomy. Goserelin should be continued until the ovaries have been removed.

- b) Postmenopausal patients whose tumours responded to tamoxifen should be continued on tamoxifen after mastectomy until relapse supervenes.

J. ASSESSMENT OF RESPONSE:

1. Response of the tumour to systemic therapy will be assessed from changes in the mean of 8 calliper measured diameters taken at 22.5° axes, converted to log volume, at weekly intervals between treatment weeks 4 and 12.
2. By using linear regression analysis the response will be graded as follows:
 - a) Significant reduction - when the probability that the regression line has deviated below the horizontal is greater than 95%.
 - b) Progression - when there is a significant increase in tumour diameter or any other clinical sign of tumour progression.
 - c) No significant change - when the slope of the regression line is intermediate between the slope of a significant reduction and the slope of a significant progression.

K. CONVENTIONAL THERAPY GROUP:

1. **LOCAL THERAPY:** Mastectomy and axillary clearance should be performed as described in para H,3 above and postoperative radiotherapy to the chest wall given if indicated as in para H,4 above.
2. **ADJUVANT SYSTEMIC THERAPY:**
 - a) Premenopausal patients with involved axillary lymph nodes should be given six 3-weekly cycles of IV CMF chemotherapy as follows:
 - Cyclophosphamide 600mg/m², I.V.
 - Methotrexate 50mg/m², I.V.
 - 5-fluorouracil 600mg/m² I.V.
 - b) All other patients should be commenced on continuous tamoxifen 20mg daily within 10 days of surgery.

L. FOLLOW-UP:

1. Patients on primary systemic therapy should be seen weekly from weeks 4-12 inclusive to evaluate response.
2. After mastectomy, patients in both groups should be reviewed at 3 monthly intervals for 3 years. Thereafter patients should be reviewed 6 monthly.

M. ETHICAL CONSIDERATIONS:

1. Although none of the treatments are novel, as a result of the experience already gained in the use of primary systemic therapy in the management of the larger operable breast cancers, we believe that it is important that this approach be tested now in a randomised way.
2. Approval of the protocol by local ethical committees before patients are entered is mandatory.
3. Verbal and written explanation of the trial must be given to patients and consent to entry obtained prior to randomization.

N. STATISTICS:

1. It is anticipated that approximately 50 patients per annum will be eligible for entry to the trial from the Longmore breast unit. The initial feasibility study should thus be completed within 2 years.
2. If initial piloting of the trial proves successful, the trial will be opened for participation by other specialist units until a total entry of 300 cases is reached. This sample size will have a 75% power to detect differences in long-term survival of 65% and 50%, in the two arms of the trial, at the 5% level of statistical significance.

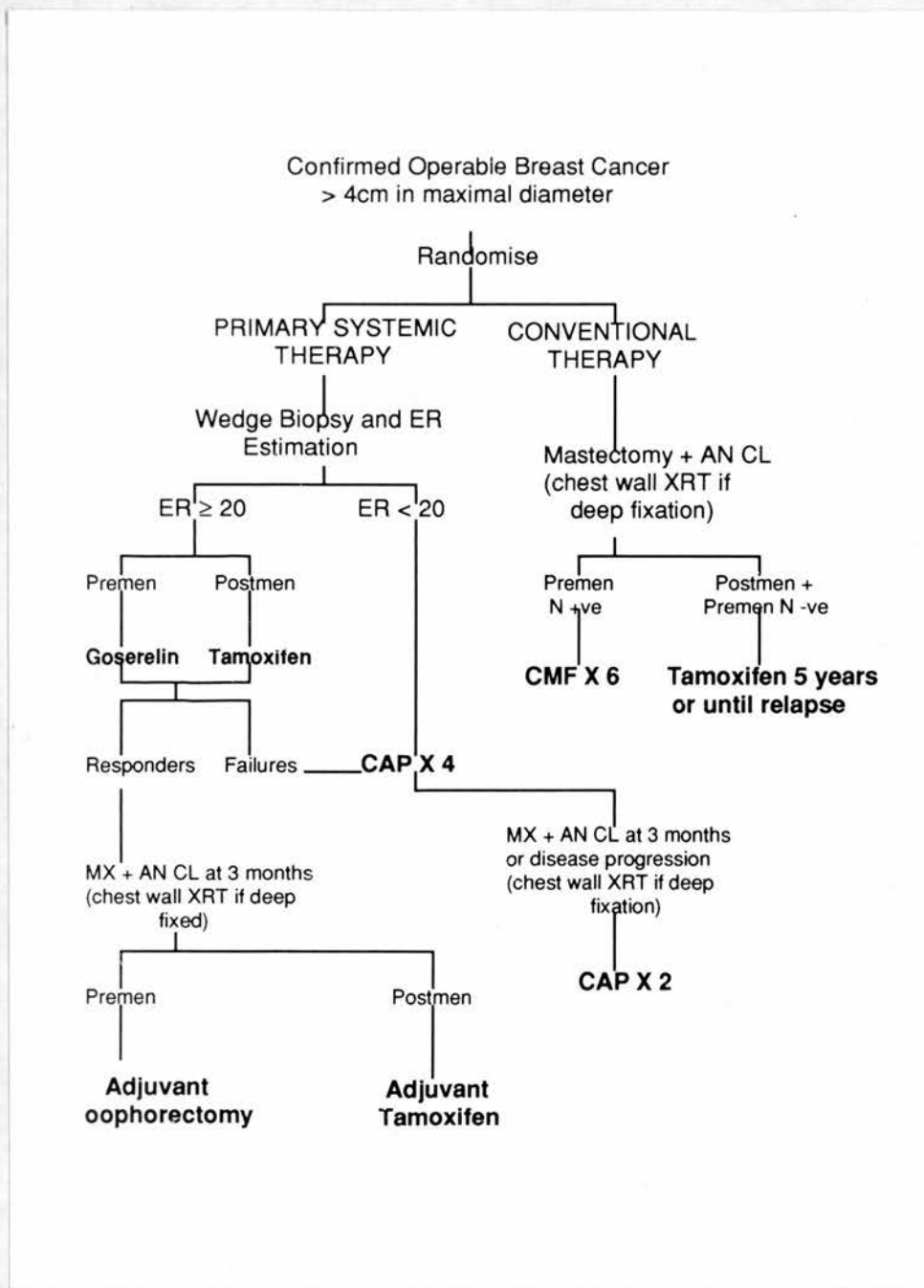


Figure 41.

Protocol for randomised controlled trial of primary systemic therapy versus conventional therapy in operable breast cancer. Premen = premenopausal, Postmen = postmenopausal, Mx = mastectomy, AN CL = axillary node clearance, CMF = cyclophosphamide 600mg/m² i.v., methotrexate 50 mg/m² i.v., 5 fluorouracil 600 mg/m² i.v., CAP = cyclophosphamide 1 gm/m² i.v. on day 1, adriamycin 50mg/m² i.v. on day 1, prednisolone 40 mgs daily for 5 days orally, N +ve = histologically proven axillary node metastasis, N -ve = axillary node histologically proven free of metastasis

Response to endocrine manipulation and oestrogen receptor concentration in large operable primary breast cancer

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Summary Forty-three patients with large (≥ 4 cm) but operable carcinoma of the breast have been treated by endocrine manipulation before definitive local surgery. This has allowed the study of the relationship between response to therapy and pretreatment oestrogen receptor (ER) concentration, as measured by a dextran-coated charcoal adsorption method. Premenopausal patients (17) were treated by surgical (4) or medical (13) oophorectomy. Post-menopausal patients (26) received either tamoxifen (10) or an aromatase inhibitor (16). Response was assessed from statistical analysis of the changes in tumour size. On completion of 12 weeks of endocrine therapy, there was significant regression of tumour size in 18 of the 43 patients. All 18 patients had tumours with ER concentrations of ≥ 20 fmol mg⁻¹ cytosol protein. Conversely all patients except one progressing on treatment had tumours with ER concentrations of < 20 fmol mg⁻¹ cytosol protein. This relationship applied for both premenopausal and post-menopausal patients. The overall response rate of patients with tumours of ER concentration ≥ 20 fmol mg⁻¹ cytosol protein was 60%.

The likelihood of response to endocrine treatment in patients with metastatic breast carcinoma is related to the concentration of oestrogen receptor (ER) protein within that tumour (McGuire *et al.*, 1975; Brooks *et al.*, 1980; Jensen, 1975; Dao & Nemoto, 1980; Oriana *et al.*, 1987). The value of ER status in predicting benefit from adjuvant endocrine treatment remains controversial. While several studies have demonstrated that only patients with ER-positive tumours have a significant survival advantage (Rose *et al.*, 1985; Fisher *et al.*, 1986; Rutquist *et al.*, 1987; Meakin, 1986; Marshall *et al.*, 1987; Bianco *et al.*, 1988), the Nato trial (Nolvadex Adjuvant Trial Organisation, 1988) has indicated that the benefit is independent of ER status. An intermediate view has been suggested by the Scottish (Scottish Cancer Trials Office (MRC) Edinburgh, 1987) and Copenhagen (Palshof *et al.*, 1985) trials, in which all patients who received tamoxifen benefited but the level of benefit was greatest in those patients with tumours of an ER concentration of ≥ 100 fmol mg⁻¹ cytosol protein. Thus the relationship between ER concentration and response of primary operable breast cancer to endocrine treatment remains uncertain.

We have previously reported (Forrest *et al.*, 1986) that the response of large but operable breast cancer to systemic therapy can be measured with precision. By obtaining a small piece of tumour before initiating systemic therapy, response can be related to the specific biochemical and histological parameters of an individual primary tumour. This paper describes the relationship between the ER concentration of large primary operable breast cancer and their response to endocrine treatment.

Materials and methods

Patient population

Between April 1985 and December 1987, 43 patients with primary operable breast cancer of mean clinical diameter greater than or equal to 4 cm were given endocrine therapy for 3 months before definitive local surgery. Initially all patients ($n=35$) were given endocrine therapy irrespective of the ER concentration of their tumour. In April 1987, however, there was a change in policy resulting from review of the results and only those patients with tumours of ER concentration of greater than 20 fmol mg⁻¹ cytosol protein received primary hormonal manipulation ($n=8$). Patients

with tumours of ER concentration 20 fmol mg⁻¹ cytosol protein or less were given four cycles of the chemotherapeutic regime CHOP (cyclophosphamide 1 g m⁻², adriamycin 50 mg m⁻², vincristine 1.4 mg m⁻², prednisolone 40 mg orally 5 days).

Patients over 70 years of age, with a history of psychiatric instability or evidence of metastatic disease on clinical, haematological, biochemical or bone scintiscan investigation were excluded from the study. Seventeen patients were premenopausal, 26 were post-menopausal, i.e. more than 1 year since their last menstrual period.

Initial assessment

At initial presentation, tumour size was assessed from both clinical and radiological examination. The mean clinical diameter was calculated from the mean of eight caliper-measured diameters taken at 22.5° axes. An incisional wedge biopsy was performed under general anaesthesia and 0.6 cm³ of tumour removed and sent for histological and biochemical evaluation.

The determination of oestrogen receptor concentration

The ER concentration of the excised tumour specimen was determined using the dextran-coated charcoal adsorption method (Hawkins *et al.*, 1981). In brief, tumour was homogenised in tris-monothioglycerol-glycerol buffer and centrifuged at low speed; portions of tumour extract were incubated at 4°C overnight with eight concentrations of ³H oestradiol \pm non-radioactive oestradiol (0.031–62.3 nM). After separation of the bound fraction by adsorption with dextran-coated charcoal and scintillation counting, the concentration of receptor sites and dissociation constant of binding were calculated by Scatchard analysis (Scatchard, 1949) using a programmed BBC microcomputer. Protein concentration was determined in a separate portion of each tumour extract by the method of Bradford (Bradford, 1976) using serum albumin as a standard, with five quality controls. Receptor concentration was finally expressed as femtomol of binding sites per mg extract protein. Quality controls consisting of pools of minced human uterus were processed at least twice per week. Intra-assay precision was 3.9% but inter-assay precision was modest (25.1%, 33.6% on two tissue pools (Hawkins *et al.*, 1987a)).

Endocrine treatment

In premenopausal patients ($n=17$), suppression of ovarian function was achieved by either surgical bilateral oophorectomy ($n=4$) or the administration of the gonadotrophin

releasing-hormone agonist goserelin (Zoladex; ICI 118630, subcutaneous implantation of 3.6 mg depot preparation at 28 day intervals, $n=13$).

Post-menopausal patients ($n=26$) received either tamoxifen (20 mg oral nocte, $n=10$), or an aromatase inhibitor ($n=16$). Initially aminoglutethimide 500 mg plus hydrocortisone 40 mg orally per day ($n=9$) was used, but recently the selective peripheral aromatase inhibitor 4-hydroxy-androstenedione (Ciba-Geigy CGP 32349, 250 mg intramuscular injection at 14-day intervals, $n=7$) has been preferred.

Assessment

During treatment, the patients were reviewed weekly by either EA or PL and the mean clinical tumour diameter was estimated. Single view mammography, in the plane known to give the best view of the tumour, was performed every 4 weeks.

Calculation of response

Assessment of response was carried out at 12 weeks by analysis of the change in mean tumour diameter, as described previously (Forrest *et al.*, 1986) with one refinement: the measurements recorded between treatment weeks 1 and 3 were disregarded to minimise any error introduced by the wedge biopsy. In brief, a regression line was calculated by least square analysis of the logs of the mean clinical diameters measured between treatment weeks 4 and 12 (Figure 1). The statistical difference between the regression line and the horizontal was ascertained by application of Student's *t* test. Response was said to have occurred when there was a reduction in tumour size, and the probability that the regression slope deviated from the horizontal was $\geq 95\%$. The appearance of lymphoedema, or a statistically significant increase in tumour size, indicated progression. Tumours with regression slopes which lay between response and progression were categorised as 'no change'.

Local therapy

Those patients who had shown a response to endocrine treatment proceeded on to mastectomy with extensive skin removal (3 cm clear of original tumour site), axillary node clearance and a latissimus dorsi myocutaneous flap reconstruction when required. Patients whose tumour remained static or in whom treatment required to be prematurely terminated due to progressive disease received four cycles of

the chemotherapeutic regime CHOP before also proceeding to mastectomy.

Results

The spectrum of tumour ER concentrations in these patients is shown in relation to four subgroups (ER-negative 0–5 fmol mg⁻¹ cytosol protein, ER-poor 5–19 fmol mg⁻¹ cytosol protein ER-moderate 20–99 fmol mg⁻¹ cytosol protein and ER-rich ≥ 100 fmol mg⁻¹ cytosol protein) in Table I. The median ER concentration in premenopausal patients was lower (30 fmol mg⁻¹ cytosol protein) than in the post-menopausal group (158 fmol mg⁻¹ cytosol protein), a reflection of the higher fraction of post-menopausal patients with ER-rich tumours. The proportion of patients within each menopausal group with ER-negative or ER-poor tumours was similar at around 30%.

Response rates

Eighteen of 43 patients (42%) had significant regression of their primary tumour following endocrine therapy (Tables II and III). The overall response rate for post-menopausal patients was slightly higher than for premenopausal patients (46% vs 35%) (Table I) but this difference was not significant ($\chi^2=0.09$, $P=0.8$). The relationship between ER concentration and response is shown in Figure 2. Although there was considerable overlap between groups, a significant relationship between ER concentration and the likelihood of response to endocrine therapy existed (Spearman rank correlation coefficient $r=0.65$, $P<0.0001$). All patients who responded to treatment had tumours with an ER concentration of greater than 20 fmol mg⁻¹ cytosol protein. Conversely all

Table I Response to endocrine therapy in relation to ER concentration and menopausal status in 43 patients with large but operable primary breast cancer

ER ^a subgroup	No. patients responding/total		% responding total
	Premenopausal	Post-menopausal	
<5	0/2	0/5	0
5–19	0/3	0/3	0
20–99	6/11	2/4	53
≥ 100	0/1	10/14	67
Total	6/17	12/26	42

^afmol mg⁻¹ cytosol protein.

Table II Clinical mean tumour diameters at treatment weeks 4 and 12 of the 18 patients who responded to endocrine therapy

Patient	Clinical mean tumour diameter (cm)		Probability of regression
	Week 4	Week 12	
E.F.	4.75	3.7	0.01
M.H.	3.6	2.0	0.003
M.W.	4.0	3.1	0.05
M.F.	3.6	2.9	0.05
J. McF.	3.1	2.1	0.03
I.C.	5.4	4.2	0.01
A.P.	4.6	3.6	0.002
M.D.	3.6	2.5	0.04
J.C.	4.3	3.4	0.02
A.A.	3.6	2.5	0.004
P.G.	4.0	2.4	0.00008
E.A.	5.0	4.2	0.01
H.C.	4.1	2.9	0.001
J.A.	4.1	0	^a
E.B.	4.3	2.7	0.004
W.S.	3.5	3.0	0.03
J.E.	4.2	3.6	0.00008
M.F.	6.9	2.0	0.0007

^aDenotes no *P* value available due to too few degrees of freedom but tumour clinically impalpable at treatment week 12.

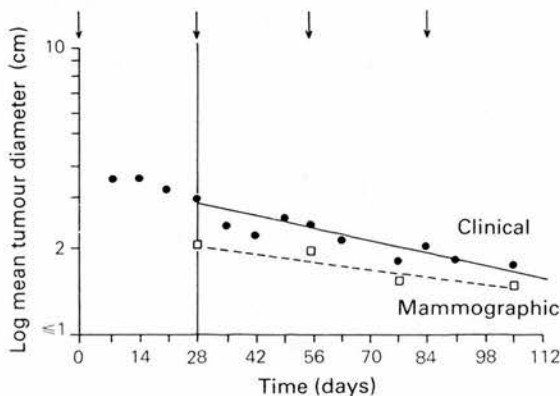


Figure 1 Response graph of a premenopausal patient with a primary operable breast cancer of ER concentration 23 fmol mg⁻¹ cytosol protein following the administration of the gonadotrophin-hormone releasing-hormone Zoladex. This was given by subcutaneous implantation of a depot preparation at 28-day intervals as indicated by the arrows. Each point represents a mean clinical diameter while each square is the mean mammographic diameter calculated from a single view mammogram. The calculated regression line had a correlation coefficient of -0.8 and a slope of -3×10^{-3} cm(log) day⁻¹. This indicates statistically significant regression ($P<0.01$, Student's *t* test).

patients who progressed on treatment, except one, had tumours with an ER concentration of less than 20 fmol mg⁻¹ cytosol protein (Figure 3). This relationship held true for both premenopausal and post-menopausal patients.

Relationship between response and disease-free survival

With a mean follow-up period of 40.6 months, eight patients have developed recurrent disease; two patients who responded to endocrine therapy and six patients who did not. Clinical response is related to disease-free survival in Figure 3. Using the generalised Wilcoxon (Breslow) survival test, there was a statistically significant difference in survival between those patients who showed a response to endocrine treatment and those who did not (*P* < 0.05).

Table III Response rates of 43 patients with large operable breast carcinoma treated with primary endocrine therapy

	Total no. of patients	ER < 20 ^a (no. patients responding/ total)	ER ≥ 20 ^a (no. patients responding/ total)
Premenopausal			
Oophorectomy	4	0/2	1/2
GnRH analogue	13	0/3	5/10
Total	17	0/5	6/12
Post-menopausal			
Tamoxifen	10	0/4	4/6
Aminoglutethimide	9	0/4	4/5
4-Hydroxyandrostenedione	7	—	4/7
Total	26	0/8	12/18
Grand total	43	0/13	18/30

^afmol mg⁻¹ cytosol protein.

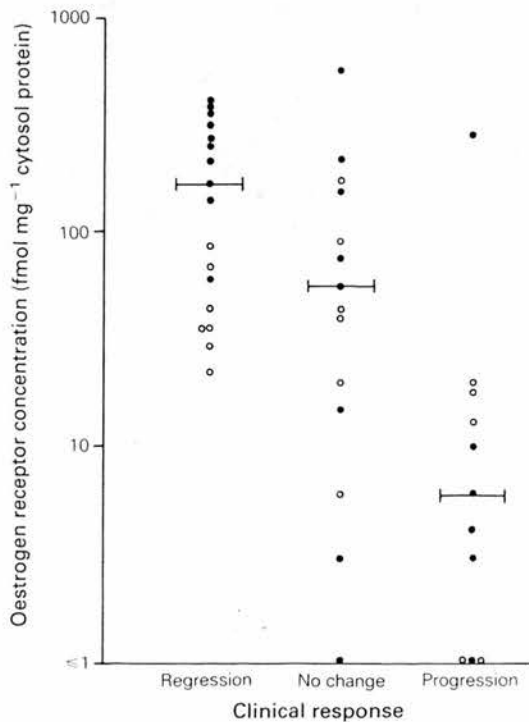


Figure 2 The relationship between the pretreatment ER concentration in 43 patients with primary operable carcinoma of the breast and clinical response to 3 months' treatment with endocrine therapy. The ER concentration was determined by the dextran-coated charcoal adsorption method. Response was assessed from statistical analysis of the regression slope (Student's *t* test, see text). The median ER concentration in each response group is shown by a bar. Open circles have been used to denote tumours from premenopausal patients and closed circles tumours from post-menopausal patients.

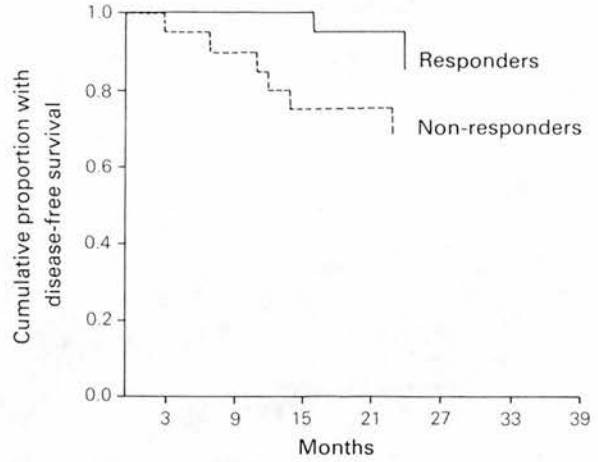


Figure 3 The relationship between disease-free survival and response to endocrine therapy in 43 patients with large but operable primary carcinoma of the breast. With a mean follow-up period of 40.6 months (range 7–43 months), there is a statistically significant difference in the survival of those patients who responded to treatment when compared to those who did not (*P* < 0.05, generalised Wilcoxon (Breslow) survival test).

Discussion

This report confirms and expands our previous reports (Forrest *et al.*, 1986; Hawkins *et al.*, 1988; Anderson *et al.*, 1989) of the relationship between ER concentration and response to endocrine therapy in primary operable breast cancer. Primary tumours with an ER concentration of less than 20 fmol mg⁻¹ cytosol protein did not respond to endocrine therapy, while 60% of those with higher ER values did. Using an immunocytochemical assay we have observed a similar relationship between ER status and response of primary tumours in elderly women treated with tamoxifen (Gaskell *et al.*, 1989). Considering that this relationship also exists for metastatic disease (McGuire *et al.*, 1975; Brooks *et al.*, 1980; Jensen, 1975; Dao & Nemoto, 1980; Oriana *et al.*, 1987), it is reasonable to expect that ER concentration should be of value in selecting appropriate adjuvant endocrine therapy. We have already discussed the confusion which surrounds the value of ER activity in predicting benefit with adjuvant tamoxifen. *In vitro* studies have suggested that tamoxifen may have antitumour actions which are independent of the oestrogen receptor (Sutherland *et al.*, 1986) and can be distinguished from nonspecific cytotoxicity by its cell cycle specific nature (Sutherland *et al.*, 1983). Thus results obtained using adjuvant tamoxifen cannot necessarily be extrapolated to other forms of endocrine therapy, e.g. oophorectomy. Further definition of the role of ER activity in predicting the benefit from adjuvant endocrine therapy is obviously required. This can only come from well conducted, controlled randomised trials designed specifically to answer this question and in which the performance of ER assays is of uniform high quality.

Systemic therapy is of major importance in the long term control of invasive breast cancer because of the high possibility of micrometastatic disease at the time of initial presentation (Brinkley & Haybittle, 1984). Since it is not yet possible to predict, on an individual basis, those patients who will benefit from a specific form of systemic therapy, there is theoretical value in giving systemic therapy as the preferred first line treatment. This would enable the response of the individual's tumour to be assessed and allow selection of appropriate systemic therapy. The availability of fine-needle aspiration techniques for diagnosis (Dixon *et al.*, 1984) and more recently ER assay (Coombes *et al.*, 1987; Hawkins *et al.*, 1988; Anderson *et al.*, 1989; Gaskell *et al.*, 1989) should lend feasibility to this approach, avoiding the need for open biopsy.

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Does the oestrogen receptor concentration of a breast cancer change during systemic therapy?

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Summary The effect of systemic therapy on tumour oestrogen receptor (ER) concentration has been studied in 88 patients with large, operable, primary tumours (total 89) of the breast. In 26 patients, tumour was not available for study on one occasion (usually post-treatment). Forty-five patients were treated initially by endocrine therapy but, of these, 13 who had failed to respond went on to receive chemotherapy also. Seventeen patients with low concentrations of ER (<20 fmol mg⁻¹ protein) were treated directly by chemotherapy. Patients underwent an incisional biopsy for confirmation of diagnosis and determination of pre-treatment ER by radioligand binding assay, followed by systemic therapy for 3 months (or 6 months for both endocrine and cytotoxic therapies). Response was assessed clinically and mammographically before mastectomy. ER concentration was then determined in the post-treatment tumour specimen. No significant change in ER concentration was seen in any treatment group except when the patients had received tamoxifen; there, receptor concentration fell to very low levels, presumably due to interference with the assay. There was no relationship between tumour response to systemic treatment and change in ER concentration. It is concluded that changes in ER concentration are unlikely to play a major role in the early response of breast tumours to systemic therapy.

Studies of the effect of therapy on the oestrogen receptor (ER) concentration of breast cancer have previously relied upon examination of different tumour deposits (Taylor *et al.*, 1982; Hamm & Allegra, 1988). Since these deposits may differ in biological characteristics, including the concentration of ER (Hoehn *et al.*, 1979; Hawkins *et al.*, 1981), this may lead to erroneous conclusions. We have previously reported the treatment of patients with large operable breast cancers by primary systemic therapy, with direct observation of response and eradication of residual local disease by planned locoregional surgery 3-6 months later (Forrest *et al.*, 1986; Anderson *et al.*, 1989). This method of treatment has allowed the study of the concentration of ER, both before and after systemic therapy, within the same tumour mass (primary tumour).

Methods

Patient population

We attempted to measure oestrogen receptor concentration, both before and after systemic therapy, in 88 patients with large (mean clinical diameter >4 cm) operable (T₂ or T₃, N₀ or N₁ and M₀) cancers of the breast: one patient had two tumours and thus there was a total of 89 tumours. In 26 patients, the tumour specimen was inadequate (see below) on one or more occasions: this left 62 patients (with 63 tumours) for study. These patients form part of a larger series which will be reported in full elsewhere (Anderson *et al.*, in preparation).

Twenty-six patients were premenopausal and 36 were postmenopausal in that it was greater than one year since their last menstrual period. The mean age of the population was 53 years (range 34-69).

Method

Before administration of systemic therapy, tumour was obtained from 62 patients for histological and biochemical studies, including ER assay, by an incisional wedge biopsy performed under general anaesthesia. Forty-one patients with ER- moderate/rich tumours (ER >20 fmol mg⁻¹ cytosol

protein) and four with ER-poor/negative tumour (ER <20 fmol mg⁻¹ protein) were initially treated by endocrine therapy. Ovarian function was ablated in premenopausal patients either surgically ($n=4$), or medically, using the luteinising hormone releasing hormone agonist, goserelin (ICI 118630 or zoladex, 3.6 mg subcutaneous depot preparation at 28-day intervals, $n=16$). Tamoxifen (20 mg per day, $n=3$) or an aromatase inhibitor (aminoglutethimide 500 mg plus 40 mg hydrocortisone acetate, $n=7$, or 4-hydroxyandrostenedione, Ciba-Geigy CGP 32349, 250 mg intramuscular injection at 14-day intervals, $n=15$) were the endocrine therapies used in postmenopausal patients. Thirteen patients who failed to respond to endocrine therapy subsequently went on to receive cytotoxic therapy (four cycles of 'CHOP': cyclophosphamide 1 g m⁻², adriamycin 50 mg m⁻², vincristine 1.4 mg m⁻² and oral prednisolone, 40 mg per day for 5 days, at 21-day intervals).

A further 17 patients with tumours of low ER concentration (<20 fmol mg⁻¹ cytosol protein) were given cytotoxic therapy (CHOP $\times 4$) as initial treatment.

During treatment, the tumour was measured weekly by clinical examination and monthly by mammographic assessment. Response was classified on the basis of linear regression analysis (Apple Macintosh Statview program) of changes in clinical tumour diameter as previously described (Anderson *et al.*, 1989) but the results have been presented in terms of a calculated tumour volume in order to give a better indication of 'tumour bulk'. Three response categories were defined: significant regression, when the probability that significant reduction in tumour size was $>95\%$; progression, when there was a significant increase in tumour size or signs of local advancement; and no change, when no significant difference in tumour size could be demonstrated.

Following 3 months of systemic therapy (6 months when patients received both endocrine and cytotoxic therapies), patients proceeded on to mastectomy and axillary lymph-node clearance. When residual tumour was present within the mastectomy specimen, a portion was selected for ER assay by the pathologist.

In both pre- and post-treatment specimens, a section was cut from the face of the tissue portion used for receptor analysis, fixed in formol-saline and stained with haematoxylin and eosin to permit histopathological confirmation of the presence of tumour. Twenty-six patients in whom either the pre- or post-treatment specimen contained $<10\%$ tumour, as assessed by the pathologist, have been excluded from the

study: these include, for example, 11 patients who achieved a complete clinical response to chemotherapy. Thus of the whole group, both pre- and post-treatment specimens were available in 62 patients (63 tumours).

Correlation of changes in ER concentration with changes in histology

To examine the correlation between any changes in ER concentration and histopathology, 12 paired (pre- and post-treatment) tumour samples were independently examined by Dr T.J. Anderson, Department of Pathology, and graded as to whether they showed major differences in morphology or not between the pre- and post-treatment specimens.

Statistical analysis

The relationship between the pre- and post-treatment specimen ER concentrations was examined using the paired *t* test after logarithmic transformation of the data.

Determination of oestrogen receptor activity

Oestrogen receptor activity was determined by saturation analysis (Hawkins *et al.*, 1975, 1981) on both the pre-treatment biopsy and post-treatment tumour from the mastectomy specimen. Quality control samples, processed 2–4 times per week, consisted of pools of finely divided uterine tissue and, on occasion lyophilised powders. The dissociation constant of binding (K_d) and receptor site concentration (P_0) were evaluated by Scatchard analysis (1949).

The soluble protein concentration in each tumour extract was determined by the method of Bradford (1976) using bovine serum albumin as a standard. Five quality controls of known value (three albumin, two mixed standard, Sigma 540–10) were also processed; assays in which the quality controls deviated by more than 10% from the expected values were repeated. Ultimately the receptor content of each tumour was expressed as fmol binding sites per mg soluble protein (P_0 protein).

The overall intra-assay precision on a pool of minced uterine tissue was 15.4% ($n = 5$). Inter-assay precision on lyophilised powders (no homogenisation step) was 17.8% ($n = 10$) at low levels (27 fmol mg⁻¹ protein) and 11.7% at higher levels (90 fmol mg⁻¹ protein); on two pools of minced uterine tissue (including homogenisation) it was 25.5% ($n = 144$) at low levels (48 fmol mg⁻¹ protein) and 17.0% ($n = 48$) at a higher level (111 fmol mg⁻¹ protein).

Results

Changes in ER concentration according to type of systemic therapy

The changes in ER concentration in the tumours from the 62 patients, separated into groups according to mode of treatment, are shown in Table I. Although the changes in individual tumours varied considerably, even within one treatment group (Figure 1), there was no significant change in receptor concentration in patients treated by surgical or medical oophorectomy, aromatase inhibitors, chemotherapy or both cytotoxic and endocrine therapies. Only the three patients treated with tamoxifen showed a significant (99%) fall in ER concentration after 3 months.

Changes in ER concentration according to response to therapy

When the patients were separated into those who achieved a significant regression to systemic therapy and those who did not, no significant change in the receptor concentration was found in either group (Table II). Six of the 62 patients have been excluded from this table because they were on tamoxifen, shown above to influence receptor levels.

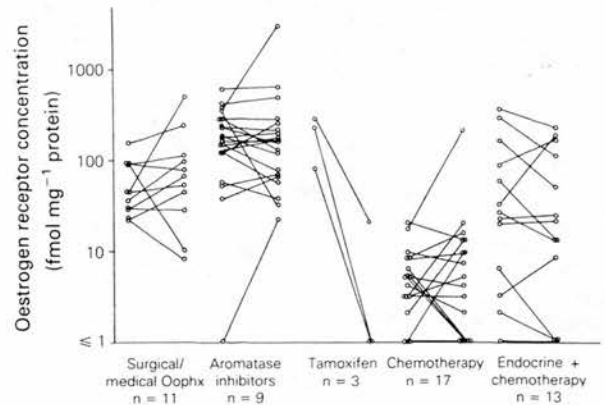


Figure 1 The changes in oestrogen receptor concentration in 63 large, operable primary breast cancers: receptor concentration was assayed by ligand-binding assay in a pretreatment wedge biopsy and again, after systemic therapy for 3 or 6 months, in tumour removed at mastectomy. Each point represents a single assay; the lines drawn join pre- and post-treatment specimens from the same patient. Only the change seen in patients on tamoxifen is significant (paired *t* test, $P < 0.05$).

Table I Changes in receptor concentration in large primary breast tumours during systemic therapy

Treatment group	Oestrogen receptor conc. (fmol mg ⁻¹ protein) ^a			
	Pre-treatment	Post-treatment	Difference	Sig. ^b
Surgical/medical oophorectomy ($n = 11$) ^c	49	60	1.2 ± 3.0	n.s.
Aromatase inhibitors ($n = 19$)	163	163	1.1 ± 2.3	n.s.
Tamoxifen ($n = 3$)	186	2	68 ± 3.6	$P < 0.05$
Chemotherapy ($n = 17$)	4	4	1.0 ± 3.5	n.s.
Endocrine & chemotherapy ^d ($n = 13$)	24	18	1.3 ± 2.1	n.s.

^aGeometric mean calculated after logarithmic transformation of (receptor concentration + 1); ± one standard deviation. ^bSignificance calculated from paired *t* test on log-transformed data. ^c n = number of tumours. For the group treated with aromatase inhibitors, 18 patients were treated, one patient having two tumours. ^dPatients on tamoxifen have been excluded.

Table II Changes in receptor concentration and tumour volume in large primary tumours according to response to systemic therapy

Response group	Treatment	Pre-treatment value ^a	Post-treatment value ^a	Difference	Significance
<i>Oestrogen receptor concentration (fmol mg⁻¹ protein)</i>					
Regression (n = 33) ^c					
Endocrine ^b (17)		102	127	1.26 ± 2.96	n.s.
Chemotherapy (13)		3	4	1.15 ± 2.69	n.s.
Endo ^b + Chemo (3)		43	34	-1.26 ± 2.89	n.s.
No significant regression (n = 23)					
Endocrine ^b (11)		79	70	-1.14 ± 3.08	n.s.
Chemotherapy (4)		8	6	-1.36 ± 7.66	n.s.
Endo ^b + Chemo (8)		33	24	-1.38 ± 2.63	n.s.
<i>Clinical tumour volume (cm³)^d</i>					
Regression	all	53.4	9.0	-6.86 ± 2.93	P<0.001
No significant regression	all	34.7	28.4	-1.25 ± 1.36	P<0.005

^aGeometric means calculated after logarithmic transformation ± one standard deviation. ^bPatients on tamoxifen have been excluded (n = 6). ^cn = number of tumours, one patient having two tumours. ^dTumour diameter was measured and response was classified as described previously (Anderson *et al.*, 1989). The results were converted to a tumour volume to give a better indication of tumour bulk, using the formula $4/3\pi r^3$, where r = mean tumour radius.

As a control, the change in tumour volume for these two response groups was also examined. As expected, the group of patients showing significant regression, taken as a whole, exhibited a highly significant decrease in tumour volume. Although the remaining patients *individually* did not show a significant reduction in tumour volume, as a group they also exhibited a small decrease.

Examination of the relationship between changes in ER concentration and change in tumour volume in individual patients (data not shown) equally did not reveal any consistent pattern.

Changes in ER concentration in relation to tumour morphology

Although most treatments were, on average, without a significant effect on ER concentration, in some individual patients there were large changes in tumour ER. In order to see if these related to tumour heterogeneity and sampling, the histological sections from 12 paired (pre- and post-treatment) tumour specimens were examined by the pathologist, in the absence of any knowledge of the ER concentration.

Of six paired tumour specimens showing a 'large' change in receptor concentration, four showed major differences in morphology between the pre- and post-treatment specimens. In contrast, none of the six paired specimens from patients showing little or no change in ER concentration exhibited a striking difference in histopathological appearance.

Discussion

This study has demonstrated that, on average, tumour ER concentration is little changed by most forms of systemic therapy. Large changes in tumour ER concentration in individual patients were probably related to tumour heterogeneity (Hawkins *et al.*, 1977a; Van Netten, 1985; Senjo *et al.*, 1986). Patients on tamoxifen, however, did show a marked fall in receptor concentration during therapy; this

was almost certainly due to interference by tamoxifen or its metabolites in the ligand-binding assay, as noted by Hull *et al.* (1983). In the present study, patients treated by medical or surgical oophorectomy showed only a slight, but insignificant rise in tumour ER concentration. In a large number of patients with fibroids, treated with the LHRH agonist, zoladex, however, a similar but *significant* rise in the concentration of ER in the uterine tissues has been observed (Lumsden *et al.*, 1989).

Previous studies in patients with breast cancer (Taylor *et al.*, 1982; Hamm & Allegra, 1988; Toma *et al.*, 1986) and in experimental animals (Vignon & Rochefort, 1976; Hawkins *et al.*, 1977b; Cho-Chung *et al.*, 1978) have shown a decrease in receptor concentration after endocrine manipulation or, as in the present study, no consistent change (Hull *et al.*, 1983; Mobbs *et al.*, 1987). The conflicting results in human breast cancer may derive from the inclusion of patients on tamoxifen (Taylor *et al.*, 1982), which causes a marked apparent reduction in ER concentration (this study and Hull *et al.*, 1983) or from the difficulties in comparing different tumour deposits (Taylor *et al.*, 1982; Hamm & Allegra, 1988).

In summary, ER concentration in breast tumours changed little after most common forms of systemic therapy, even in regressing tumours. Thus, in general, a marked change in ER concentration does not appear to be a component of the mechanism by which tumours are initially influenced by systemic therapy.

We are particularly grateful to Dr T.J. Anderson, Department of Pathology, for selecting the portions of tumour for assay, for carrying out the histopathological examination and helpful discussion. We thank the Cancer Research Campaign for support of Miss E.D.C. Anderson and Dr P.A. Levack (grant no. SP1256 to Professor Forrest). The receptor assays were performed by Miss A.L. Tesdale and Mr D. Carson, through the support of the Lothian Health Board. Miss K. Sangster and Mrs E. Killen kindly helped to check and collate the results.

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Primary systemic therapy for operable breast cancer

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Summary Eighty-eight patients presenting with operable breast cancer of 4 cm or greater in diameter (T2, T3, N0, N1, M0) have received primary systemic therapy. Response was assessed following 12 weeks of systemic therapy by linear regression analysis of changes in tumour volume. Definitive locoregional surgery (mastectomy $n = 82$, wide local excision $n = 6$) was performed on completion of systemic therapy (3–6 months). Response was observed in 24 (39%) of the 61 patients who received endocrine therapy; all 24 had tumours with an oestrogen receptor (ER) concentration of ≥ 20 fmol mg^{-1} cytosol protein. Cytotoxic therapy was reserved for patients with tumours of ER concentration < 20 fmol mg^{-1} cytosol protein ($n = 27$) or when endocrine therapy had failed ($n = 20$). Response was observed in 34 patients (72%). The overall survival rate at 3 years was 86%, with 81% remaining free from local relapse. We propose that the treatment policy outlined in this paper should now be tested against orthodox management by controlled randomised trial.

It has now been established from statistical analyses of large controlled randomised trials that the long term survival of patients with operable breast cancer can be improved by systemic endocrine or cytotoxic therapy (Early Breast Cancer Trialists' Collaborative Group 1988). These trials however have not defined which therapy is most suitable for an individual patient. Given the morbidity of cytotoxic therapy (Glass *et al.*, 1981) an unselective policy is not ideal. Furthermore the value of tumour oestrogen receptor status (ER) in selecting patients for adjuvant hormonal therapy remains controversial (Palshof *et al.*, 1985; Rose *et al.*, 1985; Fisher *et al.*, 1986; Rutquist *et al.*, 1987; Bianco *et al.*, 1988; Scottish Breast Cancer Trials Committee 1987; Nolvadex Adjuvant Trial Organisation 1988).

In 1985 we initiated a study in which local surgical treatment was delayed in patients with large, but still technically operable breast cancer until the response of the primary tumour to systemic therapy had been assessed (Forrest *et al.*, 1986). We now report our experience with primary endocrine and cytotoxic therapy in 88 such patients.

Patients and methods

Patient population

Patients were considered for entry into the study if they presented with an invasive breast carcinoma 4 cm or greater in diameter (T2, T3, N0, N1, M0). Patients with evidence of tumour fixation to skin or pectoral muscle, lymphoedema of the skin, detectable metastases on routine clinical and radiological investigations (including bone scan) or with a history of cardiac or mental instability were excluded from the study. All patients were Karnofsky grade 0.

During the 4 year period between April 1985 and April 1989, 136 patients with tumours measuring 4 cm or greater presented to the Breast Unit of Longmore Hospital, of whom 88 were included in this study. Sixteen patients failed to fulfil the selection requirements, in five cases an incisional biopsy had been performed to confirm the diagnosis and had removed a large amount of tissue, while in 13 patients the tumour was either multifocal, partly cystic, bilateral or difficult to measure reliably. Seven patients were excluded because they lived more than 50 miles from the hospital.

Only seven patients refused preoperative therapy, preferring immediate mastectomy.

The mean age of the patient population studied was 53.1 years (range 33–69 years). Thirty-eight patients were premenopausal (1 year or less since their last menstrual period) and 50 were postmenopausal. Determination of menopausal status in patients who had undergone hysterectomy was based on serum gonadotrophin levels; patients were defined as postmenopausal if their serum follicle-stimulating hormone concentration was greater than $30 \mu\text{l}^{-1}$.

Initial assessment

An initial presentation, tumour size was assessed both clinically and mammographically. Clinical diameters were calculated from the mean of eight caliper-measured diameters taken at 22.5° axes before fine needle aspiration, tumour volume was calculated by assuming that the tumour was spherical ($4/3\pi r^3$). Fine-needle aspiration was used to obtain a cytological diagnosis of malignancy. Staging assessment was then performed and involved a thorough clinical examination supplemented by haematological (erythrocyte sedimentation rate, full blood count), biochemical (urea and electrolytes, liver function tests, serum calcium, phosphate and albumin) and radiological (chest X-ray and radioisotope bone scan) investigation. Any patient with abnormal liver function tests had a liver ultrasound examination to exclude the presence of overt metastasis. The philosophy of the study was explained to all suitable patients both verbally and by written document and informed consent obtained.

Pretreatment tumour material for histological and biochemical evaluation, was obtained by incisional wedge biopsy performed under general anaesthesia. Approximately 0.6 cm^3 of tumour was removed. In order to standardise the technique this procedure was performed by one person (EDCA) in the last 50 patients, and to aid post-therapeutic localisation of the tumour area, the tumour bed was marked by ligaclips. In 15 patients, an involved axillary node was excised in preference to biopsy of the primary tumour. Formal axillary node sampling was not initially performed but has been routine in the last 29 patients.

The oestrogen receptor concentration of the pretreatment biopsy was determined by the dextran-coated charcoal adsorption method (Hawkins *et al.*, 1975, 1981).

Systemic therapy

Of the 88 patients included within this study 41 received only endocrine therapy, 27 received only cytotoxic therapy while

20 received both forms of therapy. Systemic therapy was commenced within 10 days of the wedge biopsy.

Pilot study

The first 36 patients all received primary endocrine therapy (Anderson *et al.*, 1989). In premenopausal women ovarian function was ablated initially by surgical bilateral oophorectomy ($n = 5$) and subsequently by the luteinising-hormone releasing-hormone analogue goserelin (Zoladex ICI 118630, subcutaneous implantation 3.6 mg depot preparation at 28 day intervals following 4 ml lignocaine local anaesthetic, $n = 7$). Postmenopausal women received either tamoxifen (20 mg per day, $n = 11$) or the aromatase inhibitor aminoglutethimide (500 mg plus 40 mg hydrocortisone acetate, $n = 10$). Three postmenopausal patients received goserelin as their primary therapy. Cytotoxic therapy was reserved for those patients whose tumours had failed to respond to endocrine therapy. The chemotherapeutic regimen used was four cycles of CHOP, i.e. cyclophosphamide 1 g m^{-2} , adriamycin 50 mg m^{-2} , vincristine 1.4 mg m^{-2} to a maximum of 2 g, all by i.v. bolus and oral prednisolone 40 mg per day for 5 days. The regimen was administered every 21 days. If cytopenia (WBC $< 3,000 \text{ ml}^{-3}$ or platelet count of $< 100,000 \text{ ml}^{-3}$) was presented on day 21, therapy was delayed until the cytopenia resolved. A dose adjusted course was then given.

Selective policy

Following the demonstration that no patient with an ER concentration of < 20 femtomols $\text{mg cytosol protein}^{-1}$ showed significant regression while receiving endocrine therapy (Anderson *et al.*, 1989), and indeed two thirds progressed (Table III), a more formal selective policy was instituted on 1 April 1987. Endocrine therapy thereafter was reserved only for those patients with ER-moderate/rich tumours ($\geq 20 \text{ fmol mg cytosol protein}^{-1}$, $n = 25$). Patients with ER-poor tumours (ER $< 20 \text{ fmol mg cytosol protein}^{-1}$, $n = 27$) or those patients with tumours un-responsive to endocrine therapy received cytotoxic therapy ($n = 7$). In this formalised protocol premenopausal patients received goserelin ($n = 9$) and postmenopausal patients received the selective peripheral aromatase inhibitor, 4-hydroxyandrostenedione (250 mg intramuscular injection to alternate buttocks at 14 days intervals; Ciba-Geigy CGP 32349, $n = 16$). The chemotherapeutic regimen was unchanged.

Assessment of response

Patients were reviewed weekly by one of us (EDCA). Although formal assessment of tumour response was calculated following completion of 12 weeks systemic therapy, detection of any interim signs of local progression ($n = 16$), such as *de novo* skin lymphoedema or increasing size of tumour led to immediate cessation of endocrine therapy. If progression was detected cytotoxic therapy was instituted ($n = 14$) although two patients proceeded directly to surgery. Statistical evaluation of response was by linear regression analysis (Apple Mac, Statview) of the changes in tumour volume between treatment weeks 4 to 12; earlier measurements were discarded in order to allow the reaction caused by tumour biopsy to subside (Figure 1). Response was graded as (i) significant regression (reduction in tumour size where the probability that the regression line deviated from the horizontal was greater than 95% (ii) progression (significant increase in tumour diameter where the probability that the regression line deviated from the horizontal was greater than 95% or signs of local advancement (iii) no change (regression slope intermediate to response and progression).

Alterations in tumour size were also assessed radiologically by a single mammogram, performed at 4 weekly intervals, in the view known to give the best perspective of the tumour.

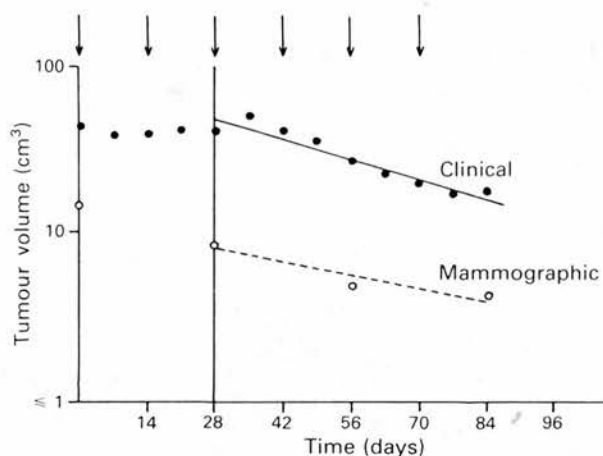


Figure 1 Response of a postmenopausal patient with an operable breast cancer of ER concentration $142 \text{ fmol mg cytosol protein}^{-1}$ following intramuscular 4-hydroxyandrostenedione (250 mg) at 14 day intervals as shown by the arrows. Each closed point represents tumour volume as calculated from the mean tumour diameter, while each open point is the volume calculated from the mean mammographic diameter. The calculated regression line had a correlation coefficient of -0.96 and a slope of $-9 \times 10^{-3} \text{ cm}^3(\log) \text{ day}^{-1}$. This indicates statistically significant regression ($P < 0.0001$, Student's *t*-test).

Side-effects

Side-effects of therapy were assessed at weekly clinical interview. The morbidity associated with cytotoxic therapy was reported using the WHO toxicity grading system (Miller *et al.*, 1981).

Definitive locoregional surgery

On completion of systemic therapy (3–6 months) mastectomy with extensive skin removal and axillary node clearance was performed in 82 patients, of whom 55 had simultaneous reconstruction by latissimus dorsi myocutaneous flaps. In six patients with complete clinical response, wide local excision of the previous tumour site was preferred; this being followed by radiotherapy in five cases. The excised specimen was submitted to histological examination.

Patients who had shown a significant response to preoperative endocrine therapy were continued on endocrine therapy following definitive locoregional surgery. Premenopausal patients proceeded to oophorectomy, postmenopausal patients received tamoxifen at a daily dose of 20 mg. Further cytotoxic therapy was not given to any patient after surgery.

Survival

The follow-up period has been expressed from the time of initiating systemic therapy to the date of analysis. The median period of follow-up was 24 months (range 4–55 months). Locoregional relapse has been defined as recurrence confined to the chest wall, breast or axilla. Supraclavicular lymph node recurrence has been classified as distant metastasis, in keeping with the staging classification for disease at initial presentation (International Union Against Cancer, 1987 TMN classification).

Results

Response to endocrine therapy

Twenty-four of the 61 (39%) patients treated by initial endocrine therapy had significant regression of their tumours (Table III). All responding tumours had an ER concentration of $\geq 20 \text{ fmol mg cytosol protein}^{-1}$. The proportion of patients achieving regression did not vary greatly in relation to the

type of endocrine therapy received or menopausal status and for the purpose of this report all patients receiving endocrine treatment have been considered together.

Of those patients responding to endocrine therapy, the median time taken to achieve half volume (T1/2) was 44 days (range 3–150 days, Figure 2). Only one tumour showed complete clinical regression within the period of the study; however all had residual invasive carcinoma detected by histopathological examination. For various reasons the duration of preoperative antioestrogen therapy was prolonged beyond 12 weeks in eight patients. Seven tumours continued to regress at the same rate while one tumour regarded as static underwent a rapid reduction in size at 5 months.

Response to cytotoxic therapy

A significant reduction in tumour volume was observed in 34 of the 47 patients (72%) who received cytotoxic therapy (Table II). Thirteen patients (27.6%) had complete clinical regression of their tumour and eight (17%) had no histological evidence of invasive carcinoma in their mastectomy or

Table I Relationship between responses to hormonal therapy and oestrogen receptor concentration of the primary tumour as determined by the dextran-coated charcoal adsorption method in 61 patients with large operable breast cancer

	No. of patients with significant regression/total		
	ER <20 ^a	ER >20 ^a	Total
Premenopausal			
Oophorectomy	0/2	2/3	2/5
Goserelin	0/3	7/13	7/16
Postmenopausal			
Tamoxifen	0/5	4/6	4/11
Aminoglutethimide	0/4	4/6	4/10
4-hydroxyandrostenedione	–	7/16	7/16
Goserelin	0/1	0/2	0/3
Total	0/15	24/46	24/61

^afmol mg cytosol protein⁻¹.

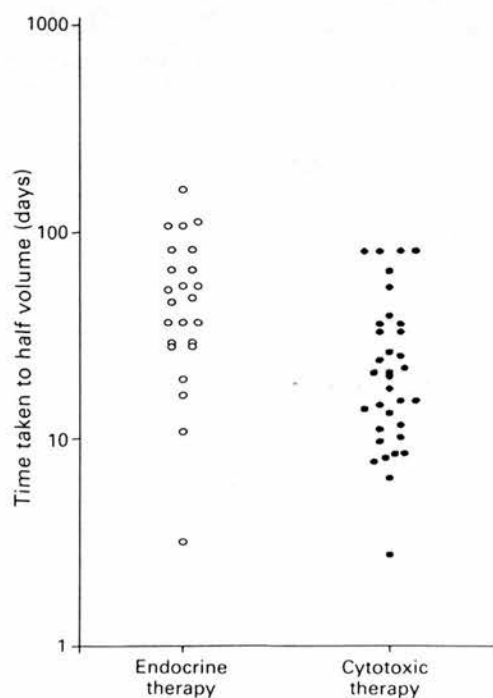


Figure 2 Graph illustrating the difference in the time taken to achieve half volume (T1/2) in tumours which responded to endocrine therapy ($n = 24$) and cytotoxic therapy ($n = 34$). The median T1/2 of tumours responding to endocrine therapy was 44 days (range 3–150 days). The median T1/2 of tumours responding to cytotoxic therapy was 20 days (range 3–77 days).

Table II Response rates in 47 patients with large operable cancers of the breast treated with four cycles of the chemotherapeutic regime CHOP (cyclophosphamide 1 g m⁻², adriamycin 50 mg m⁻², vincristine 1.4 mg m⁻² to a maximum of 2 g, all by i.v. bolus and oral prednisolone 40 mg per day for 5 days) before definitive locoregional surgery. The χ^2 test has been used to compare the proportion responding to chemotherapy following failed endocrine therapy in relation to ER concentration

	No. with significant regression/total	No. with complete clinical regression
Primary cytotoxic therapy		
ER <20 ^a	23/27	8
Following failed endocrine therapy		
ER <20 ^a	8/10	4
ER ≥20 ^a	3/10 ^b	1
Total	34/47	13

^afmol mg cytosol protein⁻¹, ^bstatistically significant $\chi^2 = 5.05$, $P = 0.025$.

Table III Relationship between the pretreatment ER concentration and response to 12 weeks endocrine therapy in 61 patients with large operable cancers of the breast. The ER concentration was determined by the dextran-coated charcoal adsorption method

ER status	Total no.	Significant regression	No change	Progression
ER – poor 20 ^a	15	0	5	10
ER – rich ≥20 ^a	46	24	16	6

^afmol mg cytosol protein⁻¹.

wide local excision specimens; five patients had no gross residual disease but invasive carcinoma was visible microscopically. No patient showed evidence of tumour progression during treatment with chemotherapy.

The rate of regression was, on average, more rapid than that achieved with endocrine therapy (median T1/2 of 20 days, range 3–77 days, Figure 2). Those patients with steeper regression slopes were more likely to achieve complete clinical response within the time scale of the study.

There was no significant relationship between significant regression to chemotherapy and the pretreatment variables of age, menopausal status, initial clinical tumour size or pathological axillary node status (Table IV). Of those who failed to respond to endocrine therapy, chemotherapy was more likely to achieve regression in ER-poor tumours (Table IV).

Survival and preoperative therapy

The overall, distant disease-free and disease-free survival of all patients within the study is shown in Figure 3. Local recurrence-free survival is shown in Figure 4. With a median follow-up of 23 months (range 4–55 months), 18 (20%) patients have relapsed, seven of whom have died as a result of their disease. Of these seven patients, three had shown failed to achieve significant regression in response to systemic therapy. In five patients relapse was locoregional alone, six patients had distant metastasis alone while 13 had evidence of both. At 3 years 86% (76–96%; 95% confidence limits) remain alive and 67% (55–79%; 95% confidence limits) remain disease-free, with 81% (70–92%; 95% confidence limits) having no evidence of local recurrence.

Toxicity

The side-effects experienced with endocrine treatment were minimal. Hot flushings were noted in seven patients (37%) receiving goserelin but were only moderately severe in two patients. Vaginal dryness was a problem in one patient. Of the 16 patients who received 4-hydroxyandrostenedione

Table IV Relationship between pretreatment variables and significant tumour regression following four cycles of preoperative cytotoxic therapy (cyclophosphamide 1 g m^{-2} , adriamycin 50 mg m^{-2} , vincristine 1.4 mg m^{-2} to a maximum of 2 g , all by i.v. bolus and oral prednisolone 40 mg per day for 5 days). Patients were designated postmenopausal if more than 1 year had elapsed since their last menstrual period. Pretreatment axillary node status as determined from histological examination for an axillary node sample was available for 29 patients. The χ^2 test has been used to compare the relationship between pretreatment variables and the proportion within each group who achieved significant regression

	No. significant regression/total no.	χ^2	P value
Tumour diameter			
< 5 cm	19/26		
5–< 6 cm	10/14	0.813	0.666
$\geq 6 \text{ cm}$	7/8		
Age			
30–39 years	3/5		
40–49 years	14/18		
50–59 years	13/19	0.939	0.816
> 60 years	4/5		
Menstrual status			
Premenopausal	19/25		
Postmenopausal	15/22	0.357	0.55
Oestrogen receptor concentration			
< $20 \text{ fmol mg cytosol protein}^{-1}$	31/37		
$\geq 20 \text{ fmol mg cytosol protein}^{-1}$	3/10	11.38	0.0007
Axillary lymph node status			
Metastasis	15/23		
No metastasis	6/6	2.88	0.09
Unavailable	5/13		

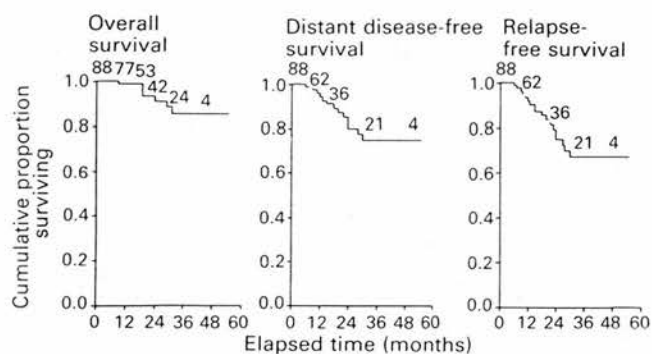


Figure 3 Overall, distant disease-free and relapse-free survival for 88 patients with large operable breast cancer treated by primary systemic therapy before definitive locoregional surgery. The median period of follow-up was 24 months (range 4–55 months).

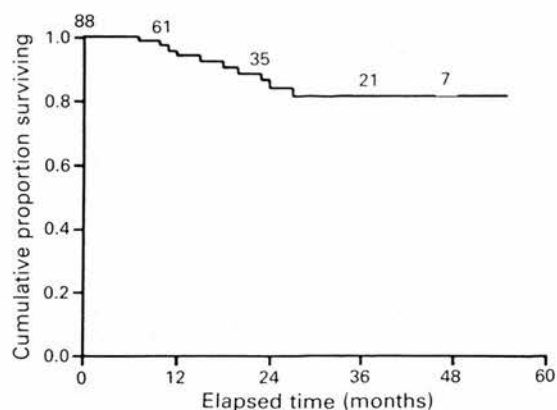


Figure 4 Cumulative proportion remaining free from local relapse in 88 patients with operable breast cancers of greater than 4 cm at diagnosis treated with primary systemic therapy before definitive locoregional surgery. The median period of follow-up was 24 months (range 4–55 months).

adverse effects included a tender lump at the injection site ($n = 4$), hot flushings ($n = 2$), erythematous rash on buttocks ($n = 1$), and a clinically insignificant, self-limiting abnormality of liver function tests ($n = 2$). Four patients who received aminoglutethimide complained of nausea and lethargy on initiation of therapy.

The chemotherapeutic regime was moderately toxic. The principle side-effects were alopecia (100%), nausea and vomiting (91% WHO grade 2 or greater), stomatitis (68% WHO grade 2), dyspepsia (20%) mild dysuria (13%) and neutropenia (26% WHO grade 2 or greater). Premature termination of therapy was required in two patients because of nonspecific toxicity and in one on account of iliofemoral thrombosis. There were no treatment related deaths and no increase in morbidity associated with definitive surgery.

Axillary lymph node status

Histological assessment of axillary lymph node status was performed in 46 patients (51%) before and in 86 patients (98%) on completion of systemic therapy. Metastatic carcinoma was detected in 33 (72%) and 42 cases (49%) respectively. Overall 56 patients (64%) had axillary metastases detectable at some stage in their management.

Comparison of pre- and post-treatment axillary node status was possible in 43 patients. Of the 33 patients in whom axillary node metastasis were detected pretreatment, 14 had no evidence of metastases following systemic therapy. Of these eight had shown significant regression during systemic therapy of which five were clinically complete. Axillary node metastases were found in only one of the ten patients in whom the pretreatment axillary node sample had failed to demonstrate metastases. This patient did not respond to 4-hydroxyandrostenedione or proceed to chemotherapy and so may represent a true progression of axillary node status.

Discussion

This study was undertaken to ascertain whether appropriate long-term systemic therapy could be selected by direct assess-

ment of primary tumour response before surgical excision. Experience of this novel form of management in 88 cases has shown it to be a feasible approach.

A biopsy of the tumour was performed prior to initiation of systemic therapy and has allowed direct correlation of oestrogen receptor concentration to individual tumour response. Of the first 36 patients treated by primary endocrine therapy, no patient with a tumour of ER concentration less than 20 fmol mg cytosol protein⁻¹ showed significant regression (Anderson *et al.*, 1989). Thereafter a change in protocol was instituted and primary endocrine therapy was reserved for those patients with tumours of ER concentration ≥ 20 fmol mg cytosol protein⁻¹. Individual responsiveness to endocrine therapy, even within ER-moderate/rich tumours however could only reliably be determined by direct observation of the effect of therapy. In this way we have selected out those patients in whom continuing endocrine systemic therapy is appropriate. Such patients would appear to have an excellent prognosis (Anderson *et al.*, 1989). Similarly direct objective assessment of tumour response to systemic therapy allows cessation of endocrine therapy where it has been demonstrated to be of no value with initiation of chemotherapy if desired.

A variety of endocrine therapies have now been tried and the proportion of patients achieving regression is similar to that documented using the same agents in advanced disease (Hawkins, 1985; Coombes, Stein & Dowsett, 1989; Nicholson & Waler, 1989). Of particular interest is the efficacy of the gonadotrophin-releasing hormone agonist, goserelin and the peripheral aromatase inhibitor 4-hydroxyandrostenedione in pre- and post-menopausal women respectively. Gonadotrophin-releasing hormone agonists produce an effect similar to oophorectomy but without operation (Nicholson & Walker, 1989) and would appear to be suitable for the primary treatment of premenopausal women. Furthermore they can be discontinued should therapy prove ineffective. The requirement for intramuscular injection of 4-hydroxyandrostenedione is a disadvantage and tamoxifen is preferred for primary therapy in postmenopausal women.

Within this study cytotoxic therapy with its greater associated toxicity was reserved for patients in whom endocrine therapy had failed or the likelihood of response to endocrine therapy was minimal (i.e. patients with ER-poor or ER-negative tumours). The proportion of such patients with tumours which were chemosensitive was high and lies within the observed range of 70–93% described with 'neoadjuvant' chemotherapy in more locally advanced breast cancers (Jacquillat *et al.*, 1988; Hortobagyi *et al.*, 1988; Swain *et al.*, 1987). Of those individual tumours directly demonstrated as endocrine-resistant however the proportion of ER-poor/negative tumours regressing with chemotherapy paralleled that of primary chemotherapy (~80%) but the efficacy in ER-rich tumours was much lower (30%). This difference in response pattern may suggest a common mechanism of failure to respond to both endocrine and cytotoxic therapies.

The response to endocrine therapy was on average, slower than that achieved with cytotoxic therapy. Within the period of the study only one patient achieved complete clinical regression of their tumour during endocrine therapy and in no patient was pathological remission complete. In contrast the response to cytotoxic therapy was occasionally rapid and in those patients with such a rapid response, complete clinical and even complete pathological response was observed. None of the parameters studied were able to define which patients were more likely to achieve such complete remission.

As this study has progressed refinements have been made to the protocol. The use of ER data to select systemic therapy has already been described. Initially axillary node sampling was not an integral part of the pretreatment assessment. Analysis of the pathological post-treatment axillary node staging of the first 43 patients demonstrated a lower

incidence (51%) of positivity than would be expected. Since clinical axillary node staging is notoriously unreliable it was felt that pathological staging of the axillary nodes should be included in the preoperative assessment if survival data was to be assessed. Of the 45 patients in whom pretreatment axillary node status was known there was a higher incidence of lymph node metastases (73%) which is more in keeping with previous studies for tumours of similar stage (Carter *et al.*, 1989). Following therapy however only 20 (44%) had detectable node metastases suggesting that the preliminary figure of 51% was not due to sample bias. While it is conceivable that axillary node sampling has simply removed the few lymph nodes with metastatic disease it is also possible that the systemic therapy has been effective in eradicating axillary metastases.

A possible benefit of primary systemic therapy, which we have not yet explored, is that it may permit conservative surgery in patients with large tumours which would otherwise require mastectomy. The usefulness of initial systemic therapy with the aim of avoiding mastectomy has been reported in a series of 57 patients with large but potentially operable breast cancers (Mansi *et al.*, 1989). With a median follow-up of 19 months these authors report similar response rates, loco-regional recurrence, distant relapse and projected overall survival rates to this study with only 18% (10/57) of patients subsequently proceeding to mastectomy. Primary systemic therapy however is not yet orthodox for operable disease and we did not believe it justified to perform less than a mastectomy in the majority of patients. In future studies a more conservative approach would be worthy of trial.

A disadvantage of preoperative systemic therapy is the potential psychological morbidity induced by leaving the tumour *in situ* while initial systemic therapy is undertaken. In general this did not prove to be a problem even in patients with nonresponsive disease. This was probably due to the fact that surgical removal of the tumour, still regarded by many patients as the critical step in their management, was still possible. Formal examination of psychological morbidity was not however undertaken during this study, but should be part of any future work.

In addition to the benefit of selecting appropriate systemic therapy, there is theoretical (Goldie & Coldman, 1979; Skipper 1964), experimental (Fisher *et al.*, 1983) and clinical (Nissen-Meyer *et al.*, 1986; Ragaz, 1986) evidence, that early administration of systemic therapy in the treatment schedule of patients with breast cancer may improve survival. The 3 year cumulative survival rate within this study was 86%, with 81% of patients remaining free of local recurrence. This compares favourably to 3 year survival rates achieved with orthodox treatment of large tumours of similar stage which range from 65–78% (Duncan & Kerr, 1976; Sorace & Lippman, 1988; Carter *et al.*, 1989). Proof requires a controlled randomised trial in which this selective approach is assessed against conventional treatment, that is mastectomy followed by combination chemotherapy in premenopausal women and tamoxifen in postmenopausal women; such a trial is currently underway in Edinburgh.

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